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Zheng-Hong Qin *Editor*

# Autophagy: Biology and Diseases

## Basic Science

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*Editor*
Zheng-Hong Qin
Jiangsu Key Laboratory of Neuropsychiatric Diseases, Department of Pharmacology and Laboratory of Aging and Nervous Diseases
College of Pharmaceutical Sciences of Soochow University
Suzhou, China

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# Editorial Commitee

## Chief Editor



## Editorial Board

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# Contributors

**Linxi Chen** Learning Key Laboratory for Pharmacoproteomics, Hunan Province Cooperative Innovation Center for Molecular Target New Drug Study, Institute of Pharmacy and Pharmacology, University of South China, Hengyang, China

**Song Chen** Translational Research Institute, Henan Provincial People's Hospital, Academy of Medical Science, Zhengzhou University, Zhengzhou, China

**Yingyu Chen** Department of Immunology, Peking University School of Basic Medical Sciences, NHC Key Laboratory of Medical Immunology, Peking University, Beijing, China

**Chao Cheng** Department of Thoracic Surgery, The First Affiliated Hospital of Sun Yat-Sen University, Guangzhou, People's Republic of China

**Bing Cui** State Key Laboratory of Bioactive Substance and Function of Natural Medicines, Institute of Materia Medica, Chinese Academy of Medical Sciences & Peking Union Medical College, Beijing, People's Republic of China

**Jun Cui** MOE Key Laboratory of Gene Function and Regulation, State Key Laboratory of Biocontrol, School of Life Sciences, Sun Yat-Sen University, Guangzhou, China

**Hui Gao** State Key Laboratory of Stem Cell and Reproductive Biology, Institute of Zoology, Chinese Academy of Sciences, Chaoyang District, Beijing, China

**Qi Gao** Department of Clinical Laboratory, Shandong Provincial Hospital, Shandong University, Jinan, Shandong, China

**Bing Han** Department of Pharmacology, School of Medicine, Southeast University, Nanjing, Jiangsu, China

**Xiao-Shuai Han** CAS Key Lab of Tissue Microenvironment and Tumor, Shanghai Institute of Nutrition and Health, Chinese Academy of Sciences, Shanghai, China

**Li-Fang Hu** Institute of Neuroscience, Soochow University, Suzhou, China

**Wanglai Hu** Translational Research Institute, Henan Provincial People's Hospital, Academy of Medical Science, Zhengzhou University, Zhengzhou, China

**Yang-Xi Hu** Department of Cardiology, Shanghai Changhai Hospital, Shanghai, China

**Zhuowei Hu** State Key Laboratory of Bioactive Substance and Function of Natural Medicines, Institute of Materia Medica, Chinese Academy of Medical Sciences & Peking Union Medical College, Beijing, People's Republic of China

**Fang Hua** State Key Laboratory of Bioactive Substance and Function of Natural Medicines, Institute of Materia Medica, Chinese Academy of Medical Sciences & Peking Union Medical College, Beijing, People's Republic of China

**Rongrong Huang** Department of Pharmacology, School of Medicine, Southeast University, Nanjing, Jiangsu, China

**Shou-Heng Jin** MOE Key Laboratory of Gene Function and Regulation, State Key Laboratory of Biocontrol, School of Life Sciences, Sun Yat-Sen University, Guangzhou, China

**Qing Jing** CAS Key Lab of Tissue Microenvironment and Tumor, Shanghai Institute of Nutrition and Health, Chinese Academy of Sciences, Shanghai, China

**Muhammad Babar Khawar** State Key Laboratory of Stem Cell and Reproductive Biology, Institute of Zoology, Chinese Academy of Sciences, Chaoyang District, Beijing, China;
University of Chinese Academy of Sciences, Beijing, China

**Wei-Dong Le** Liaoning Provincial Center for Clinical Research on Neurological Diseases, The First Affiliated Hospital, Dalian Medical University, Dalian, People's Republic of China

**Ke Li** NHC Key Laboratory of Biotechnology of Antibiotics, Institute of Medicinal Biotechnology, Chinese Academy of Medical Sciences & Peking Union Medical College, Beijing, China

**Wei Li** State Key Laboratory of Stem Cell and Reproductive Biology, Institute of Zoology, Chinese Academy of Sciences, Chaoyang District, Beijing, China;
University of Chinese Academy of Sciences, Beijing, China

**Wen Li** Department of Immunology, School of Basic Medical Science, Shandong University, Jinan, China

**Yanjun Li** College of Life Sciences, Nankai University, Tianjin, China

**Ying Li** Shanghai Institute of Organic Chemistry, Chinese Academy of Sciences, Shanghai, China

**Heng Lin** State Key Laboratory of Bioactive Substance and Function of Natural Medicines, Institute of Materia Medica, Chinese Academy of Medical Sciences & Peking Union Medical College, Beijing, People's Republic of China

**Jianping Liu** Shanghai Institute of Organic Chemistry, Chinese Academy of Sciences, Shanghai, China

**Kun Liu** State Key Laboratory of Stem Cell and Reproductive Biology, Institute of Zoology, Chinese Academy of Sciences, Beijing, China

**Zhen-Guo Liu** Department of Thoracic Surgery, The First Affiliated Hospital of Sun Yat-Sen University, Guangzhou, People's Republic of China

**Li Luo** School of Physical Education and Sports Science, Soochow University, Suzhou, China

**Zhenyi Ma** Department of Biochemistry and Molecular Biology, School of Basic Medical Sciences, Tianjin Medical University, Tianjin, China

**Lifeng Pan** Shanghai Institute of Organic Chemistry, Chinese Academy of Sciences, Shanghai, China

**Zhihao Qi** Learning Key Laboratory for Pharmacoproteomics, Hunan Province Cooperative Innovation Center for Molecular Target New Drug Study, Institute of Pharmacy and Pharmacology, University of South China, Hengyang, China

**Zheng-Hong Qin** Jiangsu Key Laboratory of Neuropsychiatric Diseases, Department of Pharmacology and Laboratory of Aging and Nervous Diseases, College of Pharmaceutical Sciences of Soochow University, Suzhou, China

**Shuang Shang** State Key Laboratory of Bioactive Substance and Function of Natural Medicines, Institute of Materia Medica, Chinese Academy of Medical Sciences & Peking Union Medical College, Beijing, People's Republic of China

**Ling Shen** Department of Pharmacology, School of Medicine, Southeast University, Nanjing, Jiangsu, China

**Rui Sheng** Department of Pharmacology, Laboratory of Aging and Nervous Diseases, Jiangsu Key Laboratory of Neuropsychiatric Diseases, College of Pharmaceutical Sciences, Soochow University, Suzhou, China

**Rick F. Thorne** Translational Research Institute, Henan Provincial People's Hospital, Academy of Medical Science, Zhengzhou University, Zhengzhou, China

**Feng Wang** State Key Laboratory of Bioactive Substance and Function of Natural Medicines, Institute of Materia Medica, Chinese Academy of Medical Sciences & Peking Union Medical College, Beijing, People's Republic of China

**Guanghui Wang** Laboratory of Molecular Neuropathology, Jiangsu Key Laboratory of Neuropsychiatric Diseases & Department of Pharmacology, College of Pharmaceutical Sciences, Soochow University, Suzhou, China

**Liang Wang** State Key Laboratory of Stem Cell and Reproductive Biology, Institute of Zoology, Chinese Academy of Sciences, Beijing, China

**Ronglin Wang** Department of Neurosurgery, Tangdu Hospital, The Fourth Military Medical University, Xi'an, China

**Rui Wang** Laboratory of Molecular Neuropathology, Jiangsu Key Laboratory of Neuropsychiatric Diseases & Department of Pharmacology, College of Pharmaceutical Sciences, Soochow University, Suzhou, Jiangsu, China

**Yan Wang** Department of Pharmacology and Laboratory of Aging and Nervous Diseases, Jiangsu Key Laboratory of Neuropsychiatric Diseases, College of Pharmaceutical Sciences of Soochow University, Suzhou, China

**Ying Wang** Department of Physiology, Chinese Academy of Medical Sciences and Peking Union Medical College, Institute of Basic Medical Sciences, Beijing, China;
Department of Molecular Orthopedics, Beijing Research Institute of Traumatology and Orthopaedics, Beijing, China

**Min Wei** Liaoning Provincial Center for Clinical Research on Neurological Diseases, The First Affiliated Hospital, Dalian Medical University, Dalian, People's Republic of China

**Mian Wu** Translational Research Institute, Henan Provincial People's Hospital, Academy of Medical Science, Zhengzhou University, Zhengzhou, China

**Yao-Xing Wu** MOE Key Laboratory of Gene Function and Regulation, State Key Laboratory of Biocontrol, School of Life Sciences, Sun Yat-Sen University, Guangzhou, China

**Hai-Dong Xu** Laboratory of Aging and Nervous Diseases, Department of Pharmacology, College of Pharmaceutical Science, Soochow University, Suzhou, China

**Xiaojie Yan** Department of Biochemistry and Molecular Biology, School of Basic Medical Sciences, Tianjin Medical University, Tianjin, China

**Jie Yang** Department of Biochemistry and Molecular Biology, School of Basic Medical Sciences, Tianjin Medical University, Tianjin, China

**Qian Yang** Department of Neurosurgery, Tangdu Hospital, The Fourth Military Medical University, Xi'an, China

**Honghong Yao** Department of Pharmacology, School of Medicine, Southeast University, Nanjing, Jiangsu, China

**Jiaojiao Yu** State Key Laboratory of Bioactive Substance and Function of Natural Medicines, Institute of Materia Medica, Chinese Academy of Medical Sciences & Peking Union Medical College, Beijing, People's Republic of China

**Jinmei Yu** State Key Laboratory of Bioactive Substance and Function of Natural Medicines, Institute of Materia Medica, Chinese Academy of Medical Sciences & Peking Union Medical College, Beijing, People's Republic of China

**Hongbing Zhang** Department of Physiology, Chinese Academy of Medical Sciences and Peking Union Medical College, Institute of Basic Medical Sciences, Beijing, China

**Hui-Ling Zhang** Department of Pharmacology, Laboratory of Cerebrovascular Pharmacology, Soochow University, Suzhou, Jiangsu, China

**Lining Zhang** Department of Immunology, School of Basic Medical Science, Shandong University, Jinan, China

**Nan Zhang** Department of Biochemistry and Molecular Biology, School of Basic Medical Sciences, Peking University Health Science Center, Beijing, China

**Yuan Zhang** Department of Pharmacology, School of Medicine, Southeast University, Nanjing, Jiangsu, China

**Tongbiao Zhao** State Key Laboratory of Stem Cell and Reproductive Biology, Institute of Zoology, Chinese Academy of Sciences, Beijing, China

**Ying Zhao** Department of Biochemistry and Molecular Biology, School of Basic Medical Sciences, Peking University Health Science Center, Beijing, China

**Ruimin Zhou** Department of Biochemistry and Molecular Biology, School of Basic Medical Sciences, Tianjin Medical University, Tianjin, China

**Xian-Yong Zhou** Department of Pharmacology, Laboratory of Cerebrovascular Pharmacology, Soochow University, Suzhou, Jiangsu, China

**Lin Zhu** Department of Neurosurgery, Tangdu Hospital, The Fourth Military Medical University, Xi'an, China

**Yong-Ming Zhu** Department of Pharmacology, Laboratory of Cerebrovascular Pharmacology, Soochow University, Suzhou, Jiangsu, China

# Part I
# History and Current Status of Autophagy Research

# Chapter 1
# History and Current Status of Autophagy Research


**Rui Sheng and Zheng-Hong Qin**

**Abstract** Autophagy is an evolutionarily conserved process in which eukaryotic bilayer membrane vesicles enclose intracellular contents and transport them to lysosomes for degradation. In the 1990s, Ohsumi et al. identified multiple autophagy-related genes in a yeast model. Functional homologues of almost all yeast autophagy-related genes were found in higher eukaryotes. In 2003, Klionsky et al. named these genes the *Atg* genes and studied the interactions between the proteins they encoded and their functions in autophagy. In April 2005, a new journal, Autophagy, was published that was edited by Klionsky. The number of autophagy research papers indexed by PubMed has increased each year. In 2016, Yoshinori Ohsumi won the Nobel Prize in Medicine or Physiology for his discovery of the autophagy mechanism. Autophagy has thus become a hot research area, which involves biology, medicine, botany and microbiology. Many researchers are actively exploring the relationship between non-selective and selective autophagy and various pathophysiological states in humans, and are studying the molecular mechanisms underlying autophagy regulation in various biological conditions, including cancer, neurodegenerative diseases, cardiovascular diseases, immune responses, development and ageing. This chapter focuses on the history and current status of autophagy research and highlights the milestones that contributed to the development of the field.

**Keywords** Autophagy · History · Lysosome · *Atg* genes · Yoshinori Ohsumi

In 1963, Christian de Duve first proposed the concept of autophagy, which is derived from Greek; 'auto' means self, and 'phagy' means eating. Autophagy is an evolutionarily conserved process in which eukaryotic bilayer membrane vesicles enclose intracellular components and transport them to lysosomes for degradation. The main function of autophagy is to degrade endogenous biomacromolecules to recycle

R. Sheng (✉) · Z.-H. Qin (✉)
Department of Pharmacology, Laboratory of Aging and Nervous Diseases, Jiangsu Key Laboratory of Neuropsychiatric Diseases, College of Pharmaceutical Sciences, Soochow University, 199 Ren Ai Road, Suzhou 215123, China
e-mail: shengrui@suda.edu.cn

Z.-H. Qin
e-mail: qinzhenhong@suda.edu.cn



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amino acids and monosaccharides, which is especially important for maintaining intracellular homeostasis and survival in the absence of nutrients. In the following two decades, due to limitations in the available techniques, the field of autophagy research progressed slowly and did not attract widespread attention. However, in the 1990s, Yoshinori Ohsumi et al. discovered autophagy in yeast and identified multiple autophagy-related genes in a yeast model. Functional homologues of almost all yeast autophagy-related genes were found in higher eukaryotes and humans, greatly promoting the development of autophagy research. In 2003, Klionsky et al. named these genes the *Atg* (AuTophaGy) genes and studied the interactions between their encoded proteins and their functions in autophagy. Science published an article in December 2004 that predicted that autophagy would become one of the six leading cutting-edge topics in science in 2005 and ranked it as the number-one topic. Since 1997, various international autophagy conferences have been held around the world. In April 2005, a new journal, Autophagy, was published and edited by Klionsky. The number of autophagy research papers published on PubMed has also increased each year. In 2016, Ohsumi won the Nobel Prize in Medicine or Physiology for his discovery of the autophagy mechanism (Mizushima 2018). All of this suggest that autophagy has attracted increasing attention and that autophagy has become a hot research area involving biology, medicine, botany and microbiology. Many researchers are actively exploring the relationship between non-selective and selective autophagy and various pathophysiological states in humans, and are studying the molecular mechanisms underlying autophagy regulation in various diseases, including cancer, neurodegenerative diseases, cardiovascular diseases, immune responses, development and ageing.

This chapter will focus on the history and current status of autophagy research and highlight the milestones that contributed to the development of the field (Fig. 1.1). The subsequent sections will detail the classification and characteristics of the different types of autophagy, the generation, transport and degradation of autophagic vesicles, the signalling pathways involved in autophagy regulation, the biological roles and functions of autophagy and the tools and methods used in autophagy research.

## 1.1 History of Autophagy Research

### *1.1.1 Early Events in Autophagy Research*

#### 1.1.1.1 The Concept of the Lysosome and Autophagy

The field of autophagy originated from the discovery of lysosomes by the Belgian cytochemist Christian de Duve. In 1955, de Duve discovered a new organelle during the process of the differential separation of liver homogenate. The organelle could enclose acidic phosphohydrolase, which is most active at acidic pH. He named the organelle the lysosome, as it represented an organelle with a lytic function.

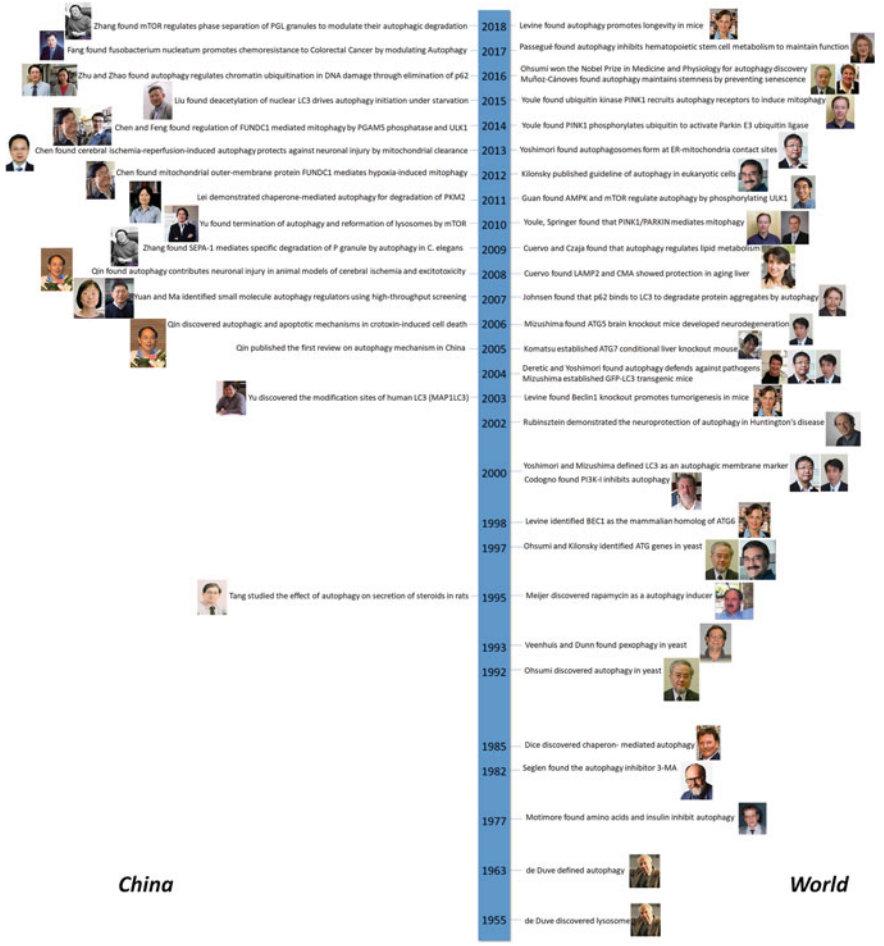


**Fig. 1.1** Milestone discoveries in the history of autophagy research. Left: History of Chinese autophagy research; Right: History of international autophagy research

In 1957, Clark observed irregularly shaped bilayer membrane vesicles enveloping amorphous materials, including mitochondria, in renal proximal tubular epithelial cells in neonatal mice with electron microscopy, which were described as 'irregular density bodies' and 'dense bodies' and were believed to be the first report of autophagosomes. In 1962, Ashfold and Porter treated rat hepatocytes with glucagon and found that membrane vesicles enclosed semi-digested mitochondria and endoplasmic reticulum. Novikoff et al. observed an increase in autophagosomes in liver tissue sections from starved rats (peripheral bodies). In 1963, they observed similar structures containing lysosomal hydrolase in the kidney, known as 'cytolysosomes', which appeared to be acid phosphatase positive and contained mitochondria, endoplasmic reticulum, ribosomes and other cytoplasmic components. Based on these

findings, in 1963, at the Ciba Foundation Lysosome Symposium held in London, de Duve gave a conference report entitled 'The Concept of Lysosomes' that described endocytosis and exocytosis and distinguished the functions of lysosomes in heterophagy and autophagy. He proposed the concept of autophagy for the first time, which described the degradation of cytoplasmic and organelles via single or bilayer membrane vesicles known as 'autophagosomes'. In 1966, a review by de Duve and Wattiaux predicted the main function of autophagy, which has since been confirmed, to consist of the feeding of nutrients through fragmentation and self-digestion when food supply is insufficient, cell differentiation and metamorphosis, intracellular clearance to promote longevity, cell self-recovery and self-clearing of dead cells. In 1974, due to the discovery of lysosomes (which are equivalent to organelles in the cell digestive system) and peroxidases (which play an important role in cellular metabolism), de Duve was awarded the Nobel Prize for Physiology or Medicine.

### *1.1.2 Lysosomes and Protein Turnover*

In the 1960s and 1970s, some researchers examined the relationship between lysosomes and protein turnover. In 1969, Schimke et al. found that the rates of protein degradation were different. The pulse-tracking of radioactive leucine, arginine and methionine was used to detect the half-life of various intracellular proteins. The half-life of different proteins ranged from a few minutes to several days, regardless of their cellular localization. Then, researchers wanted to understand how these proteins were degraded. In the 1970s, multiple investigators used the lysosomal inhibitors $NH_4Cl$, leupeptin, chloroquine and anti-protease to evaluate the role of lysosomes in the degradation of proteins in intact skeletal muscle, hepatocytes and macrophages. It was found that lysosomes preferentially degrade long-lived proteins compared with short-lived proteins. Fifty-four percent of short-lived proteins and 75% of long-lived proteins are degraded in lysosomes, and lysosomes are simultaneously responsible for the degradation of endogenous and exogenous proteins. Dice et al. found that in liver and muscle, the degradation of macromolecular proteins is faster than that of smaller proteins, the degradation of acidic proteins is faster than that of neutral or basic proteins and the degradation of glycoproteins is faster than that of non-glycoproteins. In addition, in rats with diabetes or under starvation, protein degradation is accelerated. All these changes have been confirmed to be due to the activation of non-selective autophagy.

### *1.1.3 Discovery of the Autophagy Regulation Pathway*

Early studies have shown that autophagy is regulated by amino acids and some hormones. In 1967, Deter and de Duve observed an increase in the volume and number of autophagic vacuoles and lysosomes, which are more sensitive to mechanical stress

and osmotic changes, in live rats after glucagon injection, indicating that glucagon activates autophagy. In contrast to the effect of glucagon, in 1977, Pfeifer and Mortimore demonstrated that amino acids and insulin significantly inhibit autophagy and protein turnover (Mortimore and Schworer 1977). When amino acids were reduced in rat liver perfusion fluid, the protein degradation rate increased significantly. In contrast, an increase in amino acids in solution reduced the degradation rate to one-third of that observed in control rats, indicating that autophagy-mediated protein degradation is tightly regulated by the amino acid level. Mortimore et al. further studied which amino acids inhibited autophagy, either alone or synergistically. Seven amino acids, including leucine, tyrosine, phenylalanine, glutamine, histidine, tryptophan and methionine, could inhibit autophagy. Seglen obtained similar results in cultured hepatocytes and found that leucine has the strongest inhibitory effect on autophagy. In 1981, Pfeifer et al. found that feeding inhibited autophagy in liver and heart in rats, while fasting induced autophagy. Thirty to forty percent of liver protein was degraded after 48 h of starvation. If all seven of the regulatory amino acids are removed, autophagosomes are formed within 5 min, and autophagy reaches a steady state within 20 min, which suggests that the rate of autophagosome (AV) formation is equal to the rate of AV degradation. When the amino acids are reintroduced, lysosomal degradation is accelerated, and the number of autophagosomes is significantly reduced. These studies demonstrated that glucagon activates autophagy, while insulin and amino acids inhibit autophagy, which is consistent with the influence of the regulation of these two hormones on catabolism and anabolism.

In 1982, Seglen and Gordon reported that 3-methyladenine (3-MA) inhibited autophagy (Seglen and Gordon 1982). Since then, 3-MA has become a classic autophagy inhibitor. Subsequently, the Seglen, Meijer and Codogno groups found that protein kinases, phosphatases and heterotrimeric G proteins can regulate autophagy. In 1995, the Meijer group isolated hepatocytes from starved rats and found that autophagy was reduced when amino acids were added to the medium but that ribosomal protein S6 was phosphorylated. Rapamycin, an immunosuppressant, inhibits mTOR (mammalian target of rapamycin) and prevents S6 phosphorylation to activate autophagy. This study revealed the role of mTOR signalling in autophagy regulation and demonstrated that rapamycin activates autophagy by inhibiting mTOR (Blommaart et al. 1995). It was not until 2000 that the Codogno group discovered the mechanism by which 3-MA inhibits autophagy. Type I phosphatidylinositol 3-kinase (PI3K-I) inhibits autophagy, while type III phosphatidylinositol (PI3K-III) activates autophagy. Autophagy inhibitors, including 3-MA and wortmannin, are both PI3K-III inhibitors. By using 3-MA and rapamycin, two classical regulators of autophagy, many researchers intend to find more specific autophagy regulators based on the molecular mechanisms of autophagy.

### 1.1.4 Discovery of Autophagy Types

It is well known that the typical process of macroautophagy involves a membrane structure (a phagophore or isolated membrane) that appears in the cytoplasm and extends to form a cup-shaped structure, which is then closed. This is an early autophagosome (AVi), which is surrounded by a double membrane or a multilayer membrane. Autophagosomes then fuse with lysosomes to form autophagic vacuoles, i.e. degraded autophagosomes (autolysosomes, AVd), in which the inner membrane and contents are degraded by lysosomal hydrolases. Subsequently, autolysosomes become residual, and degradation products are transported back to the cytoplasm for recycling. Autophagosomes (AVi) are surrounded by two or more membrane layers, while AVd are single layer membrane structures. In 1981, Mortimore first proposed the concept of AVi and AVd and indicated that there is a quantitative relationship between the rate of protein turnover and the fractions of early (AVi) and degraded (AVd) autophagosomes.

In the 1980s, Mortimore studied the ultrastructure of liver lysosomes and concluded that cell components can be degraded by lysosomes through two mechanisms, microautophagy and macroautophagy. Microautophagy involves lysosomal membrane invagination, which directly transports cellular components during lysosome degradation, and therefore only a small amount of the cytoplasm is degraded by microautophagy (compared to macroautophagy). Microautophagy is responsible for basal degradation, while macroautophagy regulates protein degradation by amino acids, insulin and glucagon. The vesicle membranes of some yeast, such as *Pichia pastoris* and *Hansenula polymorpha*, mediate microautophagy and can serve as models for the study of microautophagy.

In 1985, Dice et al. reported that lysosomes selectively degrade soluble proteins. The rate of the degradation of radiolabelled ribonuclease (RNase A) increases after serum deprivation, and the degradation signal residue is contained in the 'Lys-Phe-Glu-Arg-Gln' (KFERQ) motif, which is recognized by cytoplasmic Hsc70 protein. Hsc70 then interacts with the lysosomal membrane protein LAMP-2 to promote the transfer of RNaseA to lysosomes for degradation. This pathway is known as chaperone-mediated autophagy (CMA). In 2008, Cuervo et al. found that LAMP2 and CMA had protective effects against ageing in liver and studied the molecular mechanism of CMA and its functions in metabolism, ageing and disease (Zhang and Cuervo 2008).

### 1.1.5 Source of the Autophagosome Membrane

The source and formation site of the autophagosome membrane remains a challenging topic in the field of autophagy. In 1987, Seglen observed by electron microscopy that autophagosomes were formed by cup-shaped phagophores made of thick osmiophilic membranes, indicating that they are rich in lipids. In 1990, Dunn et al. presented

immunohistochemical evidence that AVi are derived from rough endoplasmic reticulum rather than the Golgi apparatus or serous membrane. In 1998, Seglen isolated autophagosomes from vinblastine-treated hepatocytes that contained markers from the endoplasmic reticulum, including protein disulfide isomerase (PDI) and GRP78 (also known as Bip), the lysosomal marker LAMP2, the cytoplasmic marker SOD and the Golgi marker p58, but no seromembranous and endosomal markers were detected. In 2013, Yoshimori et al. reported that autophagosomes form at endoplasmic reticulum–mitochondria contact sites. During starvation, the autophagosome precursor/autophagosome markers Atg14 and Atg5 relocalize to endoplasmic reticulum–mitochondria contact sites. Syntaxin 17 in the endoplasmic reticulum then binds to Atg14 and recruits it to endoplasmic reticulum–mitochondria contact sites, suggesting that these are a novel source of autophagosomes (Hamasaki et al. 2013).

In addition, the canonical autophagy pathway involves the formation of a two- or multilayer membrane to enclose contents for lysosomal degradation, but it was recently found that in non-canonical autophagy, the key upstream signal of autophagosome formation and maturation, microtubule-associated protein 1 light chain 3 (LC3), can be localized to monomembrane vesicles to drive lysosomal degradation. Moreover, multilayer or monolayer membrane structures also mediate non-degradative functions, such as the promotion of bacterial replication, the local secretion of lysosomes and the functioning of the melanosome. Therefore, to some extent, autophagy machinery is likely to mediate the vesicle shuttle mechanism, which plays a broader role in the cellular process. These novel findings greatly expand the concept and biological significance of autophagy.

## 1.2 Milestones in Autophagy Research

### *1.2.1 Discovery of Autophagy-Related Genes*

#### 1.2.1.1 Discovery of Autophagy in Yeast

Prior to 1990, autophagy research was mainly carried out in mammalian cell lines and the livers of rodents. In 1992, Yoshinori Ohsumi's group found that nutrient deficiency in Saccharomyces cerevisiae induced autophagic degradation. In Saccharomyces cerevisiae, vacuoles are the only organelles visible under phase-contrast microscopy. They are acidic and contain a variety of hydrolases and function as mammalian lysosomes. Ohsumi et al. pioneered the use of yeast strains lacking vacuolar proteases. Due to the inhibition of degradation, multiple spheroids were observed around the vacuoles under light microscopy after nitrogen starvation for 30 min, and the number slowly increased until the vacuoles were filled. Electron microscopy confirmed that these spheroids were autophagosomes with an average diameter of 500–900 nm that enclosed part of the cytoplasm, ribosomes or mitochondria. In wild-type yeast, these autophagosomes can be rapidly degraded by vacuolar

hydrolases. When the outer membrane of the autophagosome fuses with the vacuole, a single-layer membrane autolysosome is formed. The membrane dynamics are very similar to those of autophagy in mammals. Subsequently, carbon, sulphur, phosphate and single amino acid deficiency have also been demonstrated to induce similar processes.

#### 1.2.1.2 Identification and Cloning of the Yeast *Atg* Gene

Since 1992, due to the discovery of autophagy in yeast, Ohsumi and Tsukada have used yeast strains lacking vacuolar proteases to screen for autophagy-deficient mutants that cannot form autophagosomes during nitrogen deficiency by using light microscopy (Takeshige et al. 1992). They screened approximately 38 000 independent clones under starvation and finally isolated 15 autophagy-deficient yeast mutants and identified a series of autophagy genes, which are named the *Apg* genes. In 1992, Klionsky et al. transduced aminopeptidase I into the vacuoles of Saccharomyces cerevisiae and proposed the cytoplasm-to-vacuole transport (CVT) of vacuolar enzyme-aminopeptidase I (API). They found many mutants with defective transport, thereby identifying the *Cvt* gene. Veenhuis and Dunn tested peroxisome enzyme activity and identified the *Pdd* (peroxisome degradation deficiency) gene and *Gsa* (glucose-mediated selective autophagy) gene. Thumm et al. screened clones deficient in fructose-1,6-bisphosphatase (FBPase) degradation and identified the *Aut* gene.

In 1993, the Ohsumi group reported the first yeast autophagy gene, *Apg1* (now called *Atg1*), which encodes the Atg1 protein that has kinase activity and that plays a key role in nutrient starvation-induced autophagy. In 1996, the Ohsumi group reported the autophagy gene *Apg5* (now *Atg5*). In 1997, the Thumm group reported that *Aut1* (now called *Atg3*) encodes the E2-binding enzyme-modified ubiquitin-like protein Atg8. In 1998, Mizushima et al. reported the Atg12-Atg5 protein conjugation system in yeast (Mizushima et al. 1998). In 1999, yeast *Atg7* was cloned and identified. In 2000, Ichimura et al. reported that the Atg7 E1-like enzyme promotes the binding of Atg8 to phosphatidylethanolamine, indicating the role of protein lipidation in membrane kinetics during autophagy. In fact, many *Cvt*, *Atg* and *Aut* genes are allelic. For example, Atg7 (Gsa7, Cvt2) is an essential protein in the autophagy, peroxisome and CVT pathways. In 2003, these genes were named autophagy-associated genes (*Atg*) (Klionsky et al. 2003), which include genes involved in the APG, AUT, CVT, GSA and PDD pathways in yeast, plants and vertebrates. In 2010, Kanki et al. identified *Atg32* and *Atg33* by genetic screening of selective mitophagy mutants. To date, a total of 42 *Atg* genes have been identified that are highly conserved in eukaryotes and are responsible for autophagosome formation and the regulation of selective autophagy.

#### 1.2.1.3 *Atg* Gene in Mammals and Other Organisms

Researchers then identified the structural and functional homologs of yeast *Atg* genes in mammals. In 1998, Mizushima et al. identified the first mammalian autophagy genes, *Atg5* and *Atg12*, and demonstrated that the Atg12-Atg5 conjugation system is conserved in mammals. In 1999, the Levine group found that a Bcl-2 binding protein, Beclin 1, was able to induce autophagy and that Beclin 1 is the homologue of Atg6/Vps30. In 2000, Kominami group identified the mammalian homologues of the proteins in the Atg7 conjugation system, while Yosimori group identified microtubule-associated protein 1 light chain 3 (LC3 or GABARAP) as a mammalian homologue of Atg8 (Kabeya et al. 2000). There are two forms of LC3: cytosolic LC3-I and phosphatidylethanolamine-bound LC3-II. LC3-II is synthesized by multiple processes, including C-terminal cleavage, and the LC3-II level reflects the number of autophagosomes. In the presence of bafilomycin A1, LC3-II cannot fuse to lysosomes. In the presence of protease inhibitors, LC3-II cannot be degraded. Currently, LC3 is considered a classic indicator of autophagy in mammalian cells. Homologs of almost all yeast Atg proteins have been found in mammals, including two Atg1 homologs, ULK1 and ULK2, the PI3K complex, Atg14L (Atg14 homologue), p150 (Vps15 homologue), Atg9 and the WIPI-Atg2 complex. In 2002, the Ohsumi group identified most of the *Atg* gene homologues in the complex plant model organism Arabidopsis, suggesting that these genes are also involved in autophagy in plant cells. In 2010, Zhang group discovered four specific autophagy genes in *C. elegans*: *epg-2*, *epg-3/VMP1*, *epg-4/EI24* and *epg-5*. Epg-2 is unique to nematodes, while the other three genes are conserved in species that span the evolutionary pathway from nematodes to mammals. These findings strongly suggest that autophagy is a highly conserved mechanism that developed in the early stage of eukaryotic evolution.

#### 1.2.1.4 Signal Regulation in Autophagy

A breakthrough in the understanding of the autophagy regulatory signalling pathway was the discovery of the target of rapamycin kinase (TOR), which regulates cell growth, the cell cycle and protein synthesis. In 1995, the Meijer group first discovered that the TOR inhibitor rapamycin can induce autophagy in rat hepatocytes and reduce the inhibitory effect of amino acids on autophagy. They also found that amino acids stimulate the phosphorylation of the ribosomal protein S6 and that rapamycin inhibits this effect, suggesting that amino acids and TOR-regulated autophagy signals are interrelated.

In 1997, the Meijer group found that in rat hepatocytes, the PI3 K inhibitors wortmannin, LY294002 and 3-MA prevented amino acid-induced S6 phosphorylation, which appeared to be similar to the effect of rapamycin. However, these PI3 K inhibitors blocked autophagy in the absence of amino acids. In 2000, Codogno discovered that PI3P, the product of type III phosphatidylinositol (PI3 K-III), is essential for autophagy, whereas the products of type I phosphatidylinositol (PI3 K-I),

phosphatidylinositol (3, 4) diphosphate (PI(3,4)P2) and phosphatidylinositol (3,4,5)-triphosphate (PI(3,4,5)P3), inhibit autophagy. Since 3-MA and wortmannin inhibit both type I and type III PI3 K, they downregulate S6 phosphorylation and inhibit autophagy. Insulin can inhibit autophagy, and it is now known that the initial step of insulin signalling involves the activation of class I PI3 K and the production PI(3,4,5)P3 and thus inhibits autophagy. In addition, insulin activates protein kinase B (PKB, also known as AKT) through inositol 3-phosphate-dependent protein kinase 1 (PDK1) to inhibit autophagy. Other TOR-dependent mediators include stress-responsive c-Jun N-terminal kinase 1 (JNK1) and death-associated protein kinase (DAPK), which activates Beclin 1 to induce autophagy. In 2011, the Guan group reported that AMPK and mTOR regulate autophagy by phosphorylating ULK1. During starvation, AMPK directly activates ULK1 by phosphorylating Ser317 and Ser777, thereby inducing autophagy; when the nutrient level is sufficient, increased mTOR activity may induce the phosphorylation of Ser757 to block ULK1 activation, disrupting the interaction between ULK1 and AMPK. This study revealed the mechanism by which AMPK/mTOR regulates autophagy via autophagy-related genes (Kim et al. 2011).

### *1.2.2 Discovery of Selective Autophagy*

In early autophagy research, it was thought that autophagy non-selectively enclosed part of the cytoplasm for lysosomal degradation. In 1973, Bolender and Weibel discovered that the smooth endoplasmic reticulum was selectively degraded by autophagy. In 1983, Veenhuis and Dunn reported for the first time the occurrence of selective pexophagy in Hansenula. In 1985, Dice et al. reported that lysosomes selectively degraded soluble protein ribonuclease (CMA) and later found other CMA substrates, such as GAPDH, annexin, IκB and aldolase B. In 1987, Mortimore et al. reported that when hepatocytes were exposed to glucagon, ribosomes were selectively degraded by autophagy. In 2004, Lemasters et al. found that abnormal mitochondria were selectively degraded by autophagy, thus introducing the concept of mitophagy. These studies in yeast and higher eukaryotes have fully demonstrated that autophagy is selective.

#### 1.2.2.1 Cytoplasm-to-Vacuole Targeting (Cvt) Pathway

Since 1992, Klionsky has studied the cytoplasm-to-vacuole targeting (Cvt) pathway, which is responsible for the transport of vesicle proteins, including alpha-aminopeptidase I and alpha-mannosidase 1. The membrane kinetics are very similar to those observed in macroautophagy. The essential gene (*Cvt* gene) for the Cvt pathway also identified the essential gene for autophagy (*Atg*), but there are also differences between the two pathways. Cvt vesicles selectively surround the Cvt complex (comprised of α-aminopeptidase 1 complex and Ty1 virus-like particles), and Cvt

vesicles (approximately 150 nm in diameter) are much smaller than autophagosomes (approximately 500 nm in diameter). The selectivity of Cvt allows it to serve as a model system for studying selective autophagy.

Autophagy-associated proteins that mediate selective autophagy are called autophagic adaptor proteins. These adaptor proteins fall into two broad categories: ubiquitin-dependent and ubiquitin-independent. Ubiquitin-dependent proteins recognize LC3 or other Atg8 analogues by ubiquitin labelling; ubiquitin-independent proteins directly enclose organelles or pathogens. Atg11 (Cvt9/Gsa9) is the first essential protein that was discovered to be responsible for the vesicular trafficking of aminopeptidase I (CVT pathway), peroxisomes (pexophagy) and autophagy (mitophagy). Atg 11 functions as a scaffold protein to recruit other Atg proteins and also as a target for selective degradation but is not required for macroautophagy. Ubiquitin-independent adaptor proteins also include Atg19 and Atg34 (selective receptor proteins for alpha-aminopeptidase 1 and alpha-mannosidase 1 in the Cvt pathway, respectively), Atg30 (yeast pexophagy) and Bnip3L/Nix (mitophagy). p62 is a ubiquitin-dependent adaptor protein. The p62 protein (sequestosome 1, SQSTM1) binds to ubiquitinated proteins. p62 also binds to LC3 to be assembled into autophagosomes and eventually is degraded in autolysosomes as a ubiquitinated protein.

#### 1.2.2.2 Xenophagy

Xenophagy is the selective autophagy of intracellular pathogens, and its existence suggests that autophagy can act as a defence mechanism against bacteria and viruses. Streptococcus A enters cells by endocytosis. In 2004, the Yoshimori group found that streptococcus A in the cytoplasm was immediately captured by autophagosomes, which were fused with other autophagosomes to form a large membranous structure. The bacteria were then digested after fusion with lysosomes. In the same year, Deretic et al. demonstrated that autophagy is involved in innate immunity against a variety of pathogens, including *Mycobacterium tuberculosis*, streptococcus A, *Listeria monocytogenes*, *Shigella flexneri*, *Salmonella enterica*, *Legionella* and *Monoucleosis*. *Mycobacteria, Streptococcus, Listeria, Shigella* and *Salmonella* are degraded by autophagosomes, while *Legionella* and *Monoucleosis* can survive in autophagosomes and host cells. Thus, autophagy is capable of selectively degrading intracellular pathogens, but some pathogens can disrupt the autophagic pathway to maintain their survival and replication. After *Salmonella typhimurium* infects cells and is released into the cytosol, the bacterial proteins are rapidly ubiquitinated and recognized by the cargo receptors SQSTM1, CALCOCO2 and OPTN. All these cargo receptors contain a ubiquitin-binding domain and an LIR motif, which mediates the interaction between ubiquitinated bacteria and LC3/GABARAP family members to form phagophores. The binding of CALCOCO2 to bacteria also depends on the recruitment of the lectin LGALS8 to vesicles containing damaged bacteria. Currently, the autophagy of intracellular pathogens is an exciting research area and promises to lead to novel strategies that can be used against infection.

#### 1.2.2.3 Mitophagy

Mitochondrial quality control is critical for maintaining mitochondria and cellular functioning. Mitophagy and the ubiquitin-proteasome system are important for mitochondrial quality control. Mitophagy selectively removes damaged and/or redundant mitochondria to maintain mitochondrial homeostasis. In yeast, the mitochondrial outer membrane protein Atg32 is a ubiquitin-independent mitophagy receptor, and Atg32 interacts with Atg8 and Atg11 to promote mitophagy. The PINK1/Parkin pathway is the most important mechanism mediating mitophagy in most mammalian cells. The Youle and Przedborski groups, in 2008, and the Springer group, in 2010, found that mitophagy in mammals is dependent on PINK1/Parkin, and that PINK1 kinase includes the E3 ubiquitin ligase Parkin to localize to damaged mitochondria. Parkin ubiquitinates one or more mitochondrial proteins, which are then recognized by p62 and recruited by LC3 to autophagosomes. In fact, p62 and NBR1, which contain the LC3 interaction region (LIR) and the ubiquitin-related domain (UBA), are capable of binding to ubiquitin. The LIR then binds to the hydrophobic pocket of Atg8 or its homologues, and this represents the most important interaction mode of selective autophagy. The LIR is also found within Nix, Atg19, Atg32 and other possible adaptor proteins. In 2015, the Youle group further elucidated the Parkin-dependent and non-dependent pathways by which PINK1 activates mitophagy. In the absence of Parkin, PINK1 phosphorylates ubiquitin and recruits the autophagy receptors NDP52 and optineurin to directly activate mitophagy independently of Parkin, but the level of mitophagy is low. In the presence of Parkin, PINK1 recruits Parkin, NDP52 and optineurin to mitochondria and Parkin amplifies ubiquitin signals produced from phosphorylation by PINK1, thus inducing rapid and powerful mitophagy (Lazarou et al. 2015). In addition, the Sandoval group (Sandoval et al. 2008) and the Chen group (Liu et al. 2012) discovered the mitophagy receptors BNIP3L/Nix and FUNDC1, respectively, in mammalian cells. Both proteins contain the LIR domain and localize to the mitochondrial outer membrane, and both are involved in hypoxia-induced mitophagy. Because hypoxia-induced mitophagy is involved in the pathogenesis of cancer and cardiovascular disease, elucidating the roles of BNIP3L and FUNDC1 may be an important strategy for the treatment of cancer or hypoxia-related diseases.

#### 1.2.2.4 ER-Phagy/Reticulophagy

ER-phagy/reticulophagy selectively degrades the endoplasmic reticulum (ER). In 2007, Bernales first coined the term reticulophagy. In *Saccharomyces cerevisiae*, ER stress activates both reticulophagy and macrophagy. The two processes can operate independently and complement each other. Importantly, the protective effects of ER stress-induced autophagy are primarily associated with reticulophagy rather than macrophagy (Schuck et al. 2014). In 2015, two consecutive papers published in Nature reported the regulation of reticulophagy. Mochida et al. (Mochida et al. 2015) identified two novel autophagy-related proteins, Atg39 and Atg40. Atg39 is

localized in the perinuclear ER (or nuclear membrane) to mediate nucleophagy; Atg40 is localized to the cytoplasmic ER, which is capable of loading ER into the autophagosome and mediating yeast reticulophagy. Khaminets et al. (Khaminets et al. 2015) found in mouse and human cells that the reticulon protein family member FAM134B/RETREG1 mediates reticulophagy. FAM134B contains an LIR domain and a reticulon domain. The membrane protein domain binds to ER fragments, while LIR interacts with LC3 to promote the degradation of the ER by autophagy. The downregulation of FAM134B leads to ER expansion, inhibits ER turnover and sensitizes the cell to stress-induced apoptosis. Autophagy in FAM134B knockout mice decreases with age. Misfolded proteins and aggregated proteins then accumulate in the ER, resulting in sensory neuropathy. Interestingly, FAM134B is a mammalian homologue of Atg40. Thus, FAM134/Atg40-mediated selective reticulophagy controls ER morphology and renewal and is an indispensable process for maintaining cell homeostasis.

### *1.2.3 Discovery of the Biological Function of Autophagy*

The function of autophagy generally includes two aspects. First, autophagy can generate degradation products and nutrients to maintain cell survival during starvation or growth-induced demand, especially in yeast and multicellular organisms in the acute phase of nutrient limitation during short-term starvation, postpartum and pre-implantation. Second, autophagy represents intracellular quality control by removing damaged macromolecules and organelles, which is important for long-lived cells and organisms. Via these two basic functions, autophagy plays multiple roles in metabolism, development, stem cells, ageing and longevity, and cell death.

#### 1.2.3.1 Metabolism

Autophagy facilitates the catabolism of nutrients and is essential for regulating nutrients and maintaining cell homeostasis. Biomacromolecules are degraded into glucose, free fatty acids and amino acids by autophagy for cell recycling. When nutrients are deficient, cells utilize autophagy to degrade intracellular glycogen to produce energy. Autophagy also promotes the degradation of intracellular proteins to generate amino acids to maintain protein synthesis. Although the amino acids produced by autophagy degradation can be utilized to produce ATP for supplemental energy, the energy balance produced by such conversion is relatively weak, and lipids are a better source of energy. Autophagy facilitates the degradation of lipids to produce free fatty acids, which are oxidized by mitochondria to produce energy after leaving the lysosomal cavity. Cells also control lipid metabolism and prevent lipid accumulation through autophagy (Singh et al. 2009). Since autophagy plays an important role in nutrient metabolism, its dysfunction leads to a series of metabolic diseases, such as diabetes, obesity, liver disease and atherosclerosis.

#### 1.2.3.2 Ageing and Longevity

A common feature of senescent cells is the progressive accumulation of damaged proteins and organelles (such as damaged mitochondria), and a decline in autophagy may be closely related to these phenomena. The Bergamini group found that autophagy in rodent and cultured hepatocytes decreased with age, while caloric restriction (the only method known to effectively delay ageing) prevented decrease in autophagy. In 2003, the Levine group conducted the first study of autophagy-related genes during ageing and longevity. In the absence of insulin signalling, Bec-1 (Beclin 1 homologue in *C. elegans*) knockdown could inhibit *daf-2*, a gene associated with a longevity phenotype. Subsequent studies in Drosophila found that *Atg7* deletion in Drosophila shortened the lifespan, while an increase in basal autophagy levels in adult Drosophila prolonged the lifespan, thus suggesting that autophagy can prolong the lifespan in model organisms. In 2018, the Levine group further confirmed the role of autophagy in promoting longevity in mammals. By constructing transgenic mice with a *Beclin 1* mutation *(Becn1F121A/F121A)*, the interaction between beclin 1 and the negative regulator BCL-2 was reduced, and basal autophagy was increased. In these mice, the lifespan was significantly prolonged. Age-related renal and cardiac pathological changes and spontaneous tumours were also significantly reduced. Conversely, mice lacking the anti-ageing protein klotho 3 showed increased interaction between beclin 1 and BCL-2, decreased autophagy and increased premature death and infertility, while the *beclin 1* (*F121A*) mutation rescued these phenotypes. These data suggest that beclin 1-mediated autophagy prevents ageing and promotes longevity in mammals (Fernandez et al. 2018).

#### 1.2.3.3 Stem Cells

Stem cells produce key cells in the body during embryogenesis and maintain tissue repair and homeostasis during the postnatal period and adulthood, so strong quality control in these cells is essential for their survival. Autophagy is the primary cell quality control pathway and plays a key role in maintaining the multiple functions of stem cells. Autophagy facilitates the quiescence of stem cells, maintains stemness and self-renewal and mediates differentiation. In addition, autophagy helps protect stem cell function by regulating cell remodelling and metabolism. In fact, the anti-ageing effects of autophagy may be related to the regulation of stem cells. During ageing, the regeneration of stem cells declines during the progression from the normal resting state to the irreversible ageing state, and autophagy is essential for maintaining the resting state and the stemness of stem cells. In 2016, the Muñoz-Cánoves group found that autophagy was impaired in physiologically aged satellite cells. The genetic loss of autophagy in young cells caused a decrease in protein homeostasis, mitochondrial dysfunction and oxidative stress, leading to a decrease in the function and number of satellite cells (senescent state). The reintroduction of autophagy restored the regenerative function of senescent satellite cells (Garcia-Prat et al. 2016).

In 2017, the Passegué group further confirmed that autophagy maintains the function of haematopoietic stem cells. The loss of autophagy in senescent haematopoietic stem cells leads to a metabolic state characterized by mitochondrial accumulation and activation, which impairs the self-renewal and regeneration potential of stem cells. However, approximately one-third of ageing haematopoietic stem cells exhibit high levels of autophagy to maintain a low metabolic level with a strong regenerative potential, which is similar to that observed in young haematopoietic stem cells, suggesting that autophagy can eliminate mitochondria to inhibit the metabolism of young and ageing haematopoietic stem cells. These studies indicate that autophagy is important for maintaining the function and regeneration of stem cells (Ho et al. 2017).

#### 1.2.3.4 Development and Cell Death

In a study of the yeast *Atg* gene, Tsukada and Ohsumi noticed that yeast autophagy mutants could not form spores upon starvation. Subsequent studies confirmed the effects of autophagy on development in different organisms. Autophagy mutants of *Dictyostelium discoideum* show multiple developmental defects. Loss of a autophagy gene in *C. elegans* disrupts larval formation. Beclin 1 knockout mice show early embryonic death, indicating that autophagy may supply nutrients during development. In 2008, Mizushima et al. found that autophagy was induced shortly after fertilization and that autophagy was necessary for oocyte to embryo conversion. These studies suggest that autophagy is essential for early embryonic development in mice but is not required for late embryonic development.

Most studies have demonstrated the role of autophagy in cell survival, but some researchers have proposed a role of autophagy in cell death. The earliest described autophagic cell death occurs during development. In the early 1960s and the 1970s, ultrastructural studies revealed that autophagic vacuoles accumulate during the early stage of the destruction of larval tissues in Drosophila. Autophagy is known as 'type II programmed cell death', while apoptosis known as type I programmed cell death. In 2004, Yu et al. and Shimizu et al. demonstrated that the activation of autophagy can lead to cell death when apoptosis is disrupted. The interaction between autophagy (self-digestion) and apoptosis (suicide) may play an important role in different stages of development and in different diseases.

Autophagic cell death is particularly important for development because some developmental programmes require large amounts of cellular clearance. In 2007, Berry et al. demonstrated that autophagy is required for the developmental degradation of salivary gland cells in Drosophila. In 2010, McPhee et al. further demonstrated that the phagocytic receptor Draper induces autophagy during the degradation of salivary glands, but Draper does not mediate starvation-induced autophagy, which is related to survival, suggesting that Draper may be a critical checkpoint between cell death-related autophagy and cell survival-related autophagy. In addition, autophagy-mediated cell death is not limited to physiologically programmed cell death during development. Since it has the capability to degrade intact organelles, autophagy may

also mediate cell death in a variety of pathological conditions, including cancer, neurodegenerative diseases, immune responses and ageing. A complete understanding of the contradictory roles of autophagy in survival and death is critical for the clinical application of autophagy to disease treatment.

### *1.2.4 Discovery of the Relationship Between Autophagy and Disease*

Due to the importance of the autophagy pathway, mutations in a variety of autophagy-related genes have been shown to be closely related to human disease.

#### 1.2.4.1 Cancer

Cancer was the first disease found to be associated with impaired autophagy. In 1999, the Levine group identified the mammalian homologue of yeast *Atg6, Beclin 1*, and demonstrated the tumour suppressive effect of Beclin 1(Liang et al. 1999). Beclin 1 is related to the anti-apoptotic protein Bcl-2 (B-cell lymphoma 2). When Bcl-2 binds to Beclin 1, it reduces the PI3 K activity of Beclin 1-associated hVps34, thereby inhibiting autophagy (Pattingre et al. 2005). In 40–75% of human breast, ovarian and prostate cancer cases, the single BECLIN 1 allele in the chromosomal locus 17q21 is deleted. Beclin 1 knockout homozygosity in mouse embryos are lethal, while the incidence of spontaneous tumours in Beclin 1 knockout heterozygotes is increased. In 2005, Komatsu et al. demonstrated that liver-specific Atg7 knockout resulted in hepatomegaly and tumour formation, and Atg4C-deficient mice exhibited similar characteristics. These studies indicate that autophagy can inhibit tumourigenesis. However, White and other researchers found that autophagy can have a cancer-promoting effect. Amaravadi et al. conducted clinical trials of the autophagy–lysosome inhibitors chloroquine and hydroxychloroquine in human cancer. In 2007, White et al. explained the apparent paradox. First, in apoptosis-deficient cells, autophagy prevents necrosis, which may exacerbate local inflammation and promote tumour growth. Second, in autophagy-deficient tumour cells, impaired mitochondria, reactive oxygen species, protein aggregates and p62 accumulation may induce DNA damage, oncogene activation and tumourigenesis. However, in a microenvironment characterized by hypoxia and minimal nutrients, autophagy is also protective against lethal factors (such as hypoxia) and promotes tumour metastasis. Therefore, in the presence of different environmental factors or steady states, autophagy can either prevent or promote tumourigenesis.

#### 1.2.4.2 Neurodegenerative Diseases

An important piece of evidence demonstrating the relationship between autophagy and human disease is the neuroprotective effect of autophagy. In 2004, the Rubinsztein lab demonstrated that autophagy is involved in the degradation of mutant Huntington protein (aggregated Htt) in Huntington's disease. Rapamycin, which inhibits TOR to induce autophagy, increases Htt degradation and improves neurodegeneration in Drosophila and mouse chorea models. Subsequent studies showed that the activation of autophagy produces beneficial effects in almost all neurodegenerative diseases mainly by eliminating cytoplasmic aggregates, such as alpha-synuclein in Parkinson's disease and tau protein in various neurodegenerative diseases. In 2006, Mizushima group established neuron-specific Atg5 or Atg7 knockout mice and found that the level of basal autophagy in the brain was significantly reduced, whereas ubiquitinated protein and p62 accumulated. A large number of neurons were lost in the brain and cerebellar cortex of Atg 7 knockout mice, while Atg5-deficient mice developed progressive motor dysfunction(Hara et al. 2006). The Youle group found that mitochondria were significantly impaired in some types of familial Parkinson's disease and that Parkin was involved in autophagy that eliminated damaged mitochondria. It was later demonstrated that two Parkinson's disease-related genes, *PARK2* (encoding Parkin) and *PARK6* (encoding PINK1), were able to selectively recognize damaged or depolarized mitochondria and mediate mitophagy. Recent studies also confirmed that in human congenital ataxia accompanied by mental retardation, homozygous missense *ATG5* mutations cause impaired autophagy. *WDR45* (encoding WIPI4) is a mammalian homologue of yeast *Atg18* or *Atg21*. Static encephalopathy of childhood with neural degeneration in adulthood (SENDA, also known as BPAN) is a rare neurodegenerative disease, and an exome study found a *WDR45* mutation that was associated with SENDA. These studies suggest that autophagy can control the renewal of soluble cytoplasmic proteins and reduce the accumulation of abnormal proteins to prevent neurodegeneration.

#### 1.2.4.3 Infection and Immunity

Xenophagy, which refers to the degradation of bacteria through autophagy, is an important defensive mechanism against microbial invasion. As early as 1984, Rikihisa et al. reported that rickettsia infection induced autophagy. In 2004, the Yoshimori and Deretic lab reported that autophagy removed intracellular streptococcus A and mycobacteria, suggesting that autophagy is an important defensive mechanism against some bacteria (Gutierrez et al. 2004). In 2008, the Kurata and Yoshimori groups found that in Drosophila the intracellular pattern recognition receptor PGRP-LE mediates autophagy-dependent host defence pathways that are involved in the recognition and transport of *Listeria monocytogenes*. In 2009, the Randow lab further demonstrated that the human autophagy receptor NDP52 (nuclear dot protein 52 kDa) recognizes ubiquitinated Salmonella and then binds to LC3 and directs the bacteria into autophagosomes. Moreover, the encapsulation of intracellular pathogens by

autophagy is not limited to bacteria and parasites, and Liang et al. demonstrated that the enhanced expression of Beclin 1 in neurons prevents viral replication and protects mice from viral encephalitis. Autophagy also regulates inflammation and affects the development and function of lymphocytes. For example, genome-wide association studies found that a single-nucleotide polymorphism in the core autophagy gene *ATG16L1* increases susceptibility to Crohn's disease. Compared with wild-type mice, Atg16l1-deficient mice showed more severe pathology in colitis.

In addition to its role in innate immunity, autophagy also promotes acquired immunity. In 2005, Munz demonstrated that autophagy is involved in the presentation of Epstein–Barr virus nuclear antigen 1 (EBNA1) by class II MHCs. In 2009, the Desjardins group demonstrated that autophagy promotes the presentation of and responses against class I MHC antigens.

### *1.2.5 Tools and Model Organisms for Autophagy Research*

As mentioned earlier, the most important advancements in autophagy research were the discovery of autophagy in yeast in the 1990s and the identification of multiple *Atg* genes. Currently, yeast is still one of the best model organisms for autophagy research.

In 2000, the Yoshimori group found that GFPLC3 is a marker of autophagosomes, and since then, fluorescence-labelled Atg8/LC3 homologs have been used to monitor the number of autophagosomes in various species. In the 21st century, transgenic animal models have promoted the development of autophagy research. In 2004, Mizushima et al. constructed GFP-LC3 transgenic mice (Mizushima et al. 2004). Since LC3-II is localized to the autophagosome membrane and therefore GFP-positive structures are equivalent to autophagosomes or autolysosomes, the number and location of autophagosomes can be evaluated in vivo by observing the green fluorescence from GFP with fluorescence microscopy. Using this mouse model, it was found that autophagy was organ-specific during fasting. Skeletal muscle and heart showed the strongest GFP-LC3 fluorescent staining. The transgenic mice were later widely used to monitor autophagy in tissues and cells during fasting, embryogenesis, and axonal atrophy. Mizushima also successfully established the first Atg knockout mouse ($Atg5^{-/-}$), which can reproduce normally, but the neonates die within 24 h, indicating that autophagy is essential for survival in mammalian development. To date, nine Atg knockout mice have been reported. Atg3, Atg7, Atg9 and Atg16L1 knockout mice have a phenotype similar to that of $Atg5^{-/-}$; $Atg4B^{-/-}$ mice, which can survive and reproduce, but show dysfunctional body balance, which may be associated with inner ear defects. $Atg4C^{-/-}$ mice show normal development, lifespan and reproduction, but $Atg4C^{-/-}$ mice have a reduced ability to cope with long-term starvation and are more likely to die of starvation than wild-type mice. ULK1 knockout mice have mild anaemia. Beclin 1, Ambra1 and FIP200 knockout mice are unable to produce homozygous offspring. Beclin 1 heterozygote mutant ($Beclin\ 1^{+/-}$) mice

show reduced autophagy and increased cell proliferation and are prone to spontaneous tumours such as lymphoma, papillary carcinoma and hepatoma.

Since 2005, Komatsu et al. conditionally knocked out Atg5 and Atg7 in the liver, brain, heart and skeletal muscles to study the role of autophagy in organ homeostasis. After 1 day of fasting in Atg7flox/flox:Mx1 (conditional liver knockout) mice, micro-autophagic vacuoles were observed, but the organelles were not enclosed, and mitochondria had accumulated. LC3 was upregulated, but its modification and degradation were impaired. The mice also showed structural distortion of the leaflet, cell swelling, hepatomegaly, alkaline phosphatase leakage and the presence of aspartate aminotransferase and alanine aminotransferase in the blood. $Atg7^{flox/flox}$; nestin-Cre (conditional brain knockout) mice showed a lower survival rate 4 weeks after birth. Motor and behavioural defects were observed at 14 days and 21 days after birth. At 56 days after birth, neurons in the central nervous system were significantly atrophied and contained aggregated ubiquitinated proteins and inclusion bodies. All mice died within 28 weeks. These studies suggest that autophagy plays an important role in maintaining liver and brain function during growth and development.

In 2010, the Zhang lab established *C. elegans* multicellular models to study the mechanisms, regulation and functions of autophagy. They studied the role of autophagy in embryonic development and screened specific autophagy genes in *C. elegans*.

## 1.3 The Impact of Autophagy Research

### *1.3.1 Publication of Autophagy Research*

Autophagy research has been conducted for more than 50 years. Autophagy was originally thought to be a normal physiological phenomenon and did not receive widespread attention. Only 200 autophagy research papers were indexed by PubMed between 1980 and 1990 (Fig. 1.2a). From 2000 to 2009, this number reached 3,482, which was a 17-fold increase. Moreover, in a less than 10-year period from 2010 to 2018, the number of papers published has far exceeded the number published during the previous 50 years and has reached more than 30,000 articles. Based on analysis of the past decade, the number of research papers on autophagy published each year has grown from 892 articles in 2008 to 3,358 articles in 2013 and 6,591 articles in 2018 (Fig. 1.2b). These data suggest that autophagy has received extensive attention from scientists in various fields around the world in the past decade and is a hot topic in the field of scientific research today.

Among the papers on the topic of autophagy, the paper so far ranked first was published in EMBO J in 2000 by Tamotsu Yoshimori and was entitled ‘LC3, a mammalian homologue of yeast Apg8p, is localized in autophagosome membranes after processing’ (Kabeya et al. 2000). By January 2019, the total number of citations was 4,258.

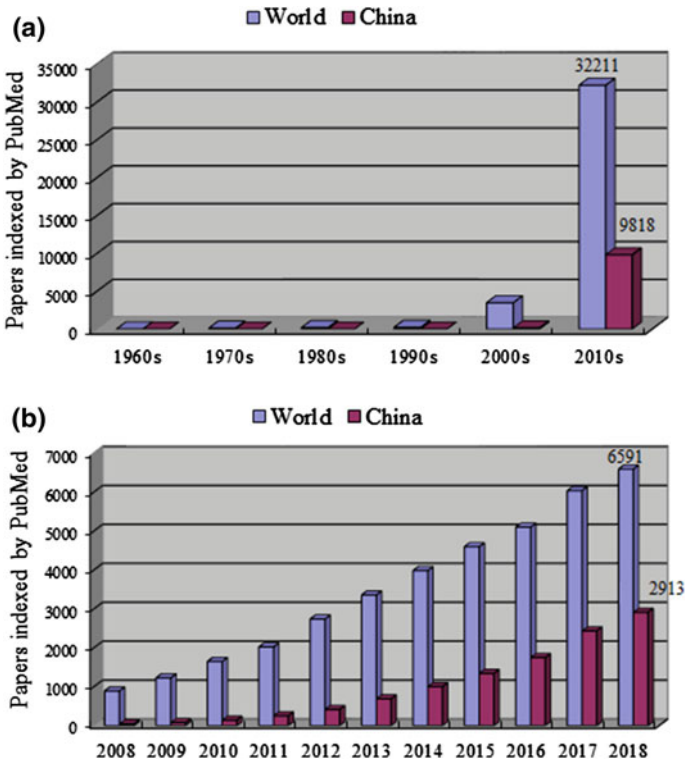


**Fig. 1.2** Number of papers on autophagy indexed by PubMed. **a** Number of papers on autophagy published every 10 years from 1960 to 2018. **b** Number of papers on autophagy published each year in the past decade. Blue: World; Red: China

### *1.3.2 Important Scientists in the Field of Autophagy*

When looking back at the 50-year history of autophagy research, it is obvious that many researchers have made outstanding contributions to the advancement of the field of autophagy (Fig. 1.1). Among them, the most famous scientists who were involved in milestones include Ohsumi, Mizushima, Yoshimori, Levine and Klionsky.

Yoshinori Ohsumi is a professor at the Tokyo Institute of Technology in Japan. His outstanding contribution to the field of autophagy was the discovery of autophagy in yeast in the 1990s. The use of this model to identify multiple autophagy genes has greatly promoted the development of the field. Thanks to these outstanding contributions, Yoshinori Ohsumi won the 2016 Nobel Prize in Medicine or Physiology.

Noboru Mizushima is currently a professor at the University of Tokyo, Japan. His laboratory not only first identified the Atg5 protein but also participated in the cloning of the autophagy marker LC3 and established Atg knockout mice. He also established GFP-LC3 transgenic mice with autophagosomes labelled with a green fluorescent protein so that they could be conveniently observed by fluorescence

microscopy. Thus, he has contributed greatly to autophagy research methodology and the understanding of the relationship between autophagy and development.

Tamotsu Yoshimori is a professor at Osaka University in Japan. His team identified LC3, a key protein involved in autophagy in mammalian cells that greatly enhanced the understanding of the mechanism of autophagy. He also found that streptococcus A, which invades host cells, could be killed by cytoplasmic autophagosomes, suggesting that autophagy participates in innate immunity. He demonstrated that autophagy participates in the elimination of misfolded proteins and prevents protein aggregation, which suggests that autophagy can prevent neurodegenerative diseases such as Alzheimer's disease.

Beth Levine is currently a professor at the Howard Hughes Medical Institute and the University of Texas Southwestern Medical Center. Her team discovered the Beclin 1 protein, which interacts with the anti-apoptotic protein Bcl-2. After studying yeast, nematodes and mice, her team found that *Beclin 1* is a homologue of the yeast gene *Atg6*. Beclin 1 deficiency in organisms inhibits autophagy, leads to tumourigenesis and increases susceptibility to bacterial and viral infection and neurodegenerative and autoimmune diseases.

Daniel J. Klionsky is a professor at the Life Science Institute at the University of Michigan. His team used *Saccharomyces cerevisiae* as a model organism to study autophagy-related genes in the cytoplasmic-vesicular transport (CVT) pathway. In 2003, he and Ohsumi et al. jointly established a universal nomenclature for the *Atg* gene. In 2005, Klionsky founded the journal 'Autophagy', which is now one of the leading journals in the biological sciences. In 2012, Klionsky co-authored publications with more than 1,200 experts in the field of autophagy research worldwide and wrote 'Guidelines for the use and interpretation of assays for monitoring autophagy', a set of guidelines for the detection of autophagy. The guidelines detailed the detection criteria, application scope and useful precautions for autophagy research, including (1) autophagy detection methods, including electron microscopy, for Atg8 (LC3), p62, mTOR, AMPK and Atg1/ULK1 and methods for detecting selective autophagy; (2) the evaluation of other methods, including the acid dye method, autophagy inhibitors and inducers and experimental models; (3) methods and challenges for the detection of autophagy in different biological model systems. These guidelines systematically summarize several classical indicators and methodologies of autophagy and has great reference value.

### *1.3.3 Autophagy Researcher was Awarded the 2016 Nobel Prize*

On 3 October 2016, the Royal Swedish Academy of Sciences presented the Nobel Prize in Medicine or Physiology to Yoshinori Ohsumi for the 'discovery of autophagy mechanisms'. As mentioned earlier, Ohsumi pioneered autophagy research in yeast,

enabling thousands of scientists worldwide to study the role of autophagy in mammalian health and disease. His research on autophagy has made a tremendous impact on the development of biology and medicine, suggesting that major discovery in basic science is essential for opening up new areas of medicine (Levine and Klionsky 2017).

## 1.4 Autophagy Journal and International Conferences

### *1.4.1 'Autophagy' Journal*

To address the rapid development of autophagy research, an important international peer-reviewed journal, Autophagy, was officially founded in April 2005. The journal quickly became an influential and important journal in the field of biological sciences. The publishing house of the journal is Landes Bioscience, and it is edited by Professor Klionsky at the University of Michigan. Many well-known scientists in the field of autophagy are the editors of the journal. Professor Hong Zhang from the Institute of Biophysics of the Chinese Academy of Sciences is the associate editor of the journal. Professor Weidong Le from Dalian Medical University, Professor Quan Chen from the Institute of Zoology of the Chinese Academy of Sciences and Professor Fucheng Lin from Zhejiang University are former or current members of the journal's editor board.

Autophagy covers the following topics: macroautophagy, microautophagy, selective organelle degradation (such as pexophagy and mitophagy) and other autophagy processes (such as chaperone-mediated autophagy); autophagy mechanisms, the structure and structural/functional relationships of autophagy proteins; regulation and signalling involved in autophagy; the role of autophagy in human health and diseases, including cancer, neurodegeneration, ageing, diabetes, myopathy and heart disease. The types of papers include original research (basic science, translational and clinical research), reviews (including comprehensive reviews and short comments), technical papers and commentaries. The goals of Autophagy are as follows: (1) to publish high-quality papers covering all aspects of autophagy research to advance the field; (2) to establish a global community of autophagy research to promote communication; (3) to provide resources to strengthen autophagy research. Autophagy has become one of the leading journals in the field of biological sciences. Its impact factor has increased year after year. The impact factor in 2018 was 11.059, and the 5-year impact factor is 11.815.

## 1.5 International Autophagy Conferences

### *1.5.1 Historical Conferences in Autophagy Research*

As mentioned above, at the Ciba Foundation Lysosome Symposium held in London in 1963, de Duve first proposed the concept of autophagy. In 1967, the first Gordon Research Conference (GRC) Lysosome Symposium was held to discuss topics such as endocytosis, lysosomal production, lysosomal disease and protein trafficking. In 1996, at the 11th session in Turku, Finland, Seglen organized the first autophagy symposium. The pioneers in the field of autophagy, including Ohsumi, Dunn, Codogno and Meijer, participated in the conference and achieved complete success. Since then, autophagy has become a new field of research.

### *1.5.2 Gordon Research Conference—Autophagy and Stress, Development and Disease*

The first 'Gordon Research Conference—Autophagy and Stress, Development and Diseases' held in 2003 brought together senior experts and new researchers in the field of autophagy worldwide. They made presentations and exchanges and promoted both yeast and mammalian autophagy research. The conference also promoted the formulation of new concepts and the development of new research directions in the field of autophagy. Subsequent GRC sessions were held in 2003, 2005, 2008, 2010, 2012, 2014 and 2016.

The most recent Gordon Research Conference, Autophagy and Stress, Development and Disease was held in Lucca, Italy, from 18 to 23 March, 2018, and was hosted by AK Simon and DC Rubinsztein. The topic of the conference was 'autophagy: basic biology, ageing and age-related diseases'. The conference focused on the novel molecular mechanisms of autophagy and its regulation in ageing and age-related diseases, with a focus on the translation of research findings into new treatments for ageing and diseases.

### *1.5.3 International Symposium on Autophagy (ISA)*

In 1997, the first International Conference on Autophagy (ISA) was held in Okazaki, Japan, and was hosted by Y Ohsumi. The meeting addressed the mechanisms of macroautophagy, microautophagy and pexophagy in yeast and mammalian cells, as well as newly discovered autophagy functions revealed in the fields of biology and pathology. The second ISA was held in Aix Lex Bains, France and was hosted by P Codogno. The third through sixth ISAs were held in Osaka (hosted by Y Uchiyama,

2002), Mishima (hosted by E Kominami, 2006), Otsu (hosted by Y Sakai, 2009) and Okinawa (hosted by T Yoshimori, 2012), Japan.

Due to the development of the field of autophagy, the scale of the ISA conference has gradually expanded and is now held approximately once every 2 years. The 8th ISA was held in Huangshan, China in March 2015 and was hosted by Professor Hong Zhang from the Institute of Biophysics of the Chinese Academy of Sciences. The 8th ISA was held in Nara, Japan from 29 May to 1 June 2017 and was hosted by N Mizushima from the University of Tokyo. More than 300 scholars from around the world participated in the conference. The theme of the conference covered a broad range of topics that addressed the basic biology of autophagy and its role in diseases.

### *1.5.4 Keystone Symposium—Autophagy*

The first Keystone Autophagy Symposium—Autophagy, Health and Diseases was held in Monterey in central California from 15 to 20 April 2007. The conference covered the emerging molecular mechanisms and concepts of autophagy and the role of autophagy in organelle degradation, physiological regulation, cell survival, cell death and disease.

Due to the increasingly far-reaching influence of autophagy, since 2013, the Keystone Symposium—Autophagy has been held every year. The most recent Keystone Symposium—'Autophagy: From Model System to Clinical Treatment' was held in Santa Fe, USA, from 17 to 21 February 2019, and was hosted by V Deretic, L Yu and LO Muphy. The goals of the conference were: (1) to compare autophagy in yeast and higher organisms with a focus on the common and dissimilar regulatory factors; (2) to explore the relationship between autophagy and energy metabolism, innate immune signalling and intimal damage; (3) to study how signalling cascades regulate selective autophagy and how substrates are recruited through receptors and molecular tags such as ubiquitin and galectin to form autophagosomes. The conference also explored how autophagy can be applied to the study of human disease and the translation of research.

### *1.5.5 European Molecular Biology Organization (EMBO) Conference—Autophagy*

The first EMBO conference on the topic 'autophagy: cell biology, physiology and pathology' was held in Ascona, Switzerland, from 18 to 21 October 2009. The organizers were M Peter of Switzerland and C Kraft and F Reggiori of the Netherlands. The topics addressed the molecular mechanisms of autophagy, selective autophagy and organelle degradation, the relationship between ubiquitination and autophagy,

autophagy signalling and membrane dynamics, autophagy regulation and physiology and the role of autophagy in health and disease.

The EMBO Conference—Autophagy is held every 2 years. The most recent EMBO Conference—Autophagy was held in Croatia from 25 to 29 September 2017, and was hosted by I Dikic, T Proikas-Ceaznne and Z Elazar. The theme of the conference was 'autophagy: from the molecule mechanism to human disease'. The conference focused on recent advances in the understanding of the molecular mechanisms of autophagy, the effects of autophagy on human disease and the potential use of autophagy as a therapeutic target.

## 1.6 History and Current Status of Autophagy Research in China

### *1.6.1 The History of Autophagy Research in China*

The earliest independent study of autophagy in China was carried out by Xueming Tang at the Shanghai Second Medical University. Starting in the 1990s, they compared basal autophagy activity in different tissues and found that the rat testis had a higher basal autophagy activity. Autophagy activity affects the secretion of steroid hormones, so Leydig cells may be an ideal cell model to study autophagy. After the 1990s, autophagy gradually became a research hot topic for scientists from all over the world. Autophagy research in China is obviously not as advanced as the current international level. However, since 2003, Chinese researchers have gradually begun to catch up. Several research groups have actively explored the signalling pathways involved in autophagy regulation, the biological function of autophagy, the relationship between autophagy and disease and the development of drugs targeting the autophagy pathway. After 2009, Chinese researchers published a number of high-level papers on autophagy in top journals, proving the strength of our scientific research in the field of autophagy to the world.

## 1.7 Regulation of Autophagy

In 2003, the Long Yu group at Fudan University cloned three homologous human *LC3* genes and found that the post-transcriptional modification site in one of them (MAP1LC3B) is Lys-122 instead of Gly-120, suggesting that different LC3 subtypes may have different regulation and function. This work was published in the Journal of Biological Chemistry and was the first high-quality article published on autophagy research in China.

The Hong Zhang group at the Beijing Institute of Life Sciences is dedicated to studying the regulation of autophagy in the multicellular organism *C. elegans*. In

2009, they published the first high-quality article on independent autophagy research in China in Cell. In the embryonic development of *C. elegans*, the components of P-particles derived from oocytes, PGL-1 and PGL-3, were selectively degraded by autophagy in somatic cells. SEPA-1 mediates this process by functioning as a receptor protein (Zhang et al. 2009). In 2010, the group identified several genes specifically involved in autophagy in multicellular organisms, including *epg-2*, *epg-3*, *epg-4*, *epg-5* and *epg-6*. Among them, epg-2 is a scaffold protein that plays an important role in the degradation of PGL particles by autophagy (Tian et al. Cell 2010). *EPG5* contains a disease-causing mutation that is involved in the systemic disease known as Vici syndrome. *EPG5* gene deficiency affects pigmentation in the retina of mice. EPG5 is an effector protein of Rab7 in late endosomes/lysosomes that can specifically mediate fusion between autophagosomes and late endosomes/lysosomes (Autophagy 2016; Mol Cell 2016; in collaboration with Professor Yingyu Chen from Peking University). EPG-3 (VMP1 is the mammalian homologue) localizes to the endoplasmic reticulum, activates the calcium channel SERCA in the ER and negatively regulates the endoplasmic reticulum–mitochondrial interaction (Mol Cell, 2017). The selective degradation of PGL-1 and PGL-3 is regulated by the arginine methylase EPG-11 (Li et al. 2013) and the concentrations of their components (Zhang et al. 2017). In 2018, the same group found that the sizes and biophysical properties of PGL particles determine the degradation efficiency of autophagy. The receptor protein SEPA-1 promotes the phase transition of PGL-1/-3, while the scaffold protein EPG-2 controls the size of PGL-1/-3 particles and promotes their conversion from a liquid to a hydrogel state. In a high-temperature environment, mTOR increases PGL-1/-3 phosphorylation and accelerates the phase transition of PGL-1/-3 particles to prevent them from being degraded by autophagy. The accumulation of PGL particles is essential for the normal embryonic development of *C. elegans* under heat stress conditions. This work revealed that during the embryonic development of *C. elegans*, mTOR acts as a receptor that senses heat stress and can control autophagic degradation due to heat stress by regulating the phase transition of PGL particles (Zhang et al. 2018). A series of research studies revealed the regulation of autophagy by EPG signals.

Professor Li Yu from the School of Life Sciences at Tsinghua University studied the relationship between autophagy and apoptosis as a member of the group of Professor Lenardo starting in 2003. He found that autophagic cell death requires Atg7 and Beclin 1, and the inhibition of caspase-8-mediated apoptosis could induce autophagic cell death. In 2010, his group found that mTOR can terminate autophagy to reconstitute lysosomes. Further research then identified spinster, which is a molecule that plays a key role in autophagosomal and lysosomal remodelling and mTOR activation. Spinster is a lysosomal efflux permease with the features of a sugar transporter, that participates in the regulation of autolysosomal remodelling via its sugar transporter activity. Mutations in the *Spinster* gene can lead to the accumulation of sugars in lysosomes, leading to lysosomal enlargement and eventually the accumulation of large autolysosomes in cells. In 2012, his group used *Saccharomyces cerevisiae* as a model organism and reported that the histone acetylase Esa1 and the deacetylase Rpd3 regulate autophagy by regulating the acetylation of the key protein Atg3. In

the same year, this group used proteomic analysis to identify the molecular mechanisms utilized by clathrin and PI(4,5)P2 in the regulation of autolysosome renewal and described the role of clathrin and its accessory proteins in membrane protein sorting, lipid conversion, autolysosomal initiation and protolysosomal production. The assembly of actin scaffolds in isolated membranes is necessary for the formation of autophagosome membranes in mammalian cells. In 2015, the same group found that actin filaments depolymerized shortly after starvation and that actin was assembled into a network within the membrane. When an inhibitor of actin polymerization was used to disrupt actin puncta or to knock down the actin capping protein CapZβ, the isolated membrane and omegasome collapsed into a mixed membrane bundle. The formation of actin puncta is PtdIns(3)P-dependent, and CapZ binds to the abundant PtdIns(3)P in the omegasome to stimulate actin polymerization, which is suggestive of the underlying mechanism of autophagosome membrane formation (Nat Cell Biol, 2015). In 2017, this team further found that the DNA damage repair protein Mec1 is specifically involved in energy deficiency-induced autophagy. Under energy-deficient conditions, Snf1 (a homologue of AMPK in yeast), the DNA damage repair protein Mec1 (a homologue of ATR in yeast) and the autophagy protein Atg1 were specifically recruited to mitochondria to regulate mitochondrial respiration and autophagy.

In 2010, Weiguo Zhu and Ying Zhao at Peking University investigated the role of FoxO1 in the induction of autophagy. Cytoplasmic FoxO1 binds to the histone deacetylase SIRT2 and remains inactive, but under stress conditions, FoxO1 dissociates from SIRT2 and is converted into activated acetylated FoxO1, which specifically binds to the autophagy protein Atg7 to activate autophagy. Animal experiments and clinical specimens also demonstrated that FoxO1-induced autophagy is one of the main causes of the antitumour effect of FoxO1. This study organically linked the epigenetic modification of histone deacetylase, autophagy and tumour suppression. In 2016, further studies by this group found that accumulated p62/SQSTM1 in autophagy-deficient cells directly binds to and inhibits histone ubiquitination, resulting in DNA damage, while p62 localized to the nucleus increases the radiosensitivity of tumour cells, thus elucidating the relationship among autophagy, epigenetic modification and DNA damage (Wang et al. 2016).

Acetylation, an important protein post-translational modification in mammalian cells, is involved in the regulation of numerous biological processes. Professor Wei Liu of Zhejiang University was dedicated to explore the regulation of acetylation modification on autophagy. In January 2015, his group described the importance of the regulation of the deacetylation of nuclear LC3 in the formation of autophagic vacuoles (Huang et al. 2015). In November 2015, his team discovered a new signalling pathway that activates the histone deacetylase Sirt1 to initiate autophagy. In August 2017, his team discovered that the acetyltransferase p300-mediated acetylation of VPS34 plays a key role in the regulation of VPS34 activity. In October 2017, his group also found that mTORC1, an important protein complex that regulates cell growth and metabolism, activates p300 by phosphorylating p300. Functional studies found that the mTORC1-p300 pathway plays an important regulatory role in autophagy

initiation and lipid production, suggesting that this pathway plays a key role in coordinating intracellular catabolism and anabolism.

### *1.7.1 Biological Function of Autophagy*

Some autophagy research groups in China have explored the biological functions of autophagy, including the complex relationship between autophagy and protein degradation, quality control, mitochondrial renewal and cell survival and death.

In 2011, Professor Qunying Lei from Fudan University demonstrated that a high concentration of glucose promotes the acetylation of lysine 305 in PKM2, a key enzyme in tumour metabolism, thereby inhibiting PKM2 activity and promoting the binding of PKM2 to the chaperone protein HSC70. Subsequently, PKM2 is degraded in lysosomes. Moreover, PKM2 acetylation can promote the accumulation of glycolysis intermediates in tumour cells, resulting in cell proliferation and tumour growth. This study revealed that the CMA degradation pathway was involved in the degradation of the key enzyme of tumour metabolism, PKM2.

Professor Quan Chen's team at the Institute of Zoology of the Chinese Academy of Sciences is dedicated to exploring the regulatory mechanisms of mitophagy and mitochondrial quality control. In 2012, they discovered a new receptor molecule, Fundc1, which mediates mitophagy in mammalian cells (Liu et al. 2012). Fundc1, localized to the outer membrane of mitochondria, interacts with LC3 through a unique conserved LIR domain to mediate hypoxia-induced mitophagy. Fundc1 phosphorylation plays a key role in the regulation of mitophagy. Normally, Fundc1 can be phosphorylated by the kinase Src. During hypoxia, the activity of the protein kinase Src is decreased, resulting in a decrease in the phosphorylation of Fundc1, thereby promoting its interaction with LC3 to induce mitophagy. Based on these studies, the group found that the activity of the Src and CK2 protein kinases was inhibited in hypoxia or impaired mitochondria, while the activity of the mitochondrial phosphatase PGAM5 was enhanced, which dephosphorylated Fundc1 and enhanced its ability to bind to LC3 to activate mitophagy (Chen et al. 2014). In 2016, his team also found that hypoxia treatment (8%) in wild-type mice for 72 h induced Fundc1-dependent mitophagy in tissues and organs. Hypoxia also induced mitophagy in platelets and decreased platelet activity. Synthetic short peptides that interfere with Fundc1-mediated mitophagy can affect mitochondrial mass in platelets and platelet activity. Moreover, Fundc1 knockout mice are insensitive to hypoxia and short peptides. Platelet mitophagy also occurs in a mouse model of ischaemia–reperfusion (I/R). Hypoxic preconditioning significantly reduced I/R-induced myocardial necrosis and improved cardiac function, thus suggesting a new mechanism that could be used for hypoxic preconditioning to reduce I/R injury. In 2018, the Chen group generated liver-conditional Fundc1 knockout mice. They induced primary hepatic carcinoma using diethylnitrosamine (DEN) and found that Fundc1 knockout in liver promoted tumourigenesis in liver. In hepatocytes, the loss of Fundc1 induced the

accumulation of damaged mitochondria in the liver. A large amount of mitochondrial DNA was released from the mitochondrial matrix into the cytoplasm to activate inflammasomes. Excessively activated inflammasomes then produced large amounts of inflammatory factors such as IL1, which stimulates phagocytes to release cytokines (TNF, IL6, etc.) to activate downstream signalling pathways such as JAK/STAT and NF-kB, which may be responsible for hyperproliferation of hepatocytes and may ultimately lead to liver cancer. These findings provide a basis for elucidating the role of mitophagy in diseases and the development of related drugs.

### *1.7.2 Relationship Between Autophagy and Disease*

The Laboratory of Ageing and Neurological Diseases, which is led by Professor Zhenghong Qin of Suzhou University, is one of the first labs that carried out research on autophagy in China. His group conducted the first National Natural Science Foundation (2003) and key projects (2009) on autophagy. He is one of the first researchers who carried out research on autophagy and the metabolism of abnormally folded proteins. He published the first review on autophagy in China that explained the molecular mechanisms and regulation of autophagy. His lab is dedicated to studying the regulation of cellular metabolism and survival by autophagy in a variety of neurodegenerative diseases, cardiovascular and cerebrovascular diseases, and cancer. The biomarkers of Huntington's disease (HD) are the formation of inclusion bodies and the presence of N-terminal mutants of Htt in the brain. His group showed that autophagy and lysosomal cathepsins play important roles in the degradation of N-terminal Htt. The inhibition of autophagy and cathepsin D aggravates the pathology of HD, and Beclin 1 is involved in the degradation of Htt. His lab also first discovered that Htt fragments can be degraded by CMA. The CMA elements HSC70 and LAMP-2A play important roles in Htt clearance. Excitotoxicity plays a role in the pathology of a variety of neurodegenerative diseases. The group found that the excitatory amino acid receptor agonist kainate and the NMDA receptor agonist quinolinic acid-induced autophagy, activated the mitochondrial apoptotic pathway and upregulated Bax/Bcl-2, TP53 and PUMA. Autophagy inhibitors and cathepsin inhibitors significantly reduced autophagy and the activation of apoptotic pathways. TP53 and DRAM1 may be the key factors involved in regulating autophagy-mediated neuronal excitotoxicity and mitochondrial dysfunction. The inhibition of TP53 and DRAM1 can inhibit excitatory neuronal injury, autophagy activation and mitochondrial dysfunction. DRAM1 affects autophagy in multiple ways. DRAM1 interacts with Bax, which enhances the acidification of lysosomes through the fusion of lysosomes with autophagosomes to eliminate autophagosomes. In 2008, the Qin group demonstrated for the first time that the autophagy pathway is involved in neuronal death induced by permanent focal ischaemia in rats. However, in 2010, the group found that ischaemic preconditioning (IPC) also activates autophagy, while the inhibition of autophagy abolishes the neuroprotection conferred by preconditioning, suggesting that autophagy may play different roles in IPC and fatal cerebral

ischaemia. The extent and time courses of autophagy activation during IPC and lethal cerebral ischaemia are different. The different roles of autophagy in IPC and lethal ischaemia are also associated with endoplasmic reticulum (ER) stress. IPC activates autophagy and upregulates molecular chaperones, thereby inhibiting excessive ER stress-mediated apoptosis in lethal ischaemia. In 2014, the group explored the relationship between autophagy and cancer. Tp53-induced glycolysis and apoptosis regulator (TIGAR) can inhibit glycolysis in lung cancer and liver cancer, leading to an increase in intracellular NADPH and a decrease in reactive oxygen species (ROS) and autophagy activity, suggesting that TIGAR inhibits apoptosis and autophagy and may thereby have a dual effect on cell death after cancer chemotherapy. In 2011, Professor Zhenghong Qin and Professor Weidong Le edited and published the first autophagy monograph in China, 'Autophagy-Biology and Disease', which was reprinted in 2015 and 2020 and has greatly promoted research on autophagy in China.

In 2009, Professor Weidong Le of Dalian Medical University reported that p53 mediates autophagy activation in a Parkinson's disease model induced by proteasome inhibitors. Autophagy activation may protect dopamine neurons. In 2011, his team used classical amyotrophic lateral sclerosis (ALS) transgenic SOD1G93A mice and found an increase in the number of autophagosomes (AVs) in the motor neurons in the anterior horn of the spinal cord in the transgenic mice. When the autophagy inducer rapamycin was used on transgenic mice, it was shown to aggravate mitochondrial damage in motor neurons, activate apoptosis and induce cell death in mice. Interestingly, rapamycin did not reduce the accumulation of intracellular SOD1 protein while activating autophagy in motor neurons that was accompanied by the accumulation of the autophagy flux protein p62, suggesting that there was an abnormality in autophagic flux in ALS transgenic mice. In 2013, based on previous work, his team found that the mTOR-independent autophagy activator trehalose can promote the fusion of autophagosomes and lysosomes, accelerate the degradation of aggregated proteins and improve mitochondrial function, thereby enhancing the survival of motor neurons and delaying the degeneration of skeletal muscle in ALS. This suggests that the autophagy agonist trehalose may be a potential therapeutic drug for ALS and other neurodegenerative diseases.

In 2017, Professor Jingyuan Fang at Renji Hospital at Shanghai Jiao Tong University first revealed that the abundance of *Fusobacterium nucleatum* was significantly increased in patients with recurrent colorectal cancer and was an important factor in the failure of chemotherapy with 5-fluorouracil and oxaliplatin. The underlying molecular mechanism was shown to involve the downregulation of the level of miRNA-18a*/4802 and the upregulation of the expression of the autophagy pathway-related protein ULK1/ATG7 through the TLR4/MYD88 pathway, leading to chemotherapy resistance in colorectal cancer cells. This study provided a new method for the clinical prediction of chemotherapy effects and the prognosis of colorectal cancer and provided important new ideas for improving therapeutic efficacy against colorectal cancer (Yu et al. 2017).

### *1.7.3 Autophagy and Drug Development*

In 2007, Junying Yuan of Harvard University and Dawei Ma of the Shanghai Institute of Organic Chemistry of the Chinese Academy of Sciences first screened autophagy drugs in China. Eight compounds that can activate autophagy were found among FDA-approved drugs. In 2011, they used high-throughput screening technology and found an effective small-molecule autophagy inhibitor, spautin-1, which can inhibit the deubiquitination of Beclin 1 by inhibiting the ubiquitin-specific peptidases USP10 and USP13. Spautin-1 then promotes the degradation of Beclin 1 and Vps34 complexes, thereby inhibiting autophagy. Moreover, Beclin 1 can also affect the deubiquitination activity of USP10 and USP13, thereby regulating p53 protein levels. This study not only provided a potential anticancer lead compound but also revealed an important molecular signalling pathway linking the tumour suppressor p53 and the autophagy-related gene *Beclin 1*.

In summary, we are pleased to find that research on the field of autophagy in China over the past decade is thriving. By December 2018, PubMed had indexed 9989 Chinese autophagy research papers. In contrast, in 2003, only two papers on autophagy published in China were indexed by PubMed (Fig. 1.2b), accounting for only 2% of the total number of international papers. In 2008, 5 years later, the number of autophagy research papers in China reached 47, accounting for 5% of the total number of international papers, and 690 papers were published in 2013, accounting for 21% of the total number of international papers. In 2018, the number was 2,913, accounting for 44.2% of the total number of international papers. These data represent an unprecedented increase in autophagy research in China. An increasing number of Chinese researchers are no longer followers in the field of autophagy research and are revealing more novel autophagy regulatory mechanisms and functions of autophagy in cell metabolism, survival and development. The relationship between autophagy and health and disease is the focus of domestic autophagy research, and Chinese researchers are pioneers in international research on autophagy topics. We look forward to Chinese researchers generating even more breakthroughs in the field of autophagy.

## 1.8 Autophagy Research Funded by the National Natural Science Foundation

In 2003, Professor Zhenghong Qin from Suzhou University acquired the first grant for research on the topic of autophagy from the National Natural Science Foundation of China (NSFC). Since 2006, the number of autophagy projects funded by the NSFC has increased each year. According to the statistics on the number of projects approved by the NSFC, 7, 27, 55, 179, 307 and 606 projects were approved in 2006, 2008, 2010, 2012, 2014 and 2017, respectively. The number of approved projects on autophagy doubled every 2 years before 2017, which demonstrates that autophagy

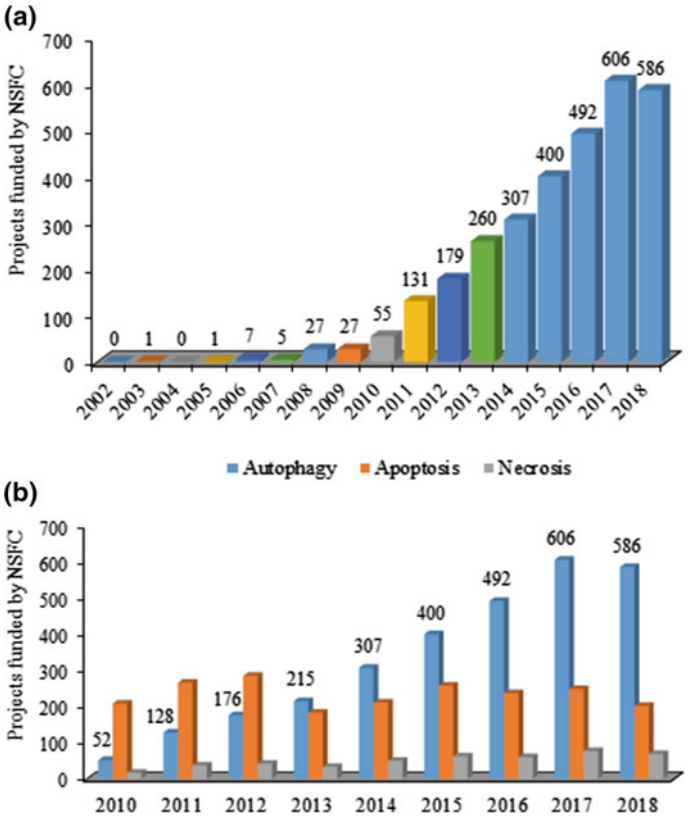


**Fig. 1.3** Number of funded projects on autophagy topic by the National Natural Science Foundation of China (NSFC). **a** The number of annual projects on autophagy topics funded by NSFC. **b** Funded projects on the topic of autophagy, apoptosis and necrosis by NSFC in 2010–2018. Blue: autophagy, orange: apoptosis, grey: necrosis

research is a hot topic in China (Fig. 1.3a). If we compare the three major cell death pathways, autophagy, apoptosis and necrosis, in terms of funding by the NSFC, it is clear that the study of autophagy is thriving and is gradually surpassing that of apoptosis and necrosis. Autophagy has become one of the most popular research fields in the life sciences, after apoptosis (Fig. 1.3b).

## 1.9 Summary

The field of autophagy research was established in 1963. Early studies found that autophagy is the main pathway for the degradation of intracellular proteins and

organelles. Amino acids and hormones regulate autophagy via the mTOR and PI3K-III pathways. The turning point in modern autophagy research was the identification of the *Atg* gene in yeast in the 1990s. Almost all yeast genes were found to have functional homologs in higher eukaryotes (including humans), particularly Atg8 (mammalian LC3), which plays a key role in the formation of autophagosome membranes. The identification of these autophagy genes and the later establishment of model organisms and transgenic animal models of autophagy have enabled researchers to accurately explore the regulatory signals and biological functions of autophagy under normal and pathological conditions. With the deepening of autophagy research, autophagy has become a hot topic in the field of life science. Researchers are discovering new connections between autophagy and other research fields at an amazing speed. Since many autophagy regulatory genes are mutated in human diseases, particularly cancer and immune and neurodegenerative diseases, there is a great potential for manipulating autophagy to prevent disease.

In the past two decades, although researchers have gained greater understanding of the molecular mechanisms of autophagy and its physiological significance, there are still many issues that need to be addressed, such as the process of autophagy, the functions of autophagy-related genes and their role in diseases and the potential of autophagy as a drug target. For example, autophagy was once considered to be suicidal pathway in which cells died by eating themselves. However, it is now recognized that autophagy has dual functions. Autophagy is primarily a cytoprotective survival mechanism. Autophagy maintains nutrient and energy homeostasis under starvation. Autophagy removes impaired proteins, dysfunctional organelles and intracellular pathogens that cause various diseases. However, the activation of autophagy may also be detrimental under some conditions. Autophagy may allow cancer cells to become resistant to chemotherapy. The overactivation of autophagy under pathological conditions may cause apoptosis or death. Therefore, a future goal is to determine the correlation between autophagy and physiological activities and pathological conditions to thereby target autophagy to treat a variety of diseases. At the same time, it is necessary to understand the regulation of autophagy and the mechanism of selective autophagy and to use more effective techniques to monitor the source, composition and dynamics of the inner membrane, lipid molecules and contents of autophagosomes. The structure and biological functions of Atg and related proteins should also be analysed. In conclusion, the study of autophagy will continue to develop rapidly and continuously. We hope that in the near future, clinicians will be able to regulate autophagy to fight disease and to maintain human health.

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# Part II
# Regulation of Autophagy

# Chapter 2
# Regulation of ATG and Autophagy Initiation


**Wen Li and Lining Zhang**

**Abstract** ATG is involved in multiple processes of autophagosome formation, including the initial phase of autophagy. The mammalian autophagy complex-ULK1 complex is composed of ULK1, FIP200, ATG13 and ATG101, and the yeast autophagy initiation complex-ATG1 complex is composed of ATG1, ATG13, ATG17, ATG29 and ATG31. After this complex is activated, it binds and phosphorylates ATG9 on the vesicles. Then PI3KC3-C1 (yeast: ATG34: ATG15: ATG6: ATG14 or mammal animal: ATG34: ATG15: BECN1: ATG14L) is recruited to the PAS. Further, ATG12-ATG5-ATG16 complex is localized on PAS (Yeast) or localized on the outer surface of the membrane (mammal) and makes binding of ATG8 (LC3) with PE to form ATG8-PE complex, promoting autophagic membrane elongation, closure and formation autophagosome and autophagosome lysosome.

**Keywords** ULK1/ATG1 complex · PI3KC3-C1 compounds · ATG8(LC3) · ATG9

## Abbreviations

| | |
|---|---|
| GABARAP | Γ-aminobutyric acid receptor-associated protein |
| GATE-16 | Golgi-associated ATPase enhancer |
| Golgi v-SNARE | Golgi vesicle-associated *N*-ethylmaleimide-sensitive factor attachment protein receptor |
| PE | Phosphatidylethanolamine |
| PI | Phosphatidylinositol phosphatidylinositol |
| PI3P | Phosphatidylinositol 3-phosphate |
| SNARE | Soluble *N*-ethylmaleimide-sensitive fusion protein attachment protein receptor |

W. Li · L. Zhang (✉)
Department of Immunology, School of Basic Medical Science, Shandong University, 44# Wenhua Xi Road, Jinan 250012, China
e-mail: zhanglining@sdu.edu.cn

W. Li
e-mail: 1227956469@qq.com



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| | |
|---|---|
| MAP1LC3 | Microtubule-associated protein 1 light-chain 3 |
| NSF | *N*-ethylmaleimide-sensitive factor |

Although autophagy-related structures were observed by electron microscopy in 1950 and autophagy is induced by glucagon or amino acid consumption in the rat liver and inhibited by insulin, the molecular mechanism of autophagy remains unclear. The discovery of autophagy-related genes (ATG) in yeast in 1990 provided a powerful genetic and molecular tool for studying autophagy. To date, more than 35 ATG genes have been identified in yeast (Wen and Klionsky 2016). The 15 core ATG genes (ATG1-10, -12, -14, -16 and -18) required for starvation-induced autophagy and selective autophagy are highly conserved in mammals.

Autophagy (usually referred to as macroautophagy) is the main mechanism by which eukaryotic cells degrade substrates larger than protein molecules, and it is also the main mechanism by which eukaryotic cells recycle cytoplasmic contents to supplement biosynthetic precursors and energy pools during periods of starvation. From yeast to mammals, the autophagy process and its mechanism are conserved. Autophagy can be selective or non-selective (Wen and Klionsky 2016). Selective autophagy removes and recycles harmful or unwanted substances from cells. These include protein aggreGATEs, damaged mitochondria, excess peroxisomes (which are harmful to cells), excess ribosomes, endoplasmic reticulum (ER) and endosomes, lipid droplets and intracellular pathogens. Uncontrolled accumulation of such substances may lead to human disease. Numerous reviews have linked autophagy to neurodegeneration, cancer, ageing, infection and other diseases. Autophagy is normally triggered by starvation and is essential to maintain the cellular supply of lipids, amino acids, carbohydrates and nucleotides. Selective and non-selective autophagy are triggered by different signals. However, these different signals converge into a pathway that initiates the membrane remodelling required to form autophagosomes.

Autophagy begins with a punctate structure in yeast, known as the proautophagosome structure (PAS, sometimes also known as the phagophore assembly site). In mammals, autophagy initiation is associated with an ER domain rich in lipid phosphatidylinositol 3-phosphate (PI(3)P) called the omegasome. Starting with a PAS or omegasome, phagocytic vesicles elonGATE into cup-shaped structures and begin to gobble up cellular material (Suzuki et al. 2007). The membranes used for phagocytic vesicular growth apparently originate from various cell reservoirs. In selective autophagy, the size of the cargo itself determines the size and shape of phagocytic vesicles. The exact mechanism is unknown, but it is clear that the actin cytoskeleton is involved. Finally, the vesicle automatically closes, and the narrow gap at the tip of the vesicle merges, resulting in complete isolation of the internal material. The outer membrane of the autophagosome then fuses with the lysosome (the vacuole in yeast or plants) to form a structure called the autolysosome. At this stage, the inner membrane and all its contents are degraded. This section will focus on the earliest steps in the autophagy process: formation of the PAS in yeast and autophagy initiation sites in mammals and the initial nucleation of phagocytic vesicles.

## 2.1 The ULK1/ATG1 Complex

### *2.1.1 Composition of the ULK1/ATG1 Complex*

The initiation of autophagy begins with activation of the ULK1 complex (ATG1 complex in yeast). In mammals, the ULK1 complex consists of ULK1 itself and the non-catalytic subunits FIP200, ATG13 and ATG101. FIP200 is a large coiled-coil protein that acts like a scaffold (a molecular weight of 200 kDa, also known as RB1CC1); ATG13 and ATG101 contain the HORMA (Hop/Rev7/Mad2) domain, which forms heterodimers. ATG13 contains a long sequence-intrinsically disordered region (IDR) after the HORMA domain, and the C-terminus of its IDR contains a motif that binds to the C-terminal EAT/tMIT domain of ULK1. Budding yeast recruits the ATG1 complex in a unique and complex way. The ATG1 subunit has the same structure as ULK1, ATG13 is conserved, but the ATG101 subunit is absent in yeast, and FIP200 is replaced by two scaffolding subunits. ATG11, which plays a role in selective autophagy, and ATG17, which plays a role in autophagic membrane expansion, further recruits two smaller subunits (ATG29 and ATG3) to participate in the assembly of the ATG1 complex.

### *2.1.2 ULK1/ATG1 Kinase Activation and Inactivation*

Autophosphorylation of the human ULK1 at Thr180 or the yeast ATG1 at Thr226 is required for activation of the ULK1/ATG1 kinase complex. The induced autophagy conditions and other subunits that are co-assembled into a complex promote their autophosphorylation. This co-assembly, in turn, increases the local concentration of ATGl molecules and promotes their mutual autophosphorylation. This is a process that occurs in both nutrient-rich conditions and selective autophagy under starvation conditions. Upon activation, ULK1 and PI3KC3-C1 can be ubiquitinated and degraded by the Cul3-KLHL20 ligase complex, thereby turning off the autophagy initiation signal (Dikic and Elazar 2018).

How does starvation trigger the Thr180 phosphorylation of ULK1? Autophosphorylation is typically promoted by dimerization of kinases or higher oligomerization. When the EAT domain of the C-terminus of ATG1 is present alone, a dimer can be formed. However, it is reported that the full-length ATG1 exists in the form of a monomer in the absence of other subunits. It is not entirely clear whether ATG1 regulates its own dimerization through its EAT domain under certain conditions. ULK1 is bridged to the scaffolding subunit FIP200 via ATG13. In yeast, ATG13 bridges with ATG11 and ATG17. Although the oligomeric state of FIP200 and ATG11 is unknown, ATG17 is a constituent dimer, and recruitment of ULK1 to FIP200 via ATG13 is the basis for ULK1 to achieve autophosphorylation.

The regulation of starvation on the formation of ATG1 complex has been studied in depth. mTORC1 (TORC1 in yeast) is a major regulator of cell growth and metabolism, and its inactivation by amino acid consumption is the main cause of autophagy. In a typical model of yeast, ATG13 is phosphorylated by TORC1 under non-starved conditions. Extensive phosphorylation results in inhibition of assembly of ATG13 with ATG1 and ATG13 with ATG17. Of the 51 phosphorylation sites within the reported yeast ATG13, 6 belong to the crystallographically defined ATG1 binding site. Mutation of all of these sites to aspartic acid reduced the binding capacity of ATG1. The effect of ATG13 phosphorylation on ATG17 binding may be that 2 of the large 51 phosphorylation sites are present at the junction of ATG13 binding to ATG17, mutations in these two phosphorylation sites significantly interfere with their binding (Itakura and Mizushima 2010). The phosphorylation regulation of the ATG17-ATG13 interaction level appears to be more variable than the interaction of ATG1-ATG13. It is widely believed that mammalian ULK1 is similar to yeast ATG1. However, the mammalian ATG13-ATG101 complex can function as an autophagy independently of the ULK1 complex.

There are other pathways that are parallel or opposite to the regulation of mTORC1/TORC1. Adenosine monophosphate (AMP) protein kinase (AMPK) upregulates autophagy by detecting an increase in cytoplasmic AMP because of energy withdrawal. AMPK directly phosphorylates multiple sites in the central IDR of ULK1, resulting in its activation. The details of how these IDR phosphorylation sites interact with the catalytic domain remain to be elucidated.

### *2.1.3 ULK1 Recruitment to the Initiation Site*

The recruitment of the ULK1 complex to the autophagy initiation site is the second regulatory step for its activation. In yeast autophagy, PAS recruitment is regulated by ATG13 phosphorylation. In yeast, ATG17 and its supporting proteins ATG29 and ATG31 are the first proteins to reach the PAS, which lays the foundation for the recruitment of ATG13 and ATG1 as described above. The EAT domain of ULK1, the locus where ATG13 binds, is essential for its recruitment to the phagocytic vesicle initiation site in human cells. This suggests that recruitment principles are similar in yeast and mammals (Cheong and Klionsky 2008).

Many other protein–protein interactions affect ULK1 localization to the phagocytic vesicle initiation site. The LC3 protein family binds the LIR/AIM motifs of ULK1 or ATG1 in human or yeast, respectively (Weidberg et al. 2010). However, many studies suggest that LC3 binding occurs downstream of ULK1 activation and that LIR/AIM motifs seem to be involved in the later stages of autophagosome biogenesis (Kabeya et al. 2004). In yeast, ATG1 is bound by a direct interaction between the HORMA domain of ATG13 and the N-terminal soluble structural domain of ATG9, and possibly, to ATG17. Bound to ATG1 is a small G protein that is a regulator of ER-Golgi and Golgi transport that helps recruit ATG1 to the PAS (Webster et al. 2016).

C9orf72 is a mutated protein in the most common genetic forms of amyotrophic lateral sclerosis (ALS) and frontotemporal dementia (FTD). Recently, it has been shown that C9orf72 is crucial for RAB1A-dependent recruitment of ULK1 to the phagocytic vesicle initiation site in human cells. C9orf72 contains a DENN domain, and in many cases, DENN domain proteins act as RAB guanine nucleotide exchange factors (GEFs). Apparently, the C9orf72 DENN binds to RAB1A but lacks GEF activity, making C9orf72 a RAB1A effector rather than a RAB1A GEF. It has been suggested that impaired recruitment of ULK1 to the autophagy initiation site in the C9orf72 mutant is the cause of its disease phenotype. Therefore, the pathways involving ATG9 and RAB1A seem to be important for the recruitment of ULK1 to the autophagy initiation site (Rao et al. 2016).

### *2.1.4 ULK1 Substrates*

ULK1 kinase transduces pro-autophagy signalling by phosphorylating many substrate proteins. The ULK1 recognition site is characterized by a preference for surrounding hydrophobic serine residues. This sequence is not uncommon. Many substrates of ULK1 include ULK1 itself and other subunits of the ULK1 complex. Other elements of the core autophagy mechanism include the PI3KC3-C1 subunit and ATG9. The regions of other proteins that connect to each other in the ULK1 complex are similar to the above regions. In the ULK1 complex, there are phosphorylation sites in ATG101 and multiple sites in FIP200 and ATG13. The ATG101 phosphorylation sites Ser11 and Ser203 are located at the beginning of the ATG101 HORMA domain, respectively, and in the flexible region just beyond the end of the HORMA domain.

The PI3KC3-C1 complex, another key autophagy initiation complex, is one of the most important and widely known targets for ULK1 phosphorylation. ULK1 phosphoric acid at Ser15 and BECN1 and other sites activates the PI3KC3-C1 complex and promotes autophagy. PI3KC3-C1 catalyses the phosphorylation of subunit ATG34 at Ser249 at the ULK1 phosphorylation site. The section on PI3KC3-C1 further discusses the consequences of these phosphorylations. The abundant PI3KC3-C1-related IDR protein AMBRA1 is another ULK1 substrate. In yeast, ATG9 is an important substrate of ATG1. ULK1 is critical for selectivity and for most autophagy. It phosphorylates the carrier protein p62 and increases its affinity for ubiquitin. ULK1 also phosphorylates FUNDC1 to promote mitochondrial autophagy. These phosphorylations, including the phosphorylation of ULK1 and the PI3KC3-C1 subunit, seem to be important in autophagy initiation, whereas in other proteins, such as ATG9, the downstream effects are more profound (Hurley and Young 2017).

## 2.2 PI3KC3-C1 Compounds

Phosphatidylinositol 3-kinase 3 (PI3KC3) produces phosphatidylinositol 3-phosphate (PI(3)P) by phosphorylating the lipid head of phosphatidylinositol. The formation of PI(3)P, an early event of autophagy initiation, is downstream of ULK1. PI3KC3 forms at least two different complexes called complex I and complex II (PI3KC3-C1 and C2). Both complexes contain catalytic subunits ATG34/ATG34, recognized protein kinases ATG15/ATG15 and BECN1/ATG6. PI3KC3-C1 contains ATG14L/ATG14, which directs the complex to the initial site of phagocytic vesicles. The PI3KC3-C1 complex promotes membrane elongation, and PI3KC3-C2 with UVRAG promotes endosomal and autophagosome maturation.

For many years, research on the PI3KC3-C1 structure progressed slowly and unsystematically. The catalytic domain and the related helical domain of ATG34, the centre-curled helical structure and the C-terminal BARA structural domain of ATG30/BECN1, as well as the WD40 structural domain of ATG15, were analysed. Recently, the complete human PI3KC3-C1 structure was analysed by electron microscopy, revealing its V-shaped structure. The X-ray crystal structures of yeast complex II showed conserved structures and domain positions, including the presence of BARA-like domains in ATG38 (yeast UVRAG).

### *2.2.1 PI3KC3-C1 Recruitment to the Preautophagosomal Structure*

To play a role in autophagy, PI3KC3-C1 needs to localize to the PAS region of yeast or the autophagy initiation site of mammals, which it achieves through its unique ATG14L/ATG14 subunit. A cysteine-rich domain near the N-terminus of ATG14L is necessary for its hunger-induced translocation to the starting site of the endoplasmic reticulum of phagocytic vesicles. The structure of this domain is unknown, as are the presumed interacting molecules at the sites of phagocytic vesicles. A C-terminal dipolar helical domain (Barkor/ATG14L autophagosome targeting sequence) known as BATS is a major class of dipolar lipid-filled receptors (ALPS) modules that is also important for complex translocation. This is primarily attributed to its ability to bond to high-curvature lipids, but it is important to note that the ALPS sequences can also bond to loosely stacked low-curvature membranes, as found in the Golgi bodies.

In addition to complex I and the autophagy specificity of PAS- and ER-targeted motifs in ATG14L, other regions of PI3KC3-C1 and -C2 do not bind to the membrane very specifically. BECN1's C-terminal BARA domain is located at the tip of the left arm, which is presumably inserted into the membrane through the aromatic fingered structure. On the other side of the V-shape is ATG34, where the K-12 helix at the end must bind to the membrane for lipid phosphorylation. On the other hand, although the C2 domain of ATG34 is speculated to bind to the membrane, the structure of complex II indicates that the C2 domain only participates in protein–protein interactions.

Finally, N-tetradecylation of ATG15 increases its contact with the membrane. The geometry of the binding of the PI3KC3-C1 and -C2 complexes to the membranes is still difficult to obtain by combining conjectural and known anchoring motifs, but this will be important to determine.

### *2.2.2 PI3KC3-C1 Regulatory Proteins*

The kinase activity of the PI3KC3-C1 complex is regulated by post-translational modifications and protein–protein interactions. The wide range of molecules interacting with the PI3KC3-C1 complex suggests that the 'two-complex' model is oversimplified. A fifth subunit, closely bound to PI3KC3-C1, is known in mammals as NRBF2 (nuclear receptor binding factor 2) and in yeast weeks as ATG38. NRBF2/ATG38 contains the mit-end triple helix (microtubule interaction and transport) domain at the N-terminus and the coiled helix domain at the C-terminus, inducing dimerization. NRBF2 can enhance its kinase activity and promote dimerization of complex I in vitro by binding to the base of complex I through interaction with the N-termini of ATG14L and BECN1. Although yeast ATG38 is also a dimer, it does not promote dimerization of the yeast PI3KC3-C1 complex. NRBF2's ability to promote mammalian PI3KC3-C1 complex dimerization and its kinase activity seems to be completely independent of each other. The mechanism of allosteric activation and the biological function of dimerization remain to be elucidated because kinase dimerization is not required for the activation of kinase activity.

Anti-apoptotic factor bcl-2 (B-cell lymphoma 2) binds to BECN1 (94) BH3 (bcl-2 homologous domain), and its binding site is close to and may even overlap with the NRBF2 domain in the complex and may even overlap. The affinity of bcl-2 was 50 times lower than that of NRBF2. Unlike NRBF2, bcl-2 is rapidly exchanged on PI3KC3-C1. Bcl-2 binding to BECN1 inhibits ATG34 kinase activity and antagonises autophagy (Ohashi et al. 2016). Since the binding site is far from the lipid kinase domain, the mechanism of this inhibition is unknown but is likely to involve tele-exchange allosteria. In addition, BECN1 can bind to other anti-apoptotic bcl-2 family members (bcl-xl, bcl-w, McL-1) through its BH3 domain. Only ER-directed bcl-2 can inhibit autophagy, while mitochondrially directed bcl-2 cannot.

AMBRA1 binds to PI3KC3-C1 in cells and promotes autophagy. The large molecular weight of this IDR protein makes it challenging to study, so the complete structure of its recombinant protein has not yet been obtained. As mentioned above, AMBRA1 is phosphorylated by ULK1 and then released by the cytoskeleton to activate PI3KC3-C1. The latest-found member of the PI3KC3-C1 interaction family is PAQR3 (progesterone and adipoQ receptor 3), a Golgi-localizable multipass transmembrane protein that has been proposed to promote autophagy by helping to assemble PI3KC3-C1. The assembly of the PI3KC3-C1 complex as a regulatory step has been studied relatively infrequently. The important genes and functions related to autophagy in mammals and yeast in Table 2.1 (Klionsky et al. 2008).

**Table 2.1** Autophagy genes implicated in autophagosome initiation

| Autophagy complex | Mammals | Function | Yeast | Function |
|---|---|---|---|---|
| ULK1/ATG1complex | ULK1 | S/T kinase | ATG1 | S/T kinase |
| | ATG13 | Regulatory subunit | ATG13 | Regulatory subunit |
| | FIP200 | Scaffold | ATG17, ATG11 | Scaffold |
| | ATG101 | Regulatory | | |
| | | | ATG29 | Budding yeast specific |
| | | | ATG31 | Budding yeast specific |
| Class III PI3-kinase complex I | ATG34 | PI kinase | ATG34 | PI kinase |
| | ATG15 | Scaffold | ATG15 | Scaffold |
| | BECN1 | Regulatory subunit | ATG6/ATG30 | Regulatory subunit |
| | ATG14L | PAS targeting | ATG14 | PAS targeting |
| | NRBF2 | Activator | ATG38 | Activator |
| ATG9 | ATG9 | Transmembrane protein localizes to small vesicles | ATG9 | Transmembrane protein localizes to small vesicles |
| RAB1 | RAB1 | Rab GTPas | Ypt1 | Rab GTPase |
| TRAPPIII | TRAPPC8 | PAS targeting | Trs85 | PAS targeting |

## 2.3 ATG12-ATG5 Ubiquitination System

ATG12 is activated by ATG7, is transferred to ATG10, and finally forms the ATG12-ATG5 complex with ATG5 (Mizushima et al. 1999). The ATG12-ATG5 complex interacts with ATG16 or ATG16L1 to form the ATG12-ATG5-ATG16L complex in the ratio of 2:2:2 (Fujioka et al. 2010). In yeast, the ATG12-ATG5-ATG16 complex is localized to the PAS but not to the intact autophagosome. Similarly, in mammals, the ATG12-ATG5-ATG16L1 complex is mainly located on the outer surface of the isolation membrane and immediately detaches from the membrane once the autophagosome forms this complex (Fujita et al. 2009). In vitro remodelling experiments have shown that ATG12-ATG5 interacts with ATG3 and significantly promotes ATG8 translocation from ATG3 to PE. Although ATG16 does not affect the acceleration effect of ATG12-ATG5 on the formation of ATG8-PE, it is very important for the formation of ATG8-PE and ATG12-ATG5. ATG16 is required to localize ATG12-ATG5 to the PAS in yeast. In mammalian cells, if ATG16L1 is artificially localized to the plasma membrane, LC3 lipidation occurs on the plasma membrane. These findings suggest that the ATG12-ATG5-ATG16(L) complex is involved in determining the location of ATG8 lipids. Further mechanistic elucidation of ATG16 function needs to be done (Fujita et al. 2009).

### *2.3.1 Ubiquitination of ATG12*

The specific binding of ATG12 and ATG8 is similar to protein ubiquitination and requires a series of enzymatic reactions. ATG7, which has E1 ubiquitination enzyme activity, can make its cysteine residue form a thioester bond with the carboxyl group of ATG12 by consuming an ATP and then transfer the thioester-containing ATG12 to ATG10, which has E2 ubiquitination enzyme activity. Finally, ATG12 can be connected to the lysine group of ATG5 to form an isomeric peptide. Analysis of the structure of ATG7 showed that it, like other E1 ubiquitination enzymes, had an adenosine acylation domain through which a homologous dimer was formed (Noda et al. 2011). In addition, ATG7 contains a unique structural domain at its N-terminus, where ATG10 and ATG3 bind in mutually exclusive ways. Combining these findings with biochemical analysis suggests that one of the dimers of ATG12 or ATG8 and ATG7 forms a thioester intermediate, which is transferred to ATG10 or ATG3 that has been bound to the same dimer (Fig. 2.1).

During ubiquitination, a lysine residue of the E2 enzyme plays a key role in the formation of a thioester bond between an asparagine (aspartic acid residue invariant) and a cysteine residue. However, a threonine residue of ATG3 corresponds to the position of asparagine, and ATG3 is highly conserved with its homologous proteins. Biochemical analysis showed that this threonine residue played a role in the transfer of ATG8 to PE, which was the same as the role of asparagine in other E2 enzymes.

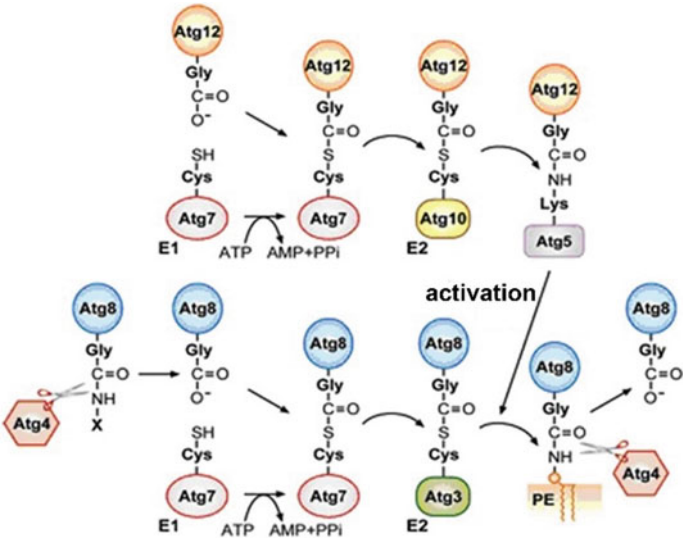


**Fig. 2.1** ATG12 and ATG8 combination system (Nakatogawa 2013). Similar to protein ubiquitination, the E1 enzyme ATG7 activates the carboxyl group of the ubiquitin-like protein ATG12 by consuming ATP to form a thioester intermediate with its cysteine residue, which is then transferred to the cysteine residue of the E2 enzyme ATG10, and finally forms a isomeric peptide with the lysine residue of ATG5. Similarly, ubiquitin-like protein ATG8 is activated by ATG7, transferred to the E2 enzyme ATG3, and finally bound to PE by amide bond

ATG3 may require these threonine residues rather than lysine residues to target the lipid PE.

However, in the ubiquitin system, the E3 enzyme determines the specificity of the substrate and the ubiquitin transfer to the substrate. In the ATG12 system, the E3 enzyme does not exist. The E2 enzyme ATG10 directly recognizes the substrate ATG5. This is reasonable considering that ATG5 is the only substrate in the ATG12 binding reaction. Similarly, in the ATG8 system, the E2 enzyme ATG3 itself recognizes the substrate PE. Under non-physiological conditions in vitro, ATG3 binds ATG8 to phosphatidylserine, and the hydrophilic head of phosphatidylserine like PE, has an amino group. Although the binding of ATG12-ATG5 enhances the E2 enzyme activity of ATG3, it does not affect the preferential selection of ATG3 substrates (Mizushima et al. 2001).

### *2.3.2 Formation of the ATG12-ATG5-ATG16 Complex*

Cells that do not produce ATG12-ATG5 and ATG8-PE complexes (such as those lacking ATG12, ATG5 or ATG10) also do not produce ATG8-PE (LC3/GABARAP-II

in mammals). This suggests a connection between the two complexes. The conjuGATE binding reaction of ATG8 in vitro can be purified by ATG8, ATG7 and ATG3 proteins or by liposomes containing PE (artificial vesicles) or ATP reconstruction. On the other hand, synchronously expressed ATG12, ATG5, ATG7 and ATG10 can form the ATG12-ATG5 complex in *E. coli*, which can be purified by this method. The addition of the ATG12-ATG5 complex into the in vitro system of ATG8 has shown that ATG12-ATG5 itself promotes the formation of ATG8-PE. Further studies showed that ATG12-ATG5 interacts with the E2 enzyme ATG3 to form a thioester bond with the catalyzed cysteine residue of ATG3 to stimulate ATG8 to transfer to PE. Therefore, the action of ATG12-ATG5 in the ATG8-conjuGATEd reaction is similar to that of the E3 enzyme in the ubiquitin system (Nakatogawa et al. 2007).

In vivo, ATG12-ATG5 forms a complex with ATG16 (ATG16L in mammals), where ATG16 forms a dimer through its C-terminus. Because the N-terminus of ATG16 interacts with ATG5, the ATG12-ATG5-ATG16 complex is a 2:2:2 heterohexamer. ATG16 is necessary to maintain the E3-like activity of ATG12-ATG5 and is essential to the localization of the complex on autophagy-related membrane structures.

### *2.3.3 Activation of ATG12-ATG5 on ATG3*

Although the basic sequences of ATG8 and ATG12 show little similarity in ubiquitin-like domains, studies on their structures showed that these proteins actually had a ubiquitin-like folding structure. In addition, it was revealed that ATG5 contains two ubiquitin-like folds. These areas reinforce the character of the ATG link system. One ubiquitin-like protein folds and binds to three other ubiquitin-like proteins as an E3 enzyme in reaction with another ubiquitin-like protein. Recent reports have successfully demonstrated how ATG12-ATG15 enhances the activation of ATG3 by E2 enzymes. ATG3 has an E2 coding region that is similar to those of other E2 enzymes. However, ATG3 is in an inactive conformation in the absence of ATG12-ATG5. The side chain-catalyzed cysteine residues of ATG3 are opposite to the threonine residues mentioned above and are directed to aspartic acid. ATG12-ATG5 causes cysteine to reorient towards threonine residues, leading to increased ATG3 activity.

### *2.3.4 The Regulation ATG12-ATG5-ATG16 Complex on ATG8-PE*

After the induction of the autophagy signal, ATG8 expression is upregulated at the transcriptional level, and the ume6-sin3-rpd3 complex combined with the promoter region of the ATG8 gene inhibits its transcription under conditions of adequate nutrition. Rim15 phosphorylates Ume6 to depolymerize its complex under conditions of

starvation, leading to the upregulation of ATG8 transcription (Bartholomew et al. 2012). The signal inducing autophagy also stimulates the formation of ATG8-PE. This is closely related to the localization of the ATG12-ATG5-ATG16 complex, which translocates from the cell membrane to the PAS region and autophagic vesicle membrane under an autophagy induction signal. It has also been reported that ATG12-ATG5-ATG16 is preferentially located on the convex membrane of autophagic vesicles in mammalian cells. ATG12-ATG5-ATG16 targets these structures (PAS, autophagic vesicle membrane) and activates ATG3 through its E3 ubiquitin-like action, leading to the production of ATG8-PE on the membrane. Since PE is a major part of the endometrium structure, ATG12-ATG5-ATG16 activation of ATG3 may be important for the production of ATG8-PE in autophagy-related membrane structures.

ATG5 and ATG16 target PAS together. In vitro analysis showed that ATG5 and ATG12-ATG5-ATG16 complexes alone (rather than ATG16 or ATG12-ATG5 alone) could bind to liposomes, indicating that ATG16 increased the membrane-binding capacity of ATG5, while ATG12 binding could inhibit this process. PI3P produced by the PI3K complex containing ATG14 is the primary condition for ATG12-ATG5-ATG16 localization. However, how ATG12-ATG5-ATG16 is positioned at the PAS and how it interacts with autophagic vesicle membranes are still unclear. Unlike ATG8, ATG12-ATG5-ATG16 is released from the membrane structure of autophagic vesicles just after their formation. The mechanism remains unclear, and PI3P may be involved in this process (Nakatogawa 2013).

## 2.4 ATG8-PE System

The synthesis of ATG8 is accompanied by an additional sequence at its C-terminus, which exposes the necessary glycine residues behind it through the action of ATG4. The binding reaction of ATG8 is catalyzed by ATG7 (ATG12 and ATG8 have a common E1 enzyme) and the specific E2 enzyme ATG3. The substrate of ATG8 is not a protein but the lipid phosphatidylethanolamine (PE). The carboxyl group of ATG8 forms an amide bond with the amino group of the hydrophilic head of PE to anchor ATG8 to the membrane (Xie et al. 2008).

By fluorescence and immune electron microscopy, whether ATG8–ll-PE in autophagy-related structures: PAS, isolation film, complete autophagosome, autophagy body (in yeast and plant cells; a vacuole and autophagosome released into the inner lining of an outer membrane-bounded vessel and vacuole) and autophagy lysosome (in mammalian cells; a fused lysosome and autophagosome). An in vitro study also provides evidence to elucidate the function of the ATG8-PE complex in autophagosome formation. In vitro, ATG8 binds to PE in the outer layer of the liposome lipid bilayer. Studies have shown that this process induces liposome semi-fusion (the fusion of the outer membrane between the two bilayer structures while the inner membrane remains independent of each other), leading to liposome aggregation. It is also confirmed that ATG8 and PE conjuGATE to form oligomers. This

evidence suggests that the ATG8-PE complexes on different membranes can interact with each other, connect the membranes, and initiate the semi-fusion between the membranes (Fig. 2.2). Most ATG8 mutants that could not form autophagosomes had significantly reduced membrane connection and semi-fusion, suggesting that the functions of these ATG8 mutants observed in vitro were similar to those observed in the process of autophagosome formation in vivo.

How is the membrane connection and semi-fusion function of ATG8-PE involved in the formation of autophagosomes? The function of cell membrane connection and semi-fusion of the ATG8 mutant was impaired, and the autophagosome formed in it was significantly smaller than that of wild-type cells, indicating that the function of ATG8-PE was important in some processes determining the size of autophagosomes, such as the process of membrane expansion. Consistent with this, the decrease in ATG8 expression level can lead to a decrease in the volume of autophagosomes. It is also possible that ATG8 could play a role at an earlier stage, that is, in the formation of hitherto unknown PAS precursors. In addition, studies have shown that abnormal forms of unclosed isolators accumulate in mammalian cells with functional defects in the binding of PE and ATG8 homologues, suggesting that in addition to the normal occurrence and development of the isolators, ATG8-PE also participates in the closure process. The specific role of ATG8-PE in the formation of autophagosomes requires further study.

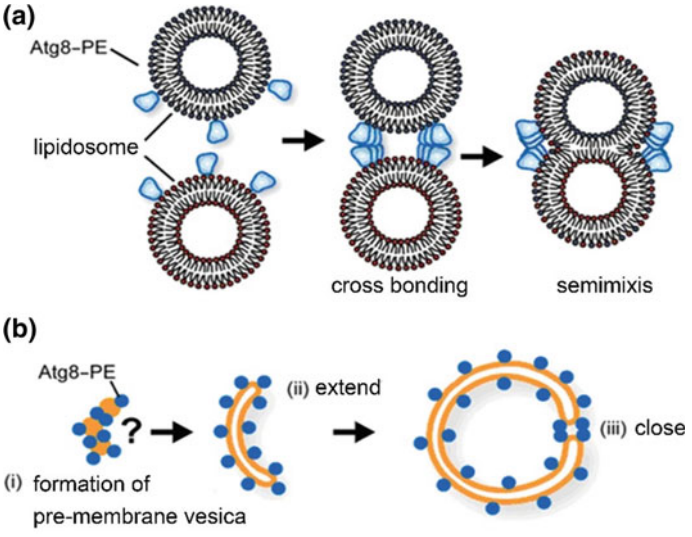


**Fig. 2.2** The role of ATG8-PE in the formation of autophagosomes (Nakatogawa 2013). **a** In vitro ATG8-PE oligomerization and initiation of liposome membrane attachment and semi-fusion. The ATG8-PE complex on the membrane of different bodies can interact with each other, connect the membranes together, and initiate the semi-fusion between the membranes. **b** ATG8-PE plays an important role in the formation (initiation, elongation and closure) of autophagosome

It should be noted that ATG8-PE-mediated semi-fusion is more sensitive to the lipid composition in liposomes than liposome aggregation is. Some studies have also confirmed that the concentration of PE in liposomes is the same as that on the membrane of the typical organelle without semi-fusion. Although this phenomenon may be because ATG8-PE does not cause semi-fusion in vivo, to clarify this point, it is necessary to detect the lipid composition of the membrane affected by ATG8-PE. In any case, semi-fusion alone cannot initiate membrane expansion. Other proteins and/or a particular lipid composition promote complete fusion in the cell. Recent studies have confirmed that in a variety of lining system-mediated membrane fusions of c2-style (soluble N-ethylmaleimide-sensitive fusion protein attachment protein receptor), proteins are necessary in the process of the formation of autophagosomes, but it is unclear whether they work together with ATG8.

Recently, on the basis of theoretical research, an intriguing isolation membrane bending model has been proposed. The membrane flexes spontaneously as it expands to a critical volume determined by three characteristics: the transverse size of the membrane, the molecular composition of the highly curved edges, and the asymmetry between the two planes. Membrane-bounded proteins such as ATG8-PE can modulate the latter two characteristics. However, the high protein concentration at the curved edge can inhibit the curvature of the isolation membrane and lead to an increase in the size of the formed autophagosome. The asymmetry of protein distribution between the two planes can promote bending, resulting in the reduction of the size of the formed autophagosome. Therefore, the density and localization of ATG8-PE on the isolation membrane can affect the volume of the autophagosome. These speculated functions of ATG8-PE are not inconsistent with the previous discussion and suggest that ATG8-PE has the ability to affect the formation of autophagosomes in a variety of ways (Slobodkin and Elazar 2013).

### *2.4.1 ATG8 Family*

Although simple eukaryotic yeast only expresses one ATG8 protein, the ATG8-homologous proteins of higher mammals form a protein family and are divided into three different subfamilies according to the homology of the amino acid sequence. These subfamilies are named after microtubule-binding protein-1 light-chain-3 (MAP1LC3), also known as LC3-aminobutyric acid receptor-associated protein (GABARAP) and Golgi-associated ATPase enhancer of 16 kDa (GATE-16). Due to the mutation and deletion of genes in the evolutionary process, the number of genes in these subfamilies in different organisms is also different. The human ATG8 protein family is composed of one GATE-16 gene, two GABARAP genes [GABARAP and GABARAPL1 (aminobutyric acid receptor-associated protein-like 1)] and four LC3 genes (LC3A, LC3B, LC3B2 and LC3C).

LC3 is the first mammalian ATG8 homologue described. Initially, light chains of microtubule-associated protein 1A and microtubule-associated protein 1B were identified in rat brain tissue. Kabeya et al. first discovered their roles in the physiological phenomenon of autophagy. They found that LC3 after completion of translation is divided into two forms: one is located in the cytoplasm as LC3-I, and the other is conjuGATEd to phosphatidylethanolamine (PE) in membrane-bounded autophagosomes as LC3-II. Their study found that the abundance of LC3-II was associated with the degree of autophagosome formation. GABARAP- and GATE-16-subfamily ATG8 homologues are considered intracellular signal transduction factors. GATE-16 can be related to the Golgi vacuole N-b maleic imide sensitive factor connection protein receptor interaction. It can also interact with N-ethylmaleimide-sensitive factor to activate ATPase (Sagiv et al. 2000). Through these interactions, GATE-16 can regulate the function of Golgi transporters in cells through the activation of NSF (N-ethylmaleimide-sensitive factor (NSF) and the activation of SNARE. In addition, studies have shown that GATE-16 is related to the reassembly of the intracellular Golgi complex after the completion of mitosis (Muller et al. 2002). GABARAP is believed to be a cytoplasmic factor that regulates the intracellular transport of the GABAA receptor 2 subunit. Subsequent research has shown that similar to LC3, GATE-16 and GABARAP must undergo translation after processing, and there are two processing forms, I and II (Leil et al. 2004).

Studies on the structure of some members of the ATG8 family have shown that the ATG8 protein has a very similar structure to ubiquitin. In addition to the five folds that make up the core of ubiquitin and are laterally linked to two helices, the ATG8 protein also contains two N-terminal helices that play an important role, possibly the regulation of protein–protein interactions.

Although the subfamilies of these proteins are structurally similar, the mammalian ATG8 protein family shows some differences in its amino acid sequence. For example, the first α-helix in LC3 is basic, whereas in gf-16 and GABARAP, it is acidic. In LC3, the surface of the second α-helix is acidic, whereas in GATE-16 it is neutral and in GABARAP it is basic. However, in mammalian ATG8 proteins, conserved regions of proteins may play an important role in maintaining the typical function of these proteins. As in the binding to mechanical proteins, different structures of various ATG8 proteins may have different specific effects on target proteins and reflect their different functions. Indeed, studies have shown that mammalian ATG8 homologues have multiple configurations with different functions and can regulate different parts of the autophagy process. In addition, the difference in ATG8 protein distribution in tissues indicates that some members of the ATG8 protein family have tissue specificity. For convenience, ‘ATG8’ will be used to refer to all members of the ATG8 family. At the same time, to distinguish the ATG8 protein of eukaryotic yeast from the mammalian ATG8 homologues, a specific name will be chosen when needed.

### 2.4.2 *Ubiquitination of ATG8*

The function of the ATG8 protein is related to its membrane binding state. It is found in cells as free cytoplasmic components (ATG8-I) tightly bound to membranes (ATG8-II). ATG8-II is located in the inner and outer layers of the autophagosome membrane. ATG8 recruitment of autophagosomes depends on a series of post-translational modifications (Fig. 2.3). Newly made ATG8 is cleaved at the C-terminus

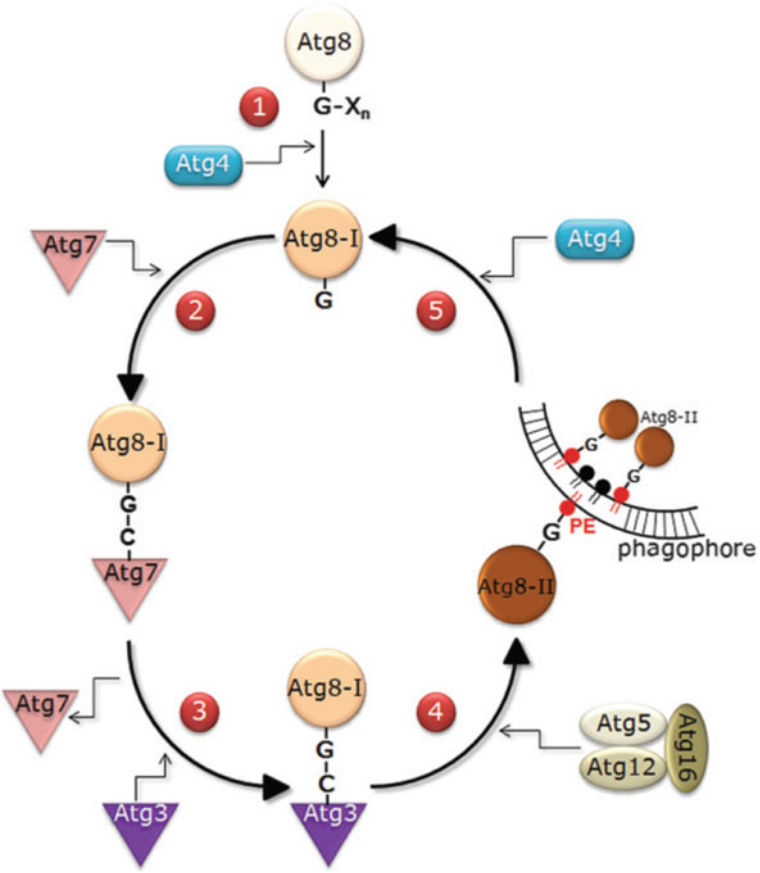


**Fig. 2.3** ATG8 processing (Slobodkin and Elazar 2013). ATG8 was hydrolyzed by ATG4 cysteine protease, leading to the formation of ATG8-I with a glycine residue hidden at the C end (1). The exposed glycine residue forms a thioester bond with the cysteine residue of the E1 enzyme ATG7 (2). E2 enzyme ATG3 binds ATG8-I by forming a thioester bond (3). Under the action of ATG5-ATG12-ATG16 complex, glycine residues at the C end of ATG8 I bind to PE through amide bond, causing the formation of ATG8-II closely related to membrane (4). ATG8—PE bound to autophagy extracorporal membranes can be cleaved and released by ATG4 to form free ATG8 (5)

by the constitutively expressed cysteine protease ATG4. ATG4 is actually homologous proteins (ATG4a-d or autophagin1 through autophagin4) that show a sequence similar to that of the ATG4 monomer in yeast (Scherz-Shouval et al. 2007). This proteolysis produces mature ATG8-I with a glycine residue exposed at the C-terminus. Subsequently, ATG8-I covalently binds to phosphatidylethanolamine through the interaction of amino amides of phosphatidylethanolamine with the glycine at the C-terminus. ATG8 combined with PE (ATG8-II) binds to the autophagosome membrane. This esterification reaction involves the regulation of ubiquitin-like ATG7, which ACTS is an E1 activase, and ATG3, which ACTS as an E2 ligase (Yu et al. 2012). Finally, ATG4 can attack and dissociate ATG8, which is attached to the autophagy extracorporeal membrane, from its lipid. This makes the combination of ATG8 and the autophagosome membrane a reversible process. The free ATG8 can cycle and participate in the binding reaction again. In contrast, ATG8-PE trapped in autophagy will be decomposed upon the fusion of lysosomes. Binding and dissociation of ATG4 are both necessary for normal autophagy (Sawa-Makarska et al. 2014).

ATG8's regulation of the interaction between ATG12 and ATG5 is the leading condition for the recruitment of ATG8 into autophagosomes. Similar to the ubiquitination system, ATG12 is first activated by ATG7 of the E1-like enzyme, then transferred to ATG10 of the E2-like enzyme, and finally, glycine residues at the C-terminus of ATG12 covalently bond to lysine residues within ATG5 through isopeptide bonds. The ATG12-ATG5 conjuGATE binds ATG16, which can form homologous oligomers by forming coiled spirochetes, allowing ATG16 to cross-link multiple ATG12-ATG5 complexes to form a single protein complex. The location of the ATG12-ATG5-ATG16 complex indicates the lipid site of ATG8 and can also promote the lipid reaction by promoting ATG8 transport from ATG3 to PE.

ATG8 recruitment to autophagosomes is mediated by additional post-translational modifications. LC3 was found to be phosphorylated by PKA. It was further found that LC3 in neurons was dephosphorylated after autophagy was induced. The binding of dephosphorylated LC3 to the membrane was enhanced, but the formation of LC3-positive autophagosomes was inhibited by the expression of a pseudophosphorylated LC3 mutant. Finally, acetylation of ATG8 and other ATG proteins by acetyltransferase p300 may negatively regulate their activity.

### *2.4.3 ATG4 Degrades ATG8*

As mentioned above, ATG4 cuts the C-terminus of ATG8, leaving exposed a glycine residue, which is important for binding to other substances. In addition, ATG4 also acts as a dissociative enzyme, blocking the binding of ATG8 to the amide of PE, thereby dissociating the protein from the membrane. This reaction is very important for the reuse of ATG8, which plays a role in autophagosomes, and ATG8 can be found on mature autophagosomes and autophagolysosomes (ATG8-PE can be transferred to the lysosome membrane or vacuole membrane by fusion with the autophagosome

extracorporal membrane) (Sawa-Makarska et al. 2014). Moreover, ATG4's shearing of ATG8 can also occur on autophagic vesicles, which can positively or negatively affect the formation of a membrane. On the other hand, the non-functional ATG8-PE is protected from degradation by ATG-4. The existence of this mechanism needs to be further explored.

In conclusion, the local production of ATG8-PE in autophagy-related membrane structures should be realized by the localization of the ATG12-ATG5-ATG16 complex on these membranes. However, recent studies have shown that this mechanism is not absolute: binding enzymes in the cytoplasm largely incorrectly produce ATG8-PE in various intracellular membrane structures. Therefore, a new role of ATG4 has been proposed: ATG4 eliminates those useless ATG8-PE, thus providing a cytoplasmic, unbound ATG8 pool, which is necessary for the formation of ATG8-PE in the correct position.

## *2.4.4 Functions of ATG8 Family*

### 2.4.4.1 Role of ATG8 in Autophagy Body Formation

In yeast, it was initially found that the formation of autophagosomes was seriously impaired in the mutant with ATG8 deletion, suggesting that ATG8 plays an important role in the development of autophagosomes. Later, the important role of ATG8 protein in autophagy regulation was found in both yeast and mammals. Starvation induced the expression of ATG8 in yeast. The number of ATG8 lipids on autophagosomes increased significantly after autophagy was induced, which was significantly related to the formation of autophagosomes. The level of ATG8 determined the level of autophagy and could control the size of autophagosomes (Ichimura et al. 2004).

Research into the role of ATG8 in mammals was initially hampered by the presence of multiple homologous proteins. Overexpression of catalytic ATG4B in mammalian cells was found to inhibit autophagy by sequestering non-lipid ATG8 homologues. In these cells, the accumulation of overexpressed mutant ATG4B and unclosed autophagosome precursors suggests that the lipidation of ATG8 homologous proteins is necessary for the formation of autophagosomes in mammalian cells (Fujita et al. 2008). Although LC3 mediates the elongation of phagocytic vesicles, the GABARAP and GATE-16 subfamilies may be involved in some of the downstream responses during the maturation of autophagosomes (Shpilka et al. 2011).

Ohsumi and his colleagues established an ATG8-PE-conjuGATEd system using liposomes in vitro. They found that aliphatic ATG8 mediated the localization and semi-fusion of liposomes. It was further found that deletion mutation of ATG8 in yeast lead to impaired membrane localization and semi-fusion activity, which further affect the formation of autophagosomes. These phenomena indicate that ATG8 plays a role in membrane remodelling and can promote the growth and maturation of the autophagosome structure. Subsequently, it was found that two ATG8 homologs in mammals, LC3 and GATE-16, can promote localization and membrane fusion. These

activities are mediated by positively charged amino acids in the N-terminal helix of LC3 and hydrophobic amino acids of GATE-16, respectively (Sou et al. 2008).

In addition to the key role of ATG8-PE in autophagy, ATG4-mediated ATG8 lipid removal, namely, the release of ATG8 autophagosome membrane, is a necessary and critical step for autophagy to proceed. If ATG8 lipid removal is blocked, it will be unable to localize on the vacuolar membrane, and the number and size of autophagosomes will be reduced, which has an inhibitory effect on the occurrence of autophagosomes. The possible explanation for this is that the combination of ATG8 and PE occurs not only in the structure of autophagic small body precursors but also at other sites in the cell. The release of ATG8-PE from these sites is critical for the increasing demand for ATG8 in the process of autophagy induction. In addition, the dissociation of ATG8-PE is also necessary in the maturation of the autophagosome and its fusion with the vacuole.

It has been reported that the lir-dependent interaction between ATG8 and autophagy core proteins plays a regulatory role. The interaction of ATG8 with the complex of the essential autophagy regulator ATG1 (in yeast) and ULK1 (unequal 51-like kinase 1), which is directly mediated by LIR, contributes to the formation of the autophagosome. Similarly, ATG8 was found to interact with a few GAPS of the Rab protein (a GTP-activated protein), all of which included TBC (Tre2, Bub2 and Cdc16) regions. The Rab small GTPase is an evolutionarily conserved membrane transport protein; some members of this family are involved in regulating autophagy-related sharing and fusion. In addition to its role in identifying degradation targets, ATG8 can be used as a scaffold protein to promote the aggregation of key complexes on the surface of autophagy.

Autophagy has long been considered a non-selective autodegradation process. However, recent experimental data have presented conclusive evidence of another pattern of autophagy, the process of selective inclusion by lysosomal degradation. Some substrates that can be specifically eliminated by autophagy include protein inclusions caused by aggreGATE or misfolded proteins, damaged organelles (such as peroxisomes, mitochondria, redundant endoplasmic reticulum (ER), ribosomes), bacteria and viruses.

In addition to its role in the biological origin of autophagosomes, the ATG8 protein is also a central factor mediating the selective classification of cellular inclusions into autophagosomes. This process is largely completed by the interaction of autophagy receptors, which connect autophagy substrates to autophagy-related ATG8. Autophagy receptors are mainly degraded by autophagy. The inclusion body classification directly mediated by ATG8 was first elucidated in the autophagy-related CVT pathway in yeast. ATG8 was found to interact with ATG19, a receptor for CVT inclusions that cavitates their target. In studies on the role of LC3 in removing ubiquitin molecules in mammals, the classification function of LC3 inclusions has also been revealed. In addition to its basic role in proteasome-degrading proteins, ubiquitin can also serve as a selective degradation signal, namely, lysosomal targeting of various types of autophagy substrates, including protein polymers, membrane-bounded organelles and microorganisms. The removal of this ubiquitination substrate depends

on a group of autophagic receptors containing the ubiquitin-binding domain (UBD), which can bind to ATG8 proteins simultaneously.

In mammals, autophagy receptors include p62/SQSTM1 and Brca1 (Nbr1) neighbours (autophagy involved in ubiquitin-mediated protein degradation). The interaction of autophagy receptors with ATG8 homologues is mediated by a short linear molecule called LIR (LC3 interaction region), consistent with the W/F/y-x-x-l/I/V structure surrounded by at least one acidic residue.

#### 2.4.4.2 LC3 (ATG8) Is Used as a Tool to Monitor the Autophagy Process

ATG8 has a specific correlation with precocious intact autophagosomes, and since the binding ATG8 is related to the number of autophagosomes, these proteins are widely used as specific markers for monitoring autophagosomes and autophagosome activities. A variety of experimental methods have been used to detect and quantify ATG8 proteins in yeast and mammals, including Western blotting, fluorescence microscopy, and flow cytometry.

Autophagy starts with the conversion of soluble ATG8-I to PE membrane-bounded ATG8—increasing II transformation. ATG8-II accumulation is a sign of autophagy (Kabeya et al. 2000). Fat-soluble ATG8 can be distinguished from the fat-soluble form as a result of the fat-soluble ATG8 electrophoretic moving speed, although its molecular weight is larger. However, when electrophoresis is run at a lower level, due to the ATG8-II (related to the inner autophagy body membrane) fusion with the lysosome bubble interface and degradation, lower ATG8-II levels tend to occur, and autophagy is reduced. Therefore, to accurately measure the activity of autophagy, the level of ATG8 should be the determining factor for the presence or absence of lysosomal degradation inhibitors. In yeast, the difference between fat-soluble and non-fat-soluble forms of ATG8 is more complex due to their nearly identical SDS-PAGE motility. The most commonly used experiment to detect autophagy in the body is the GFP-ATG8 treatment experiment. This experimentally dependent application of heterotopic N-terminally GFP-labelled ATG8 (not C-terminally labelled because this is hydrolyzed by ATG4). With the induction of autophagy, GFP-ATG8 that is bound to the endothelial autophagosome membrane is released into the cytoplasm. Although ATG8 can be rapidly degraded by proteases in the cytoplasm, relatively stable GFP remains intact and leads to the accumulation of free GFP. The accumulation of free GFP indicates an increase in autophagic flow.

By fluorescence microscopy, GFP-labelled ATG8/LC3 can also be used as a functional indicator for autophagy detection in cultured cells and transgenic organisms. Using specific antibodies, endogenous ATG8 proteins can be detected in immunocytochemistry or immunohistochemistry. Since it is endogenous, transfection is not required, avoiding the false appearance caused by human factors. ATG8-related

autophagic cell membranes and autophagosomes look like bright, colloidal, dispersed tear drops. It should be noted that the accumulation of ATG8-positive spots in cells does not completely represent the induction of autophagy but may reflect the autophagy inhibition caused by blocking the flow of autophagosomes (in the process of lysosomal degradation, the inhibition of the lysosomal pathway leads to the reduction of degradation in the ATG8 autophagosome). This possibility can be identified with lysosomal degradation inhibitors. In the presence of inhibitors, the increase in the abundance of ATG8 can reflect active autophagy, while no change in ATG8 level after lysosomal inhibitor treatment may indicate the blocking of autophagy flow. In addition, fluorescence can be used to analyse both autophagy induction and autophagy flow without drug treatment. However, the GFP signal is sensitive to lysosomal acidity or proteolytic conditions, and the RFP is more stable. Thus, prior to fusion with lysosomes, the yellow signal (from localization of GFP and fluorescence of RFP) overlaps with the autophagosome structure. Autophagosomes that have been bound to lysosomes show red spots from RFP without GFP signalling.

Flow cytometry can be used to analyse autophagy in living mammalian cells. The induction of autophagy leads to a decrease in lysosomal activity dependence in all cell signals of fluorescence-labelled ATG8. This simple method requires accurate automated analysis of a large number of cells to obtain the data.

Note: although the ATG8 family is routinely used to study autophagy, approaches based on these proteins are different. The number and processing of ATG8 are organized and cell dependent. In addition, ATG8 may be involved in the process of non-autophagic cells. Therefore, it is recommended to use other methods, such as electron microscopy (EM), and a more direct method to detect autophagy flow by monitoring the degradation of the autophagy matrix.

## 2.5 ATG9 and Formation of Autophagosomes

Among the ATG core proteins, ATG9 is the only transmembrane protein. Although most downstream ATG protein aggreGATEs require ATG9, its function remains a mystery. In yeast, ATG9 is concentrated in microvesicles with a diameter of 30–60 nm (ATG9 vesicles), which are localized in the PAS and cytoplasm. The localization of ATG9 on PAS requires Golgi vesicles. Yeast ATG9 also exists in isolated membrane structures and autophagosomes. In mammals, part of ATG9 is located on the tubular network structure and endosome on the opposite side of the Golgi body. When directly observed by fluorescence microscopy, starvation-induced ATG9 vesicles are dissociated from the tubular network structure on the opposite side of the Golgi body and co-localized with LC3-labelled autophagosomes for a short time. Mammalian ATG9 exists in vesicles and tubular structures similar to yeast ATG9. Since ATG9 is located on membranous vesicles, these vesicles may provide membrane structures for the biogenesis of autophagosomes (Lamb et al. 2016).

In mammals, the co-localization of ATG9 and LC3 was observed by fluorescence microscopy. However, immune electron microscopy could not detect ATG9 in autophagosomes. Mammalian ATG9 cannot stabilize and enter into independent membrane structures and autophagosomes, although it can interact with these structures briefly. In contrast, yeast ATG9 can be detected on isolated membrane structures and autophagosomes, suggesting that ATG9 vesicles can be incorporated into these membranes. Interestingly, only a fraction of ATG9 vesicles are recruited during the formation of autophagosomes in yeast. Since only a small portion of ATG9 vesicles (30–60 nm diameter) provide lipids for autophagosomes (300–900 nm diameter), it is likely that other membrane sources exist. However, the fusion of ATG9 vesicles may provide a membranous platform for the formation of isolated membrane structures. In another study, immune electron microscopy detected ATG9 in a cluster of vesicles and tubules, suggesting that the formation of independent membrane structures began with the fusion of these clustered vesicles or tubules. The ATG9 vesicular fusion mechanism may include the yeast Rab protein Ypt1 and the GEF complex (TRAPPIII complex), which is also required by autophagy and identified in purified ATG9 vesicles. In addition, the SNARE proteins required for the formation of autophagosomes in yeast are associated with ATG9 vesicular fusion (Sogawa et al. 2018).

Recent structural analyses of yeast ATG17 have provided insights into the mechanisms of ATG9 vesicular fusion. ATG17 forms a crescent-shaped structure with a curvature of approximately 10 nm. Two ATG17 molecules combine at the C-terminus to form an S-shaped homologous dimer (Hurley and Young 2017). Since the radius of the ATG17 crescent is similar to that of the ATG9 vesicles, the authors suspected that the ATG17 homologous dimer acts as a scaffold for two ATG9 vesicles and promotes their fusion. In this study, however, the ATG17-ATG31-ATG29 complex was bound to a liposome. It should be noted that the vesicles used in these experiments were synthetic liposomes and did not contain ATG9. In view of the previously reported interaction of ATG1-dependent ATG9-ATG17, the authors suggested that other molecules on ATG9 or ATG9 vesicles may contribute to ATG17 binding to ATG9. In addition, they found that yeast ATG1 has a membrane-bounded domain at the C-terminal, similar to mammalian ULK1, which is directly homologous. They named this binding domain the early autophagy target region. The vesicular binding capacity of this domain may further facilitate ATG9 vesicular fusion. However, much of this is hypothetical and should be further tested. The important mammalian and yeast autophagy-related genes and functions are summarized in Table 2.2.

**Table 2.2** The important mammalian and yeast autophagy-related genes and functions

| Mammal autophagy-related genes | Yeast autophagy-related genes | The function of gene products |
|---|---|---|
| ATG3 | Aut1/ATG3 | Autophagosome formation mediates LC3 modification and ATG5-ATG12 binding |
| ATG4/autophagins | Aut2/ATG4 | Autophagosomes are formed by shearing the glycine exposed at the c-terminal to assist the modification of LC3 |
| ATG5 | ATG5 | Autophagosome formation, located on the membrane forming a new autophagosome, forms a complex with ATG12 |
| *BECN1* | ATG30/ATG6 | Autophagy induces or autophagosome formation, which forms a complex with type III PI3K kinase ATG34 |
| ATG7 | ATG7 | Autophagosome formation mediates the binding of ATG5-ATG12 and the modification of LC3 |
| MAP-LC3 | Aut7/ATG8 | Autophagosomes are formed and located on the septa of autophagosomes |
| ATG10 | ATG10 | Autophagosome formation mediates the binding of ATG5-ATG12 and promotes the modification of LC3 |
| ATG12 | ATG12 | Autophagosome formation, located on the membrane forming a new autophagosome, and ATG5-shaped A compound |
| ATG16L | ATG16 | Autophagosome forms and connects with ATG5-ATG12 to form a polymer |

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# Chapter 3
# Regulation of Autophagy by mTOR Signaling Pathway


**Ying Wang and Hongbing Zhang**

**Abstract** Autophagy plays a crucial role in maintaining cellular homeostasis, and is closely related to the occurrence of variety of human diseases. It is known that autophagy occurs in response to various environmental stresses such as nutrient deficiency, growth factor deficiency, and hypoxia. Induced autophagy eliminates the damage caused by these stresses and returns to normal levels when the stresses are relieved. To comprehend the induction of autophagy under various stress conditions and the effects of autophagy on the life processes of cells, it is necessary to understand how autophagy is regulated. Many studies have shown that a number of signal transduction pathways are involved in the regulation of autophagy. Among these pathways, some pathways converge at the target of rapamycin (TOR), a highly conserved kinase important for autophagy regulation. This review will focus on the role of TOR signaling pathway in the regulation of autophagy.

**Keywords** TOR · Autophagy · Regulation

## Abbreviation

| | |
|---|---|
| AKT | v-akt murine thymoma viral oncogene homolog |
| AMBRA1 | Autophagy/beclin-1 regulator 1 |
| AMPK | AMP-activated protein kinase |
| Arf1 | Adenosine diphosphate ribosylation factor 1 |
| ATG | Autophagy-related genes |
| DAP1 | Death-associated protein 1 |
| FKBP12 | FK506-binding protein, 12 kDa molecular weight |

Y. Wang · H. Zhang (✉)
Department of Physiology, Chinese Academy of Medical Sciences and Peking Union Medical College, Institute of Basic Medical Sciences, Beijing, China
e-mail: hbzhang@ibms.pumc.edu.cn

Y. Wang
Department of Molecular Orthopedics, Beijing Research Institute of Traumatology and Orthopaedics, Beijing, China



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| | |
|---|---|
| FRB | FKBP12-Rapamycin Binding |
| GEF | Guanine exchange factor |
| HAT | Histone acetyltransferase |
| IGF1 | Insulin-like growth factor1 |
| IRS | Insulin receptor substrates |
| MITF | Microphthalmia-associated transcription factor |
| mTOR | Mammalian target of rapamycin, which has been renamed as mechanistic target of rapamycin |
| PDK1 | 3-Phosphoinositide-dependent protein kinase 1 |
| PDK2 | 3-Phosphoinositide-dependent protein kinase 2 |
| PI3K CI | Phosphatidylinositol 3-kinase ClassI |
| PI3K C III | Phosphatidylinositol 3-kinase Class III |
| PKK | Phosphoinositide 3-kinase-related kinase |
| RB1CC1 | RB1-inducible coiled-coil protein 1 |
| REDD1 | Regulated in development and DNA damage 1 |
| TFEB | Transcription factor EB |
| TFE3 | Transcription factor E3 |
| TOR | Target of rapamycin |
| TORC1 | Target of rapamycin complex 1 |
| TORC2 | Target of rapamycin complex 2 |

## 3.1 Overview of mTOR Signal Transduction Pathway

### *3.1.1 mTORC1 and mTORC2*

TOR is a highly conserved serine/threonine-protein kinase that belongs to the phosphoinositide 3 kinase-related kinase (PKK) family. It functions as a central regulatory protein that integrates signals originating from intracellular and extracellular changes in nutrient content, energy level, growth factors and so on. TOR regulates cell growth, proliferation, and protein synthesis by activating its downstream effectors.

TOR was initially identified as the target of rapamycin, which is a macrolide antifungal agent with structural similarity to FK506. TOR is involved in the regulation of cellular autophagy. Even under nutrient-rich conditions, rapamycin can induce autophagy. TOR exists not only in yeast, but also in multicellular animals, such as flies (dTOR) and mammals (mammalian target of rapamycin, mTOR, which has been renamed as mechanistic target of rapamycin in recent years). In yeast, TOR is encoded by two genes, *TOR1* and *TOR2*, which share 80% overall amino acid similarity. In contrast to yeast, other multicellular eukaryotes have only one single *TOR* gene. In eukaryotes, TOR is functionally homologous to yeast TOR2. In multicellular eukaryotes, TOR can interact with other regulatory proteins to form at least two distinct complexes, target of rapamycin complex 1 (TORC1) and target of rapamycin

complex 2 (TORC2), respectively. Although these two complexes contain the same catalytic subunit-TOR, they phosphorylate different downstream targets and thus show different cellular functions.

#### 3.1.1.1 mTORC1

In mammalian cells, TORC1 plays a key role in regulating autophagy, ribosomal biosynthesis, and protein synthesis. mTORCl is a multiprotein complex composed of mTOR, RAPTOR, MLST8 (or GPL), PRAS40, and DEPTOR. The amino terminus of mTOR contains multiple tandem HEAT repeats. Each HEAT motif consists of about 40 amino acid residues that form into a pair of antiparallel α-helix structurally. This repeat sequence forms a superhelix structure, mediates protein–protein interaction and plays an important role in the localization of mTOR in cells. In the middle of the carboxyl terminal of mTOR is the catalytic domain. The region immediately upstream of the catalytic domain is the 12 kDa FK506-binding protein (FK506-binding protein, 12 kDa molecular weight, FKBP12)-rapamycin binding (FRB, FKBP12-rapamycin binding) domain, which is bound by the FKBP12-rapamycin complex to inhibit the activity of mTOR. On the amino side of FRB is the FAT domain containing about 500 amino acid residues, which may interact with the FATC domain at the carboxyl terminus of mTOR to form into a spatial structure, thereby regulating the activity of the catalytic domain of mTOR. FATC domain is essential to maintain the kinase activity of mTOR. Mutation or deletion of any amino acid residue in this domain may ablate mTOR kinase activity. Between the catalytic domain of kinase and FATC domain is the negative regulatory domain (NRD) of mTOR. Exclusion of the NRD domain can enhance the kinase activity of mTOR. RAPTOR has a highly conserved molecular structure with a molecular weight of 150 kDa. There are three HEAT repeats and seven WD-40 repeats at its amino- and carboxyl terminuses, respectively. RAPTOR seems to interact with mTOR through multiple contact sites. It also contains multiple docking sites for mTOR downstream effectors, 4E-BP1 and S6K1. RAPTOR functions as a "bridge" molecule to link the kinase domain of mTOR with the downstream substrates that control regulation of the metabolic processes such as protein translation and ribosome synthesis. The 36 kDa MLST8 is structurally highly conservative, which contains seven WD-40 repeats (GPL). It binds specifically to the catalytic domain of mTOR kinase and stabilizes the complex composed of mTOR and RAPTOR. PRAS40 is a proline-rich AKT substrate protein with a molecular weight of 40 kDa, which can inhibit mTOR kinase activity. DEPTOR is also an important component of mTORCl, which interacts with mTOR through its PDZ domain, thereby inhibiting mTOR kinase activity.

#### 3.1.1.2 mTORC2

In mammalian cells, TORC2 is known to consist of mTOR, RICTOR, SINl, MLST8, and DEPTOR. Deletion of each subunit is sufficient to impair the kinase activity of

mTORC2. Previous studies have reported that mTORC2 is insensitive to rapamycin, but in recent years, long-term treatment of cells with rapamycin can also indirectly inhibit the function of mTORC2. mTORC2 functionally acts as phosphorylated 3-Phosphoinositide-dependent protein kinase 2 (PDK2) that phosphorylates AKT1 at Ser473 site. RICTOR protein consists of 1,708 amino acid residues with a molecular weight of about 200 kDa. The mTOR-RICTOR complex is involved in regulating the structure of cytoskeleton proteins. It can phosphorylate a series of amino acid residues in AKT1 and activate AKT1 in coordination with PDK1. SIN1, also known as MIP1, is a key subunit of mTORC2, which maintains the integrity of the mTOR-RICTOR complex and regulates the phosphorylation of AKT1 at Ser473 site.

### *3.1.2 Upstream Signals that Regulate the Activity of mTORC1*

At present, little is known about the upstream regulating signals of mTORC2, so we will mainly introduce the upstream regulatory architecture of mTORC1 here.

#### 3.1.2.1 Intracellular and Extracellular Stimulus Signals

Amino acids, especially leucine, glutamine, and arginine, are essential signals for the activation of mTORC1, as when amino acids are deficient, growth factors or other stimuli cannot activate mTORC1 effectively. Growth factors, such as insulin or insulin-like growth factor 1 (IGF1), play an important role in promoting cell survival, growth, and metabolism. In the past decades, a series of studies have demonstrated the importance of mTORC1 signaling pathway in regulating growth factor-mediated cell effects. In addition, the activity of mTORC1 is closely related to the energy status of cells. Stress signals, for example, hypoxia, can also regulate the activity of mTORC1.

#### 3.1.2.2 TSC Complex-RHEB

The TSC complex-RHEB pathway can transmit a variety of upstream signals, such as RTK-PI3K/PTEN-AKT, RAS-RAF-MEK-ERK (-RSK), and the signal from the cellular energy sensor AMP-activated protein kinase (AMPK) (for convenience, see the second section of this chapter for detailed information), thereby, controls the activity of mTOR, which, in turn, regulates cell growth and proliferation.

RHEB is a small GTP-binding protein that shuttles between an inactive GDP-bound form and an active GTP-bound form to regulate mTOR activity. When RHEB binds to GTP, it can activate mTORC1 through directly interacting with the kinase domain of mTOR. When GTP is hydrolyzed to GDP, RHEB activation of

mTOR catalytic function is terminated. TSC1 and TSC2 interact to form heterodimer (TSC1/TSC2) to indirectly inhibit mTOR activity by negatively regulating the activity of RHEB via the GTPase-activating protein (GAP) activity of TSC2. Activation of extracellular signal-regulated kinase 1/2 (ERK1/2), protein kinase B (AKT), and p90 ribosomal S6 kinase (RSK1) can phosphorylate and inactivate TSC1/TSC2 complex, which thereby promotes the activity of mTORC1.

#### 3.1.2.3 AMPK

The energy level regulates MTORC1 through AMPK. The falling cellular energy status enhances the phosphorylation and activation of AMPK by LKB1, which in turn, suppresses mTORCl activity through phosphorylating and activating TSC2 or directly binding on RAPTOR, the key subunit of mTORC1.

### *3.1.3 Downstream Targets of mTOR Complex*

#### 3.1.3.1 Downstream Targets of mTORC1

There are two best-characterized substrates of mTORC1, 4E-BP1, and S6K1, which are usually used to evaluate the activity of mTORC1. 4E-BPl (eIF-4E-binding protein 1) is a negative regulator of translation, and can be phosphorylated and inactivated by mTORC1. The inactivated 4E-BP1 dissociates with eIF-4E, thereby abrogating its inhibitory effect on eIF-4E function. EIF-4E binds with eIF-4A and eIF-4G to form eIF-4F complex, which binds to the cap structure present at the 5' end of mRNAs and promotes cap-dependent translation initiation. EIF-4E can regulate the translation of many proteins, such as CYCLIN D, MYC, and RAS, which are closely related to cell growth, proliferation, and cell cycle regulation. S6K1 is a serine/threonine kinase with multiple phosphorylation sites, which is directly or indirectly regulated by mTORC1 and participates in the regulation of autophagy. S6K1 can phosphorylate and activate 40S ribosomal protein S6. Activated S6 can improve the translation efficiency of 5′ terminal oligopyrimidine tracts (TOPs) type of RNA. 5′TOP mRNA accounts for 15–20% of the total intracellular RNA. It encodes many components needed for protein translation, such as ribosomal protein, prolongation factor, and polyA-binding protein.

#### 3.1.3.2 Downstream Targets of mTORC2

Members of the AGC protein kinase family, such as AKT, SGK1, and PKC, are known substrates of mTORC2 and play important roles in regulating cytoskeleton remodeling and autophagy.

## 3.2 Role of mTOR Signaling Pathway in the Regulation of Autophagy

### *3.2.1 Role of mTORC1 and Its Regulatory Signals in Autophagy Regulation*

#### 3.2.1.1 Amino Acids

Amino acids are biologically important environmental factors that regulate cell growth and proliferation. They also act as negative feedback inhibitors of autophagy. Deprivation of amino acids can effectively stimulate autophagy. This inhibitory effect of amino acids on autophagy is highly conserved from yeast to mammals. Amino acids regulate autophagy through a variety of signal transduction pathways. Among these is the mTORC1 signaling pathway. However, for a long time, it is not clear how amino acids are communicated to mTORC1. Until recent years, the intracellular signal transmission mechanism has been gradually revealed with the publication of a series of papers from several research groups.

Unlike other stimulus signals, amino acids regulate mTORC1 through a TSC complex-independent pathway. In mouse embryonic fibroblasts with TSC2 deletion, the activity of mTORC1 can be regulated by amino acid levels, but is no longer affected by growth factor deficiency, suggesting that amino acid signals may be transmitted in a way independent of the TSC pathway. It is noteworthy that exogenous amino acids do not activate mTORC1 in cells constitutively expressing RHEB-GDP mutants, suggesting that RHEB is involved in the regulation of mTORC1 activity by amino acids.

Recent studies have shown that the activation of mTORC1 mediated by amino acids (including leucine, arginine, and glutamine) occurs mainly in lysosomes. This activation process requires the participation of RAG GTPase, RAGULATOR, vacuolar $H^+$-adenosine triphosphatase (v-ATPase), SLC38A9, and KICSTOR (Fig. 3.1a). Identification of RAG small GTPases in Drosophila melanogaster and mammalian cells was a major breakthrough in deciphering the regulation of mTORC1 activity by amino acids. RAG small GTPases (RAGA-RAGD in mammals; GTR1 and GTR2 in yeast) are recognized as the sixth subfamily of Ras-related GTPases. In mammalian and yeast cells, both RAG and GTR proteins function as heterodimers, which consist of a GTR1-like factor (RAGA or RAGB) and a GTR2-like factor (RAGC or RAGD). In nutrient-rich conditions, the presence of amino acids causes RAG GTPases heterodimers to switch from the inactivated state (RAGA/B • GDP-RAGC/D • GTP) to active state (RAGA/B • GTP-RAGC/D • GDP). RAG small GTPases functionally signal amino acid sufficiency to mTORC1. The intracellular amino acid level directly affects the nucleotide loading state of RAG heterodimers, which in turn influences the affinity of RAG heterodimers with mTORC1 (Shen et al. 2017). RAG small GTPases do not directly activate mTORC1; rather they help localize mTORC1 to lysosome

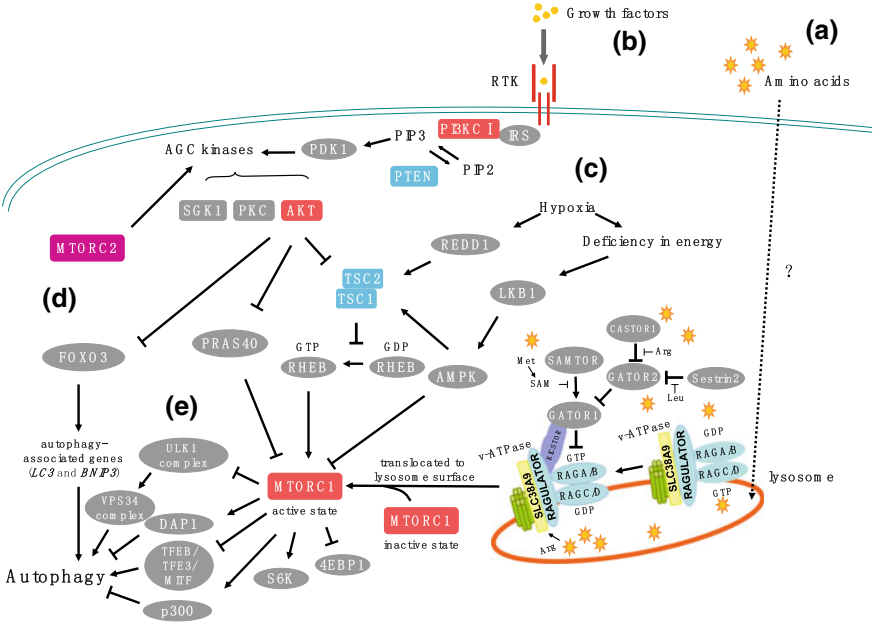


**Fig. 3.1** The role of mTORC1 and mTORC2 in autophagy regulation. **a** The activation of mTORC1 mediated by amino acids occurs mainly on the surface of lysosomes. This activation process requires the participation of RAG complexes, RAGULATOR, v-ATPase, SLC38A9, and KICSTOR. **b** Growth factor activates AKT through PI3K CI. Activated AKT1 further activates mTORCl in two ways. **c** Hypoxia inhibits the activity of mTORC1 in two ways. **d** mTORC2 blocked FOXO3 activation by activation of AKT. Activated FOXO3 induces autophagy formation by enhancing the transcription of two autophagy-related genes, LC3 and BNIP3. **e** mTORCl affects autophagy by regulation of ATG1/ULK1 complex, DAP1, TFE/MITF transcription factors, and acetylation mediated by p300 (see details in the text)

in response to amino acids. Under amino acids-deficient conditions, mTORC1 distributes in the cytoplasm; upon amino acids stimulation, active RAG complex promotes the translocation of mTORCl to the lysosome surface where RHEB resides. So far, several factors have been demonstrated to regulate RAG GTPases, including GATOR1 complex (NPRL2, NPRL3, and DEPDC5), the GAP for and inhibitor of RAGA/B; GATOR2 complex (Milos, WDR24, WDR59, SEHL1L, and SEC13), a positive regulator of unknown molecular function (Bar-Peled et al. 2013). RAGULATOR is a pentameric complex composed of five members, namely P18, P14, MP1, C7ORF59, and HBXIP, which are encoded by *LAMTOR1*, *LAMTOR2*, *LAMTOR3*, *LAMTOR4*, and *LAMTOR5* genes, respectively. Among these constituents, P18 is a lysosome membrane-anchored protein, which functions as a scaffold for recruitment of MP1-P14 and C7ORF59-HBXIP heterodimers to the lysosome and form the RAGULATOR complex. RAGULATOR has a direct interaction with C-terminal dimeric Roadblock domains, rather than N-terminal GTPase domains of RAG GTPase via

two binding sites, namely the α1 helix of P18 and the two helices side of MP1-P14 (Zhang et al. 2017). This direct interaction between RAGULATOR and RAG complex helps anchor RAG GTPases on the surface of lysosome. RAGULATOR was once regarded as a guanine exchange factor (GEF), which could promote the transformation of RAGA/B • GDP to RAGA/B • GTP and activate RAG complex (Bar-Peled et al. 2012). However, information on biochemical data furnished based on recently published literature has shown that the RAGULATOR complex alone does not function as a GEF for the RAG GTPases and speculated that RAGULATOR complex might function as a scaffold for an unknown GEF which could interact preferentially to the inactive RAG GTPases (Zhang et al. 2017). v-ATPase is necessary for amino acids to activate mTORC1. In Drosophila and mammalian cells, v-ATPase interacts with RAG GTPase and RAGULATOR on the lysosome surface, thereby regulating the activity of mTORC1. Studies have shown that the signal transduction of amino acids to mTORC1 begins in the lysosomal lumen and transmits information between v-ATPase and the lysosomal lumen through the "internal-external" mechanism, i.e., the amino acids accumulated in the lysosomal lumen are transported outside the lysosomal lumen through v-ATPase, and then mTORC1 was enriched and activated on the lysosomal surface (Zoncu et al. 2011). SLC38A9, a lysosomal transmembrane protein with homolog to amino acid transporter, is identified as an important arginine sensor in the lysosome lumen. On the one hand, arginine, at concentrations found in lysosomes, promotes the interaction of SLC38A9 with Rag-Ragulator complex, while on the other it stimulates SLC38A9 to efflux essential amino acids from lysosomes to the cytosol, especially leucine, which hereby further activates mTORC1. KICSTOR complex, composed of four proteins, KPTN, ITFG2, C12orf66, and SZT2, is a lysosome-associated negative regulator of mTORC1 signaling (Wyant et al. 2017). KICSTOR localizes to lysosomes where it binds and recruits GATOR1, but not GATOR2, to the lysosomal surface; and is necessary for the interaction of GATOR1 with its substrates, the Rag GTPases, and with GATOR2 (Wolfson et al. 2017).

The cytosol amino acid sensors upstream of mTORC1 have eluded scientists for many years. Sestrin2 and CASTOR1 represent the very few reported amino acid sensors in the cytosol whose sensing and transmitting mechanisms have been adequately defined. Sestrin2 serves as a cytosolic leucine sensor for mTORC1 pathway, while CASTOR1 functions in parallel with SLC38A9 to regulate mTORC1 in response to arginine. Sestrin2 and CASTOR1 bind leucine and arginine, respectively, at physiological concentrations and activate mTORC1 through disrupting the Sestrin2- or CASTOR1-GATOR2 interactions (Wolfson et al. 2016; Chantranupong et al. 2016). Apart from Sestrin2, leucyl-tRNA synthetase (LARS), a protein synthesis enzyme, also serves as a leucine sensor for mTORC1 pathway. It has been reported that LARS acts as an initiating "ON" switch by controlling GTP hydrolysis of RAGD, whereas Sestrin2 functions as an "OFF" switch via GTP hydrolysis of RAGB in the RAG GTPase–mTORC1 axis (Lee et al. 2018).

Leucine and arginine are well-established activators of mTORC1 signaling. Recently, it has been reported that methionine can also activate mTORC1 signaling. Unlike leucine and arginine, which bind directly with sensors upstream of mTORC1,

methionine can be sensed indirectly via S-adenosylmethionine (SAM) by SAMTOR that inhibit mTORC1 signaling through interacting with GATOR1. It has been demonstrated that SAM disrupts the association of SAMTOR with GATOR1, thereby activating mTORC1 signaling (Gu et al. 2017).

Amino acids (such as glutamine) can also regulate the activity of mTORC1 through a RAG-RAGULATOR complex independent pathway. In RagA/B-deleted cells, glutamine activates mTORCl via the adenosine diphosphate ribosylation factor-1 (Arf1) GTPase. This process is dependent on v-ATPase (Nguyen et al. 2017).

Whether the amino acids transported to lysosomes originate from intracellular or extracellular environments remains unclear. However, the increase of intracellular amino acid concentration mediated by Cycloheximide, an inhibitor of protein synthesis, can enhance the activity of mTORC1, suggesting that intracellular amino acids can accumulate in lysosomal lumen.

#### 3.2.1.2 Growth Factors

Growth factor signaling induces kinase activation of mTORC1 via a process that begins at the plasma membrane with the transduction of a signal evoked by protein hormones like insulin, via tyrosine kinase receptors (RTKs), and activation of Class I phosphatidylinositol 3-kinase (PI3KC I). PI3KC I, in turn, activates AKT1, which phosphorylates and inhibits TSC, a complex with GAP activity toward the RHEB GTPase, which directly promotes kinase activity of mTORC1. Similar to mTORCl, the activity of mTORC2 is also regulated by growth factor. However, little is known about the exact mechanism involved. One possible mechanism is that the activation of mTORC2 requires the involvement of ribosomes. In addition, mTORC2 can bind to the lysosome surface in a PI3KC I-dependent manner.

PI3KC I is a heterodimer composed of P85 regulatory subunit and P110 catalytic subunit. The activity of PI3KC I is strictly controlled by many mechanisms. In general, inactive P85-P110 complex is dispersed in the cytoplasm of resting cells. Once activated by environmental stimuli, such as growth factors, RTK enriches P85-P110 complex to plasma membrane and activates it through directly binding to the SH2 domain of P85. In some cases, P85-P110 complex can also be indirectly combined with RTK via insulin receptor substrate IRS1/IRS2. Activated PI3KC I exert its catalytic activity by phosphorylating PI (4,5) P2 to yield a second messenger—PI (3,4,5) P3, which recruits Pleckstrin homology domain (PH) domain-containing proteins, such as serine/threonine kinase AKT1 to the plasma membrane, where it is phosphorylated and activated by 3-Phosphoinositide-dependent kinase 1 (PDK1). PTEN can dephosphorylate PI (3,4,5) P3 to generate PI (3,4) P2 and PI (4,5) P2, and thus quench PI3KC I signaling.

AKT1 is the cellular homologue of the v-AKT oncogene transduced in an oncogenic murine retrovirus. It is a 59 kDa serine/threonine kinase that has a kinase catalytic domain, a PH domain and a regulatory domain. The kinase domain is constituted by glycine-rich residues and a T-loop that consists of threonine 308 (Thr308) and Serine 473 (Ser473) residues for the activation of AKT1. An important moiety

of AKT1 is the PH domain that consists of about 100 amino acid residues and mainly mediates the binding between AKT1 and PI (3,4,5) P3. AKT1 is activated by a multistep process involving PI (3,4,5) P3-mediated recruitment of AKT1 and other molecules to the inner surface of the plasma membrane, where they undergo conformational changes that allow for protein–protein interactions that activate the AKT1-mediated signaling pathway. PDK1 is recruited to the membrane in a PI (3,4,5) P3-dependent manner, where it phosphorylates AKT1 at Thr308 residue, which is critical to AKT1 catalytic activity. The phosphorylation of Ser473 at the end of the regulatory domain maximizes the activity of AKT1. Ser473 of AKT1 can be phosphorylated by various kinases such as mTORC2, PDKl, DNA-dependent protein kinase (DNA-PK), integrin-binding kinase (ILK), ILK-related kinase, and AKT1 itself. Among them, mTORC2 has the strongest ability to phosphorylate AKT Ser473. Activated AKT1 further activates mTORCl in two ways, one by directly phosphorylating PRAS40 (a kinase-suppressing protein in mTORCl) to activate mTORCl, the other by phosphorylating TSC2 at Ser939 and Thr1462 residues to inhibit the hydrolysis of GTP-RHEB by TSC complex.

Continuous activation of mTORCl eventually leads to negative feedback suppression of RTK signal, which is mediated by many downstream components of mTORCl signaling pathway, including S6K1 and mTORCl itself.

As mentioned above, growth factors mainly inhibit autophagy by activating PI3KC I-AKT-TSC complex-RHEB-mTORCl signaling pathway (Fig. 3.1b). Deficiency in growth factor triggers autophagy. When growth factors are scarce, the number of nutrient transport-related receptors such as glucose receptors, low-density lipoprotein receptors, and amino acid transporters on the cell surface decreases accordingly, thus leading to the reduction in the intracellular nutrients supply, which, in turn, induces autophagy to allow cells to maintain a supply of nutrients and energy adequate for their survival.

In drosophila, S6K was found to induce autophagy, but was not necessary for the initiation of autophagy. In addition, S6K may contribute to maintaining the basic level of autophagy by feedback inhibition of PI3KC I-mediated insulin signaling in mammalian cells. When nutrients are adequate, S6K can maintain a certain basic level of autophagy by phosphorylation and negative feedback inhibition of insulin receptor substrates (IRS). However, when the cells are at the early stage of nutrient deficiency, the activity of S6K kinase is still relatively high for a period of time, which will further inhibit the activity of IRS, thereby inhibiting the activity of mTORC1 and inducing autophagy. When nutrients have been depleted for a long time, the activity of S6K gradually decreases. Inhibition of S6K kinase activity leads to weakness in the negative feedback inhibition of IRS so that IRS maintains a certain activity. The activity of PI3KC I was thereby maintained at a certain level, and ultimately the autophagy level was not too high to cause damage to the cells. However, this mechanism of cell survival is limited. When nutrients are persistently deficient in the environment, the activity of mTORC1 is persistently decreased, leading to autophagy-induced cell death. However, no change in autophagy rate was observed in the rhabdomyosus of S6K deficient mice, so more studies are needed to elucidate the role of S6K in autophagy.

#### 3.2.1.3 Stress

Hypoxia induces autophagy by inhibiting the activity of mTORC1. Cells treated with low concentration of oxygen for a certain time can inhibit the insulin-mediated activation of mTORC1; with the increase of oxygen concentration, the activity of mTORC1 also increases, indicating that hypoxia can reversibly inhibit the activity of mTORC1 in a given time. Hypoxia inhibits the activity of mTORC1 in two ways, one is through reducing ATP/AMP ratio-mediated AMPK signaling pathway, the other is through transcriptional activation of regulated in development and DNA damage 1 (REDD1). REDD1 can activate TSC complex through binding to 14-3-3, an inhibitor of TSC complex (DeYoung et al. 2008) (Fig. 3.1c).

### *3.2.2 The Bidirectional Role of mTORC2 in Autophagy Regulation*

mTORC2 is also involved in the regulation of autophagy. mTORC2 is a bidirectional regulator of autophagy. On one hand, mTORC2 indirectly inhibits autophagy through AKT1/FOXO3a axis. FOXO3a is a transcription factor that is necessary and sufficient for the induction of autophagy. RICTOR, a key subunit of mTORC2 complex, mediates mTORC2 interaction with AKT1 and activate AKT1 by phosphorylating it at the Ser473 residue. Activated AKT1 promotes the phosphorylation in Thr32 of FOXO3a, which hereby results in translocation of FOXO3 from the nucleus, thus inhibiting the transcription of two autophagy-related genes, *LC3* and *BNIP3* (Chen et al. 2013) (Fig. 3.1d). This effect is not prevented by rapamycin. In breast cancer cells, IFN-4 binding protein can promote phosphorylation of AKT1 at the Ser473 site by activating mTORC2, which further inhibits its downstream transcription factor FOXO3a, and ultimately inhibits the induction of autophagy. Thus, the mTORC2/AKT1/FOXO3a signaling pathway has a crucial role in the suppression of autophagy in many cellular settings. On the other hand, since AKT1/mTORCl signaling pathway functions as an inhibitory regulator of autophagy, IGF1, which is upstream of AKT1/mTORCl signaling pathway, should inhibit autophagy theoretically. However, the opposite is true. Studies have shown that this process may be related to the regulation of mTORC2 on microfilament cytoskeleton and endocytosis-related pathways. Similarly, deletion of RICTOR inhibits the activity of PKCα/β to some extent, and destroys the microfilament skeleton and inhibit the endocytosis, thus affecting the nucleation stage of autophagy. These phenomena indicate that mTORC2 plays an important role in the maturation and transportation of autophagic vesicles.

In conclusion, unlike mTORCl, mTORC2 has a bidirectional role in the regulation of autophagy. Therefore, the use of mTOR inhibitors or activators requires careful consideration.

## 3.3 The Main Mechanisms for mTORC1 Regulation of Autophagy

In the past decade, we have made great efforts to study the mechanism of mTORCl regulating autophagy. Now, researchers have filled the gaps with new findings that will be recommended in detail (Fig. 3.1e).

### *3.3.1 mTORC1 Negatively Controls Autophagy Through Inhibitory Phosphorylations of the Components of the Key Autophagy-Related ULK1 Complex and VSP34 Complex and Through Regulation of ATG5-RACK1 Protein Complexes*

There are two complexes involved in the initiation of autophagy, namely ATG1/ULK1 (unc-51 like kinase 1) complex and VPS34/PI3K C III (phosphoinositide 3-kinase class III) complex. ATG1/ULK1 complex is the upstream regulator of PI3K C III complex, and is usually regarded as a "bridge" between nutritional signals and autophagy. *ATG1* (autophagy-related gene 1) is the first autophagic gene cloned successfully in yeast. It encodes a serine/threonine protein kinase, which is similar to ULK1, a mammalian homolog protein, and has an essential role in autophagy induction. Under both nutrient-rich and nutrient-starved conditions, the majority of ULK1 exists as part of a macromolecular complex containing ATG13, FIP200, and ATG101. The interaction between ULK1 and these components is essential for the maintenance of the stability and kinase activity of ULK1. FIP200, also named RB1-inducible coiled-coil protein 1 (RB1CC1), is a protein that interacts with focal adhesion kinase FAK. The homolog of FIP200 in yeast is ATG17. ATG13 is regarded as a bridge between ATG1 and ATG 17 in yeast cells. Studies have shown that ATG13 and FIP200 can enhance the kinase activity of ULK1. ATG101 is relatively conserved in various eukaryotes and has no obvious homology with other ATG proteins. ATG101 interacts with ATG13 and plays an important role in autophagy regulation (Ganley et al. 2009).

Activated ULK1 complex promotes the translocation of PI3K C III complex from dynein motor complex to endoplasmic reticulum (ER) by phosphorylation of autophagy/Beclin 1 regulator 1 (AMBRA1), a component of PI3K C III complex. VPS34 complex (homologous to PI3K C III complex) in ER phosphorylates PI to PI (3,4,5) P3, thus promoting the initiation of autophagy nucleation (Di Bartolomeo et al. 2010). In addition, ULK1 also activates VPS34 complex and induce autophagy through phosphorylation of Beclin 1, a member of VPS34 complex (Russell et al. 2013).

mTORC1 inhibits autophagy through direct phosphorylation of ULK1 and ATG13 in ULK1 complex and ATG14 in ATG14-containing PI3K C III complex, by which

it sequesters the ULK1 complex and ATG14-containing PI3K C III complex in an inactive state. Autophagy can be promoted by AMP-activated protein kinase (AMPK), which is a key energy sensor and regulates cellular metabolism to maintain energy homeostasis. mTORC1 and AMPK regulate autophagy through direct phosphorylation of ULK1. A molecular mechanism for regulation of ULK1 in a nutrient-dependent manner has been demonstrated. Under glucose starvation, AMPK promotes autophagy by directly activating ULK1 via phosphorylation of Ser317 and Ser777 residues. Under nutrient sufficiency, active mTORC1 prevents ULK1 activation by phosphorylating ULK1 at Ser757 site and disrupting the interaction between ULK1 and AMPK (Kim et al. 2011). This coordinated phosphorylation is essential for ULK1 in autophagy induction. In addition, mTORC1 also indirectly inhibits the stability of ULK1 and eventually leads to the inhibition of autophagy. This process is associated with the phosphorylation of AMBRA1.

The E3-like ATG12-ATG5-ATG16 protein complex that catalyzes conjugation of the MAP1LC3 (microtubule-associated protein 1 light chain 3, a mammalian homolog of yeast Atg8) protein to lipids controls autophagic vesicle expansion. In this protein complex, ATG5 is indicated as a convergence point for autophagy regulation. A recent study has demonstrated that RACK1 (receptor-activated C-kinase 1, GNB2L1), a WD40 repeat protein with a seven-bladed-propeller structure, is a novel ATG5 interactor and required for mTOR inhibition and starvation-induced autophagy. Thus, RACK1-ATG5 association control is one of the mechanisms used by the mTORC1 signaling pathway to regulate autophagy (Erbil et al. 2016). However, whether RACK1 is a direct target of mTORC1 needs to be determined.

### *3.3.2 DAP1, a Substrate of mTORC1, Negatively Regulates Autophagy*

It has been confirmed that ULK1 complex, as a branch downstream of mTORC1, is a positive regulator of autophagy. The question is whether there are negative regulators of autophagy downstream of mTORC1. Keren et al. pointed out for the first time that death-associated protein 1 (DAP1) participates in the negative regulation of autophagy.

DAP1 is highly conserved and widely expressed in various tissues and cells. *DAP1* is transcribed into a single 2.4-kb mRNA that codes for a proline-rich protein consisting of 102 amino acid residues. However, due to the lack of confirmed functional motifs and sequences homologous to other proteins with known functions, the prediction and study on the function of DAP1 has been limited for years. Until 2010, Koren et al. (2010) proved that DAP1 was a phosphorus-containing protein with increased electrophoretic mobility under amino acid starvation. By the use of alkaline phosphatase of the calf intestine, they found that the increase of electrophoretic mobility was due to dephosphorylation of DAP1, and further confirmed that the phosphorylation level of Ser3 and Ser51 in DAP1 protein decreased under

amino acid starvation. The above changes were similar to that in the phosphorylation level of S6K, a downstream effector protein of mTOR. Further studies demonstrated that mTOR is a specific kinase of DAP1. Finally, Koren et al. confirmed that either knockdown of DAP1 or transfection of plasmids expressing an Ser3 and Ser51 phosphorylation sites mutant contributed to amino acid starvation-induced autophagy in Hela cell line stably expressing GFP-LC3. So far, Koren et al's research has proved for the first time that DAP1, the substrate molecule of mTOR, functions as an inhibitory regulator of autophagy. It is worth noting that Yahiro et al. (2014) also found that DAP1 inhibits autophagy in Hela cells mediated by mycotoxin of Bacillus subtilis. Nevertheless, the molecular mechanism of DAP1 regulating autophagy needs further investigation.

### *3.3.3 mTORC1 Inhibits Autophagy by Preventing the Nuclear Translocation of Lysosomal Biogenesis-Related Genes*

Lysosomes are membrane-enclosed vesicles containing more than 60 hydrolases for the degradation and recycling of essential nutrients to maintain cellular homeostasis. The lysosomal degradation pathway regulates a range of cellular functions such as autophagy, phagocytosis and endocytosis.

Lysosomal biogenesis is controlled by the TFE/MITF family of transcriptional factors, master regulators including transcription factor EB (TFEB), transcription factor E3 (TFE3), and microphthalmia-associated transcription factor (MITF). In the presence of amino acids, active RAG GTPase heterodimers bind and recruit mTORC1 complexes to lysosome membranes where RHEB proteins reside and activate them. Meanwhile, TFEB, TFE3, and MITF transcription factors are recruited by active RAG GTPases to lysosomes, and phosphorylated by mTORC1. Phosphorylation by mTORC1 triggers the binding of 14-3-3 proteins to the transcription factors, which results in their retention in the cytosol. In contrast, upon amino acid starvation, RAG GTPases turn to the inactivated state, leading to the inactivation of mTORC1. In such conditions, the ULK1 complexes are reactivated and induce autophagy. Furthermore, TFEB, TFE3 and MITF transcription factors can be dephosphorylated and then translocate into the nucleus to trigger the transcription of autophagy- and lysosomal-related gene (such as *atg9B*, *UVRAG*, *CLCN7*, *ATP6V1H,* and *MCOLN1*) (Martina et al. 2012; Ozturk et al. 2019; Martina et al. 2014).

### *3.3.4 mTORC1 Inhibits Autophagy Through Direct Phosphorylation and Activation of Acetyltransferase P300*

Acetylation is one of the most important mechanisms for posttranslational regulation of various cellular functions. Accumulating evidence suggests that acetylation can regulate autophagy. Histone acetyltransferase (HAT) p300 and the related CREB-binding protein (CBP) are evolutionarily conserved and traditionally function as transcription coactivators by acetylating core histones and nuclear non-histone proteins. It has been suggested that p300, but not CBP is a key acetyltransferase in the regulation of autophagy. Knockdown of p300 reduces acetylation of several ATG proteins such as ATG5, ATG7, ATG8, and ATG12, leading to induction of autophagy in nutrient-rich conditions.

A recent research has identified p300 as a direct phosphorylation substrate of mTORC1. mTOR phosphorylates and activates p300 at four serine residues (Ser2271, Ser2279, Ser2291, and Ser2315) in the C-terminus domain, resulting in suppression of starvation-induced autophagy (Wan et al. 2017). These findings have established a direct and strong regulatory relationship between mTORC1 and p300 and the role of regulation in the control of cell autophagy.

#### 3.3.4.1 Perspectives

MTORC1 is a key growth regulatory kinase complex that mediates amino acid signaling, growth factor signaling, hypoxia signaling, and energy signaling. MTORC1 signaling pathway is deregulated in many diseases, such as cancer and diabetes. Although it has been well recognized that mTORC1 can negatively regulate autophagy, the mechanism of mTORC1 regulation of autophagy activity is not entirely clear. In recent years, the discovery of new downstream targets of mTORC1, such as DAP1, TFE/MITF transcription factor family, and p300, explained, at least in part, how autophagy is regulated by mTORC1. However, if other regulatory mechanisms exist remains to be further explored across subcellular organelles, cell types, tissues, or species.

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# Chapter 4
# AMPK and Autophagy


**Yanjun Li and Yingyu Chen**

**Abstract** AMPK is an evolutionarily conserved serine/threonine-protein kinase that acts as an energy sensor in cells and plays a key role in the upregulation of catabolism and inactivation of anabolism. Under various physiological and pathological conditions, AMPK can be phosphorylated by an upstream kinase and bind to AMP or ADP rather than ATP, leading to its activation. Activated AMPK regulates a variety of metabolic processes, including autophagy. AMPK promotes autophagy directly by phosphorylating autophagy-related proteins in the mTORC1, ULK1, and PIK3C3/VPS34 complexes or indirectly by regulating the expression of autophagy-related genes downstream of transcription factors such as FOXO3, TFEB, and BRD4. AMPK can also upregulate the autophagic degradation of mitochondria (mitophagy), as it can induce fragmentation of damaged mitochondria in the network and promote the translocation of the autophagy machinery to damaged mitochondria. In this section, we will detail the molecular structure of AMPK, how its activity is regulated, and its pivotal role in regulating autophagy and mitophagy.

**Keywords** AMPK · Autophagy · Mitophagy

## Abbreviation

| | |
|---|---|
| ACC | Acetyl-CoA carboxylase |
| ACSS2 | Acetyl-CoA synthetase 2 |
| ADaM | Allosteric drug and metabolite |
| ADP | Adenosine diphosphate |
| AICAR | 5-aminoimidazole-4-carboxamide riboside |
| AMP | Adenosine monophosphate |

Y. Li
College of Life Sciences, Nankai University, Tianjin, China

Y. Chen (✉)
Department of Immunology, Peking University School of Basic Medical Sciences, NHC Key Laboratory of Medical Immunology, Peking University, Beijing, China
e-mail: yingyu_chen@bjmu.edu.cn



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| | |
|---|---|
| AMPK | AMP-activated protein kinase |
| ATP | Adenosine triphosphate |
| ATG | Autophagy-related gene |
| BMDM | Bone marrow-derived macrophages |
| BRD4 | Bromodomain-containing protein 4 |
| CAMKK2 | Calmodulin-dependent protein kinases kinase 2 |
| CARM1 | Coactivator-associated arginine (R) methyltransferase 1 |
| CBD | Carbohydrate-binding domain |
| CBS | Cystathionine β-synthase |
| CHOP | C/EBP homologous protein |
| CCCP | Carbonyl cyanide 3-chlorophenylhydrazone |
| DDIT3 | DNA damage-inducible transcript 3 |
| DRP1 | Dynamin-related protein 1 |
| FAS | Fatty acid synthase |
| FOXK | Forkhead box protein K |
| FOXO3 | Forkhead box O3 |
| FUNDC1 | FUN14 domain-containing protein 1 |
| GAP | GTPase-activating protein |
| GBD | Glycogen-binding domain |
| GDP | Guanine dinucleotide phosphate |
| GLUT4 | Glucose Transporter 4 |
| GPAT | Glycerol phosphate acyltransferase |
| GSK3β | Glycogen synthase kinase 3 beta |
| GTP | Guanine trinucleotide phosphate |
| HSF1 | Heat shock factor 1 |
| IGF-1 | Insulin-like growth factor 1 |
| LKB1 | Liver kinase B1 |
| MAPKK | Mitogen-activated protein kinase kinase |
| MEF | Mouse embryonic fibroblasts |
| MFF | Mitochondrial fission factor |
| MO25 | Mouse protein 25 |
| mtDNA | Mitochondrial DNA |
| mTOR | Mammalian target of rapamycin |
| NBS | Nucleotide-binding site |
| NES | Nuclear export sequence |
| NMT1 | N-myristoyl transferase 1 |
| NRF2 | Nuclear factor erythroid 2-related factor 2 |
| PAQR3 | Progestin and adipo-Q receptor 3 |
| PGC1α | PPARγ coactivator 1α |
| PI3P | Phosphatidylinositol 3-phosphate |
| PIK3C3 | Phosphatidylinositol 3-kinase catalytic subunit 3 |
| PIK3R4 | Phosphoinositide 3-kinase regulatory subunit 4 |
| PINKl | PTEN induced kinase 1 |
| PKA | Protein kinase A |
| PKB | Protein kinase B |

| | |
|---|---|
| PKC | Protein kinase C |
| PKD | Protein kinase D |
| PP2A | Protein phosphatase 2A |
| PPARγ | Peroxisome proliferator-activated receptor γ |
| PPM1E | Protein Phosphatase, $Mg^{2+}$/$Mn^{2+}$-Dependent 1E |
| PRKAA | AMP-activated protein kinase catalytic subunit alpha |
| PRKAB | AMP-activated protein kinase subunit beta |
| PRKAG | AMP-activated protein kinase subunit gamma |
| RACK1 | Receptor for activated C kinase 1 |
| RB1CC1 | RB1-inducible coiled-coil 1 |
| RHEB | Ras Homolog Enriched in Brain |
| RNS | Reactive nitrogen species |
| ROS | Reactive oxygen species |
| SKP2 | S-phase kinase-associated protein 2 |
| STK11 | Serine/Threonine kinase 11 |
| STRAD | Ste20-related adaptor protein |
| TAK1 | Transforming growth factor-β-activating kinase 1 |
| TCA | Tricarboxylic acid cycle |
| TFEB | Transcription Factor EB |
| TNFSF10 | TNF superfamily member 10 |
| TRAIL | TNF-related apoptosis inducing ligand |
| TSC | Tuberous sclerosis complex |
| TZD | Thiazolidinediones, |
| ULK1 | Unc-51-like kinase 1 |
| UVRAG | Ultraviolet radiation resistance-associated gene protein |

Adenylate-activated protein kinase (AMP-activated protein kinase, AMPK) is an evolutionarily conserved serine/threonine protein kinase that is expressed extensively in all eukaryotic cells. Under physiological or pathological conditions such as ischemia, hypoxia, starvation, electrical stimulation, and heat shock, AMPK is activated through a combination of multiple phosphorylation by upstream kinases and increased AMP and ADP levels. Activated AMPK further acts on different downstream target proteins and signal transduction pathways to upregulate catabolic processes (such as glucose absorption, glycolysis, fatty acid oxidation, and mitochondrial formation) and shut down anabolic processes (such as the synthesis of proteins, fatty acids, or cholesterol). Such effects maintain energy balance and the homeostasis of the internal environment (Hardie et al. 2016). AMPK plays an important role in regulating the normal physiological functions of the liver, skeletal muscle, adipose tissue, islet beta cells, heart and central nervous system. It has become a biological target for the treatment of obesity, diabetes, cardiovascular disease, tumor and other metabolic diseases and inflammatory diseases.

## 4.1 Structure and Distribution of AMPK

### *4.1.1 Molecular Structure of AMPK*

AMPK is a heterotrimeric complex composed of a catalytic α-subunit and two regulatory subunits (β and γ). Vertebrates possess multiple isoforms of each subunit encoded by different *PRKAA* (5′-AMP-activated protein kinase catalytic subunit alpha), *PRKAB* (5′-AMP-activated protein kinase subunit beta), and *PRKAG* (5′-AMP-activated protein kinase subunit gamma) genes. In humans, the α subunit consists of two subtypes, α1 and α2, encoded by the *PRKAA1* and *PRKAA2* genes, respectively. The molecular weight of the α subunit is 63 kDa. There are two β-subunits, β1 and β2, encoded by the genes *PRKAB1* and *PRKAB2*, and three γ-subunits, γ1, γ2, and γ3, encoded by *PRKAG1*, *PRKAG2,* and *PRKAG3* genes, respectively. Each AMPK complex consists of one α subunit, one β subunit and one γ subunit. Theoretically, these subunits produce 12 different AMPKαβγ complexes: α1β1γ1, α1β1γ2, α1β1γ3, α1β2γ1, α1β2γ2, α1β2γ3, α2β1γ1, α2β1γ2, α2β1γ3, α2β2γ1, α2β2γ2, and α2β2γ3. These complexes are distributed in different tissues or cell types and in different subcellular locations inside cells.

The N-terminus of the AMPKα subunit contains a typical serine/threonine-protein kinase domain (α-KD), which is the active center of the AMPK complex. α-KD is further divided into a small N-terminal lobe (N-lobe) and a larger C-terminal lobe (C-lobe). The C-lobe contains a conserved threonine site (α1 is Thr172, α2 is Thr174) whose phosphorylation is necessary for its kinase activity. Phosphorylation of the Thr172 site is usually used as a marker of AMPK activation. There is an autoinhibitory domain (α-AID) downstream of α-KD. When α-KD is expressed in cells alone, AMPK composed of α-AID and α-KD exhibits complete catalytic activity. When α-KD is expressed in cells alone, AMPK shows complete catalytic activity. When α-AID and α-KD are coexpressed simultaneously, α-AID can block α-KD, thus downregulating the activity of AMPK. α-AID is followed by a flexible α-linker that contains two conserved motifs termed α-regulatory subunit-interacting motif 1 (α-RIM1) and α-RIM2. Following the linker is the subunit-interacting C-terminal domain (α-CTD), which is a globular domain that terminates with a nuclear export sequence (NES). Immediately preceding this NES is a serine/threonine-rich loop (the ST-loop) of approximately 50 residues, which harbors proposed phosphorylation sites for AKT, PKA, GSK, etc.

The AMPKβ subunit contains a flexible, glycine-rich segment with a myristoylation site at its N-terminus, followed by a carbohydrate-binding domain (β-CBD), also known as the glycogen-binding domain (β-GBD), of approximately 80 amino acids. The AMPKβ subunit contains β-linker regions and C-terminal domains (β-CTD). Myristoylated AMPKβ has been reported to cause localization of AMPK to lysosomes upon glucose starvation (Zhang et al. 2014). An alternative role of N-myristoylation of the β subunits is to assist in the binding of AMPK to mitochondrial membranes, where AMPK functions in the selective removal of damaged mitochondria by autophagy (Liang et al. 2015). The β-CTD forms the core of the heterotrimeric

complex, bridging the α and γ subunits. The β-CBM allows AMPK to bind to glycogen. In addition, there is a cleft between the β-CBD and the N-lobe of the α-KD. This cleft remains stable through an interaction between the phosphorylated Ser108 residue in the β-CBD and the conserved Lys29 and Lys31 residues in the N-lobe. This site has been referred to as as the allosteric drug and metabolite (ADaM)-binding site for ligands such as A769662 (allosteric AMPK activators) (Xiao et al. 2013). The human AMPKβ1 (Ser108A) mutant can block the binding of A769662 to ADaM sites and inhibit the allogenic activation of AMPK.

The three alternative γ subunits contain variable N-terminal regions that interact with the β-CTD. The N-terminus is followed by four tandem repeats of a motif known as the CBS1–4 (Cystathionine β-synthase–4) repeat, which contains binding sites for regulatory nucleotides including AMP, ADP, and ATP. The four CBS repeats in the AMPKγ subunits form a flattened disk shape, and the center contains four potential nucleotide-binding sites (NBS1–4). CBS1, 3, and 4 are functional, whereas in CBS2 of mammalian AMPKγ1, the ribose-binding Asp residue is replaced by an Arg, and no nucleotide binding has been observed. Human AMPKγ1 (R299G) mutants or human AMPKγ2 (R531G) mutants can block the binding of AMP to AMPK γ subunits and inhibit the activation of AMPK.

### *4.1.2 Tissue Distribution and Cell Localization of AMPK*

Different isoforms of AMPK subunits have different tissue distribution. AMPKα1 is primarily distributed in the kidney, heart, liver, brain and islet β cells. AMPKα2 is mainly expressed in the heart, liver, skeletal muscle, and neuroplexus of the brain. AMPK β1 is mostly distributed in the liver, while AMPK β2 is mainly expressed in skeletal muscle and the myocardium. AMPKγ subunit activity is predominantly affected by the binding of AMP to AMPK. AMPKγ1 and γ2 are found in the liver, kidney, heart, lung, skeletal muscle, and pancreas, and AMPKγ3 is distributed in skeletal muscle and is related to glucose uptake and mitochondrial function.

AMPKα1 mainly localizes in the cytoplasm, while AMPKα2 is mostly located in the nucleus. The AMPKγ subunit localizes to muscle fibers, whereas myristoylation of the β subunit enables AMPK to localize to the outer membrane of mitochondria and lysosomes.

## 4.2 Regulation Mechanism of AMPK Activity

The AMPK complex is activated by phosphorylation of Thr172 by upstream kinases, as well as allostery caused by AMP binding.

### 4.2.1 *Regulation of AMPK by the LKB1-STRAD-MO25 Complex*

One of the upstream kinases for AMPK is the tumor suppressor LKB1, also known as STK11. It is a member of the serine/threonine-protein kinase family encoded by the *LKB1* gene. Similar to AMPK, LKB1 functions as a constitutive heterotrimer with two other subunits, the STE20-related kinase STRAD (Ste20-related adaptor protein) and the scaffolding protein MO25 (mouse protein 25). MO25 is also known as CAB39 (calcium-binding protein 39). Studies in mice in which *Lkb1* is genetically inactivated have revealed that LKB1 is responsible for the majority of AMPK activation under energy stress in most mammalian tissues examined, including liver and muscle. Studies in cells devoid of *LKB1* have indicated that LKB1 mediates the majority of the activation of AMPK in response to mitochondrial insults and low-energy.

LKB1 can constitutively activate AMPK, and the level of phosphorylated LKB1 (p-LKBl) is positively correlated with AMPK activity. The key molecular step in the LKB1-mediated activation of AMPK is its phosphorylation at Thr172 within the catalytic α subunit, and this process is facilitated by the binding of AMP to the γ subunit of AMPK (Fig. 4.1). It has been shown that the binding of AMP to the AMPKγ subunit further stimulates its activation by LKB1.

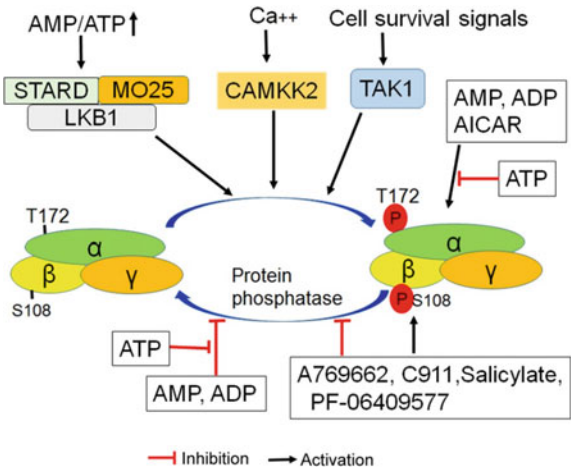


**Fig. 4.1 Regulation of AMPK complex activation**. AMPK is activated by the phosphorylation of T172 (phosphorylation sites indicated by red circles) by the upstream kinases including LKB1 complex, CAMKK2, and TAK1. Triple mechanism by which AMP (and ADP) contributes to AMPK activation. (1) AMP-binding to AMPK promotes T172 phosphorylation by LKB1; (2) AMP-binding (and ADP binding at higher concentrations) inhibits T172 dephosphorylation; and (3) AMP binding causes allosteric activation. All three effects of AMP (and the single effect of ADP) are antagonized by binding of ATP. Drugs that activate AMPK include the AMP mimetic AICAR and several small-molecule allosteric activators, such as A769662, C991, salicylate, and PF-06409577. Phosphorylation of S108 within β1 increases the binding affinity of these drugs. S108 of β1 can be autophosphorylated even in the absence of T172 phosphorylation

### 4.2.2 Regulation of AMPK by CAMKK2 (Calmodulin-Dependent Protein Kinases Kinase 2)

The expression of CAMKK2 (also known as CAMKKβ) is particularly high in central nervous tissue but low in the testis, spleen, and lung. CAMKK2 is also expressed in bone marrow-derived medullary cells and peritoneal macrophages.

AMPK can be directly phosphorylated at Thr172 in response to calcium flux by the calcium-sensitive kinase CAMKK2, thus linking calcium signaling with the regulation of energy metabolism by AMPK. CAMKK2 is composed of N-terminal, C-terminal, and Ser/Thr-binding regions. Increased intracellular $Ca^{2+}$ activates CAMKK2, which binds to AMPK and leads to direct phosphorylation of the latter at Thr172 (Fig. 4.1). The activation of AMPK by CAMKK2 is independent of LKB1 and nucleotide levels. Upstream extracellular signals such as insulin, lipopolysaccharide (LPS), amino acids, hormones, and glucose can bind to the corresponding receptors, and then induce an increase in intracellular $Ca^{2+}$ levels and accumulation of the $Ca^{2+}$/CaM targeting CaMKK2. The increased binding between $Ca^{2+}$/CaM and CaMKK2 leads to the activation of CaMKK2 kinase. CaMKK2-dependent activation of AMPK contribute to the regulation of energy balance, particularly in the brain, liver, and adipose tissue. Inactivation of *CAMKK2* in mammalian cells can completely block the activation of AMPK. In *LKB1*-deficient cells, elevated $Ca^{2+}$ can promote the phosphorylation and activation of AMPK through the CAMKK2 pathway, thereby leading to the maintaining of some AMPK activity. This CAMKK2-dependent activation of AMPK can explain the residual AMPK activity observed in *LKB1*-null tumors.

### 4.2.3 Regulation of AMPK by TAK1 (Transforming Growth Factor-β-Activating Kinase 1)

TAK1 is widely regarded as a mitogen-activated protein kinase (MAPKK). It was first reported in 2006 that TAK1 also phosphorylates the Thr172 of AMPK α, suggesting that TAK1 may be the third upstream kinase of AMPK. A large amount of data has accumulated over a decade of research, and TAK1 is now considered to be an AMPK upstream kinase that plays a role in a specific environment or in stress signals (e.g., survival signal) (Neumann 2018). It has been demonstrated that TRAIL can induce protective autophagy in epithelial cells through AMPK activation by TAK1. In *Helicobacter pylori*-infected gastric epithelial cells, inactivation of TAK1 not only inhibits the phosphorylation of AMPK but also inhibits autophagy and cell survival.

### 4.2.4 Regulation of AMPK by AMP, ADP, and ATP

In addition to the abovementioned kinases, the activity of AMPK is precisely regulated by the ratio of AMP/ATP and ADP/ATP in the cells. AMP, ADP, and ATP can bind to the γ regulatory subunit of AMPK. When cells are in a normal physiological state, the ratio of ATP to ADP and AMP is high. High levels of ATP bind to AMPK and inhibit its activity. The cellular contents of ADP and AMP are increased in response to energy stress. Numerous studies have revealed that binding of AMP and/or ADP to AMPK activates the kinase via three complementary mechanisms: (1) AMP-binding promotes the phosphorylation of AMPK at Thr172 by the LKB1 complex. This phosphorylation event can increase AMPK activity up to 100-fold in vitro, although the fold activation in intact cells is usually more modest. It has been suggested that binding of ADP also promotes phosphorylation of AMPK by CaMKK2; (2) AMP binding causes a conformational change that inhibits the dephosphorylation of Thr172 by protein phosphatases. ADP binding also have such effect. (3) The binding of AMP to the AMPK γ subunits leads to allosteric activation of the AMPK complex. Thus, large amounts of AMPK can be activated even in the presence of physiological concentrations of ATP. All three effects of AMP (and the single effect of ADP) are antagonized by the binding of ATP (Fig. 4.1).

### 4.2.5 Regulation of AMPK by Protein Phosphatase

Accumulated evidence indicates that phosphorylated AMPK is rapidly inactivated in vivo via dephosphorylation by protein phosphatase (PP), which is an important component of the regulation of AMPK activity. The protein phosphatase PP2A (protein phosphatase 2A) can interact with AMPK and promote the dephosphorylation of the AMPK Thr172 site to achieve negative regulation of AMPK (Joseph et al. 2015). In the low-energy state, the upregulated AMP/ATP ratio can reduce the inhibitory effect of PP2A on AMPK, thus increasing the phosphorylation of AMPK. It is particularly notable that this inhibition is generated by inhibiting the contact of PP2A with phosphorylated Thr172 residues. The magnesium/manganese ion-dependent protein phosphatase 1E (PPM1E) is also a phosphatase of AMPK and interacts with AMPK and promotes its dephosphorylation.

### 4.2.6 Regulation of AMPK by Other Protein Kinases

Phosphorylation of AMPK at Thr172 is essential for the activation of AMPK and is often used as a indicator for its activity. A growing number of studies indicate that phosphorylation of the ST-loop by other kinases results in the regulation and inhibition of AMPK in various tissues (Hardie 2014). For example, in the heart, skeletal

muscle, and liver, insulin or IGF-1-stimulated the phosphorylation of rat AMPKα1 at Ser485 and human AMPKα2 at Ser491 within the ST-loop by AKT/PKB. Protein kinase A (PKA) has been reported to phosphorylate these sites in human diploid fibroblasts in response to lysophosphatidic acid. PKD1 inhibits AMPKα2 through phosphorylation of Ser491 and impairs insulin signaling in skeletal muscle cells. In human endothelial cells, VEGF-stimulated PKC phosphorylates AMPKα1 at Ser487, which exhibits a strong inverse correlation with insulin sensitivity in human muscle. GSK3β (glycogen synthesis kinase 3β) can phosphorylate Thr481 and Ser477 within the ST-loop, which further inhibits AMPK by promoting Thr172 dephosphorylation. In the hypothalamus, S6K (p70S6 kinase) has been reported to inhibit food uptake by phosphorylating the Ser491 site in AMPKα2. PKA was also found to phosphorylate AMPKα Ser173/Ser175 (within the activation loop and adjacent to AMPKα1 Thr172, the residue equivalent to AMPKα2 Thr174 in humans), which blocks subsequent Thr172/Thr174 phosphorylation. Although the mechanism of AMPK inhibition is not entirely clear, it seems that these inhibitory phosphorylation sites reduce the phosphorylation of Thr172/174, either by physically interfering with its phosphorylation or by promoting its dephosphorylation. Collectively, these phosphorylation events may represent an important mechanism for keeping AMPK activity low during periods when anabolic metabolism is required.

In addition to the regulation of phosphorylation described above, the activity of AMPK is also affected by acetylation/deacetylation, ubiquitination/deubiquification, SUMOylation, and subcellular localization.

## 4.3 Activators and Inhibitors of AMPK

In view of the key role of AMPK in energy metabolism, AMPK has become a potential target for the treatment of diabetes, obesity and cardiovascular disease. At present, some AMPK activators are being tested in clinical or preclinical studies. These activators are classified as direct and indirect activators based on their different mechanisms of action.

### *4.3.1 Direct Activators of AMPK*

#### 4.3.1.1 AICAR (5-Aminoimidazole-4-Carboxamide Ribonucleoside)

AICAR was the first identified AMPK activator and has been widely used as a research tool in AMPK-related experimental studies. AICAR is an adenosine analog that can be phosphorylated by adenosine kinases to produce AICAR monophosphate ZMP. Similar to AMP, ZMP binds to AMPK at the same sites as AMP and mimics all effects of AMP on the AMPK system. It can bind to CBSl or CBS3 sites of AMPK γ subunits and causes both allosteric activation and protection against Thr172

dephosphorylation. This effect does not affect the ratio of AMP/ATP in cells. Human γ1 (R299G) or γ2 (R531G) mutants can inhibit the activation of AMPK by AICAR. Animal experiments have shown that AICAR can activate AMPK in different tissues and promote the translocation of glucose transporter 4 (GLUT4) to the cytoplasmic membrane, thus increasing the transport of glucose to the cell, the utilization of glucose, and oral glucose tolerance, and lowering the concentration of blood sugar.

#### 4.3.1.2 Thienopyridone A769662

A769662 is a synthetic compound identified from a high-throughput screen for allosteric activators of purified AMPK. A769662 acts in an AMP-independent manner. Unlike AMP binding to the AMPKγ subunit, A769662 can bind to the carbohydrate-binding domain (CBD) of the AMPKβ1 subunit and promote self-phosphorylation of the β1 subunit at Serl08. After entering the ADaM-binding site of AMPK, it directly activates AMPK, mimicking the effects of AMP to cause allosteric activation and protect against Thr172 dephosphorylation. These compounds that bind to ADaM sites are known as allogenic activators. The detailed molecular mechanism by which the binding of compounds to the ADaM site causes allosteric activation remains unclear. Importantly, A-769662 neither inhibits the mitochondrial oxygen consumption rate nor increases AMP/ATP and ADP/ATP ratios in intact cells. A769662-induced activation of AMPK is abolished by a mutation within AMPKβ1 (S108A) and in *AMPKβ1* knockout mice. The combination of AMP and A769662 can synergistically produce a hyperactivated state of AMPK, and A-769662 itself will produce the largest effect.

#### 4.3.1.3 Compound 911 (Benzimidazole) and Compound C13/C2

Compound 911 can also selectively activate the AMPK β 1 subunit by binding to the ADaM site. Compound 991 is five- to tenfold more potent than A-769662 in activating AMPK, as assessed by allosteric activation and protection against Thr172 dephosphorylation. Compound 991 efficiently activates AMPK in isolated rodent skeletal muscle without altering cellular AMP/ATP and ADP/ATP ratios.

Compound 13 is a phosphonate diester that is taken up by cells and converted by cellular esterases into C2, an AMP analog. This compound shows improved specificity toward AMPK and is a potent allosteric activator of the kinase. C2 is two—three orders of magnitude more potent as an allosteric activator of AMPK than AMP and four orders of magnitude more potent than ZMP. It is structurally different from A-769662, and the activation of AMPK by compound C2 does not require the presence of the AMPKβ subunit CBD. It may induce allosteric activation by competitive binding to the same CBS site in the AMPKγ subunit of AMP. Compound C2 cannot activate the mutant AMPK complex containing AMPKγ2 (R531G). Additionally, compound C2 shows a preference for AMPKα1-containing complexes. Similar to

AMP, compound C2 can inhibit the Thr172 dephosphorylation of AMPKα1. In contrast, C2 is a partial agonist of AMPKα2-containing complexes and fails to protect against dephosphorylation. It may be a tissue-specific AMPK activator.

#### 4.3.1.4 PF-06409577 (Compound 7)

PF-06409577, also known as Compound 7, is a potent and selective activator of AMPK for the potential treatment of diabetic nephropathy. It is a potent allosteric activator of both human and rat AMPKβ1-containing complexes. It also inhibits the dephosphorylation of Thr172 by the protein phosphatase PP2A. As a specific indole acid-based direct AMPK activator, the molecular mechanism of the activation of AMPK by PF-06409577 is similar to that of A769662 and Compound 911, which involves binding to the ADaM site (Cameron et al. 2016). Remarkably, treatment with PF-06409577 promotes dose-dependent activation of AMPK in whole kidney tissue and improves renal functions in ZSF-1 obese rats, a model of diabetic nephropathy.

#### 4.3.1.5 Salicylate

Salicylate is the natural product from which aspirin is derived. Acetylsalicylic acid, or aspirin, is rapidly decomposed into salicylate following its adsorption from the intestinal tract in vivo. Similar to A769662 or Compound 911, activation by salicylate is selective for AMPKβ1-containing complexes and is abolished by an S108A mutation in AMPKβ1, so these compounds all appear to bind at the same ADaM site. Salicylate administration in vivo increased the activity of AMPK in the liver and adipose tissue of mice and promoted fatty acid oxidation in wild-type mice, but not in AMPKβ1 knockout mice, confirming the involvement of AMPKβ1-containing complexes in the mechanism of action of the drug. Recent studies have indicated that salicylate can activate AMPK by directly interacting with the AMPKβ1 subunit drug-binding site (ADaM) in bone marrow-derived mouse macrophages (BMDMs), mouse livers and primary human hepatocytes, and *ex vivo* prostate and lung cancer cells. It has also been suggested that pharmacological activation of the AMPKβ1-containing complex in macrophages may be beneficial in the early stage of atherosclerosis. In addition, therapeutic concentrations of salicylate and metformin can act synergistically to promote insulin sensitivity and tumor cell death.

### *4.3.2 Indirect Activators of AMPK*

Some physiological hormones and compounds from natural plants such as leptin, adiponectin, resveratrol, berberine, quercetin, and oligomycin can activate AMPK. Data indicate that these molecules reduce the production of ATP, increase the ratios of AMP/ATP and ADP/ATP, and subsequently activate AMPK indirectly. This effect

is achieved mainly by inhibiting mitochondrial respiratory chains or ATP synthase. Notably, AMPK activation by resveratrol, berberine, and quercetin is reduced or eliminated in cells expressing AMP/ADP-insensitive AMPK mutants, such as human γ1 (R299G) mutants or human γ2 (R531G) mutants, indicating that dysfunction of the mitochondrial respiratory chain might be the upstream reason for the AMPK activation effects of these phytochemicals. 2-Deoxyglucose (2-deoxyglucose) can reduce the production of ATP by inhibiting glycolysis and indirectly activating AMPK.

Metformin (metformin), which is widely used in the treatment of type II diabetes, is also an agonist of AMPK. Metformin significantly activates AMPK α 1 and α 2 in isolated rat muscles, accompanied by increased glucose uptake. Metformin activates AMPK by blocking the electron transport of complex I in the mitochondrial respiratory chain, inhibiting the oxidative phosphorylation of mitochondria, reducing the synthesis of ATP in cells, and increasing the ratio of AMP/ATP in cells (Foretz et al. 2014). Metformin can also promote the exonuclear transport of LKB1 to the cytoplasm, thus promoting the phosphorylation and activation of AMPK.

Thiazolidinedione (TZD) is a peroxisomepro-liferator-activated receptor γ (PPAR γ) agonist that can indirectly activate AMPK, increase insulin sensitivity, and improve insulin resistance. These effects can maintain the balance of glucose and lipid metabolism, and TZD has been widely used in the treatment of type II diabetes.

### *4.3.3 AMPK Inhibitors*

A large number of studies conducted in vivo and in vitro have shown that high-sugar, high-fat and high-protein diets can lead to a decrease in AMPK activity in tissues. An increase in glycogen can inhibit the activity of AMPK in skeletal muscle of rats. This effect is independent of the change in the AMP concentration. The main mechanism is that glycogen binds to the CBD of the AMPKβ subunit and inhibits the conformational change in AMPK, thus reducing its activity. AMPK phosphorylation and protein expression are usually deficient in skeletal muscle, heart, liver, hypothalamus, and other tissues in mice fed a high-fat diet.

Compound C/Dorsomorphin is a widely used ATP-competitive AMPK inhibitor showing cell permeability and reversibility. Structural elucidation revealed that compound C strongly binds a phosphorylated state mimic T172D mutant kinase domain of the human AMPKα subunit. Compound C also blocks AICAR cellular uptake through competition for adenosine transporter-binding sites, which largely accounts for its suppressive effects on AICAR-mediated AMPK activation. However, this compound is not a specific target of AMPK. Numerous studies have reported that Compound C has significant "off-target" or AMPK-independent cellular effects (Dasgupta and Seibel. 2018).

SBI-0206965 is a new pyrimidine derivative that was recently discovered to act as a direct inhibitor of AMPK complexes (Dite et al. 2018). Data from in vitro

studies indicate that SBI-0206965 inhibits AMPK with 40-fold greater potency than compound C. The crystalline structure of the AMPK kinase domain/SBI-0206965 complex indicates that SBI-0206965 partially overlaps with the active site of ATP in a mixed-type inhibitory manner. SBI-0206965 not only competitively inhibits ATP but also inhibits the binding of AMPK to the substrate. In addition, SBI-0206965 is an inhibitor of ULK1. This compound provides a useful tool for studying the physiological effects of AMPK and ULK1.

## 4.4 AMPK Regulation of Autophagy

AMPK plays the central role in cell energy metabolism. It is able to regulate a wide variety of metabolic processes either by directly acting on metabolically relevant proteins or by indirectly influencing gene expression. The downstream enzymes inhibited by AMPK include mammalian target of rapamycin (mTOR), acetyl-CoA carboxylase (ACC), fatty acid synthase (FAS), glycerol phosphate acyltransferase (GPAT), and PPARγ coactivator 1α (PGC1α), which are key regulators of protein, fatty acid, glycerolipid, and mitochondrial synthesis, respectively. Such effects maintain an adequate energy balance and homeostasis of the internal environment. Autophagy, as a self-protective mechanism observed in cells under stress, is also regulated by AMPK. AMPK is able to promote autophagy by acting differentially at different levels of autophagy regulation through specific phosphorylation of components of autophagy-related protein complexes.

### *4.4.1 AMPK Localizes to the Late Endosomes/Lysosomes*

As the final degradation site of autophagic substrates, late endosomes/lysosomes play an important role in the clearance of autophagosomes. Recent studies have shown that lysosome-localized AXIN, a scaffolding protein, can interact with AMPK, which recruits AXIN and LKB1 to the surface of lysosomes to form a stable AXIN-AMPK-LKB1 complex (Zhang et al. 2013). In response to energy stress, AMP (not ADP) binds to the AMPKγ subunit, thus increasing the affinity of AMPK-AXIN, promoting its interaction with AXIN-LKB1, increasing the formation of the AMPK-AXIN-LKB1 complex, and Thr72 phosphorylation of AMPK by LKB1. It is worth noting that the N-myristoylation of the AMPKβ subunit is required for the interaction between AMPK and AXIN driven by AMP. Additionally, at least some portion of AMPK may already be located in late endosomes/lysosomes due to the myristoylation of its β subunit.

The lysosomal v-ATPase-Ragulator complex contains p18/LAMTOR1, p14/LAMTOR2, MP1/LAMTOR3, C7orf59/LAMTOR4, and HBXIP/LAMTOR5. This complex plays an important role in the docking of AXIN-LKB1, allowing the formation of a stable “supercomplex” of v-ATPase-Ragulator-AXIN/LKB1-AMPK

on the surface of late endosomes/lysosomes and, ultimately, the activation of AMPK by LKB1. Indeed, knockout of AXIN abolishes the formation of this complex as well as the phosphorylation and activation of AMPK in response to glucose starvation. This mechanism can occur without any changes in cellular AMP/ATP or ADP/ATP ratios. Importantly, adenovirus-based knockdown of Axin in the mouse liver impairs AMPK activation and exacerbates fatty liver after starvation, underscoring an essential role of AXIN in AMPK activation.

In response to nutrients such as amino acids and glucose, the lysosome is an important platform for mTORC1 activation by the v-ATPase-Ragulator-Rag GTPase complex. Lysosomal mTORC1 shows a competitive relationship with AMPK. Therefore, as a dual sensor with sufficient or deficient energy/nutrition, the V-ATPase-Ragulator complex primarily depends on which signal is dominant. The lysosomal docking site for AXIN/LKB1-mediated AMPK activation, which in turn inactivates the Rag GTPase–Ragulator complex, releases mTORC1 from the lysosomal surface and suppresses mTORC1 signaling under glucose starvation conditions. Considering that the lysosome is a recycling center for autophagic cargos, the recruitment of both AMPK and MTORC1 to the lysosome may be critical for the integration of nutrient/energy signaling with autophagy (Zhang et al. 2014).

## *4.4.2 Regulation of Nonselective Autophagy by AMPK*

### 4.4.2.1 AMPK Antagonizes MTORC1 to Regulate ULK Complex Activity

AMPK activation can induce the autophagic process through two different mechanisms: inhibition of the mammalian target of rapamycin (mTOR) protein kinase complex and direct phosphorylation of ULK1 (Unc-51-Like Kinase 1, a mammalian orthologue of Atg1).

There are two different two different mTOR complexes, known as mTORC1 and mTORC2. mTORC1 promotes protein synthesis, lipid biogenesis, cell growth, and anabolism and inhibits cellular catabolism by preventing autophagy. The relationship between mTORC2 and autophagy is not obvious. Most of the signals that affect mTORCl involve interference with TSC (tuberous sclerosis complex) and the small GTP enzyme RHEB (Ras Homolog Enriched in Brain). RHEB binds and activates mTORC 1 in a GTP-binding manner. The TSC complex, which is composed of TSCl and TSC2, shows GTP enzyme activity and can hydrolyze GTP and inactivate GTPase RHEB. Thus, it can inhibit mTORCl activity by regulating RHEB.

When energy levels are low or there is a starvation situation, AMPK is phosphorylated, and activated AMPK inhibits the activity of mTOR in two ways: (1) AMPK directly phosphorylates TSC2 at its T1227 and S1345 sites, which promotes the GAP (GTPase-activating protein) activity of the TSC1/TSC2 complexes. RHEB-GTP is transformed into an inactive RHEB-GDP state, and mTORC1 activation mediated by RHEB is turned off. (2) AMPK directly phosphorylates the Ser772 and

Ser792 sites of RAPTOR, increases 14-3-3 protein binding to RAPTOR, hinders the binding of RAPTOR to MTOR or MTOR substrates, and subsequently inhibits the mTOR signaling pathway (Fig. 4.2). Simultaneously, inactivated mTOR removes the phosphorylation inhibition of ULK1 at Ser757 and induces the binding of ULK1 to AMPK, which is one of the main mechanisms whereby AMPK increases autophagy.

AMPK positively regulates ULK1 activity to induce autophagy under glucose deficiency. AMPK can interact with the Ser/Pro rich region (AA 654-828) of ULK1 and directly phosphorylate ULK1 multiple sites (Table 4.1). Such as human Ser467, Ser556, Thr575, and Ser638 and mouse Ser317 and Ser777. This subsequently leads to a conformational change in ULK1, which promotes the interaction of ULK1 with other components of its complex, such as ATG13, ATG101, and FIP200, and

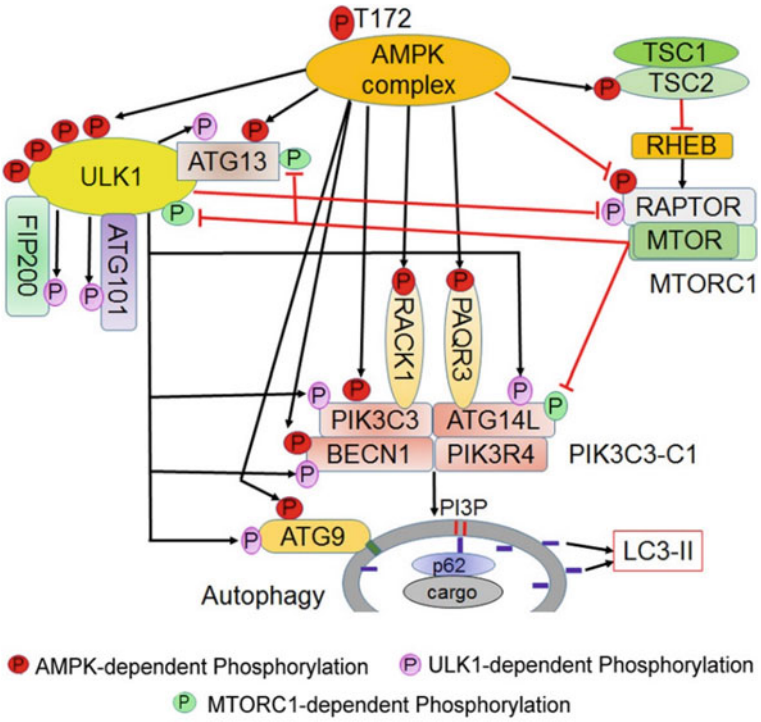


**Fig. 4.2 Regulation of autophagy by AMPK, mTORC1, and ULK1 complexes**. AMPK-mediated phosphorylation events are indicated in red, mTOR-mediated phosphorylation events in green, and ULK1-mediated phosphorylation events are in purple. Upon activation, AMPK phosphorylates TSC2 and RAPTOR, which results in a decrease in mTORC1 activity. AMPK phosphorylates ULK1 and promotes its activity. Conversely, activated ULK1 phosphorylates ATG101, ATG13, and FIP200 and further increases the activity of the ULK1 complex. ATG13 is also phosphorylated by AMPK and mTORC1. PIK3C3-C1 components, RACK1 and PAQR3 are shown with their reported phosphorylation. PIK3C3-C1 mediates the production of PI3P at the surface of the isolation membrane. ATG9 is an integral membrane protein localized at the autophagosomal membrane. It can be phosphorylated by both AMPK and ULK1

**Table 4.1** Regulation of autophagy relevant proteins by AMPK (Tamargo-Gómez and Mariño 2018)

| Protein | Phosphorylation sites(s) | Stage of autophagy | Autophagy function |
|---|---|---|---|
| ULK1 | Ser467(H, M, R)<br>Ser555(M, R)/Ser556(H)<br>Thr574(M, R)/Thr575(H)<br>Ser637(M, R)/Ser638(H)<br>Ser 317(M),Ser777(M) | Autophagy initiation | Component of the ULK1complex |
| PIK3C3 | Thr163(H, M, R)<br>Ser165(H, M, R) | Autophagosome biogenesis | Component of the PIK3C3 complex |
| BECN1 | Ser91(H, M, R)<br>Ser94(H, M, R)<br>Thr388(H, M, R) | Autophagosome biogenesis | Component of the PIK3C3 complex |
| RACK1 | Thr50(H, M, R) | Autophagosome biogenesis | Promotes the assembly of PI3KC3 complex |
| PAQR3 | Thr32(H, M) | Autophagosome biogenesis | Facilitates the formation of PI3KC3 complex |
| RAPTOR | Ser722(H, M)<br>Ser792(H, M) | Regulation of autophagy | Negative regulator of autophagy |
| MTOR | Thr2446 (H) | Regulation of autophagy | Negative regulator off autophagy |
| TSC2 | Ser1342(H, M, R)<br>Thr1227(H, R) | Regulation of autophagy | Negative regulator of MTOR |
| ATG9 | Ser761(H, M, R) | Autophagosome elongation | Participates in the recruitment of lipids to the isolation membrane |
| ATG13 | Ser224(M)/Ser225(H) | Autophagy initiation | Component of the ULK1 complex |

H, human; M, mouse; R, rat

increases the activity and stability of the ULK1 kinase. Activated ULK1 also phosphorylates ATG13, ATG101, and FIP200, further increasing the activity of the ULK1 complex. ULK1 cannot be activated when *AMPK*-knockout MEFs (mouse embryonic fibroblasts) are subjected to glucose deprivation, and ULK1-knockout cells or cells subjected to reconstitution with nonphosphorylatable mutants are also defective in autophagy induction following AMPK activation. These observations demonstrate the functional importance of ULK1 phosphorylation by AMPK in autophagy induction. In addition, the Ser556 site of ULK1 is also a 14-3-3-binding site, and this site increases the interaction between ULK1 and 14-3-3 and promotes autophagy during cell starvation or administration of AMPK activators such as AICAR. Activated AMPK can also phosphorylate the Ser225 (mouse Ser224) site of ATGl3, which is an inhibitory phosphorylation site. This phosphorylation can inhibit autophagy induced by long-term starvation, indicating negative regulation of the ULK1 complex by AMPK.

The interaction between AMPK and ULK1 is regulated by mTORC1. Under nutrient-rich conditions, mTORC1 phosphorylates ULK1 at Ser758 (mouse Ser757), which is an inhibitory phosphorylation modification. This site is located in the region of AMPK binding to ULK1 (AA 711-828), thereby inhibiting the interaction between AMPK and ULK1. Similarly, mTORC1 can also phosphorylate the Ser258 site of ATGl3 (mouse Ser259) (Puente et al. 2016), which can then dissociate from the ULK1 complex, and can reduce their affinity for each other and decrease the activity of the ULK1 complex, thus decreasing the rate of autophagosome formation. Under starvation conditions, the activity of mTORC1 is decreased, and the inhibitory phosphorylation of ULK1 and ATG13 by mTORC1 is simultaneously reduced. Such effects can promote the interaction of AMPK and ULK1 and increase the activity of ULK1 and the formation of the ULK1-ATG13-FIP200 complex. As a result, AMPK and mTORC1 coordinately regulate ULK1 to induce autophagy in response to cellular nutrient levels.

Accumulated reports indicate that there is bidirectional regulation between AMPK/mTORC1 and ULK1. Upon energy starvation, activated ULK1 can phosphorylate AMPK. The phosphorylation sites of AMPK in rats include Ser360/Thr368, Ser397, and Ser486/Thr488 of the AMPKα1 subunit; Ser38, Thr39, Ser68, and Ser173 of the AMPKβ2 subunit; and Ser260/Thr262 and Ser269 of the AMPKγ1 subunit. The above phosphorylation events lead to downregulation of AMPKα phosphorylation at Thr172 and reduce the activation of AMPK (Löffler et al. 2011). ULK1 can also negatively regulate mTORC1. In nutrient-rich conditions, ULK1 interacts with RAPTOR and induces multisite phosphorylation of RAPTOR at sites such as Ser696, Thr706, Ser855, Ser859, Ser863, Ser877, and Ser792. ULK1-dependent RAPTOR phosphorylation has been shown to inhibit both the interaction of mTORC1 with its substrate and the kinase activity of mTORC1 (Dunlop et al. 2011). On the other hand, it has been reported that overexpression of ULK1 can induce autophosphorylation of mTORC1 at Ser2481 and increase the catalytic activity of MTORC1. The existence of such a transient feedback mechanism implies that AMPK, ULK1, and mTORC1 form a signaling triad that fine-tunes the energy/nutrient response to maintain the dynamic balance of autophagy (Dunlop and Tee 2013).

#### 4.4.2.2 AMPK Regulates PI3KC3/VPS34 Complex Activity

PIK3C3/VPS34 forms at least two distinct complexes, PI3KC3-C1 and PI3KC3-C2, which are required for different stages of the autophagy process. The core complex in both PIK3C3-C1 and PIK3C3-C2 contains the catalytic subunit PIK3C3/VPS34, the pseudokinase VPS15/p150 (PIK3R4), and BECN1 (Beclin 1, a mammalian homolog of yeast ATG6). Depending on the subcellular context, this core complex binds to either ATG14L/Barkor or UVRAG (UV radiation resistance-associated gene protein) in a mutually exclusive manner defining the functions of PI3K3C3-C1 and PI3K3C3-C2, respectively. The PI3K3C3-C2 complex functions not only in autophagy (especially in autophagosome maturation and autolysosomal tubulation) but also in endosome trafficking and multivesicular body formation. In the case of the PI3K3C3-C1

complex, it appears to be more specific to autophagy, especially for autophagosome nucleation. ATG14L causes PI3KC3-C1 to function as an early upstream regulator of autophagosome formation by directing the complex to phagophore initiation sites, in which it promotes the production and localization of PI3P (Phosphatidylinositol 3-phosphate) and recruits other downstream autophagy-related molecules and as a result of the formation of autophagosomes.

A growing body of evidence has indicated that AMPK participates in the regulation of the PIK3C3/VPS34 lipid kinase complex upon glucose starvation. AMPK interacts with and phosphorylates BECN1 at Thr388 and increases BECB1 binding to VPS34 and ATG14L, which promotes higher autophagy activity upon glucose withdrawal compared with control group. Similarly, AMPK phosphorylates mouse BECN1 at Ser91 and Ser94, which increases autophagosome formation under nutrient stress conditions (Kim et al. 2013). In the same energy-deficient conditions, AMPK phosphorylates PIK3C3 at the Thr163 and Ser165 sites, which inhibits the non-autophagy activity of PIK3C3. ATG14L functions to target the PIK3C3-C1 complex to the phagophore. In the presence of ATG14L, inhibitory phosphorylation of PIK3C3 is suppressed, but the phosphorylation of BECN1 is greatly increased to activate the complex. ATG14L binding may induce conformational changes in the PIK3C3 complex to mask the availability of Thr163/Ser165 in PIK3C3 and promote the availability of Ser91/Ser94 in BECN1 for phosphorylation by AMPK. Hence, in autophagy-promoting conditions, AMPK activation both increases the activity of pro-autophagic PIK3C3 complexes and inhibits the formation of other different PIK3C3 complexes involved in autophagy-independent processes. Such effects may ensure an adequate level of PIK3C3, which is involved in autophagy. In addition, PIK3C3-C1 is a downstream target of the ULK1 kinase. The AMPK-dependent activation of ULK1, which also phosphorylates PIK3C3, ATG14L, and BECN1 (Table 4.1), further increases the activity of PIK3C3-C1 complexes (Fig. 4.2).

In addition to its activity toward components of the different PIK3C3 complexes, AMPK can also influence their composition by phosphorylating other proteins that are relevant to the formation or stability of PIK3C3 complexes. A recent study documented that PAQR3 (progestin and adipo-Q receptor member 3) specifically promotes ATG14L-containing PI3KC3-C1 complex formation as a scaffold protein, leading to complex activation. PAQR3 appears to connect the PI3KC3-C1 complex with cellular energy status via its Thr32 phosphorylation by AMPK. RACK1 (Receptor for activated C kinase 1) is an ATG14L/PIK3C3 scaffolding protein that participates in the formation of the autophagosome biogenesis complex upon its phosphorylation by AMPK at Thr50. Thr50 phosphorylation of RACK1 increases its direct binding to PIK3R4, Atg14L, and Beclin 1, thus promoting the stability and pro-autophagic activity of PIK3C3 complexes (Fig. 4.2).

In addition to its direct activity toward autophagy-initiating complexes, AMPK can influence autophagic activity by specific phosphorylation of ATG9, a transmembrane protein involved in autophagosome biogenesis by supplying vesicles that contribute to autophagosome elongation. In fact, AMPK mediates the phosphorylation of Ser761 of ATG9, which is also the phosphorylation site of ULK1. Under basal conditions, this phosphorylation is maintained at a low level by ULK1 and AMPK.

However, upon the induction of hypoxic stress (low glucose and oxygen), activated AMPK bypasses the requirement for ULK1 and increases S761 phosphorylation, resulting in increased 14-3-3 interactions and recruitment of ATG9 (and ATG9-containing vesicles) to LC3-positive autophagosomes, thus increasing autophagosome biogenesis.

### *4.4.3 Transcriptional Regulation of Autophagy by AMPK*

AMPK activation leads to selective regulation of a subset of genes involved in autophagy and lysosomal function (Table 4.2). In fact, under stress conditions, AMPK interacts with the FOXO3 (Forkhead box O3) transcription factor and directly phosphorylates multiple sites, including Thr179, Ser399, Ser413, Ser439, Ser555, Ser588, and Ser626. These phosphorylation events promote the nuclear translocation of FOXO3 and its transcriptional activity, in addition to promoting the transcription of downstream autophagy-related genes (Table 4.2) such as *ULKl, PIK3C3/VPS34, BECNl, ATG4, LC3, ATGl2,* and *BNIP3*. The other two members of the FOX transcription factor family, forkhead box protein K1 (FOXK1), and forkhead box protein K2 (FOXK2), compete with FOXO3 to inhibit autophagy-related gene transcription. (Bowman et al. 2014). Under nutrient-rich conditions, mTORC1 phosphorylates FOXK1 and FOXK2, and phosphorylated FOXK proteins then translocate to the nucleus, where they compete with FOXO3 to bind to the same genomic regulatory sites and inhibit autophagy genes. Under nutrition or energy deficiency, AMPK is activated. Activated AMPK further phosphorylates FOXO3 and concomitantly represses mTORC1 activity via the phosphorylation of TSC2 and RAPTOR. Such effects induce the translocation of FOXK1 and FOXK2 from the nucleus to the cytoplasm, thus indirectly promoting the maximal activation of FOXO3 targets.

**Table 4.2** Transcriptional regulation of autophagy through AMPK phosphorylation (Tamargo-Gómez and Mariño 2018)

| Transcription factor | Phosphorylation sites(s) | Target gene(s) |
|---|---|---|
| FOXO3 | Ser399(H) Ser413(H) Ser555(H), Ser588(H) Ser626(H), Thr179(H) | *ATG4B, GABARAPL1, ATG12, ATG14, GLUL, MAP1LC3, BECN1, PIK3CA, PIK3C3, ULK1, BNIP3, FBXO32* |
| CHOP | Ser30 (H, M, R) | *ATG5, MAP1LC3* |
| TP53 | Ser15(H)Ser18(M) | *AEN, DRAM1, DAPK1, PRKAB1,PRKAB2, PTEN, IGFBP3, TSC2* |
| HSF1 | Ser121(H, M, R) | *ATG7* |
| NRF2 | Ser550(M, R) Ser558(H) | *SQSTM1* |
| TP73 | Ser426(H) | *ATG5, DRAM1, ATG7, UVRAG* |

H, human; M, mouse; R, rat

These transcriptional repressors may therefore function to prevent overactivation of autophagy by suppressing gene transcription.

Transcription factor EB (TFEB) is considered a master transcriptional regulator of autophagy, as it upregulates a subset of autophagy and lysosome genes and activates the whole autophagy-lysosome pathway. TFEB binds to DNA sequences known as Coordinated Lysosomal Expression And Regulation (CLEAR) elements, which are present in numerous autophagy and lysosome genes such as *ATG9, LC3, UVRAG, LAMP1,* and *VPS11* and induces their expression. This effect increases autophagosome formation, promotes the fusion of autophagosomes with lysosomes, and increases lysosome function and biogenesis. TFEB is usually located in the cytoplasm. However, stress conditions such as starvation or lysosomal dysfunction result in the translocation of TFEB to the nucleus, where it promotes the transcription of its target genes. The activity and nuclear translocation of TFEB are closely related to its phosphorylated state. In nutrient-rich conditions, mTORC1 can phosphorylate TFEB at the Ser211 site, and the 14-3-3 protein then binds to phosphorylated TFEB to sequester it in the cytoplasm (Martina et al. 2012). In conditions of starvation, when AMPK is active and mTOR is inhibited, dephosphorylation of TFEB drives its import into the nucleus, where it turns on the CLEAR network of genes, which is required for autophagy.

In addition to the control of TFEB *via* suppression of mTORC1, two additional mechanisms by which AMPK may activate TFEB-dependent gene expression have been reported. (1) Upon glucose starvation, activated AMPK phosphorylates FOXO3, phosphorylated FOXO3 has been shown to inhibit the expression of SKP2 (S-phase Kinase-associated Protein 2), which is part of the SCF E3 ubiquitin ligase complex that degrades the histone demethylase CARM1 (Coactivator-Associated arginine (R) Methyltransferase 1). Such effects caused upregulation of CARM1 levels, which acts as a coactivator of TFEB to promote the expression of lysosomal genes. (2) AMPK also phosphorylates acetyl-CoA synthetase 2 (ACSS2) at Ser659 and induces its translocation to the nucleus. Then, ACSS2 interacts with TFEB and increases local acetyl-CoA and histone H3 acetylation at TFEB target gene promoters for transcriptional activation (Li et al. 2017).

BRD4 (Bromodomain-containing protein 4) functions as a transcriptional repressor of the autophagy-lysosome pathway. It is recruited to the promoter regions of various autophagy and lysosome genes and represses gene expression under nutrient-rich conditions. During nutrient starvation, activated AMPK causes SIRT1 activation. It then facilitates BRD4 dissociation from promoters, driving the transcription of autophagy and lysosome genes (Sakamaki et al. 2017). Additionally, AMPK binds and phosphorylates other transcription factors, such as Ser15 (mouse Ser18) of TP53, Ser30 of CHOP (C/EBP homologous protein)/DDIT3 (DNA damage-inducible transcript 3), Ser121 of HSF1 (heat shock factor 1), Ser558 (mouse and rat Ser550) of NRF2 (nuclear factor erythroid 2-related factor 2), and S426 of TP73. These phosphorylation events positively or negatively regulate the expression of downstream autophagy target genes and autophagy.

### *4.4.4 AMPK Regulates Mitophagy*

Mitochondria are the main site of ATP production through cell respiration processes such as oxidative phosphorylation and the tricarboxylic acid cycle (TCA), which plays an important role in regulating cell metabolism. Mitochondria are also sites where many free radicals are produced, such as reactive oxygen species (ROS) and reactive nitrogen species (RNS). Free radicals are not only necessary for signal transduction in cells but also attack mitochondrial DNA (mtDNA), lipids, and proteins, leading to mitochondrial damage. The accumulation of damaged mitochondria results in more ROS, eventually leading to an imbalance of redox, further resulting in exacerbated mitochondrial damage. Therefore, clearance of damaged mitochondria by autophagy is essential to maintain mitochondrial homeostasis.

#### 4.4.4.1 Mitochondrial Localization of AMPK

There are at least two well-characterized substrates of AMPK in mitochondria: mitochondrial fission factor (MFF) and acetyl-CoA carboxylases 2 (ACC2). MFF and ACC-2 localize to the mitochondrial outer membrane and are exposed to the cytoplasm, where they can be contacted by freely floating cytosolic AMPK, making it possible for AMPK to exist at or close to mitochondria. When cellular energy is low, activated AMPK phosphorylates ACC2 at the Ser221 site (inhibitory phosphorylation), leading to inhibition of fatty acid synthesis. MFF is the primary receptor for the dynamin-like protein DRP1 on the mitochondrial outer membrane, which mediates the constriction of mitochondria during fission. AMPK can interact with MFF and phosphorylate it at the Ser155 and Ser173 sites, subsequently recruiting Drp1 from the cytosol to the mitochondrial outer membrane. Upon recruitment, Drp1 promotes the constriction and fission of mitochondria. Thus, AMPK acutely regulates the shape of the mitochondrial network during energy stress by phosphorylating MFF and controlling the localization of DRP1.

Some experimental evidence suggests that myristoylation of the β-subunit of AMPK causes AMPK to localize to mitochondria, and the myristoylation-deficient form of AMPKβ1 (AMPKβ1G2A) is not present in mitochondria. Similarly, inhibition of myristoylation effectively abrogates the membrane recruitment of the AMPK complex. It is likely that myristoyl-CoA protein N-myristoyl transferase 1 (NMT1), but not NMT2, mediates AMPKβ N-myristoylation (Liang et al. 2015). When mitochondria are damaged (such as through CCCP treatment), an approximately threefold increase in AMPK in mitochondrial extracts is observed, suggesting that AMPK is recruited to the mitochondria in response to mitochondrial damage. Additionally, Ser555 phosphorylation of ULK1 by AMPK has been shown to induce the recruitment of ULK1 to mitochondria during mitophagy, suggesting that localized signaling of ULK1 at mitochondria regulates the removal of damaged mitochondria. Given the central role of mitochondria in energy production, it can be hypothesized that a

mitochondrial pool of AMPK may sense and respond more rapidly and efficiently to changes in energy status.

#### 4.4.4.2 Regulation of Mitophagy by AMPK

In addition to regulating nonselective autophagy, AMPK is also associated with the selective autophagy process of mitophagy, in which damaged or stressed mitochondria are engulfed by autophagosomes and transported to lysosomes for degradation. Mitophagy is required to remove unhealthy mitochondria in a timely manner and to maintain steady mitochondrial turnover. In mammals, AMPK deficiency results in aberrant accumulation of the autophagy adaptor SQSTM1/p62 and defective mitophagy (Egan et al. 2011). SQSTM1/p62 binds to specific cargo targeted for autophagy-mediated degradation. In particular, SQSTM1/p62 is bound to dysfunctional mitochondria targeted for mitophagy and is involved in mitochondrial aggregation and clearance. Moreover, the number of mitochondria per cell is significantly increased in AMPK-deficient cells, suggesting that AMPK plays an important role in mitochondrial turnover. Under conditions of stress such as mitochondrial depolarization or inhibition of mitochondrial ATP synthesis, mitochondrial fragmentation is increased by increasing mitochondrial fission rates and/or reducing mitochondrial fusion rates, which is beneficial to facilitate mitophagic processes. Rotenone and antimycin A are inhibitors of the respiratory chain and are also potent activators of AMPK complexes. They can induce mitochondrial fragmentation, which requires the activity of AMPK. In addition, AMPK activation by direct small-molecule activators (such as A769662) in the absence of mitochondrial damage is sufficient to induce mitochondrial fission. Mechanistically, activated AMPK phosphorylates MFF and recruits Drp1 from the cytosol to the mitochondrial outer membrane (Toyama et al. 2016). Upon recruitment, Drp1 promotes the constriction and fission of mitochondria, a prerequisite for the engulfment of mitochondria by mitophagy. The deletion of phosphorylated MFF inhibits mitochondrial fission and hinders mitophagy, which indicates that the AMPK-MFF/DRP1 pathway is necessary for mitochondrial autophagy.

It has been reported that specific phosphorylation of ULK1 at Ser555 by activated AMPK is crucial for ULK translocation to damaged mitochondria in response to stress conditions such as hypoxia or FCCP treatment. Translocated ULK1 further phosphorylates FUNDC1 (FUN14 domain-containing protein1), which is a mitophagy receptor located in the external mitochondrial membrane, at the Ser17 site. This effect allows interaction between FUNDC1 and LC3, resulting in mitochondrial degradation by mitophagy. Therefore, AMPK-mediated phosphorylation of ULK1 is essential for mitophagy initiation under stress conditions such as hypoxia (Wu et al. 2014).

Recent studies have shown that activated AMPK can recruit the PIK3C3 complex and the ATG12-ATG5-ATG16 complex to mitochondria, where it mediates the elongation of the isolation membrane and the maturation of the autophagosome when mitochondria are depolarized. Notably, AMPK interacts with the ATG16 complex

and mediates the retention of the ATG16 complex in damaged mitochondria. Thus, the lipidation of LC3 and the formation of autophagosomes are promoted through the AMPK-ATG16 complex pathway in response to mitochondrial stimuli. Therefore, the recruitment of AMPK in mitochondria may act as a sensor of mitochondrial damage, resulting in temporal and spatial proximity of the autophagy core machinery to the damage site, promoting the assembly of autophagosomes. Because PIK3C3 and BECN1 are direct substrates of AMPK (Table 4.1), the relationship between their phosphorylation and mitophagy requires further study.

Another pathway controlling mitophagy is the PINK1/Parkin-mediated removal of depolarized mitochondria (Nguyen et al. 2016). PINKl is a serine/threonine-protein kinase and a sensor of mitochondrial polarity. Under steady-state conditions, PINKI is transported to the outer mitochondrial intermembrane space and rapidly degraded. Loss of the mitochondrial membrane potential results in the accumulation of PINK1 on the outer membrane and stabilizes it. Stabilized PINK1 recruits the E3 ubiquitin ligase Parkin from the cytoplasm to the mitochondria and phosphorylates it, leading to the activation of Parkin. PINK1 in turn ubiquitinates diverse mitochondrial outer membrane proteins that are involved in the sequestration of mitochondria in an isolation membrane by interacting with adaptor or receptor proteins such as SQSTM1/P62. This pathway requires complete mitochondrial depolarization to be activated and is therefore different from the basal surveillance of mitochondria by the AMPK–ULK1 axis. Whether AMPK and ULK1 participate in the regulation of mitophagy through the PINK1-Parkin pathway remains to be further studied. However, it is worth noting that the proton ionophore CCCP, an inducer of PINK1/Parkin-mediated mitophagy, can effectively induce AMPK activity by inhibiting the synthesis of ATP in mitochondria. Therefore, some downstream targets of AMPK and/or ULK1 may be involved in the PINK1/Parkin signaling pathway, but further experimental evidence is needed to prove this.

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# Chapter 5
# Beclin 1, Bcl-2 and Autophagy


**Hai-Dong Xu and Zheng-Hong Qin**

**Abstract** Beclin 1 is the first mammalian autophagy protein identified as a novel Bcl-2-interacting protein. Subsequent studies have demonstrated that this landmark protein is essential for autophagy. By investigating the interaction between Bcl-2 and Beclin 1, key molecular mechanisms of mammalian autophagy regulation have been discovered. In this chapter, we will first review the discovery of Beclin 1 and then focus on the mechanisms of Bcl-2 and Beclin 1 regulation and their effect on autophagy regulation. Finally, we summarize the evidence related to the interaction of Bcl-2 and Beclin 1 and the involvement of these proteins in human diseases such as cancers, neurodegenerative diseases and infectious diseases.

**Keywords** Beclin 1 · Bcl-2 · Autophagy · PI3K

Autophagy is an ancient and conserved pathway that exists in all eukaryotic cells. During autophagy, cellular components or organelles are delivered to lysosomes for degradation and reuse. In 1963, the Belgian scientist Christian de Duve coined the word "autophagy". After nearly 70 years of research, we are just beginning to understand some of the key processes of autophagy. The important mechanisms involved in this process have been mainly discovered in the single-cell fungus *Saccharomyces cerevisiae* (Takeshige et al. 1992). In the early 1990s, Yoshinori Ohsumi and his colleagues first observed the existence of autophagic bodies in yeast. In the vacuoles (functionally equivalent to the lysosomes of eukaryotic cells) of protease-deficient cells, nutrient deprivation induces autophagic bodies to accumulate. These autophagic bodies contain cytoplasmic ribosomes, coarse endoplasmic reticulum, mitochondria, lipid granules and glycogen granules, which are morphologically indistinguishable from the cytosol (Takeshige et al. 1992). This is the earliest observation of non-selective autophagy in yeast. Using the characteristics of autophagy in yeast, several research groups began to use gene mutations to screen

H.-D. Xu (✉) · Z.-H. Qin (✉)
Laboratory of Aging and Nervous Diseases, Department of Pharmacology, College of Pharmaceutical Science, Soochow University, Suzhou 215123, China
e-mail: hdxu01@suda.edu.cn

Z.-H. Qin
e-mail: qinzhenhong@suda.edu.cn



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for genes involved in autophagy (also known as *atg* genes), which led to the discovery of the first yeast autophagy gene atg5 in 1996. Subsequently, a series of autophagy-related genes were identified in yeast that were involved in the initiation, elongation and maturation of autophagy. As yeast autophagy genes and those in mammals are highly homologous, some mammalian autophagy genes were also cloned. In 1999, the Levine group discovered the first mammalian autophagy-related gene, Beclin 1, the mammalian homologue of yeast atg6 (Aita et al. 1999). The authors demonstrated that Beclin 1 could restore autophagy activity in autophagy-deficient breast cancer cells (Liang et al. 1999). Since then, many yeast autophagy-related genes have been shown to have homologues in mammals, which not only indicates that autophagy is an evolutionarily highly conserved process but also that the key mechanisms of autophagy elucidated in yeast are of important significance for the study of autophagy in mammals. In 2016, the Japanese scientist Yoshinori Ohsumi won the Nobel Prize in Physiology or Medicine for his pioneering work on autophagy in yeast. Now, we recognize that autophagy not only is a response to starvation but also involves a wide range of processes within the cell. Autophagy dysfunction is closely related to many diseases. This chapter focuses on the discovery of the autophagy gene Beclin 1 in mammals and its roles in autophagy regulation and disease.

## 5.1 The Discovery of Beclin 1 and Its Role in Autophagy

In January 2014, Dr. Beth Levine, a researcher at the Howard Hughes Medical Institute and Director of the Center for Autophagy Research at the UT Southwestern Medical Center, received the 2014 Stanley J. Korsmeyer Award from American Society for Clinical Investigation in recognition of her discovery of the genetic regulation of autophagy in mammalian systems, and subsequent work on autophagy pathways has influenced our understanding of many diseases. The demonstration of autophagy regulation in mammalian systems is mainly related to the discovery of Beclin 1.

### *5.1.1 The Discovery of Beclin 1*

The discovery of Beclin 1 was due to the anti-apoptotic protein Bcl-2, the founding member of the Bcl-2 family proteins. The Bcl-2 (B cell lymphoma-2) gene was first discovered in the t(14;18) chromosomal translocation breakpoint of B cell follicular lymphoma (Tsujimoto et al. 1985), and its transcriptional activity is greatly enhanced by the promoter and enhancer of immunoglobulin heavy chain gene localized on chromosome 14. The discovery of Bcl-2 introduced a new category in the apoptosis field, that is, overexpression of Bcl-2 does not promote cell proliferation but prevents cell death. In 1998, the Levine group found that Bcl-2 can reduce the replication of Sindbis virus and virus-induced apoptosis in the mouse brain. While exploring the

molecular mechanism responsible for this observation, they identified a new 60 kDa coiled-coil Bcl-2-interacting protein using the yeast two-hybrid system. Because it has a coiled-coil domain (so "in" suffix) and interacts with Bcl-2 (Becl), it was named Beclin (Liang et al. 1998). The results from the yeast two-hybrid assay also demonstrated that Beclin 1 interacts not only with Bcl-2 but also with Bcl-xl and that the interaction site is present in the 262–450 aa of the Beclin 1 protein. Similar to Bcl-2, overexpression of Beclin 1 in neurons inhibits the replication of Sindbis virus and reduces the apoptosis of the central nervous system (CNS) neurons caused by fatal Sindbis virus infection in vivo (Liang et al. 1998). These studies demonstrate that Beclin 1 is a novel Bcl-2-interacting protein and has a protective role in anti-viral host defences.

### *5.1.2 Beclin 1 Induces Autophagy and Inhibits Tumourigenesis*

In 1999, the Levine group reported the role of Beclin 1 in autophagy (Liang et al. 1999). The Beclin 1 gene is located at a tumour susceptibility locus on chromosome 17q21, which is mono-allelically deleted in 40–75% of sporadic human breast cancers and ovarian cancers. The human breast cancer cell line MCF7 was originally derived from a breast cancer patient with a heterozygous deletion of the 17q21 locus, and the level of Beclin 1 in this cell line was almost undetectable. Stably expressing Beclin 1 enhanced the autophagic activity and degradation of long-lived proteins in MCF7 cells, indicating that Beclin 1 rescues functional autophagy. MCF7 cells overexpressing Beclin 1 displayed several morphologic characteristics similar to those of a less malignant phenotype, including flatter appearance and larger size. In addition to significantly impaired proliferative ability and clonality, overexpression of Beclin 1 itself does not cause cell death. Experiments in nude mice also demonstrated that the ability of MCF7 cells stably expressing Beclin 1 to form tumours was significantly reduced in vivo. These results indicate that Beclin 1 is a negative regulator of mammalian cell growth and tumour formation. Beclin 1 is widely expressed in breast tissue, but the expression level of Beclin 1 is dramatically reduced in tissues of breast cancer patients (Liang et al. 1999). Compared with a previous study showing that autophagy can be induced by the inhibition of the TOR-S6K signalling pathway, this study identified the first mammalian autophagy gene. More importantly, the role of Beclin 1 in autophagy induction and cell proliferation inhibition linked autophagy and anti-tumour together for the first time, indicating that autophagy defects may be an important cause of tumourigenesis. Thus, this study started a new research field for the mechanism of cancer.

## 5.2 Molecular Mechanism of Beclin 1-Regulated Autophagy

The earliest knowledge of autophagy came from yeast. Many proteins in yeast have homologous proteins in mammals, so studies of autophagy in mammals are mostly based on discoveries in yeast. How Beclin 1 regulates autophagy is no exception. Beclin 1 is a mammalian homologue of yeast Atg6/vps30, although the amino acid sequences of Beclin 1 and Atg6/vps30 differ greatly (24.4% sequence identity and 39.1% sequence similarity). Similar to yeast, in mammalian cells, Beclin 1 regulates autophagy by forming complexes with different proteins.

### *5.2.1 Beclin 1 and the Class III PI3K Complex*

#### 5.2.1.1 Beclin 1 and the Class III PI3K form a Complex

PI3K is a family of enzymes that phosphorylate the 3′-hydroxyl group on the inositol ring of phosphoinositides. PI3K regulates many physiological functions by producing phosphorylated phospholipids, that is, signalling molecules including PtdIns(3,4,5) triphosphate and PtdIns(3,4) diphosphate. PI3K is widely involved in intracellular signal transduction pathways, including mitotic responses, cell differentiation, apoptosis, cytoskeletal reorganization and secretory and endocytic pathways of cell membrane flow. PI3K is mainly divided into three classes. Class I PI3K consists mainly of a catalytic p110 subunit and a p85 subunit. The p85 subunit contains an SH2 motif that binds to a phosphorylated tyrosine residue, thus linking the catalytic subunit to the tyrosine kinase signalling pathway. Class I PI3K phosphorylates PtdIns(4,5) diphosphate to produce PtdIns(3,4,5) triphosphate. Class II PI3K is a large kinase (>200 kDa) containing a C2 domain at its carboxy terminus. This kinase phosphorylates PtdIns and PtdIns(4) monophosphate but does not phosphorylate PtdIns(4,5) diphosphate in vitro. Class III PI3K is a homologue of Vps34p in yeast that phosphorylates PtdIns as a substrate to produce PtdIns(3) monophosphate. Yeast cells have only class III PI3K.

The discovery of Beclin 1 has attracted widespread interest in the autophagy field. In 2001, Yoshimori's group at Osaka University reported that Beclin 1 interacts with the phosphatidylinositol 3-kinase (PI3K) complex in the trans-Golgi network (TGN) (Kihara et al. 2001a). Since Beclin 1 is a homologue of Vps30p/Apg6p in mammals, Tamotsu Yoshimori and colleagues wanted to explore whether Beclin 1 also forms a complex with PI3K in mammals. Indeed, the interaction between Beclin 1 and PI3K was confirmed by co-immunoprecipitation and cross-linking experiments. Beclin 1 plays an important role in the production of PtdIns(3)P monophosphate, suggesting that Beclin 1 is essential for both autophagy and lysosomal enzyme transport. All forms of Beclin 1 can form a complex with PI3K, and approximately 50% of PI3K is free and does not form a complex with Beclin 1. Membrane-associated Beclin 1 and

PI3K are primarily localized in the TGN and partially localized in late endosomes (Kihara et al. 2001a). This study demonstrates that the Beclin 1–PI3K complex can produce PtdIns(3)P monophosphate in the TGN and plays an important role in autophagosome formation and sorting of lysosomal proteins.

#### 5.2.1.2 Beclin 1 Regulates Autophagy by Forming Complexes with Different Proteins

In yeast, there are at least two Vps34–PI3K complexes, one containing Vps34, Vps15, Vps30/Atg6 and Apg14, which play a role in autophagy, and the other containing Vps34, Vps15, Vps30/Atg6 and Vps38, which play a role in carboxypeptidase Y (CPY) sorting (Kihara et al. 2001b). Beclin 1 and Vps34 form a complex, suggesting that PI3K complexes that regulate autophagy are also likely to exit in mammals. Indeed, several groups independently demonstrated the presence of complexes that specifically regulate autophagy in mammalian cells, including hVps34, hVps15, Beclin 1 and Atg14L/Atg14/Barkor. In mammals, the counterparts of Vps34, Vps15 and Vps30/Atg6 in yeast are Vps34, p150 and Beclin 1, respectively. The homologue of Vps38 in mammals is UVRAG, which is mainly present in the endosome, while Atg14 is localized on the autophagosome membrane, and the two cannot exist in the same complex (Itakura et al. 2008). Barkor, also known as Atg14, shares an 18% consensus sequence and a 32% similarity sequence with Atg14 in yeast. Barkor can compete with UVRAG for binding to Beclin 1 and activate autophagy (Sun et al. 2008). Two other groups found that both Atg14L and Rubicon can bind to Beclin 1 and regulate autophagy. Atg14L can compete with UVRAG for binding to Beclin 1, while Rubicon binds only to a portion of UVRAG. Rubicon is primarily localized to endosomes and lysosomes, and downregulation of Rubicon promotes autophagosome maturation and endocytic pathways (Matsunaga et al. 2009). These studies suggest that the Beclin 1–hVps34–hVps15 core complex regulates the initiation and endocytic pathways by forming different complexes with Atg14L, UVRAG and Rubicon (Fig. 5.1).

### *5.2.2 Regulation of Autophagy by the Bcl-2–Beclin Complex*

Apoptosis and autophagy are two strictly regulated biological processes in the cell that play a key role in maintaining tissue homeostasis and development. Early studies classified cell death into three forms: apoptosis, autophagic cell death and necrosis. Autophagy is a double-edged sword that has protective and damaging effects, and this raises one key question: Is autophagy involved in cell death or can autophagy cause cell death? In theory, autophagy can remove damaged organelles, toxic metabolites and pathogens and produce energy to maintain cell survival under nutrient-deficient conditions, all of which can promote cell survival. On the other hand, excessive self-digestion and degradation of important intracellular components can lead to

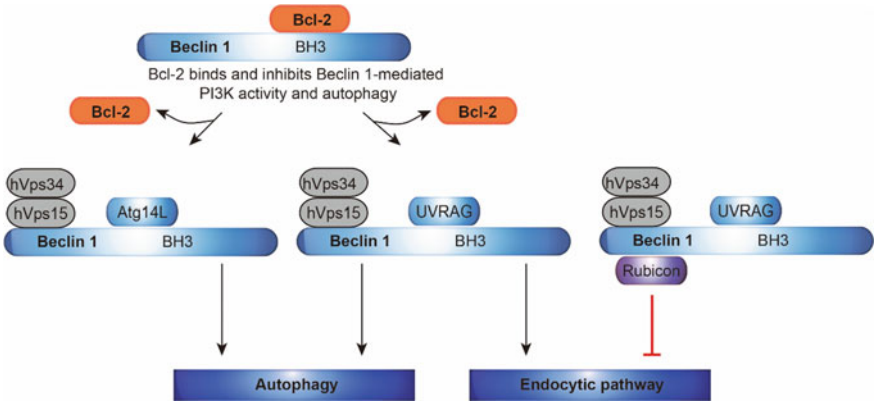


**Fig. 5.1** Model of autophagy and endocytic pathways regulated by Bcl-2–Beclin 1 and III PI3K complexes. Beclin 1 binds to Bcl-2 under normal conditions (top schematic); when Bcl-2 and Beclin 1 dissociate, Beclin 1 forms different complexes with class III PI3K, including complexes containing Atg14L, hVps34, hVps15 and Beclin 1 that can induce autophagy, and complexes containing UVRAG, hVps34, hVps15 and Beclin 1 that promote the endocytic pathway and activate autophagy. Rubicon can inhibit the endocytic pathway activated by UVRAG, hVps34, hVps15 and Beclin 1 (modified with permission from Fig. 1 of Levine et al. Trends Cell Biol. 2015, 25(9): 533–544)

cell death. To date, there is no evidence showing that activation of autophagy can lead to cell death under physiological conditions, so many scientists believe that "autophagic cell death" does not exist and usually refer to autophagic death as "cell death accompanied by autophagy". Consequently, the relationship between autophagy and apoptosis has become a hot topic with the interaction between the anti-apoptotic protein Bcl-2 and the autophagy protein Beclin 1 representing a potential target for exploring the mechanism between apoptosis and autophagy.

#### 5.2.2.1 Bcl-2 Interacts with Beclin 1 and Inhibits Beclin 1-Mediated Autophagy

The interaction between Bcl-2 and Beclin 1 was first discovered in yeast two-hybrid experiments (Pattingre et al. 2005). Both human Bcl-2 and Kaposi's sarcoma-associated herpes virus-encoded vBcl-2 interact with Beclin 1. Bcl-2 family proteins include anti-apoptotic proteins and pro-apoptotic proteins, all of which contain the Bcl-2 homology (BH) domain. Bcl-2 binds to BH3-only pro-apoptotic proteins and inhibits their pro-apoptotic ability. It was found that Beclin 1 also contains the BH3 domain. When the BH3 domain of Beclin 1 is deleted or the BH3 receptor domain of Bcl-xl is mutated, Beclin 1 cannot interact with Bcl-xl. Moreover, when the BH3 domain of Beclin 1 or the BH3 receptor domain of Bcl-xl is mutated, Bcl-xl cannot inhibit Beclin 1-induced autophagy. Meanwhile, knockdown or downregulation of

the BH3-only protein Bad reduced starvation-induced autophagy, while overexpression of Bad induced autophagy (Maiuri et al. 2007). These results indicate that Bcl-2/Bcl-xl can bind to the BH3 domain of Beclin 1 through its BH3 receptor domain. Therefore, these studies suggest that Bcl-2 not only inhibits apoptosis by binding to pro-apoptotic proteins containing the BH3 domain but also regulates autophagy by binding to the BH3 domain of Beclin 1.

In 2005, the Levine group found that wild-type Bcl-2 inhibited Beclin-1-dependent autophagy in yeast and mammalian cells, whereas defective Bcl-2 which cannot bind to Beclin 1 does not have such function. Moreover, the Beclin 1 mutant that does not bind to Bcl-2 induces a higher level of autophagy than wild-type Beclin 1 and can cause cell death (Pattingre et al. 2005). Therefore, Bcl-2 is not only an anti-apoptotic protein but also exerts an anti-autophagy function through interaction with Beclin 1. Bcl-2 can maintain autophagy levels within physiological range. Based on these findings, they proposed a model in which autophagy, as an adaptive response, is necessary for maintaining homeostasis under conditions of nutrient deprivation and other cellular stresses. Blocking autophagy or deleting autophagy genes can cause cell death under stress stimulation. However, if the induction of autophagy exceeds the normal physiological range, hyperactivated autophagy can also lead to cell death. The Beclin 1–Bcl-2 complex plays a rheostat function in the cell and ensures that autophagy levels are maintained within the physiological levels (Fig. 5.2) (Pattingre et al. 2005). Our group also found that serum starvation induces autophagy and the upregulation of Bcl-2 in neuroblastoma SH-SY5Y cells. Downregulation of Bcl-2

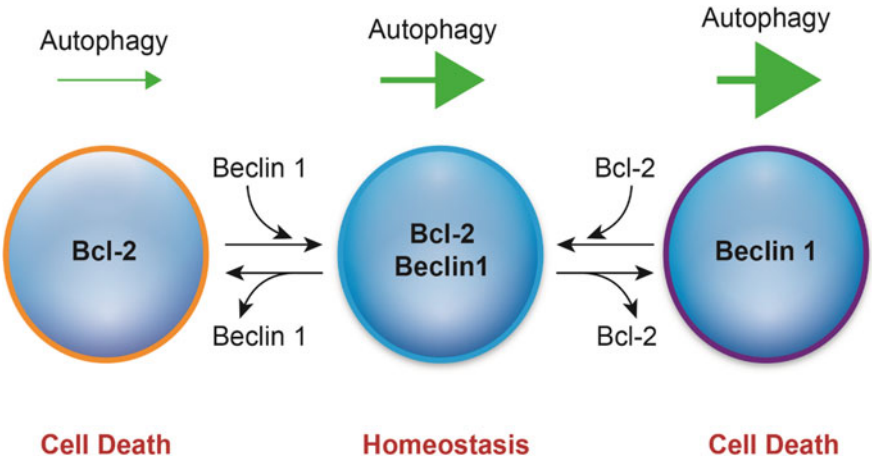


**Fig. 5.2** Model of autophagy regulated by the Bcl-2–Beclin 1 complex. Under normal conditions, the dynamic interaction of the Bcl-2–Beclin 1 complex regulates autophagy within a normal range to maintain homeostasis (middle). Loss of the autophagy gene Beclin 1 decreases autophagy activity to an abnormal level, leading to cell death under nutrient deprivation and other stress conditions (left). Conversely, loss of Bcl-2 induces autophagy overactivity that can also lead to cell death (right) (modified with permission from Fig. 7 of Pattingre et al. Cell, 2005, 122, 927–939)

overactivates autophagy and causes cell death upon serum starvation, while inhibition of autophagy or downregulation of Beclin 1 partially rescues cell death (Xu et al. 2013). Our study suggested that the protective effect of autophagy is dependent on Bcl-2 under serum starvation and that downregulation of Bcl-2 leads to the overaction of autophagy and induces cell death under serum starvation. Unlike Bcl-2 family members that regulate apoptosis mainly in mitochondria, the Bcl-2–Beclin 1 complex regulates autophagy in the endoplasmic reticulum. Similar to apoptosis, the interaction between Bcl-2 and Beclin 1 is regulated by a variety of signals, including apoptosis, indicating that the regulation of apoptosis and autophagy may share the same mechanism.

#### 5.2.2.2 Regulation of the Bcl-2–Beclin 1 Complex by Bcl-2 Phosphorylation

Early studies on Bcl-2 phosphorylation suggest that it plays a role in apoptosis. Bcl-2 phosphorylation blocks its binding to other BH3-only proteins. Studies on protein structures indicate that Bcl-2 binds to Beclin 1 through its BH3 domain, which suggests that Bcl-2 phosphorylation may inhibit its binding to Beclin 1. Yong-Jie Wei et al. found that starvation can induce the phosphorylation of Bcl-2 in multiple sites including threonine 69, serine 70 and serine 87 in its non-structural loops. Phosphorylated Bcl-2 dissociates with Beclin 1 and induces autophagy (Wei et al. 2008). Thus, phosphorylated Bcl-2 loses its ability to inhibit autophagy. JNK1 (c-Jun N-terminal protein kinase 1) but not JNK2 phosphorylates Bcl-2, dissociates the Bcl-2–Beclin 1 complex and induces autophagy. Starvation-induced JNK1 mainly phosphorylated Bcl-2 localized at the endoplasmic reticulum (Wei et al. 2008). This landmark study reveals the molecular mechanisms underlying autophagy regulation in higher eukaryotes.

Exercise can also induce Bcl-2 phosphorylation, disrupting the Bcl-2–Beclin 1 complex and inducing autophagy (He et al. 2012). It is well known that exercise has health benefits. In fact, a lack of exercise is associated with many chronic diseases such as diabetes. To study the role of autophagy in exercise, the Levine group trained GFP-LC3 transgenic mice to run on a treadmill. LC3 is a marker of autophagosomes and forms punctate structures on the autophagosome membrane. They found that the skeletal muscle and myocardium of mice had GFP-LC3 punctate structures after 300 m of training over 30 min indicating autophagy activation. The autophagosome formation peaked at approximately 900 m over 80 min of exercise. Exercise also dissociates the Bcl-2–Beclin 1 complex. To further investigate the mechanism, they produced a Bcl-2 mutant mouse in which three phosphorylation sites were mutated, Thr69Ala, Ser70Ala and Ser84Ala. This mouse is referred to as the Bcl-2 AAA mouse. The basal autophagy levels in AAA mice are normal, while under stress conditions, the induction of autophagy is impaired due to the mutant Bcl-2 failing to dissociate from Beclin 1. Under starvation or acute exercise conditions, Bcl-2 could not be phosphorylated in Bcl-2 AAA knock-in mutant mice, and thus Bcl-2–Beclin 1 could not dissociate and induce autophagy. Bcl-2 AAA mice showed a decrease

in acute exercise tolerance and abnormal glucose metabolism. The authors further found that long-term training improved glucose intolerance in wild-type mice due to a high-fat diet but could not improve glucose intolerance in Bcl-2 AAA mice (He et al. 2012). Therefore, exercise can also induce autophagy via regulating Bcl-2 phosphorylation, but the signalling pathway involved in exercise is still unknown.

#### 5.2.2.3 Bidirectional Regulation of the Bcl-2–Beclin 1 Complex by Beclin 1 Phosphorylation

In addition to Bcl-2 phosphorylation, which regulates the Bcl-2/Bcl-xl/Beclin 1 complex, Beclin 1 can also be phosphorylated by a variety of kinases to regulate autophagy. It is worth noting that the regulation of autophagy by Beclin 1 phosphorylation is bidirectional. Depending on the upstream signal kinase involved, phosphorylation of Beclin 1 can activate autophagy or inhibit autophagy.

##### Phosphorylation of Beclin 1 Activates Autophagy

The death-inducing kinase (DAPK) could interact with Beclin 1. Overexpression of DAPK phosphorylates the threonine 119 site of the BH3 domain of Beclin 1 and promotes the dissociation of Beclin 1 and Bcl-xl, thus inducing autophagy (Zalckvar et al. 2009). However, it is unclear whether endogenous DAPK is necessary for the dissociation of the Beclin 1–Bcl-xl complex, and if so, under what conditions. The role of DAPK in cell death and tumour suppression is also unclear. Another kinase capable of phosphorylating T119 of Beclin 1 is serine/threonine Rho kinase 1 (ROCK1). The interaction between ROCK1 and Beclin 1 was discovered by proteomics methods. Under nutrient deprivation conditions, ROCK1 phosphorylates T119 of Beclin 1 and causes its dissociation from Bcl-2. Autophagy induction is impaired in ROCK1 knockout mice (Gurkar et al. 2013). These results indicate that ROCK1 is a key regulator upstream of Beclin 1.

Two members of the p38 mitogen-activated protein kinase (MAPK) family, MAPKAPK2 (MK2) and MAPKAPK3 (MK3), also phosphorylate the serine 90 site in the BH3 domain of Beclin 1. Phosphorylation at this site is important for the tumour suppressive effect of Beclin 1 in MCF7 breast cancer cells (Wei et al. 2015). MK2/MK3 is able to phosphorylate the S90 site of Beclin 1 in vitro. However, its interaction with Beclin 1 has not been confirmed. A remaining question in Beclin 1 phosphorylation-mediated autophagy is whether phosphorylation of Bcl-2 and Beclin 1 occurs synergistically or independently during autophagy activation.

##### Phosphorylation of Beclin 1 Inhibits Autophagy

Phosphorylation of the serine/threonine site in the BH3 domain of Beclin 1 does not always activate autophagy. The Sadoshima group reported that the pro-apoptotic

kinase Mst1 inhibits autophagy and impairs protein quality control (Maejima et al. 2013). Mst1 is induced under stress and phosphorylates the T108 site of the BH3 domain of Beclin 1. In contrast to phosphorylation of T119, phosphorylation of T108 enhances the interaction between Beclin 1 and Bcl-2/Bcl-xl and stabilizes the Beclin 1 homodimer which inhibits the PI3K kinase activity of the Atg14L–Beclin 1–Vps34 complex and ultimately inhibits autophagy. Moreover, Mst1 enhances its interaction with Bcl-2/Bcl-xl by phosphorylating Beclin 1 which activates Bax activity and induces apoptosis (Maejima et al. 2013). This study suggested that different phosphorylation sites in Beclin 1 have different effects on the interaction of Beclin 1 with Bcl-2/Bcl-xl. Mst1 can regulate autophagy and apoptosis by phosphorylating Beclin 1 and regulating the interaction between Bcl-2, Beclin 1 and Bax. Whether Beclin-1-mediated autophagy is directly involved in the apoptotic pathway remains unclear, and the function of Mst1 in autophagy and apoptosis is still under-studied.

#### 5.2.2.4 A BH3-Only Protein/Mimetic Induces Autophagy by Disrupting the Bcl-2–Beclin 1 Complex

Another pathway that regulates Bcl-2/Bcl-xl interactions and activates autophagy includes competition for BH3-domain-only proteins (such as Bad and EGL-1 in *C. elegans*) or BH3 peptide mimetics (such as ABT737), which competitively disrupt the interaction between Bcl-2/Bcl-xl and Beclin1 (Maiuri et al. 2007). The affinity between Bcl-2/Bcl-xl and Beclin 1 is low; therefore, if the abovementioned BH3 protein or peptide binds to Bcl-2/Bcl-xl with higher affinity, then the interaction between Bcl-2/Bcl-xl and Beclin 1 can be easily disrupted. This mechanism may exist under physiological conditions since downregulation of Bad or knockout of EGL-1 can impair starvation-induced autophagy in mammals and nematodes, respectively. BH3 mimetic peptides such as ABT737 have been shown to induce apoptosis, but it is unclear how much such function can play a role in tumour suppression. If BH3 mimetics that selectively induce autophagy can be designed that can inhibit the anti-autophagy activity of the Bcl-2 family proteins without affecting their anti-tumour activity, these may be of strong therapeutic value against tumours, neurodegenerative diseases and ageing.

### *5.2.3 Regulation of Beclin 1 by Tumour-Related Signalling Pathways*

As mentioned before, Beclin 1 was originally identified as a tumour suppressor and is capable of inducing autophagy, which inherently links autophagy to tumourigenesis. However, the mechanism of how Beclin 1 induces autophagy and inhibits tumours remains unclear. At present, the role of autophagy in tumourigenesis is controversial, partially because of the overlap between tumour-associated signalling pathways and

autophagy signalling pathways. Indeed, many signalling pathways can affect both tumourigenesis and autophagy. For example, the class I PI3K signalling pathway that can target downstream mTOR signalling and regulate autophagy is the frequently aberrant signalling pathway in tumours. However, in addition to inhibiting autophagy, mTOR has many other functions, such as the regulation of protein synthesis. Furthermore, except for oncogenic signals, mTOR is also regulated by many other signals, such as stress, amino acids and energy status. Therefore, the real role of oncogenic signalling suppression of autophagy via mTOR in tumourigenesis is unclear.

#### 5.2.3.1 Akt Phosphorylates Beclin 1

Akt itself can inhibit autophagy by activating mTOR. It has been recently discovered that Akt interacts with Beclin 1 and inhibits autophagy by phosphorylating the serine 234 and serine 295 sites of Beclin 1 (Wang et al. 2012). A Beclin 1 mutant (S234A/S295A) unable to be phosphorylated by AKT enhances autophagy, reduces anchorage-independent growth and inhibits Akt-driven tumourigenesis. Further studies have revealed that Beclin 1 interacts with 14-3-3 and vimentin, one of the intermediate filament proteins. Binding with these two proteins decreases Beclin 1-mediated autophagy. Akt could phosphorylate Beclin 1 and promote its interaction with 14-3-3/vimentin. Thus, Akt inhibits autophagy and promotes tumourigenesis by phosphorylating Beclin 1, which is achieved by regulating the Beclin 1/14-3-3/vimentin complex and autophagy. This study suggests that oncogene signalling can directly target Beclin 1 and inhibit autophagy activity.

#### 5.2.3.2 EGFR Phosphorylates Beclin 1

Cell surface receptors can integrate external environmental stimuli to regulate intracellular processes. Epidermal growth factor receptor (EGFR) tyrosine kinase has recently been found to directly regulate autophagy (Wei et al. 2013). Activated EGFR interacts with Beclin 1 and phosphorylates multiple tyrosine residues, Y229, Y233 and Y352 of Beclin 1, which promotes the formation of Beclin 1 homodimers and binding to Rubicon. Interaction with Rubicon inhibits Beclin 1-associated Vps34 kinase activity and eventually inhibits autophagy. In non-small cell lung cancers (NSCLCs), EGFR is mutated and constitutively activated. Treatment with EGFR tyrosine kinase inhibitor (TKI) can disrupt the phosphorylation of Beclin 1 on tyrosine sites and its binding to the inhibitor Rubicon, thereby restoring autophagy activity. Constitutively phosphorylated Beclin 1 mutant protein enhances the formation of Beclin 1 homodimers. Xenografting of the Beclin 1 mutant in NSCLCs inhibits autophagy and promotes tumour formation and cell proliferation, and it makes tumour cells partially resistant to treatment with EGFR tyrosine kinase inhibitors. Therefore, Beclin 1 is not only a tumour suppressor but also prevents tumour progression and regulates chemotherapy responses.

#### 5.2.3.3 AMPK Phosphorylates Beclin 1

Liver kinase B1 (LKB1)/AMPK is an important tumour suppressor that targets multiple downstream signalling molecules including p53 and mTOR. AMPK differentially regulates Vps34 complexes. AMPK inhibits the activity of the Vps34 complex by phosphorylating threonine 163/serine 165 of Vps34. Upon glucose starvation, AMPK upregulates Vps34 complex activity and activates autophagy by phosphorylating the serine 93 and serine 96 sites of Beclin 1 (Kim et al. 2013). Therefore, AMPK regulates autophagy by affecting Beclin 1–Vps34 complex activity to cope with different nutrient conditions.

### *5.2.4 Beclin 1 Ubiquitination*

In addition to phosphorylation, Beclin 1 can also be regulated by ubiquitination. Autophagy participates in innate and adaptive immune processes. Toll-like receptor 4 (TLR4) is capable of recruiting Beclin 1 and inducing autophagy. In macrophages, tumour necrosis factor receptor-associated factor 6 (TRAF6)-mediated ubiquitination of lysine 63 (K63) linkage is critical for TLR4-induced autophagy. The two TRAF6 binding motifs within Beclin 1 facilitate the binding of TRAF6 and its ubiquitination. The lysine 117 site in the BH3 domain of Beclin 1 is the major site for K63-linked ubiquitination. Deubiquitinating enzyme A20 reduced K63-mediated ubiquitination of Beclin 1 and attenuated autophagy activation. Interferon γ and interleukin 1 can also induce K63-linked Beclin 1 ubiquitination and induce autophagy (Shi and Kehrl 2010). Beclin 1 ubiquitination-regulated autophagy plays an important role in the inflammatory response. Another player that has a role in Beclin 1 ubiquitination is WASH (Wiskott-Aldrich Syndrome Protein (WASP) and SCAR homologues) (Xia et al. 2013). WASH interacts with Beclin 1 and inhibits the ubiquitination of Beclin 1. The E3 ligase Ambra1 promotes K63-linked ubiquitination at the lysine 473 site of Beclin 1, which is necessary for the induction of autophagy during starvation. Ubiquitination of Beclin 1 at the lysine 473 site enhances its binding to Vps34 and promotes Vps34 activity. WASH downregulates Vps34 activity and inhibits autophagy by inhibiting ubiquitination of Beclin 1. Our group found that sphingosine kinase 2 (SPK2) binds to the BH3 domain of Bcl-2 and releases Beclin 1 during ischaemic pre-conditioning, thereby activating autophagy and protecting nerve cells from ischaemia (Song et al. 2017). Our study suggested that certain kinases may also activate autophagy by directly regulating the Bcl-2–Beclin 1 complex independent of post-transcriptional modifications.

### 5.2.5 The Discovery of Beclin 2 and Its Function

Beclin 1 is not alone in the protein family, as Beclin 2 was recently identified in mammals as a homologue of Beclin 1. Sharing 57% sequence identity (He et al. 2013), Beclin 2 also contains a BH3 domain and a coiled-coil domain. Therefore, similar to Beclin 1, Beclin 2 also interacts with the class III PI3K complex and Bcl-2 to regulate autophagy. Beclin 2 differs from Beclin 1 in its N terminus. The N terminus of Beclin 2 binds to G protein-coupled receptor-associated sorting protein 1 (GASP1) and functions in the endocytic transport pathway. Beclin 1 does not have this ability. Beclin 2 regulates endocytosis primarily by promoting the degradation of GASP1-related G-protein coupled receptors (GPCRs), including the δ-opioid receptors, the cannabinoid type I receptors and the non-recycling mutant β-adrenergic receptors. Beclin 2 selectively degrades the GPCRs by lysosomes independent of the class III PI3K complexes (He et al. 2013). This suggests that Beclin family proteins play roles in both autophagy and endocytosis. The close relationship between the mammalian autophagy-lysosomal pathway and the cell membrane-based endocytic pathway allows mammals to adapt to the complex external environment and timely adjust the distribution and number of cell membrane receptors to maintain cell homeostasis.

### 5.2.6 Beclin 1 Interactome

In recent years, functionally distinct Beclin 1–PI3K complexes have been identified, and the mechanism of the interaction between Beclin 1 and the autophagy inhibitor Bcl-2/Bcl-xl has also been studied. These studies suggest that under different physiological and pathological conditions, Beclin 1 is likely to play different roles by interacting with different components. The increasing discovery of new Beclin 1 interaction members has led to the use of the name “Beclin1 interactome” by some scholars (He and Levine, 2010).

## 5.3 The Significance of Beclin 1–Bcl-2-Regulated Autophagy in Diseases

Beclin 1 is conserved in different species, such as plants, moulds, nematodes, fruit flies, mice and humans. Dysfunction of Beclin 1 increases susceptibility to diseases such as cancer, Alzheimer’s disease, Huntington’s disease and microbial pathogenicity, affecting apoptotic cell clearance and normal development. The unanswered question is whether these phenotypes are the direct result of autophagy defects or the result of other unknown dysfunctions of Beclin 1.

### 5.3.1 The Role of Beclin 1 in Cancer

Recent studies have shown that autophagy can both inhibit and promote tumours. In either case, autophagy disorders have a strong correlation with tumourigenesis.

As mentioned before, some signalling pathways of autophagy and tumourigenesis overlap with each other. Tumour suppressor genes such as PTEN, TSC1 and TSC2 can inhibit the mTOR pathway and activate autophagy, while the proto-oncogenes class I PI3K and Akt can activate mTOR and inhibit autophagy activation. Akt could also directly phosphorylate Beclin 1 and inhibit autophagy. The widely studied tumour suppressor p53 is frequently mutated in human cancer. Following DNA damage induction, p53 can activate autophagy possibly by activating AMPK and inhibiting mTOR or by inducing the lysosomal protein DRAM and activating autophagy. The death-related kinase DAPK also activates autophagy and apoptosis, but it is usually methylated in tumours and not expressed, suggesting that the loss of DAPK may play a role in tumourigenesis. The proto-oncogene Bcl-2/Bcl-xl is highly expressed in human tumours due to dysregulated transcription. It is generally believed that Bcl-2/Bcl-xl can inhibit apoptosis and eventually promote tumourigenesis by inhibiting mitochondrial membrane permeability and the release of apoptotic factors.

Beclin 1 is the first gene that connects autophagy and human tumours (Liang et al. 1999). Beclin 1 is localized to tumour-sensitive sites and is mono-allelically deleted in most human breast, ovarian and prostate cancers. It has also been reported that the expression of Beclin 1 decreases in breast, ovarian and brain tumours. Overexpression of Beclin 1 inhibits tumour cell growth and tumour cell formation in vivo. The mono-allelic deletion of Beclin 1 may play an important role in tumour formation, as direct knockout or mutation at a single Beclin 1 locus predisposes to the development of sporadic tumours such as lymphoma, lung tumour, liver tumour, precancerous tissue damage and accelerates the carcinogenic effect of hepatitis B virus in mice. Immortalized kidney and epidermal cells isolated from Beclin 1 heterozygous deficient mice are more tumourigenic than cells isolated from wild-type mice.

Although autophagy gene defects are prone to causing tumourigenesis, little is known about what role autophagy plays in tumour suppression. Increasing evidence has shown that autophagy inhibits tumours but not through promoting cell death or survival. Epithelial tumour cells with Beclin 1 and atg5 single or biallelic knockouts showed no reduction in death but an increase in cell death under metabolic stress conditions, possibly due to autophagy defects affecting cell survival. In Atg gene-deficient cells, the phenomenon of increased cell death and enhanced tumourigenicity indicates that the ability of autophagy to promote survival does not necessarily promote tumourigenesis.

The group of Eileen White found that impaired autophagy promotes chromosomal instability and that dysregulated autophagy fails to maintain metabolic balance, which increases DNA damage, gene amplification and aneuploidy (Mathew et al. 2007). Therefore, the authors propose two possibilities for autophagy defects to promote tumours. First, when tumour cells cannot undergo apoptosis under metabolic

stress, autophagy may prevent cell necrosis, which may aggravate local inflammation and increase tumour growth rates. Second, Atg gene deletion promotes chromosomal instability in cells under metabolic stress, leading to proto-oncogene activation and tumour formation. Immortalized epithelial cells with Atg gene deletion show increased DNA damage, centrosome abnormalities, aneuploidy, chromosome number and structural abnormalities, and gene amplification, especially under ischaemic stress conditions. In recent years, researchers have favoured the view of a double-edged sword role of autophagy in tumours. In normal tissues, autophagy could inhibit tumourigenesis by eliminating harmful substances and maintaining homeostasis. However, in some tissues with highly developed tumours, nutrient stress often occurs, and autophagy can degrade macromolecules and buffers the metabolic pressure of tumour tissues, thus maintaining the survival of tumour tissues under stress. Therefore, activation of autophagy in normal tissues can inhibit tumour formation, but inhibition of autophagy may be beneficial to the treatment of advanced tumours. Tumours are increasingly recognized as a metabolic disease, and many tumour treatments can induce autophagy directly or indirectly. However, the role of autophagy activation in cancer therapy remains unclear. An in-depth study of the role of Beclin 1 in tumours will shed light on the mechanism of tumourigenesis.

### *5.3.2 The Role of Beclin 1 in Neurodegenerative Disease*

It is widely accepted now that abnormalities of the autophagy-lysosomal pathway are one of the underlying causes of neurodegenerative diseases. However, the real role of autophagy is far more complicated and still unclear. The role of Beclin 1 in neurodegenerative diseases represents just one aspect of autophagy. Alzheimer's disease (AD) is characterized by amyloid plaques, neurofibrillary tangles and extensive cerebral cortical neuronal loss. It is generally believed that the protein level or activity of Beclin 1 is decreased in AD. Beclin 1 can be cleaved by caspase, and the cleavage site affects its function in AD. Beclin 1 can also be sequestrated from the cytosol by aggregated tau tangles. It has been reported that the expression of anti-apoptotic proteins such as Bcl-2/Bcl-xl in the brain increases with age, and Beclin 1 can also lose its function by an enhanced interaction with these inhibitors. For example, a recent report showed that the Beclin F121A mutant protein, which decreases the interaction with Bcl-2, accelerates amyloid oligomer clearance and improves cognitive function in an AD model (Rocchi et al. 2017). In addition, many inflammasome-related proteins interact with Beclin 1 and inhibit autophagy. For example, NLRP4 has a high affinity to and interacts with Beclin 1 through its NACHT domain to inhibit autophagy; other proteins such as NLRP3 also have similar functions. Knocking out NLRP3 reduces amyloid β-aggregates and improves memory and behaviour in APP/PS1 transgenic mice, and Beclin 1 is thought to play a role. Huntingtin (Htt) is the causative protein of Huntington's disease. Early studies in our laboratory found that autophagy regulates the processing of the amino terminal huntingtin fragment and its lysosomal-dependent degradation (Qin et al. 2003). Further study found that Beclin 1 regulates

the accumulation of amino terminal Htt fragments in the cells (Wu et al. 2012). These studies suggest that Beclin 1-mediated autophagy plays an important role in many neurodegenerative diseases characterized by abnormal protein aggregation.

### 5.3.3 *The Role of Beclin 1 in Embryonic Development and Infectious Diseases*

Beclin 1 plays an important role in development. The embryos lacking Beclin 1 had delayed development and were smaller on 7.5 days. The isolated Beclin 1 knockout embryonic stem cells showed a reduced ability to clear cells at the centre of the embryo, and numerous cell deaths occurred in the late stage of embryogenesis. Further studies have found that during embryonic cavitation, Beclin 1 and Atg5 can maintain ATP homeostasis in apoptotic cells through autophagy, which is necessary for the appropriate "eat-me" signal in apoptotic cells (Qu et al. 2007). Therefore, in Beclin 1 knockout embryos, apoptotic cells cannot produce appropriate "come-get-me" signals and cannot be eliminated. The true cause of the numerous cell deaths in embryos is still unclear.

Autophagy plays an important role in infectious diseases, and changes in the expression of autophagy-related genes have a great impact on virus invasion. A previous study showed that Beclin 1 overexpression can protect from the lethal effect of Sindbis virus. On the other hand, pathogenic microorganisms can also invade the body by altering autophagy activity. Herpes simplex virus-1 (HSV-1) can induce lethal brain inflammation in mice, during which a virus-encoded neurovirulence factor binds to the host's Beclin 1 and inhibits autophagy activity. When this viral factor is mutated, the pathogenicity of HSV-1 is dramatically reduced (Orvedahl et al. 2007). In contrast to neurodegenerative diseases mainly caused by impaired autophagic activity, the virus can directly abolish the host's autophagy defence system and cause disease, even though the autophagy function of the host is normal. Selective disruption of the interactions between microbial virulence factors and autophagy proteins in the host may be a new anti-microbial treatment strategy.

## 5.4 Summary

In the past 10 years, Beclin 1 has been identified, and its important function in autophagy has been elucidated. Many Beclin 1-interacting proteins and new members of the Beclin 1–PI3K complex have been identified, a new mechanism of Bcl-2/Bcl-xl/Beclin 1 complex regulation has been discovered, and novel inhibitors of Beclin 1 have been identified. Those studies demonstrate that Beclin 1 functions not only during autophagosome formation but also during autophagosome and endosome maturation. The interaction between Beclin 1 and its positive and negative regulators

at different times and locations is likely to regulate the above process. In the next few years, knowledge of Beclin 1 and its interacting partners will be further amplified. Using genetic and proteomic screening, more Beclin 1-interacting proteins will be identified, and Bcl-2–Beclin 1 interactions are the cornerstone of these studies. These studies will deepen our understanding of the molecular mechanisms that maintain cell homeostasis through fine-tuning autophagy levels and will ultimately provide a therapeutic basis for clinical autophagy-deficient diseases.

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# Chapter 6
# TP53, TP53 Target Genes (DRAM, TIGAR), and Autophagy


**Wanglai Hu, Song Chen, Rick F. Thorne and Mian Wu**

**Abstract** The tumor suppressor gene Tp53 encodes p53, a pivotal transcription factor with a broad target gene repertoire. Induction and stabilization of p53 during DNA damage and oncogene activation function to induce cell cycle arrest, apoptosis, or senescence. These actions are a failsafe to counteract carcinogenesis but Tp53 also plays a key role in regulating different aspects of cell metabolism including autophagy. Autophagy or cellular "self-eating" involves the dismantling and remodeling of cellular components, activities which are fundamental in maintaining cellular homeostasis and in supporting cell growth. After providing an historical overview of Tp53 research, the purpose of this chapter is to review the different mechanistic aspects of Tp53's role in autophagy and to highlight the key challenges which lie ahead. Tp53 functions are regulated by tight control of its cellular levels and notably, Tp53 can be both an activator or inhibitor of autophagy. Under stress conditions such as nutrient depletion or hypoxia, Tp53 contributes to autophagic activation by inhibiting mTOR signaling. Alternatively, p53 can interact with death-associated protein kinase 1 (DAPK1), acting to stabilize nuclear p53 amongst other functions including activation of the key autophagic mediator, Beclin-1. Under normal physiological conditions, Tp53 can inhibit autophagosome formation but stress conditions can also result in Tp53-mediated promotion of autophagy, demonstrating that Tp53 actions are highly context dependent. Tp53 target genes also play key opposing roles in autophagy induction or inhibition such as DRAM and TIGAR, respectively. Finally, the role of Tp53 mutants in autophagy regulation are discussed.

**Keywords** p53 · p53-targeted genes · Autophagy · DRAM · TIGAR

W. Hu · S. Chen · R. F. Thorne · M. Wu (✉)
Translational Research Institute, Henan Provincial People's Hospital, Academy of Medical Science, Zhengzhou University, Zhengzhou 450003, China
e-mail: wumian@ustc.edu.cn



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## 6.1 Overview of Tp53 Research

The Tp53 gene encodes the transcription factor p53 which exhibits mutations or deletions in more than half of human tumors. Tp53 is considered a key tumor suppressor gene because of its function in cell cycle regulation, apoptosis, and DNA repair, and consequently, the regulation of Tp53 during tumorigenesis has come under intense scrutiny. Numerous studies have now shown that the biological role of p53 extends beyond preventing tumor formation and development, but touches on many other aspects of cellular homeostasis including autophagy, the subject of this review.

Autophagy or literally "self-eating" is a critical cellular process which plays a key role in maintaining cell growth and metabolism. In addition to its role in normal physiology, autophagy also significantly contributes to pathological processes such as cancer. The regulation of autophagy is dependent on different molecular mechanisms including p53 although this is highly context dependent, dictated by the cellular microenvironment and stress involved along with the subcellular localization of p53. In this chapter, we will focus on the mechanisms whereby p53 can both promote and inhibit autophagy in response to different stress conditions.

### *6.1.1 Discovery and Main Biological Functions of Tp53 Gene*

#### 6.1.1.1 Tp53 Gene Discovery Opens up a New Field of Cancer Research

After being first identified in the late 1970s, TP53 research has been ongoing for more than 35 years. Prior to this discovery, most tumor research focused on cell transformation caused by viruses. At that time, p53 was found to be elevated in many tumor types and moreover, it was shown to bind to the SV40 virus large T antigen, thus p53 was first considered to be a mediator of tumorigenesis. However, subsequent studies proved this perspective wrong since the p53 protein expressed by cancers was a mutant form having lost tumor suppressor activity, its bona fide function. Nevertheless, the discovery of p53 "opened the curtain" on a completely new field of cancer research which remains highly active to this day. Indeed, more than 93,000 research papers involving Tp53 are currently included in PubMed.

#### 6.1.1.2 Primary Functions of Tp53

The Tp53 gene encodes the transcription factor p53 whose main biological function involves a timely response to various external stresses by transactivation of many downstream target genes (Fig. 6.1). Mutations which activate proto-oncogenes along with more general DNA damage can directly activate p53, making the p53 protein more stable and promoting its transcriptional activity. Activation of p53 can induce cell cycle arrest, regulate autophagy, accelerate DNA repair, regulate cell metabolism,

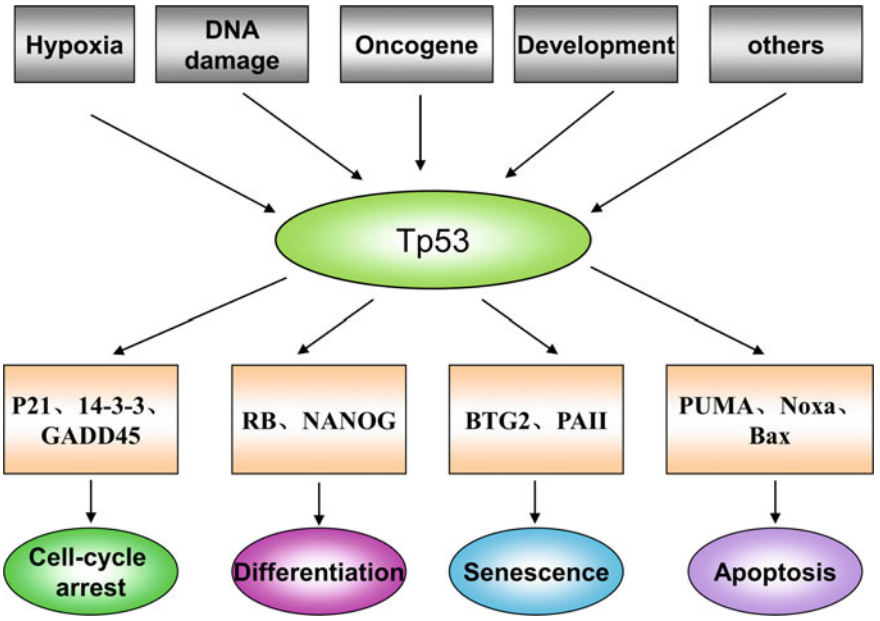


**Fig. 6.1** Primary functions of Tp53. Hypoxia, proto-oncogene mutations, and DNA damage can directly activate Tp53, promoting its transcriptional activity. Transactivation of Tp53 target genes, such as p21, can induce cell cycle arrest, senescence, and apoptosis; Tp53 can regulate autophagy, accelerate DNA repair, and regulate cell metabolism

and promote cell apoptosis. These responses prevent cell abnormalities and DNA replication errors in order to prevent the malignant transformation of cells, thereby inhibiting the formation of tumors.

## *6.1.2 Structure and Subcellular Localization of Tp53 Protein*

### 6.1.2.1 The Basic Structure of Tp53 Protein

The molecular weight of the p53 protein is 43,700 Daltons (43 kDa) based on its amino acid residues. However, the p53 name comes from its apparent molecular weight of approximately 53 kDa observed in SDS gel electrophoresis. The difference in molecular weight between the predicted and observed size is due to the large amount of proline residues in the p53 protein, which retards the migration speed in gel electrophoresis. This "slow-down effect" can be observed in many species, including humans, rodents, frogs, and fish. In humans, the TP53 gene is located on the short arm of chromosome 17 and is highly conserved amongst different species although no p53 homologue occurs in yeast. The protein structure of p53 consists of three main domains and shows features common to most transcription factors. Each

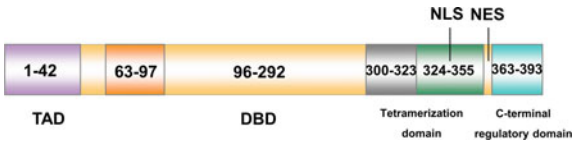


**Fig. 6.2** Schematic view of the domain structure of Tp53. The protein structure of Tp53 consists of 3 main domains: the N-terminal domain or transcription-activation domain (TAD) is mainly responsible for binding to and activating other transcription factors; the central DNA-binding domain (DBD) is responsible for binding to the promoter region of the target gene; the C-terminal domain of Tp53 is a multifunctional domain that includes (i) signal sequences responsible for the nuclear export and nuclear localization of Tp53; (ii) an oligomerization domain responsible for the formation of Tp53 tetramers; and (iii) the C-terminal regulatory domain

domain plays different roles in p53 function; the N-terminal domain or transcription-activation domain (TAD) is mainly responsible for binding to and activating other transcription factors while the central DNA-binding domain (DBD) is responsible for binding to the promoter region of the target gene. The promoter DNA sequence supporting p53 binding is relatively conserved, and its basic framework is RRRCWWGYYY (n) RRRCWWGYYY, (R for purine nucleotides, W for adenine or thymine, Y for pyrimidine nucleotides, and n for a sequence of 0–13 single nucleotides). The C-terminal domain of p53 is a multifunctional domain that includes (i) signal sequences responsible for the nuclear export and nuclear localization of p53; (ii) an oligomerization domain responsible for the formation of p53 tetramers; and (iii) the C-terminal regulatory domain (Fig. 6.2).

#### 6.1.2.2 Subcellular Localization of Tp53 Protein

The human p53 protein is composed of 393 amino acids and naturally exists as a tetramer, the formation of which involves amino acid residues at positions 334–356 in its C-terminal domain. P53 is found in both in cytoplasm and nucleus although this distribution is dynamic with fluxes occurring between both nuclear and cytoplasmic compartments. For example, the C-terminal of p53 in the nucleus can be ubiquitinated by MDM2 and transported to the cytoplasm. Notably, both pools of p53 are important in the regulation of autophagy although predominantly nuclear-localized p53 appears most significant with autophagy regulation occurring in a transcription-dependent manner. Conversely, cytoplasmic p53 also regulates autophagy in a transcriptionally independent manner (Green and Kroemer 2009).

## 6.2 Tp53 and Autophagy

### *6.2.1 Promotion of Autophagy by Tp53*

#### 6.2.1.1 The Role of Tp53 in Autophagy Mediated Through mTOR Signaling

Rapamycin, is a macrocyclic antibiotic that was first studied as an antifungal drug but later found to have immunosuppressive effects. The mammalian target of rapamycin (mTOR) is a macromolecular protein complex of 289 kDa with atypical serine/threonine (Ser/Thr) protein kinase activity. mTOR exists in two different complexes: mTORC1 and mTORC2. mTORC1 mainly includes mTOR, raptor (regulatory-associated protein of mTOR), and MLST8 (mammalian lethal with SEC13 protein 8), which is sensitive to rapamycin inhibition; whereas, mTORC2 that is resistant to rapamycin inhibition mainly consists of mTOR, MLST8, RICTOR (rapamycin-insensitive companion of mTOR), and MSIN1 (mammalian stress-activated protein kinase-interacting protein 1).

In response to both intracellular and extracellular signals, mTOR plays important roles in biological processes including apoptosis and autophagy along with regulating cell growth through the engagement of multiple downstream signaling pathways. mTOR primarily functions to regulate translation of downstream target proteins through phosphorylation of p70S6K (p70 ribosomal protein S6 kinase), S6K1 (ribosomal protein subunit 6 kinase 1), and EIF4EBP1 (eukaryotic initiation factor 4E-binding protein 1). In the context of autophagy, mTOR signaling acts as a negative regulator, since its activation can inhibit the initiation of autophagy. Moreover, given that most of the downstream target genes of p53 can inhibit mTOR signaling, activation of p53 serves to promote the initiation of autophagy. The following section focuses on the mechanistic roles played by p53 in the promotion of autophagy via the inhibition of mTOR signaling via AMPK, IGF-BP3, and REDD1.

P53 Inhibits mTOR Signaling by Activating AMPK

Adenosine monophosphate-activated protein kinase (AMPK) is an evolutionarily conserved cellular energy sensor, which modulates intracellular energy metabolism and maintains energy homeostasis. AMPK inhibits mTOR activity by phosphorylating TSC1 and TSC2 in the tuberous sclerosis complex to promote autophagy. P53 can inhibit the mTOR signaling pathway by activating AMPK through both direct and indirect transcriptional mechanisms.

First, p53 itself can directly regulate the expression of the β1 and β2 subunits of AMPK through transcriptional increases and the increased expression of β1 and β2 subunits thereby increases AMPK activation (Feng et al. 2007). P53 can also upregulating TSC2, which can inhibit mTOR activity and promote autophagy. The p53 target

genes Sestrin1 and Sestrin2 elicited following genotoxic stress are also AMPK activators, which can also promote autophagy in cells with normal p53 function. Sestrin1 and Sestrin2 bind to TSC1, TSC2, and AMPK to facilitate Thr 172 phosphorylation of AMPK, thereby activating AMPK and promoting autophagy (Budanov and Karin 2008).

In the case of energy stress such as that induced by nutrient deficiency or hypoxia, LKB1 (Liver kinase B1) can activate AMPK by directly phosphorylating the AMPK α subunit. Recently, Shackeford et al. demonstrated that p53 can also inhibit mTOR signaling pathway and induce autophagy by directly phosphorylating AMPK by a mechanism similar to LKB1 activation of AMPK. Strikingly, AMPK can also directly phosphorylate p53 on Ser15, causing p53-dependent cell cycle arrest and cell senescence to provide a protective response to metabolic stresses such as glucose deficiency (Jones et al. 2005). Together, these phenomena indicate that the interconnection between AMPK/mTOR signaling and p53-control of autophagy are tightly controlled.

## Tp53 Inhibits mTOR Signaling by Activating IGF-BP3

Insulin-like growth factor-1 (IGF-1) is a peptide growth hormone produced by the liver in response to growth hormone (GH). IGF-1 exerts its biological effects through binding at the cell membrane to receptor protein-tyrosine kinases (RPTKs) whose extracellular portion contains a ligand-specific binding domain. The RPTK intracellular domain consists of a catalytic domain with tyrosine-protein kinase activity and autophosphorylation sites. After ligand binding, the intracellular domain of the receptor is phosphorylated, triggering a conformational change to expose binding sites to recruit downstream signaling proteins to the receptor's intracellular domain. The composition of the assembled signaling complexes dictates the activation of specific signal transduction pathways, which then drives different cellular responses. In the case of IGF-1, it binds to its receptor (IGF-1 receptor, IGFR) to activate the mTOR pathway. Insulin growth factor binding protein 3 (IGF-BP3) can also bind to IGF-1 with high affinity and competitively block its binding to IGF-1R, thereby blocking the IGF-1-mediated activation of mTOR signaling. Notably, IGF-BP3 expression is upregulated through activation of p53 following intracellular stresses including hypoxia, DNA damage, and proto-oncogene conversion. Increased levels of IGF-BP3 competitively inhibit the binding of IGF-1 to IGF-1R, thereby blocking IGF-1/AKT signaling. This ultimately inhibits mTOR activation and promotes the initiation of autophagy.

Under cellular stress, the negative regulation of p53 on IGF-1/AKT and mTOR signaling pathways not only promotes autophagy, but also depletes the cell surface expression of growth factor receptors, thereby slowing cell growth and differentiation, and preserving and reusing intracellular resources. This mechanism is also involved in the damage repair process of cells or tissues.

Tp53 Inhibits the mTOR Pathway by Activating REDD1

Regulated in development and DNA damage responses (REDD1) was initially discovered by several groups as a stress response gene induced in the absence of oxygen and DNA damage. The REDD1 gene is highly conserved and encodes a protein with a predicted molecular weight of 25 kDa but interestingly the protein does not contain any presently known structural or functional domains. Deletion of REDD1 in Drosophila does not affect survival during normal development, but it does diminish survival under hypoxic and starvation conditions, indicating that REDD1 can regulate cell growth under stress conditions. Both Redd1 and the highly related gene Redd2 gene appear to both regulate cell growth in response to a variety of stresses. In addition, there is a high mutation frequency of REDD1 observed in human cancers, which proposes REDD1 to be a potential tumor suppressor gene since weakening of its growth control role under hypoxia and following DNA damage may favor tumor development.

The regulatory link between p53, REDD1, and autophagy includes the following aspects. First, REDD1 acts upstream of TSC1-TSC2 to negatively regulate mTOR signal transduction; second, under hypoxia the upregulation of hypoxia inducible factor 1 (HIF-1) by p53 in turn promotes the expression of REDD1 which by inhibition of mTOR activity can promote autophagy.

#### 6.2.1.2 DAPK1 (Death-Associated Protein Kinase 1) Mediates the Promotion of Autophagy by Tp53

The DAPK family consists of three closely related serine/threonine proteases, namely DAPK1, ZIP, and DRP1, as shown in Fig. 6.3. DAPK1 was first found to mediate apoptosis induced by interferon gamma. It is the largest protein in the family with a molecular weight of 160 kDa and consists of multiple functional domains. In addition to serine/threonine-protein kinase domains, DAPK1 also contains one death domain, two P-rings, and eight ankyrin repeats, which mediate the interaction between DAPK1 and other proteins. The cytoskeleton binding domain determines the localization of DAPK1 to actin filaments. ZIP can shuttle between the cytoplasm and nucleus by virtue of its nuclear localization signal (NLS), whereas DRP1 is mainly cytoplasmic. ZIP and DRP1 can also mediate oligomerization of proteins through their respective “leucine zipper” and “tail” at the C-terminal of the protein. DAPK1 and DRP1 also possess a calmodulin-binding domain which is absent in ZIP. Therefore, DAPK1 and DRP1 can respond to intracellular calcium concentration changes. Besides being subject to calcium-dependent regulation, autophosphorylation of Ser308 in DAPK1 and DRP1 ensures that they remain inactive in normal cells.

DAPK1 is a potential tumor suppressor protein, which can participate in the regulation of apoptosis and autophagy in a Tp53-dependent or nondependent manner. In most malignant tumors, the expression of DAPK1 is silenced due to promoter methylation.

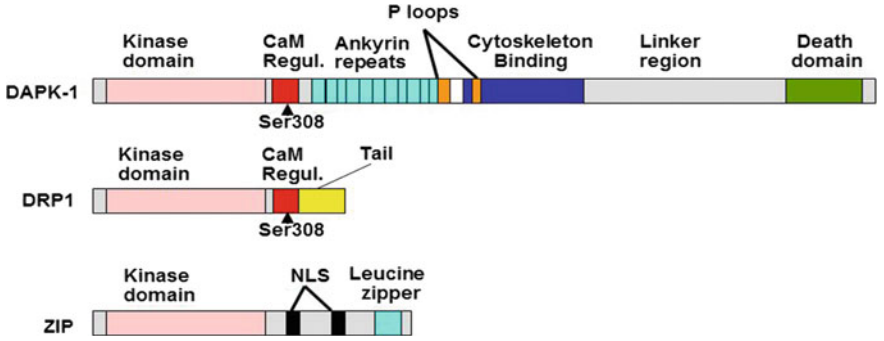


**Fig. 6.3** Schematic view of the domain structure of DAPK1 family proteins. DAPK1 is the largest protein in the family with a molecular weight of 160 kDa and consists of multiple functional domains. In addition to serine/threonine-protein kinase domains, DAPK1 also contains one death domain, two P-rings, cytoskeleton binding domain, calmodulin-binding domain, and eight ankyrin repeats, which mediate the interaction between DAPK1 and other proteins. DRP1 contains serine/threonine-protein kinase domains, calmodulin-binding domain and one "tail" at the C-terminal of the protein; C-terminal of ZIP contains leucine zipper and nuclear localization signal (NLS)

## DAPK1 Integrates with Microtubule-Binding Protein MAP1B and Facilitates Autophagy

Microtubule-associated proteins (MAPs) are defined as proteins that adhere to tubulin polymers and contribute to the assembly and function of the microtubule network. There are at least two domains in the structure of MAPs: one is the alkaline binding domain supporting microtubule interactions, and the other is the acidic extension domain, which extends outside microtubules to facilitate interactions with other cellular components. MAPs can be divided into two main types according to their sequence characteristics: type I (MAP1A and MAP1B) and type II (map2, map4, and Tau). The main function of MAPs is to participate in the aggregation of microtubules, improving their stability and providing structural interfaces for the transport of intracellular substances in the form of microtubule transport vesicles and particles.

MAP1B, also known as the "converter" of LC3 (microtubule-associated protein 1 light chain 3, LC3), can function to inhibit autophagy. DAPK1 can bind to microtubule-binding protein MAP1B and enhance autophagy by promoting the formation of autophagic vesicles and the accumulation of autophagic bodies. Under certain conditions, the level of DAPK1 gene is upregulated in a p53-dependent manner, and binding sites for p53 are found in the promoter of the DAPK1 gene in human and mouse genomes. By oligonucleotide immunoprecipitation, p53 was found to bind to the promoter region of DAPK1 and promote its expression. Moreover, the activity of DAPK1 kinase depends on the presence of p53. The ubiquitination signal site of MDM2 in the DNA-binding domain of p53 is the docking site of calmodulin kinases including DAPK1 (Harrison et al. 2008).

### DAPK1 Phosphorylates Beclin1 and Promotes Autophagy

Beclin1 was first discovered by Liang et al. in rats with lethal Sinbis viral meningitis and is a key autophagy-related protein. The BECN1 gene encodes a protein of approximately 60 kDa, which contains a BH3 domain which mediates binding to BCL2 family antiapoptotic proteins, which in turn inhibits its activity. Indeed the dissociation of Beclin1 from BCL2 family proteins is crucial for its function in promoting autophagy. Therefore, the binding between Beclin1 and BCL2 family proteins must be precisely regulated.

Both BCL2 and BCL-XL contain a "pocket" structure that combines with BH3 domain of Beclin1. Complexes between BCL2/BCL-XL and Beclin1 are usually regulated by two types of phosphokinases. First JNK1 can mediate threonine 69, serine 70, 87 (T69, S70, and S87) phosphorylation in BCL2, which dissociates Beclin1 from BCL2 to promote autophagy. Alternatively, Beclin1 may be phosphorylated at threonine 119 by DAPK1 which is located in the Beclin1 BH3 domain, and is a crucial site for the dissociation of Beclin1 from its binding with BCL-XL. Similarly, threonine 119 the phosphorylation of Beclin1 can also reduce binding to BCL2 (Zalckvar et al. 2009a, b).

### DAPK1 Stabilizes P53 to Facilitate Autophagy

ARF (human p14ARF; mouse p19ARF) is a tumor suppressor encoded by INK4a/ARF gene cluster. The frequency of ARF mutations or deletion is second only to P53 in human tumors and is highly correlated with tumorigenesis. The most important function of ARF is to activate p53 signaling by inhibition of HDM2/Mdm2 (ubiquitin ligase of Tp53, human HDM2, and mouse Mdm2). HDM2/Mdm2 is a key regulator of the p53 protein: Mdm2 degrades Tp53 at the posttranslational level, while Tp53 upregulates the level of Mdm2 at the transcriptional level, which constitutes a feedback loop and exerts fine regulatory control over each other. ARF functions to retain HDM2/Mdm2 in the nucleus and thus inhibits the degradation of p53.

DAPK1 also promotes the accumulation and activation of p53 in the nucleus in an ARF-dependent manner, and promotes autophagy through a positive feedback loop involving DAPK1-ARF-p53 (Martoriati et al. 2005). In addition, DAPK1 can also promote autophagy through ARF through a non-p53-dependent way. The regulation of autophagy by ARF is also very complex, depending on the cellular state and microenvironment. Wild-type ARF can induce autophagy through p53-dependent and nondependent mechanisms. In addition, there is an N-terminal deleted isomer protein of ARF, called short mitochondrial ARF (smARF), with translation initiation occurring in the middle of the ARF mRNA. Compared to ARF, the half-life of smARF is short, and overexpression of smARF can trigger autophagy by altering the membrane potential of mitochondria (Reef et al. 2006).

### *6.2.2 Inhibition of Autophagy by Tp53*

From the earliest stages of autophagy-related research, it was clear evidence that p53 induced and promoted autophagy. However, later studies have also shown that p53 can inhibit autophagy. Here, we describe the inhibition of autophagy by p53 under both physiological and pathological conditions.

#### 6.2.2.1 Inhibition of Autophagy by Tp53 Under Physiological Conditions

Tp53 Inhibits Autophagy by Binding to and Inhibiting RB1CC1/FIP200

RB1CC1 (RB1-inducible coiled-coil protein 1), also known as FIP200 (FAK family kinase-interacting protein of 200 KD), is a mammalian protein homologous to Atg17 (a key protein involved in autophagy in yeast). RB1CC1/FIP200 can shuttle between the nucleus and cytoplasm, and regulate cell growth, differentiation, migration, apoptosis, and autophagy through engaging multiple signaling pathways. RB1CC1/FIP200 inhibits cell cycle progression by inducing expression of RB1, and can also activate mTOR signaling through inhibiting Pyk2 and FAK1 in the cytoplasm. RB1CC1 is also involved in terminal differentiation of many cell types, including hepatocytes, cardiomyocytes, and skeletal muscle cells. In addition, RB1CC1/FIP200 can also act as a tumor suppressor. It is reported that deletion mutations in RB1CC1/FIP200 occur in approximately 20% of breast cancer cases. In yeast, Atg17 is essential for the initiation of autophagosome formation. RB1CC1/FIP200 functions similarly in mammalian cells, regulating autophagy by affecting the stability and phosphorylation levels of ULK1.

P53 inhibits autophagy by binding to RB1CC1/FIP200: the interaction occurs between p53 and the N-terminus (1–154 amino acids) of RB1CC1/FIP200 and serves to stabilize p53 (Morselli et al. 2011). In addition, RB1CC1/FIP200 can form a complex with p53 in the nucleus, which facilitates p53-mediated transcriptional regulation of RB1, CDKN1A, and CDKN2A. Notably, the inhibition of autophagy through RB1CC1/FIP200 is limited to wild-type p53, and mutants of p53 such as p53K382R cannot bind to RB1CC1/FIP200 and consequently do not inhibit autophagy. The role of p53 in inhibiting autophagy by RB1CC1/FIP200 is associated with mTOR signaling, but the mechanism(s) remains unclear and need further study. However, it has been speculated that the functional link may involve a role for RB1CC1/FIP200 in the formation of the PI3K complex.

Inhibition of Autophagy by PKR

Double-stranded RNA (dsRNA) molecules exert multiple biological functions within the cell. The background levels of endogenous dsRNA influences a number of cellular

physiological activities, including gene expression, RNA editing, amongst others. The translation of dsRNA is regulated by natural antisense RNA and microRNAs. The production of massive amounts of dsRNA is thought to be a signal that cells are at risk. Several studies have shown that high levels of dsRNA production in cells result from interference with normal RNA metabolism of RNA under three conditions: (i) the effects of genotoxic stress; (ii) abnormal transcription of damaged DNA; and (iii) viral infection.

The effect of the accumulation of dsRNA in the cell is very complex and presently poorly understood. Currently, the best-known mechanism is mediated by the interferon-induced, double-stranded RNA-activated protein kinase, or protein kinase RNA-activated (PKR). The main function of PKR is to phosphorylate the eukaryotic translation initiation factor 2α (eIF2α) and to rapidly shut down protein translation. PKR can prevent translation and can (at least in some cases) induce autophagy in response to intracellular dsRNA accumulation. In addition, PKR can also limit the spread of viruses by inhibiting translation, which is also considered to be one effective antiviral mechanism.

Grinberg et al. found that cell lysates containing wild-type p53 are capable of catalyzing the degradation of nucleotides of dsRNA and ssRNA from the 3′-terminus. However, the exonuclease activity is lost when p53 is deleted in cells. Thus, it is speculated that p53 may inhibit autophagy by reducing the stability of dsRNA in cells and the accumulation of dsRNA and activation of PKR (Galluzzi et al. 2010). However while wild-type and mutant p53 can both play inhibitory roles in autophagy, only wild-type p53 promotes nuclease activity, thus further exploration is needed regarding the function of p53 in dsRNA degradation.

#### 6.2.2.2 TP53 Represses Autophagy Under Stress

In the absence of stress, the cellular levels of p53 are normally very low. When cells are exposed to stressors such as hypoxia, starvation, or DNA damage, p53 becomes stabilized and activated by different mechanisms. This permits p53 to participate in the regulation of apoptosis, senescence, and autophagy.

##### Degradation of p53 Promotes Autophagy Under Conditions of Nutrient Deprivation

The specific mechanism by which p53 inhibits autophagy under conditions including lack of nutrients, tunicamycin-induced endoplasmic reticulum stress, and mTOR inhibition is still not clear.

It has been shown that when cells are stimulated by mTOR inhibitors (rapamycin) or other conditions that induce autophagy (e.g., auxotrophic factors, tunicamycin-induced endoplasmic reticulum response), p53 is degraded by the HDM2-dependent proteasome, and the decrease in intracellular p53 content causes an increase in intracellular autophagy. When p53 degradation is blocked by proteasome inhibitors

(MG132) or knockdown of HDM2 by RNA interference, the initiation of autophagy is inhibited. This effect of p53 can be observed in many types of cells, such as colon cancer cells, cervical cancer cells, and fibroblasts. More specific experiments were conducted to further confirm the inhibitory effect of p53 on autophagy. Treatment of cells with both Nutlin-3 and RITA (HDM2 antagonists) can stabilize p53, thereby inhibiting autophagy induced by nutrient deficiency or mTOR inhibitors. However, the same two antagonists of HDM2 could not block the autophagy induced by p53-deficient cells under the same conditions, demonstrating that these two antagonists of HDM2 do not have the ability to inhibit autophagy by themselves, but exert an inhibitory effect on autophagy through p53. In addition, autophagy initiation can also be inhibited by overexpression of p53 in these cases. When oxygen and nutrients are deficient, the enhanced autophagy can help maintain high levels of ATP, thereby promoting the survival of p53-deficient tumor cells.

Tp53 Suppresses Autophagy Through AMPK Signaling Pathway

When gene knockout or related inhibitors were used to reduce p53 protein expression or inhibit its function, it is found that p53 has the role of inhibiting autophagy in the cytoplasm. Moreover, this inhibition is also associated with AMPK activity, but the specific mechanism is not clear.

The inhibitory effect of p53 on autophagy is also closely related to cell cycle progression. The p53-mediated inhibition on autophagy occurs mainly in the G0/G1 phases of the cell cycle, rarely in the S phase and even less in the G2/M phase. For distinct cell types, the role of p53 in regulating autophagy is also different. In nonnucleated cells, p53 has a strong inhibitory effect on autophagy. In addition, the cellular localization of p53 appears strongly related to its inhibition of autophagy. When the nuclear localization signal sequence is deleted and p53 accumulates in the cytoplasm there is a strong inhibitory effect on autophagy. Conversely, when its nuclear export signal sequence is deleted and p53 accumulates in the nucleus, the inhibitory effect on autophagy disappears.

## 6.3 TP53 Target Genes (DRAM, TIGAR), and Autophagy

### *6.3.1 Autophagy Regulatory Protein DRAM is Induced by P53 to Boost Autophagy*

DRAM (damage-regulated autophagy modulator) is a phylogenetically ancient lysosomal protein with evolutionary links to animals and plants. DRAM plays an important role in the crossroad between Tp53-induced autophagy and cell apoptosis. DRAM is highly conserved across many diverse species including humans, mice,

fruit flies, zebrafish and nematodes. There exist multiple mammalian DRAM homologues. DRAM1 is one of the most studied. In human, DRAM1 gene encodes a protein of 238 amino acid residues, structurally containing six hydrophobic transmembrane domains, and a latent signal peptide sequence directed to the endoplasmic reticulum at its N-terminus. DRAM1 localizes in lysosomes or endosomes. It is one of the constituent proteins of the lysosomal membrane and plays a crucial role in the formation of autophagic lysosomes. DRAM1 is involved in the promotion of autophagy by p53 since when DRAM1 is absent, the promoting effect of Tp53 is significantly weakened. By analyzing the genomic sequence of DRAM1, it was found that there is a conserved p53 binding site in the first intron and p53 was shown to bind to this site and directly transcribe DRAM. Therefore, DRAM is a target gene directly regulated by p53. In the case of DNA damage, p53 can directly upregulate DRAM mRNA level and induce increased DRAM expression (Crighton et al. 2007).

When DRAM1 is highly expressed in cells, it was found by electron microscopy that a large number of autophagic vesicles with a bilayer membrane structure accumulate. Moreover, overexpression of DRAM1 also increased the punctate distribution of the autophagy marker LC3 in the cytoplasm representing the formation of autophagic lysosomes. DRAM1 is not only essential for autophagy triggered by Tp53 overexpression or DNA damage caused by Tp53 accumulation, but is also indispensable for Tp53-induced apoptosis. Although DRAM alone has a weak ability to induce apoptosis, if DRAM is absent in DNA damage or other apoptotic stimuli, Tp53-induced apoptosis will be severely weakened, suggesting that Tp53-induced apoptosis depends on the existence of DRAM.

At present, the specific mechanism of how DRAM causes the accumulation of autophagosomes and how it promotes the formation of autophagosomes is still not completely clear. DRAM is localized only to lysosomal membranes, and lysosomes are believed to only play late-stage roles in autophagosome formation. Since lysosomes do not function at the initial stage of autophagosome formation, it is still unclear how DRAM influences autophagy initiation events.

Li Yen Mah et al. reported that DRAM1 encodes two important splice variants, DRAM1-SV4 and DRAM1-SV5. They lack exons 4–5 and 4–6 and localize to peroxisomes and autophagosomes, respectively. Overexpression of DRAM1-SV4 and DRAM1-SV5 induces significant autophagosome accumulation although the effect on promoting autophagy is weaker than that of wild-type DRAM1 (Mah et al. 2012).

DRAM1 has two other homologues DRAM2 and DRAM3. DRAM2 localizes to lysosomes and is involved in autophagy. Overexpression of DRAM2 promotes autophagy. In contrast, knockdown of DRAM2 inhibits starvation-induced autophagy (Yoon et al. 2012).

DRAM-3 is expressed in a broad spectrum of normal tissues and tumor cells, but different from DRAM-1, DRAM-3 is not induced by p53 or DNA-damaging agents. Overexpression of DRAM3 induces autophagosome accumulation and enhances autophagic flux. Silencing of DRAM3 significantly inhibits autophagic flux (Mrschtik et al. 2015). No DRAM homologues were found in simple organisms such as yeast and bacteria, suggesting that DRAM mainly plays roles in autophagy regulation in higher organisms.

### 6.3.2 *Inhibition of Autophagy Through Synergistic Effect of Tp53 and Target Gene TIGAR*

TIGAR (TP53-induced glycolysis and apoptosis regulator) is an important factor involved in p53-induced apoptosis in glycolysis. It is also a target gene of p53, located on the short arm of chromosome 12. Potential p53 binding sites (BS1 and BS2) are present upstream of the first exon and first intron, of TIGAR, respectively. After treatment with adriamycin, activated p53 binds to both BS1 and BS2 but compared with BS1, BS2 has a stronger transcriptional regulatory effect on TIGAR transcription. However, TIGAR protein levels can also be enhanced by p53 induction (Bensaad et al. 2006). TIGAR functions to reduce intracellular fructose-2,6-bisphosphatase activity, thereby inhibiting glycolysis and activating the pentose phosphate pathway. These conditions increase the intracellular NADPH level, and glutathione is inhibited to reduce intracellular reactive oxygen species (ROS) levels which inhibits autophagy.

### 6.3.3 *Tp53 Regulates Autophagy Through Other Targeted Genes*

#### 6.3.3.1 Tp53 Promotes Autophagy by Targeting PTEN

PI3K (phosphatidylinositol-3-kinase) is an important class of signal transduction mediators, which are principally composed of a p110 catalytic subunit usually in combination with a regulatory subunit. PI3K possesses both serine/threonine kinase activity together with phosphatidylinositol kinase activity. According to the structural characteristics of the p110 subunit and also substrate specificity, PI3K can be divided into three types: type I with PI, PIP and PIP2 as substrates, type II with PI and PIP as substrates, and type III with PI only as a substrate. Type I can be further classified into either type IA or IB according to their respective p110 catalytic subunits. Type IA includes p110α, p110β, and p110δ that mainly receives signals from tyrosine kinase receptors. The single IB p110 subunit is p110γ, which alternatively engages with G-protein-coupled receptors. PI3K can be activated in response to stimulation by extracellular growth factors and hormones, and activated PI3K can phosphorylate phosphoinositides in the cell membrane.

AKT is a serine/threonine-protein kinase which binds to PI-3, 4P2, PI-3, 4, and 5P3 under the synergistic action of phosphoinositide-dependent protein kinase (PDK), resulting in phosphorylation of Ser473 and/or Thr308 of AKT, which are essential for AKT activation. mTOR which belongs to the family of phosphoinositide kinase-associated kinases (PIKKs) and is a downstream substrate of PI3K/AKT. Activated AKT can exert a wide range of biological effects by promoting phosphorylation of mTOR, including the promotion of autophagy.

PTEN (phosphatase and tensin homology deleted on chromosome 10) is a tumor suppressor gene selectively expressed in certain cells or tissues. PTEN was originally thought to be involved in tumor formation, cell proliferation, and adhesion, and genetic deletion syndrome is prone to occur after its mutation. In contrast to PI3K function, PTEN, a PIP3-phosphatase, is considered to be an inhibitor of PI3K. PTEN can convert PIP3 to PIP2 by dephosphorylation, thus reducing the activation of AKT, thereby preventing all downstream signal transduction regulated by AKT phosphorylation.

The PTEN gene promoter region contains a binding site for the p53 protein and PTEN expression levels can be directly regulated by p53 transcriptional activity. After overexpression of p53 or activation by other conditions, p53 can directly interact with the p53 binding site in the PTEN promoter, leading to a significant upregulation of PTEN mRNA and consequently PTEN protein levels. This acts to suppress PI3K activity, for example, in acute myeloid leukemia cells where PTEN expression is highly expressed there is a negative correlation with PI3K activity. In this manner, PTEN can promote autophagy by inhibiting the PI3K/AKT/mTOR signaling pathway. The links between p53, PTEN and mTOR-mediated regulation of autophagy are presently an active area of investigation in autophagy research.

## Tp53 Induces Mitophagy by Targeting PUMA, BAX, BAD, and BINP3

PUMA belongs to the BH3 only domain subclass of the BCL2 family. Its expression is regulated by Tp53 and plays an important role in Tp53-dependent cell apoptosis. PUMA induces apoptosis by activating BAX and increasing the permeability of the outer mitochondrial membrane. Toward regulating autophagy, PUMA can bind with BCL2/BCL-XL through its BH3 domain, and competitively release Beclin1 from Beclin1-BCL2/BCL-XL complexes to induce mitophagy. Point mutation of the key amino acids in the BH3 domain of PUMA that prevent BCL2/BCL-XL binding results in the functional loss of PUMA in promoting mitophagy. The regulation of PUMA on mitochondrial autophagy also depends on the presence of BAX/BAK. Interestingly, overexpression of BAX alone functions similarly to PUMA. Notably, when mitophagy induced by PUMA or BAX is inhibited artificially, the cell response to apoptosis can be weakened, indicating that in some specific cases, this selective induction of mitochondrial autophagy plays a role in promoting apoptosis (Yee et al. 2009).

BAD, another target protein regulated by Tp53, also contains a single BH3 domain with a structure similar to Beclin1. It can compete with Beclin1 for binding to BCL2/BCL-XL, and dissociate Beclin1 from this complex, thus promoting autophagy. However, the autophagy induction caused by Tp53-mediated upregulation of BAD occurs only in mitochondria, i.e., mitochondrial autophagy, but not in endoplasmic reticulum or other organelles. This may be related to the fact that most of the proteins of BCL2 family are located in mitochondrial membranes.

BNIP3 (BCL2/adenovirus E1B 19-kDa-interacting protein 3) was initially identified by the yeast two-hybrid method along with BNIP1 and BNIP2. BNIP1 and

BNIP2 are located in the nuclear membrane and endoplasmic reticulum, respectively, while BNIP3 is located in mitochondria. This localization dictates that BNIP3 participates in the regulation of apoptosis and autophagy through mitochondrial signaling pathways. BNIP3 has a potential BH3 domain in its structure and a transmembrane domain in C-terminal. However, the amino acid sequence of BH3 domain of BNIP3 is somewhat distinct from that of the BH3 domains of other BCL2 family members. BNIP3 is closer to BAX in structure, endowing BNIP3 with apoptotic activity and ready heterodimer formation with BCL-XL (Zhang and Ney 2009).

Daido et al. first described in malignant glioma cells that BNIP3 was involved in ceramide-induced autophagic death. Ceramide treatment induced the upregulation of BNIP3 expression, leading to mitochondrial depolarization and autophagy. Subsequent studies have confirmed that BNIP3 can participate in the regulation of mitochondrial autophagy. Under hypoxia, BNIP3 promotes autophagy and protects cells from necrosis (Feng et al. 2011). However, it is still unclear what molecular mechanism(s) BNIP3 uses to promote mitochondrial autophagy although there are a number of theoretical models proposed for different cell types and stimulation conditions. One of the most recently proposed and accepted explanations is that there is a "dialogue" between BNIP3-induced autophagy and cell apoptosis. Since both BNIP3 and Beclin1 are thought to bind to BCL2 or BCL-XL through their BH3 domains, BNIP3 can compete with Beclin1 for binding. Consequently, BNIP3 may release Beclin1 to promote autophagy. However, this hypothesis does not entirely support all experimental findings. First, autophagic mechanisms in reticulocytes lacking BNIP3 are entirely normal, indicating that the core mechanism of autophagy can function in the absence of BNIP3. However, BNIP3 may only play a role in the mitochondrial outer membrane and hence there may be other ways of activating autophagy in reticulocytes.

### Tp53 Regulates Autophagy by Targeting Noncoding RNA

Noncoding RNAs can be divided into structural noncoding RNA (ncRNA) and regulatory ncRNAs. The former mainly includes transfer RNA (tRNA), ribosomal RNA (rRNA), and nucleolar small molecule RNA (snoRNA). The latter can be further divided into (1) short-chain ncRNAs, commonly microRNAs (miRNAs) and (2) long-chain noncoding RNAs (lnRNAs).

MiRNAs are a double-stranded RNA molecules with a length range of 20–24 nucleotides that play important regulatory roles by targeting and modifying gene expression in cells. In response to different environmental stimuli both in vitro and in vivo, miRNAs can regulate the expression of multiple genes, or finely regulate the expression of a specific gene through a combination of multiple miRNAs, thus forming a complex network. So far, there are about 28,645 miRNA molecules discovered in animals, plants, and viruses. Most miRNA genes are present in the genome in the form of single copies, multiple copies, or clusters. The inhibitory effect of microRNA on target gene expression depends mainly on the degree of complementarity between the miRNA and the sequence of the gene transcript it targets. Should

the miRNA be fully complementary to the target gene sequence, miRNA binding can directly initiate cleavage and destabilization of the target gene mRNA molecule, similar to the principles of small interfering (si)RNA. However, if the miRNA is only partially complementary to the target gene sequence, binding of the miRNA acts to inhibit translation of the target gene without affecting mRNA stability. It has been demonstrated that p53 transcriptional regulation of microRNAs affect autophagy. MiR-34a is a target gene of p53 and its expression is regulated by p53 transcription. Yiqing Zheng et al. found that miR34a could inhibit autophagy by targeting ATG9A expression while Jiqin Lian et al. reported that microRNA-34a/34c-5p could inhibit rapamycin-induced autophagy by targeting ATG4B expression (Xu et al. 2012; Zhai et al. 2013; Liao et al. 2014).

The term lncRNA is applied to noncoding RNA molecules of more than 200 nucleotides but this description belies their great diversity in structure and function. In recent years, the number of studies showing that lncRNAs regulate important biological functions have grown exponentially. LncRNAs exhibit plasticity in nature and can bind with DNA and RNA through base-pairing interactions. Some lncRNAs participate in gene regulation, and others can bind to proteins through specific structural motifs to play a role in guiding molecules, scaffold molecules, and inducing molecules, or directly regulate the high-level structural forms and gene expression of chromatin. The understanding of how lncRNAs can regulate autophagy is still in its infancy. Some progress has been made with the identification of lncRNAs such as NBR2, MALAT1, and MGE3, which have been found to be involved in the regulation of autophagy at different stages (Yang et al. 2017). Notably, Kun Wang et al. found that the lncRNA CAIF can reduce myocardial infarction injury by inhibiting cell autophagy. The specific mechanism was shown to be blocking p53-mediated myocardin transcription, highlighting the relationship between p53, lncRNAs, and autophagy (Liu et al. 2018). However, it has not been established yet whether those lncRNAs which are directly regulated by p53 are involved in autophagy.

## 6.4 Other Mechanisms of Autophagy Regulation by Tp53

### *6.4.1 Regulation of Mutant Tp53 on Autophagy*

#### 6.4.1.1 Mutation Types and Common Forms of Tp53

Forms of gene mutation mainly include deletion of gene fragments, coding shifts caused by insertions, missense mutations caused by point mutation and loss of heterozygosity (LOH). All of these mutation classes have been observed with Tp53 but more than 80% of reported observations involve missense mutations at a single nucleotide. Moreover, 97% of these missense mutations are concentrated in the DNA-binding domain (DBD) of Tp53. Each amino acid in the DBD domain can theoretically mutate to produce corresponding mutants, but the mutation frequencies

of six sites are very high, namely, R175, G245, R248, R249, R273, and R282. These so-called called hot spot mutations are closely related to tumorigenesis.

Mutant Tp53 can be structurally divided into two types: the first type such as R273H are defective in DNA binding, where the mutation weakens p53's DNA-binding capacity; alternatively, there are the conformation mutants such as such as R175H that significantly alter the conformational structure of wild-type p53 after point mutation. It is worth noting that in recent years, the Tp53 gene has been shown to express many different isoforms arising through different transcription initiation sites and also alternative splicing of mRNA. These include Tp53α, Tp53β, Tp53γ, △40Tp53α, △40Tp53β, Δ40Tp53γ, Δ133Tp53α, Δ133Tp53β, and Δ133Tp53γ. The functions of these isoforms are less well studied but they also are widely involved in numerous Tp53-related biological activities.

#### 6.4.1.2 Regulation of Autophagy by Tp53 Mutants

Tp53 mutants have been shown to be involved in the regulation of autophagy in several ways. First, since mutation of Tp53 changes the stability and transcriptional activity of wild-type p53, this affects the p53-mediated regulation of autophagy. Second, the heteroligomeric complexes formed between mutant and wild Tp53 through their C-terminal tetramerization domains can inhibit wild p53 activity and thereby influence autophagy regulation. Third, many mutants of Tp53 alter the cellular localization of p53 and as mentioned earlier, the localization of p53 in the cytoplasm or nucleus determines its activity as a regulator of autophagy.

### *6.4.2 Regulation of Autophagy by Tp53 Family Protein Tp73*

Tp53 family members include Tp53, Tp63, and Tp73. The overall structure of these proteins is highly evolutionarily conserved from Drosophila to humans. All contain a central DNA-binding domain, a transcriptional activation domain in their N termini and oligomerization domains in their C termini. Similar to Tp53, the Tp73 gene can also be transcribed from a promoter located in its third intron. The Tp73 gene expresses at least seven C-terminal splice variants and four N-terminal splice variants and in total at least 29 different splicing forms. Notably, Tp73 can specifically bind to mutant Tp53 and inhibit its transcriptional activity. Moreover, the short arm 36 region of chromosome 1 where Tp73 is located is also mutated in many cancer cells. It has been speculated that Tp73 may act as a defense signal against DNA damage and prevent cells from inducing tumorigenesis due to genetic instability.

Tp73 along with its main isomeric forms, Tp73α, Tp73β, and Tp73γ, can induce autophagy. The ability of Tp73γ to induce autophagy appears more prominent, although the mechanism is not clear. Studies have shown that Tp73 can induce the expression of the autophagy-related protein DRAM, but nevertheless, Tp73-induced

DRAM expression is not necessary for Tp73-induced autophagy. Moreover, an N-terminal deletion mutant of Tp73 (NTp73) which lacks gene transcription abilities, can compete with Tp53 and Tp73 for DNA-binding sites and inhibit the transcription function of Tp53 and Tp73. NTp73 alone could not induce autophagy, but it could inhibit the formation of autophagosomes induced by Tp73 and Tp53. However, NTp73 did not inhibit or regulate the autophagy induced by nutrient deficiency. While Tp73-induced autophagy does not depend on DRAM, it may depend on other target genes regulated by Tp73. Interestingly the presence of p53 is not a requirement for Tp73-induced autophagy.

## 6.5 Perspectives

The Tp53 gene has been the focus of cell biology and basic medical research since it was discovered in the late 1970s. The understanding of Tp53's biological functions has evolved from the original "genome guardian" to the now recognized "pleiotropic regulator" involved in a variety of physiological and stress responses. Autophagy is considered to be a highly conserved metabolic mechanism from lower organisms to higher organisms. It plays an important role in maintaining the stability of the internal environment, growth and development, cell differentiation, and endogenous or exogenous stimulation of response cells.

The regulatory effects of Tp53 on autophagy are very diverse, as shown in Table 6.1. There are still many unsolved mysteries. For example, why nuclear-localized p53 promotes autophagy, presumably to allow cells to cope with stress, while cytoplasmic p53 is associated with inhibition of autophagy and apoptosis. The clarification of the specific molecular mechanism(s) behind these phenomena is yet to be fully explored, but it is certain that Tp53 plays an important regulatory role in the process of autophagy.

It is also intriguing that Tp53 can regulate opposing processes by transcriptional regulation. For example, Tp53 promotes the expression of transcriptional cell cycle regulatory protein p21, which arrests the cell cycle at G0/G1 phase by inhibiting G1-specific cyclin kinase. However, Tp53 can also effectively transcribe BAX, PUMA, NOXA, and other apoptotic proteins in BCL2 family and promote cell apoptosis.

The selection of target genes for Tp53 transcriptional regulation is influenced by different posttranslational modifications including phosphorylation, acetylation, sumoylation, and ubiquitination. It has been proposed that changes in posttranslational modification of p53 or interactions with as yet unidentified proteins (located in the nucleus or cytoplasm) determine whether p53 plays a role in promoting or inhibiting autophagy. Another hypothesis is that p53, in response to cell pressure, first activates mechanisms that promote cell survival (such as cell cycle arrest and autophagy) and then attempts to restore cell homeostasis, but when this fails to be restored, the cell initiates lethal processes such as apoptosis and autophagy inhibition. Presently, these mechanisms are speculation that urgently needs to be confirmed by more in-depth research.

**Table 6.1** Overview of Tp53 regulation of autophagy

| | Regulation pathway | | Mechanism |
|---|---|---|---|
| Tp53 | Promoting autophagy | Through mTOR | Activating AMPK |
| | | | Activating IGF-BP3 |
| | | | Activating REDD1 |
| | | Through DAPK1 | Binding MAP1B |
| | | | Phosphorylating beclin1 |
| | | | Increasing Tp53 protein stability |
| | Inhibiting autophagy | Under physiology condition | Binding and inhibiting RB1CC/FIP200 |
| | | | Through PKR |
| | | Under stress | Tp53 degradation promoting autophagy upon nutrient deprivation |
| | | | Inhibiting autophagy through AMPK pathway |
| Tp53 target gene | DRAM | | Promoting auto-lysosome formation |
| | TIGAR | | Inhibiting glycolysis, decreasing ROS, inhibiting autophagy |
| | PTEN | | Inhibiting PI3K/AKT/mTOR, Promoting autophagy |
| | PUMA, BAX, BAD, BNIP3 | | Releasing beclin1, promoting autophagy |
| | Noncoding RNA | | Regulating the autophagy-related gene expression |
| Other mechanism | Mutant Tp53 | | Affecting wild-type Tp53 |
| | Tp73 | | Unknown mechanism |

Autophagy, like apoptosis and cell senescence, is a very important biological process. Many major human diseases, including tumors, obesity, neurodegenerative diseases, and diabetes, are associated with abnormal autophagy. Deeper exploration of the mechanism of autophagy may therefore be helpful in elucidating the causes and etiology of these diseases. In the case of tumors, autophagy is closely related to the occurrence and development of tumors along with the sensitivity of tumors to chemotherapeutic drugs or radiotherapy. Of interest, autophagy in tumor cells leads to two opposite outcomes. On the one hand, autophagic death of cancer cells inhibits the proliferation of tumors. On the other, autophagy can act to stabilize and protect the internal environment of cancer cells against adverse microenvironments such as hypoxia. Autophagy also allows cancer cells to adapt and survive the actions of chemotherapeutic drugs, thus contributing to the further progression of cancers. At present, the mechanism of autophagy regulation in tumors is still not clear, particularly which stages of autophagy play regulatory roles in tumorigenesis and tumor development.

Studies on the regulation of Tp53 and its family on tumorigenesis have a long history and this has expanded to include the role of Tp53 in autophagy regulation. Targeting Tp53 using gene therapy has become an important direction in the field of anticancer research, so it is particularly important to understand the molecular mechanisms of how Tp53 is involved in autophagy. At present, the mode and target of action of p53 and related proteins in mTOR signaling, along with the "molecular switch" mechanism, whereby p53 plays different roles in autophagy are hot research topics. In addition, the contribution of p53-mediated autophagy in therapeutic approaches against cancers also needs to be urgently determined. Further study of these problems is expected to open up new opportunities for cancer treatment and reveal new treatment targets. Understanding the occurrence of autophagy at different stages of tumorigenesis and development will help us target Tp53 (including overexpression, knockout, modification, etc.) in order to intervene or treat tumors.

It is also worth mentioning that autophagy plays an important role in the maintenance, self-renewal, and directional differentiation of various types of stem cells, including embryonic stem cells, hematopoietic stem cells, and neural stem cells. Tp53 also plays an important role in self-renewal of induced pluripotent stem cells (iPSC) and embryonic stem cells. Indeed, the functions of p53 have been identified as major obstacles during the process of cell reprogramming. It will be an important direction in this field to determine whether p53 affects cell reprogramming or cell differentiation by regulating autophagy and if so to uncover the specific molecular mechanisms involved. It is hoped that further research will provide solutions for obtaining induced pluripotent stem cells more safely and efficiently. The regulation of p53 on autophagy affects the antiaging activity of stem cells, which may also provide new ideas and strategies for the prevention and treatment of aging-related diseases.

## 6.6 Summary

The regulation of Tp53 on autophagy is multilevel, complex, and delicate. On the one hand, Tp53 can promote autophagy. First, Tp53 can promote autophagy by activating AMPK pathway, inducing the expression of IGF-BP3 and activating REDD1. Second, Tp53 can also act on microtubule-binding protein MAP1B, phosphorylated Beclin1 and enhance the stability of Tp53 protein in an ARF-dependent manner through transcriptional regulation of DAPK-1. On the other hand, Tp53 can also inhibit autophagy in different ways under physiological or stress conditions. In addition, Tp53 can directly regulate its downstream target genes DRAM, TIGAR, PTEN, BCL2 family proteins (PUMA, BAX, BAD, and BNIP3), and noncoding RNA to participate in autophagy regulation. TP53 mutant or TP73 can also affect autophagy under certain circumstances. At present, although we have accumulated some knowledge about p53 or its target gene in the regulation of autophagy, the details of many mechanisms are still not fully clear and need to be further studied. For example, Tp53 also regulates autophagy through AMPK signaling, which can promote autophagy and also inhibit autophagy.

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# Chapter 7
# Ca(2+) Ion and Autophagy


**Yang-Xi Hu, Xiao-Shuai Han and Qing Jing**

**Abstract** Controlled by a strict mechanism, intracellular calcium (Ca(2+)) is closely related to various cellular activities, including the regulation of autophagy. Researchers believed that under normal or stress state, Ca(2+) has a positive or negative regulation effect on autophagy, the mechanisms of which are different. This bidirectional role of Ca(2+), promotive or suppressing in the regulation of autophagy under different conditions remains controversial, so as the potential mechanisms. Several studies reported that Ca(2+) promotes autophagy through plenty of ways, like inositol 1,4,5-trisphosphate receptor (IP3R) and beclin1 pathway, calmodulin-dependent kinase kinase beta (CaMKKβ)–AMPK–mTOR pathway, mitochondrial energy metabolism-related Ca(2+) uptake, lysosome's regulation of Ca(2+) signal, and so on. Others thought Ca(2+) may inhibit autophagy through IP3R and beclin1–Bcl-2 complex and the AMPK–mTOR pathway, either. It seems to be still a long way to thoroughly understand the truth of Ca(2+) and autophagy.

**Keywords** Calcium · Ca(2+) · Autophagy · IP3R · AMPK

## Abbreviations

| | |
|---|---|
| $[Ca(2+)]_{cyt}$ | Cytoplasmic calcium concentration |
| $[Ca(2+)]_{ER}$ | Endoplasmic reticulum calcium concentration |
| 2-APB | 2-Aminoethoxydiphenyl borate |
| AMP | Adenosine monophosphate |
| AMPK | Adenosine 5′-monophosphate (AMP)-activated protein kinase |
| Atg | Autophagy associated gene |

Y.-X. Hu
Department of Cardiology, Shanghai Changhai Hospital, 168 Changhai Road, Shanghai 200433, China

X.-S. Han · Q. Jing (✉)
CAS Key Lab of Tissue Microenvironment and Tumor, Shanghai Institute of Nutrition and Health, Chinese Academy of Sciences, 320 Yueyang Road, Shanghai 200031, China
e-mail: qjing@sibs.ac.cn



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| | |
|---|---|
| ATP | Adenosine triphosphate |
| Ca(2+) | Calcium ion |
| CaMKK | Calmodulin-dependent protein kinase kinase |
| cAMP | Cyclic adenosine monophosphate |
| CICR | Calcium-induced Ca(2+) release |
| DAPK | Death-associated protein kinase |
| DRAM | Damage-regulated autophagy modulator |
| Epac | Exchange protein directly activated by cAMP |
| GFP | Green fluorescent protein |
| GPN | Glycine-L-phenylalanine amide-naphthalene |
| IMPase | Inositol monophosphatase |
| $IP_3$ | 1,4,6-inositol triphosphate |
| $IP_3R$ | 1,4,5-inositol triphosphate receptor |
| LC3 | Microtubule-associated protein 1 light chain 3 |
| LRRK | Leucine-rich repeat kinase |
| mTOR | Mammalian target of rapamycin |
| NAADP | Nicotinic acid adenine dinucleotide phosphate |
| NAD | Nicotinamide adenine dinucleotide |
| NADH | Reduced form of nicotinamide-adenine dinucleotid |
| OCR | Oxygen consumption rate |
| PDH | Pyruvate dehydrogenase |
| PLC | Phospholipase C |
| PTP | Permeability transition pore |
| ROS | Reactive oxygen species |
| SERCA | Sarco/endoplasmic reticulum Ca(2+) ATPase |
| siRNA | Small interfering RNA |
| SNAREs | Soluble N-ethylmaleimide-sensitive factor attachment protein receptor |
| TPCs | Two-pore channels |
| TRP | Transient receptor potential channel |
| TRPML | Transient receptor potential channel, mucolipin subfamily |
| ULK | Uncoordinated 51-like kinase |
| VDAC | Voltage-dependent anion-selective channel |

The metabolism and function of cells usually require the participation of multiple cellular components. Studies have shown that intracellular calcium (Ca(2+)) is closely related to various cellular activities, and intracellular-free Ca(2+) is controlled by a strict mechanism. In 1993, for the first time, Gordon et al. reported the regulation of Ca(2+) on autophagy. In the following 20 years, the researchers conducted an in-depth study of the role of Ca(2+) in autophagy. Currently, it is clear that the intracellular Ca(2+) signaling could participate in the regulation of autophagy. However, the regulation of autophagy by Ca(2+) under different conditions remains controversial. Different studies hold different views on whether Ca(2+) promotes or inhibits autophagy. There are also different views on the exact mechanism by which

Ca(2+) regulates autophagy. At present, it is generally believed that under normal or stress state, Ca(2+) has a positive or negative regulation effect on autophagy, the mechanisms of which are different. This chapter will introduce the roles of Ca(2+) in promoting and inhibiting autophagy, the molecular mechanisms behind and relationship between the two effects.

## 7.1 Ca(2+) Promotes Autophagy

### *7.1.1 The Role of Intracellular Ca(2+) and Endoplasmic Reticulum in Autophagy*

As early as 1993, Gordon et al. have described the role of intracellular Ca(2+) in autophagy. The endoplasmic reticulum acts as an important organelle for Ca(2+) storage in cells, the role of which in the regulation of Ca(2+) on autophagy cannot be ignored. It is well known that endoplasmic reticulum stress can trigger autophagy and is regulated by the accumulation and release of endoplasmic reticulum Ca(2+), which is similar to the regulation of apoptosis. Ca(2+) from the endoplasmic reticulum was promoted to release by the calcium mobilizers (including ionomycin, adenosine triphosphate (ATP), vitamin D3 and its analogs, cadmium ions, resveratrol and hypericin combined with photodynamic therapy (PDT)) and increase the cytoplasmic Ca(2+) concentration, [Ca(2+)]cyt, which induces autophagy (Fig. 7.1).

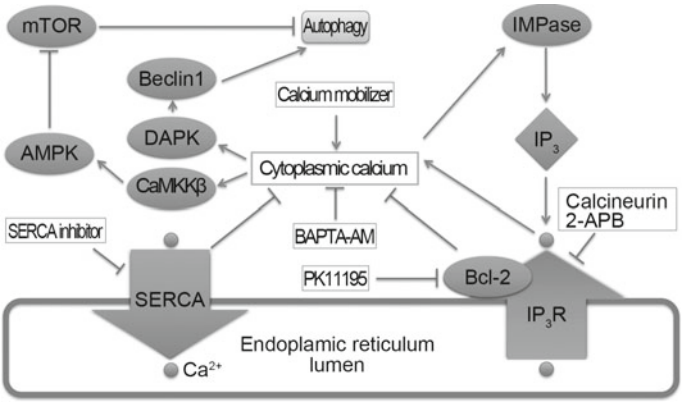


**Fig. 7.1** Intracellular mechanism by which calcium ions promote autophagy. mTOR: mammalian target of rapamycin; AMPK: 5′-adenosine-dependent protein kinase; DAPK: death-associated protein kinase; CaMKKβ: calmodulin-dependent kinase kinase β; SERCA: calcium of the endoplasmic reticulum ATPase; IMPase: inositol monophosphorylase; IP3: inositol 1,4,5-triphosphate; IP3R: inositol 1,4,5-trisphosphate; 2-APB: 2-aminoethyl phenylborate

In the optic nerve injury model, inhibition of the membrane Ca(2+) channel can prevent the increase of Ca(2+) concentration in the axon, thereby inhibiting the occurrence of autophagy. Extracellular or intracellular Ca(2+) buffering can counteract the autophagy induced by exogenous calcium phosphate precipitation. In Ca(2+)-induced autophagy, the endoplasmic reticulum is the main structure for Ca(2+) storage and one of the important organelles involved in the induction of autophagy.

In the study, the sarcoplasmic or endoplasmic reticulum calcium-ATPase (SERCA) inhibitors thapsigargin and alisol B can cause [Ca(2+)]cyt rise, which then promotes autophagy (Fig. 7.1). The thapsigargin is the most commonly used irreversible inhibitors of SERCA, and its roles on autophagy have a dose- and time-dependent effect. Studies have shown that treatment with 1 μM thapsigargin for 10 min and a 6-h recovery period can significantly enhance Ca(2+)-dependent autophagy, while higher doses or longer treatment may lead to side effects such as the inhibition of secretory pathway Ca(2+)-ATP enzyme (SPCA), accumulation of Ca(2+) in the Golgi apparatus, inhibition of Ca(2+) influx due to Ca(2+) channel barrier, and so on. In addition, endoplasmic reticulum stress is closely related to unfolded protein response, and autophagy induced by thapsigargin can also occur in cells lacking unfolded protein response, suggesting that Ca(2+) can directly induce autophagy without endoplasmic reticulum stress.

To monitor intracellular Ca(2+) activity, the researchers introduced BAPTA-AM. BAPTA is a selective Ca(2+) chelating agent whose acetomethyl ester derivative is BAPTA-AM which can be easily loaded into cells. BAPTA-AM is a fast and effective intracellular Ca(2+) buffer that prevents the activation of autophagy in cells, suggesting that cytoplasmic Ca(2+) plays an important role in the induction or activation of autophagy (Fig. 7.1). Further studies have also found that intracellular GFP-LC3 punctate fluorescent particles, which represent autophagosomes in cellular immunofluorescence, can be inhibited by BAPTA-AM, suggesting that Ca(2+) is critical for the formation of autophagosomes. In addition, nutrient deficiencies or starvation-induced autophagy can also be inhibited by BAPTA-AM, suggesting that other autophagy induction conditions such as starvation or rapamycin may also activate autophagy through the Ca(2+) signaling pathway.

Recent studies have found that BAPTA-AM can inhibit the activation of autophagy by chelation of intracellular Ca(2+), suggesting that Ca(2+) plays an important role in the induction or activation of autophagy. Further studies found that BAPTA-AM treated cells could not form autophagosomes, suggesting that Ca(2+) plays an essential role in the formation of autophagosomes. In addition, autophagy induced by nutrient deficiency or starvation can also be inhibited by BAPTA-AM, suggesting that other autophagy induction conditions such as starvation or rapamycin may also activate autophagy via the Ca(2+) signaling pathway. By establishing a tunicamycin-induced endoplasmic reticulum stress model in mouse liver and human cell lines, the researchers found that calreticulin stimulated the formation of autophagosomes and increased autophagic flow, revealing an endoplasmic reticulum stress-mediated upstream signal activated by autophagy. Under endoplasmic reticulum stress conditions, calreticulin induces autophagy by interacting with microtubule-associated protein 1A/1B-light chain 3 (LC3). Further studies revealed that the region that

interacts with LC3 in calreticulin is required for calreticulin-mediated activation of autophagy and reduction of endoplasmic reticulum stress (Yang et al. 2019).

### 7.1.2 Inositol 1,4,5-Trisphosphate Receptor (IP3R) and Beclin1

The role of inositol 1,4,5-trisphosphate receptor (IP3R) in the endoplasmic reticulum is also important. It is a Ca(2+) release channel that widely presents in cells, and mainly locates in the endoplasmic reticulum. IP3R includes three isoforms, which are IP3R-1, IP3R-2 and IP3R-3. The functional IP3R is a tetramer comprising four 310 kDa subunits, and the largest part of the receptor faces the cytoplasm. Thus, many cytoplasmic proteins, including protein kinases and phosphatases, can directly bind to and regulate IP3R, which in turn control the Ca(2+) flow. Studies have found that cadmium-induced Ca(2+) mobilization is achieved through the action of IP3R (Fig. 7.1). It can trigger the formation of autophagosomes, through inhibiting IP3R by chemical substances or knocking out IP3R by siRNA, or suppressing the activity of IP3R by inhibiting the synthesis of inositol 1,4,5-triphosphate (IP3) by lithium ions, demonstrating that inhibition of IP3R activity activates autophagy. However, researchers blocked the IP3R with 2-aminoethyldiphenylborate (2-APB), and found that the autophagy induced by cadmium ions was inhibited. Similarly, calcineurin, an inhibitor of IP3R, should theoretically inhibit autophagy after inhibition of the enzyme. However, after knocking down calcineurin with siRNA, the activity of IP3R was increased, which promoted the cadmium-induced autophagy. The very different results above suggest that IP3R may have a dual role in the regulation of autophagy. Since 2-APB is not a highly selective IP3R inhibitor, blocking IP3R with it can also affect Ca(2+) channels and SERCA. 2-APB may also act through SERCA and other pathways, thereby playing an opposite role in autophagy. In addition, whether calcineurin can act as a modulator of IP3R is also highly controversial. Therefore, as to whether IP3R activates or inhibits autophagy, the academic community still holds two views. In the next section, we will continue to introduce the inhibitory effect of IP3R on autophagy.

It is mentioned that Ca(2+) can also directly play a role in the process of inducing autophagy (Fig. 7.1). Studies in HEK293 cells have found that phosphate precipitation of Ca(2+) can induce autophagy, a process that is beclin1-dependent. Beclin1 can promote the formation of autophagosomes, and the autophagy promoted by beclin1 can be inhibited by Bcl-2 and Bcl-xL proteins. This is also an important node for autophagy and apoptosis (Mehrpour et al. 2010). Proteins only containing the BH3 domain in the Bcl-2 family are pro-apoptotic, except beclin1, which possesses a BH3 domain, and does not trigger apoptosis but activates autophagy. So beclin1 may have a protective effect on cells by antagonizing a variety of pro-apoptotic substances. In addition, beclin1 also interacts with death-associated protein kinases (DAPK) and IP3R. DAPK phosphorylates beclin1 and promotes the dissociation of beclin1 from

Bcl-2-like proteins, which in turn induces autophagy. The interaction of beclin1 with IP3R is the basis for the functional association between endoplasmic reticulum and mitochondria at the onset of autophagy (Mehrpour et al. 2010).

### *7.1.3 Calmodulin-Dependent Kinase Kinase Beta (CaMKKβ)–AMPK–mTOR Pathway*

The 5′-ATP-dependent protein kinase (AMPK)–mTOR pathway is a very important signaling pathway in the development of autophagy (Fig. 7.1). Autophagy induced by various stressors acts through this pathway. Ca(2+) activates AMPK via calmodulin-dependent kinase kinase β (CaMKKβ), further inhibiting mTOR and activating autophagy (Fig. 7.1).

Mammalian target of rapamycin (mTOR), especially mTOR complex 1 (mTORC1), is a central link in the regulation of autophagy. An important mediator of mTORC1 activity is AMPK. AMPK is phosphorylated and activated by its upstream kinase LKB1 and calmodulin-dependent kinase kinase β (CaMKKβ). Activated AMPK promotes autophagy by phosphorylating and activating the TSC1/TSC2 complex to inhibit mTORC1 activity. Targeting inhibition of CaMKKβ by siRNA or chemical inhibitors (STO-609 and complex C) can inhibit the occurrence of autophagy, which verifies the views above. In addition, autophagy could also be promoted by the phosphorylation and activation of ULK1/2 kinase induced by AMPK (Cardenas and Foskett 2012).

CaMKKβ is associated with Ca(2+) signaling, mTOR and autophagy. A sustained increase in [Ca(2+)]cyt activates autophagy, which is achieved by the mechanisms involved in CaMKKβ and AMPK and involves inhibition of the mTORC1 signal. Amyloid beta also regulates autophagy via the CaMKKβ–AMPK mechanism. Overexpression of leucine-rich repeat kinase-2 (LRRK2) activates the Ca(2+)-dependent CaMKKβ–AMPK pathway, resulting in a sustained increase in autophagosome formation (Gomez-Suaga et al. 2012). Nutritional deficiencies can trigger IP3R-mediated release of Ca(2+) from the endoplasmic reticulum, causing an increase in [Ca(2+)]cyt, which is essential for starvation-induced autophagy. Therefore, the Ca(2+)-dependent CaMKKβ–AMPK–mTOR pathway plays an important role in autophagy induced by various stress conditions.

In studying the neuroprotective effects of propofol (PPF) in cerebral ischemia-reperfusion (I/R), it was found that PPF antagonizes glucose deprivation and reoxygenation by regulating Ca(2+)/CaMKKβ/AMPK/mTOR autophagy pathway. The primary rat cerebral cortical neurons were cultured with oxygen-glucose deprivation and reoxygenation (OGD/R) to simulate brain I/R injury in vitro. It was found that OGD/R exposure notably caused autophagy induction, reflected by augmented LC3-II/LC3-I ratio and beclin1 expression, decreased p62 expression, and increased LC3 puncta formation. Moreover, OGD/R exposure induces an increase in intracellular Ca(2+) concentration ([Ca(2+)]i). After treatment of neurons with

PPF, PPF was found to significantly antagonize OGD/R-triggered cell damage, autophagy induction and elevation of [Ca(2+)]i. Further studies have found that PPF inhibits autophagy through the Ca(2+)/CaMKKβ/AMPK/mTOR pathway and alleviates OGD/R-triggered neuronal damage (Sun et al. 2018).

### *7.1.4 Mitochondrial Energy Metabolism and Ca(2+) Uptake*

Like the endoplasmic reticulum, mitochondria store Ca(2+), but its regulation mechanism is different from that of the endoplasmic reticulum. The mitochondrial Ca(2+) uptake is through a Ca(2+) highly selective ion conductance channel. In mitochondria, the movement of Ca(2+) is regulated by mitochondrial membrane proteins. The transmembrane unidirectional transporter on the mitochondrial inner membrane is involved in the formation of a proton gradient between the mitochondria and the cytoplasm. This unidirectional transporter mediates mitochondrial Ca(2+) uptake and works in both high conductance and low conductance modes. It is activated by the Ca(2+) microdomain adjacent to the endoplasmic reticulum and mitochondria. Because of its large capacity, Ca(2+) can accumulate in mitochondria, which can protect cells under certain conditions.

Accumulation of Ca(2+) in mitochondria increases the activity of the electron transport chain to induce the formation of more ATP and make more oxygen into water. However, these processes are accompanied by an increase in free electron leakage which will result in the formation of superoxide ions. The superoxide molecules, free radicals and peroxides produced are collectively referred to as reactive oxygen species (ROS). When ROS accumulate, it can cause cell damage by oxidizing cellular components. Therefore, the accumulation of Ca(2+) in mitochondria enhances energy production and forms ROS. The buffer system possessed by the mitochondrial matrix prevents excessive accumulation of Ca(2+). Once the cytoplasmic Ca(2+) restores its resting level, the mitochondrial Na+/Ca(2+) exchange pump will pump Ca(2+) back into the cytoplasm, causing Ca(2+) to return to the endoplasmic reticulum or clear out cells. Ca(2+) can also be released from mitochondria through permeable transport pores (PTP). PTP has two functional states: one is a low-conductivity mode that allows for transient opening and is therefore considered to be related to Ca(2+) wave amplification; the other high-conductivity mode is all-or-nothing, capable of releasing large amounts of Ca(2+).

Ca(2+) is also capable of modulating the activity of mitochondrial enzymes. Three key metabolic enzymes in mitochondria are enhanced by Ca(2+), including pyruvate dehydrogenase, alpha-ketoglutarate dehydrogenase and isocitrate dehydrogenase, which results in more reduction of NAD to reduced NADH. Also, NADH is an important raw material supplier for the mitochondrial respiratory chain. In addition, Aralar1, vitamin P and aspartate/glutamate carriers are all regulated by Ca(2+), which enhances aerobic metabolism when stimulated. Owing to the presence of a voltage-dependent anion channel (VDAC), the extracellular membrane of the mitochondria is unobstructed when Ca(2+) is taken up into the mitochondria. However,

Ca(2+) penetration into the mitochondrial inner membrane is rate-limiting and is mediated by the mitochondrial calcium uniporter (MCU). MCU is a subunit of the selective Ca(2+) channel pore form and was once considered as a Ca(2+) unidirectional transporter. Normally breathing mitochondria produce a high transmembrane negative voltage during electron transfer, whereas mitochondria uptake of Ca(2+) by the MCU is driven by this transmembrane negative voltage. Studies of mitochondrial suspensions and filaments (a mitochondria without an outer membrane) indicate that mitochondrial uptake of Ca(2+) is typically between 1 and 100 μM. However, submicromolar levels of Ca(2+) elevation by IP3-related agonists trigger a large increase in mitochondrial Ca(2+), which is mediated by tight junctions between the mitochondria and the endoplasmic reticulum (Cardenas and Foskett 2012).

### *7.1.5 Lysosomal Regulation of Autophagy as a Ca(2+) Signal Regulator*

There are many acidic Ca(2+) reservoirs in eukaryotic cells, including endosomes, lysosomes, secretory granules, Golgi, and so on, which regulate the uptake of Ca(2+) by Ca(2+) pumps or Ca(2+) exchangers and promote the formation of V-type ATPase-mediated acidic Ca(2+) separation. The permeabilized Ca(2+) channels of these depots belong to the family of transient potential receptors, which are two-pore channels (TPCs) or transient receptor potential channels (TRP). Among them, lysosomes are strongly acidic organelles (pH 4–5) and are ubiquitous in cells. Lysosomes are not only important organelles for removing components of digested cells during autophagy, but also have attracted attention as Ca(2+) storage and Ca(2+) signal sites (Smaili et al. 2013).

Lysosomes can directly fuse with endosomes, phagosomes and plasma membranes, which are important in the process of autophagy. When the protein complex or organelle is engulfed by the double-membrane vesicles, the lysosome begins to initiate, and the phagocytic vacuole then fuses with the lysosome. The processes involving fusion and division, such as endocytosis, membrane transport and autophagy, are regulated by Ca(2+), and the increase of [Ca(2+)]cyt is necessary for the fusion of lysosomal–lysosomal, lysosome–autophagosome and lysosomal–cell membranes (Smaili et al. 2013).

Ca(2+) storage and release in lysosomes are regulated by a variety of molecules. Glycine-L-phenylalanine-naphthylamide (GPN) was found to induce osmotic swelling of lysosomes and release their contents into the cytosol, including Ca(2+). Carbmycin A1, a V-type ATPase inhibitor, can alter the transmembrane pH gradient of acidic organelles and block Ca(2+) uptake. For the release of Ca(2+) from lysosomal storage, nicotinic acid adenine dinucleotide phosphate (NAADP) is an important regulator and acts as a second messenger. NAADP is a ubiquitous Ca(2+) releasing factor involved in the regulation of cell function, including fertilization, cell proliferation and differentiation, insulin secretion, nitrogen oxide signaling and muscle

contraction. NAADP causes Ca(2+) response by mobilizing the initial Ca(2+) burst, and then Ca(2+)-induced Ca(2+) release (CICR) in the endoplasmic reticulum further amplifies this response. The study found that a two-pore channel (TPCs) may be a member of the NAADP receptor family, and the TPCs model receptor of NAADP has the effect of inducing Ca(2+) release from acidic organelles (Ruas et al. 2010). The NAADP/TPC2/Ca(2+) signal inhibits the fusion between autophagosomes and lysosomes by alkalizing the pH of lysosomes, thereby preventing autophagic flux (Choi and Kim 2014).

The Ca(2+) separation mechanism is an important regulator of autophagy. TRP-lipoprotein-1 (TRPML1) is involved in a mutant of the TRP gene and is a non-selective Ca(2+) channel that causes lysosomal storage abnormalities, suggesting that Ca(2+)-dependent fusion is required for normal lysosomal transport mediated by TRPML1. TRPML3 is a selective Ca(2+) channel regulated by extracellular H+, which mediates Ca(2+) signaling and changes in organelle membrane potential or pH. Overexpression of TRPML3 leads to upregulation of autophagy, whereas knockdown of TRPML3 leads to downregulation of autophagy (Kim et al. 2009). Upon induction of macroautophagy/autophagy, TRPML3 is recruited and provides Ca(2+) for the fusion process in autophagosome biogenesis. Palmitoylation of the C-terminal region of the TRPML3 protein is required for the dynamic transport and function of TRPML3 in autophagy. Palmitoylation not only regulates the transport of TRPML3 into the autophagy structure but also regulates the autophagic flux in autophagy. Nutritional starvation activates TRPML3 to release Ca(2+) and increases TRPML3 palmitoylation levels. However, disruption of TRPML3 palmitoylation levels only abolished starvation-induced TRPML3 activation without affecting its channel activity. The transport and channel functions of TRPML3 are regulated in the context of autophagy, and palmitoylation is a prerequisite for TRPML3 to function as Ca(2+) channels in autophagosome formation (Kim et al. 2018). This suggests that Ca(2+) is important and regulated at the level of autophagosomes, which plays an important role in the fusion of autophagosomes and lysosomes.

## 7.2 Ca(2+) Inhibits Autophagy

### *7.2.1 IP3R and Beclin1–Bcl-2 Complex*

The inhibitory effect of Ca(2+) on autophagy is mainly concentrated on the inositol 1,4,5-triphosphate receptor (IP3R). In the cytoplasmic, the production of inositol 1,4,5-triphosphate (IP3) increases, when cells are stimulated by hormones, growth factors or antibodies. Then IP3R mediates the release of Ca(2+) from the endoplasmic reticulum into the cytoplasm (Fig. 7.2). In addition to IP3, IP3R can be directly activated when [Ca(2+)]cyt < 300 nM, and IP3R is inhibited conversely when [Ca(2+)]cyt is high.

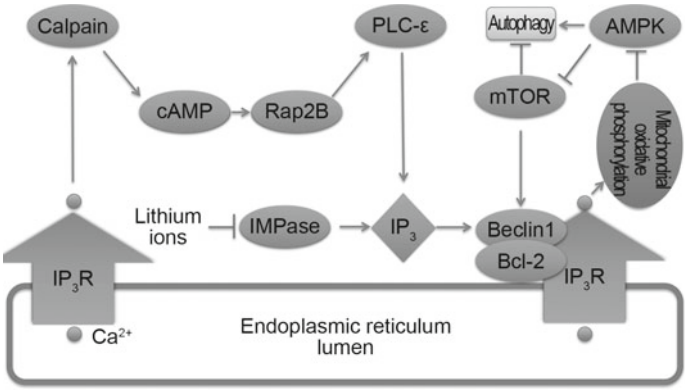


**Fig. 7.2** Intracellular mechanism by which calcium ions inhibit autophagy. IP3: inositol 1,4,5-triphosphate; IP3R: inositol 1,4,5-trisphosphate receptor; cAMP: cyclic adenosine monophosphate; IMpase: inositol monophosphorylase; PLC: phospholipase C; mTOR: mammalian target of rapamycin; AMPK: 5′-adenosine-dependent protein kinase

Lithium ion can inhibit inositol monophosphorylase (IMPases), thereby reducing IP3 levels and promoting autophagy, while increasing inositol can reduce the stimulating effect of lithium ions on autophagy (Sarkar et al. 2005). Through this pathway, the mTOR inhibitor rapamycin and lithium ions can synergistically stimulate autophagy (Fig. 7.2). The level of autophagy in chicken DT40 B lymphocytes was significantly higher than that in the wild type, and inhibition of autophagy by IP3R was confirmed in the cells knocked out of the IP3R triploid. Ectopic expression of IP3R-1 or IP3R-3 in DT40-KO cells can restore autophagy levels, while XeB does not increase autophagy levels in DT40-KO cells. In addition, expression of other endoplasmic reticulum Ca(2+) release channels, such as ryanodine receptors, does not restore elevated levels of autophagy, which further demonstrates the important role of IP3R in inhibiting autophagy (Decuypere et al. 2011a).

Low levels of IP3-mediated Ca(2+) transport to mitochondria promote oxidative phosphorylation, and cells lacking this pathway have weakened bioenergetics where the autophagy can be sensed and activated by AMPK. In cells in which IP3R activity is inhibited, cells are incubated with methylpyruvate, which reduces the NADH equivalent by oxidation to drive oxidative phosphorylation and ATP production, block autophagy and reduce AMPK activity. Inhibition of mitochondrial uptake of Ca(2+) by specific Ca(2+) unidirectional transporter blocker Ru360 or by RNAi knockdown MCU in cells expressing IP3R can reduce oxygen consumption, activate AMPK and induce non-mTOR-dependent autophagy. This is similar to the effect of using XEB or knocking down IP3R, suggesting that their target is on the same biochemical pathway (Cardenas and Foskett 2012).

Beclin1 promotes assembly of autophagosomes by forming a complex of type III PI3 kinase Vps34 and p150. In the early synthesis of autophagosomes, beclin1 interacts with Vps34 to play an important role. There is a suggestion that IP3R

buffers beclin1 and inhibits its binding to Vps34, thereby inhibiting autophagy. However, different opinions suggest that glucocorticoids can induce autophagy in lymphocytes, which reduces IP3R-mediated [Ca(2+)]cyt by a typical mTOR-dependent pathway. After inhibiting IP3R by XeB and phospholipase C inhibitor, or knocking out IP3R(DT40-KO), the autophagic flow of the cells is enhanced and exhibits basal autophagy, which supports the opinion that non-mTOR-dependent pathways act on autophagy. Re-expression of recombinant rat IP3R-3 in DT40-KO cells reversed the already elevated levels of autophagy; whereas in DT40-KO cells, mutant IP3R-3 without ion channel activity could not downregulate autophagy. Mutant IP3R, which is normally channel-gated but does not allow Ca(2+) to pass through in DT40-KO cells, also failed to inhibit autophagy that has been upregulated. Since both mutant and wild-type IP3R bind to beclin1, there is no difference in beclin1–Vps34 complex between wild-type cells and DT40-KO cells. Therefore, blocking the binding of beclin1 to Vps34 is not the reason why IP3R inhibits autophagy, and IP3R-mediated Ca(2+) release is necessary for the inhibition of autophagy.

There is another perspective in the academic community about the way IP3R interacts with beclin1. Some scholars believe that there are complexes of IP3R, beclin1 and Bcl-2 in the process of autophagy (Fig. 7.2). In this complex IP3R acts as a scaffold protein, binding to beclin1 and Bcl-2, respectively. It is therefore believed that IP3Rs promote the formation of the Bcl-2–beclin1 complex, which inhibits autophagy activation. Because beclin1 and Bcl-2 combine to form a complex, the free beclin1 is reduced, and in general, its ability to activate autophagy is reduced. Based on the complex model, the specific binding of IP3R using XeB can lead to the disintegration of the complex, thereby activating autophagy. This effect of XeB can be blocked by overexpressed Bcl-2, whereas inhibition of Bcl-2 by siRNA can abolish the interaction of IP3R–beclin1. In addition, the lack of IP3Rs can lead to the separation of beclin1 from Bcl-2, which in turn stimulates autophagy. Therefore, it can be considered that there is an interaction between the three substances in the complex. However, no difference in Bcl-2 and beclin1 interaction was observed between wild-type and DT40-KO cells, and no changes in the interaction between beclin1 and IP3R were observed under the action of XeB. It is worth noting that the separation of beclin1 from IP3R occurs only at a relatively late timepoint (6 h after the processing), which explains why some studies have not been able to observe this phenomenon. However, based on the theory of this scaffold protein, the role of IP3R in autophagy does not involve the function of Ca(2+) channels. In fact, knocking out beclin1 with siRNA did not affect agonist-induced Ca(2+) release. In this complex, beclin1 binds to the IP3 core in IP3R and has no effect on the Ca(2+) release.

Several studies have shown that the expression of the anti-apoptotic proteins Bcl-2 and Bcl-xL can inhibit autophagy. In many cases, this is attributed to its direct interaction with the BH3 domain of beclin1, which directly and effectively isolates beclin1 from the autophagy. In the above IP3R–Bcl-2–beclin1 complex model, IP3R acts only as a scaffold, promoting the interaction of Bcl-2 and beclin1, maintaining autophagy inhibition, and not relying on its channel function. Some researchers have also suggested that Bcl-2 inhibits autophagy by reducing the content of Ca(2+) in the endoplasmic reticulum. However, since the magnitude of the Ca(2+) flow through

IP3R is dependent on [Ca(2+)]ER, the reduction of [Ca(2+)]ER can reduce Ca(2+) transport to the mitochondria, thereby promoting autophagy. Bcl-2 is an important regulator of endoplasmic reticulum Ca(2+) levels and can inhibit Ca(2+)-induced autophagy. The most prominent evidence for the inhibition of autophagy by Bcl-2 is that autophagy is inhibited after overexpression of Bcl-2 in the endoplasmic reticulum. The ability to induce autophagy by PK11195 was also first mediated by inhibition of Bcl-2, followed by an increase in [Ca(2+)]ER (Gastaldello et al. 2010). However, since calcium ionophore can mobilize extracellular Ca(2+) and is not completely dependent on the release of endoplasmic reticulum Ca(2+), Bcl-2 does not inhibit calcium ionophore-induced autophagy. The inhibitory effect of Bcl-2 on autophagy involves the release of Ca(2+) from the endoplasmic reticulum. This ability to regulate intracellular Ca(2+) homeostasis and thereby inhibit autophagy is another pathway to inhibit autophagy in addition to direct inhibition of beclin1 activity.

It is reported that Bcl-xL can directly act on all subtypes of IP3R and maintain its sensitivity to low IP3 concentrations in resting cells, which promotes low levels of Ca(2+) release. In some studies, a decrease in [Ca(2+)]ER after overexpression of Bcl-2 or Bcl-xL was observed, which may be related to the above mechanism. Importantly, anti-apoptotic Bcl-2 family members enhance IP3R-mediated Ca(2+) signaling, thereby enhancing apoptosis resistance and enhancing cell bioenergy adaptation. These mechanisms provide optimal bioenergetics and then inhibit autophagy (Decuypere et al. 2011b). Thus the mechanism by which Bcl-2 and Bcl-xL inhibit autophagy involves their ability to enhance IP3R-mediated low levels of Ca(2+) transport to the mitochondria.

### *7.2.2 The AMPK–mTOR Pathway*

Regarding the role of IP3Rs and Ca(2+) in inhibiting autophagy, the AMPK–mTOR pathway may be involved (Fig. 7.2). Khan and Joseph suggested that there was no change in AMPK and Akt activity after downregulating mTOR activity in DT40-KO cells. However, more elaborate studies have found increased consumption of carbohydrates and oxygen, increased pyruvate dehydrogenase and AMPK activity in DT40-KO cells, suggesting that there is a mechanism for IP3R-mediated basic Ca(2+) release into the mitochondria, which in turn increases the bioenergy and ATP production in mitochondria (Decuypere et al. 2011a). In DT40-KO cells, either knocking or inhibiting IP3R, these basic Ca(2+) signals are eliminated, resulting in an increase in the AMP/ATP ratio, which activates and stimulates autophagy. However, it is interesting to note that in this study, mTOR activity was not affected, suggesting an atypical AMPK-dependent autophagy pathway. These different findings in DT40-KO cells again show the diversity of these cells (Decuypere et al. 2011a).

A variety of hypotheses suggest that mTOR plays an important role in this new autophagy pathway, either dependently or independently. Based on this, some

researchers have suggested that there are some internal connections in multiple pathways. In T lymphocytes, the induction of autophagy by dexamethasone is closely related to Fyn-mediated phosphorylation of IP3R and Ca(2+) oscillation, suggesting that dexamethasone stimulates autophagy through this pathway (Harr et al. 2010). However, dexamethasone also reduces mTOR activity, which may result in changes in IP3R phosphorylation and activity. mTOR may thus have a direct impact on IP3R. When IGF-1 stimulates AR4-2J cells, mTOR phosphorylates IP3R-2, resulting in increased Ca(2+) oscillations, and this effect can be abolished by rapamycin (Regimbald-Dumas et al. 2011). Furthermore, in RINm5F cells, mTOR acts on IP3R-3 and phosphorylates it, while rapamycin and other mTOR inhibitors can attenuate this phenomenon, leading to agonist-induced and IP3-induced Ca(2+) release downregulation (Fregeau et al. 2011). Therefore, it is of great interest to investigate whether this mTOR-dependent IP3R phosphorylation is associated with the regulation of autophagy. This also means that autophagy induced by rapamycin may not only result in inhibition of mTOR, but may also trigger changes in IP3R activity (Decuypere et al. 2011a). This possible association between Ca(2+)-dependent autophagy and mTOR-dependent autophagy can be used to explain the difference in autophagy between mTOR-dependent and non-dependent mechanisms.

Regardless of the downstream factors of Ca(2+)-induced autophagy, studies have proposed a meaningful upstream regulatory factor in the Ca(2+)/IP3R-mediated autophagy pathway. The researchers proposed a novel mTOR-dependent autophagy-inducing complex. In these complexes, some L-type Ca(2+) channel inhibitors are capable of inducing autophagy. Evidence suggests that intracellular Ca(2+) can activate calpain, which can form cyclic adenosine monophosphate (cAMP) by mediating the alpha subunit division of G protein and adenylyl cyclase activation. An increase in cAMP concentration stimulates IP3R production via a Rap2B-dependent pathway and a phospholipase C (PLC)-ε-dependent pathway, which further increases IP3R-mediated Ca(2+) release, suggesting a positive feedback loop in Ca(2+)-suppressing autophagy.

Recent studies have found that BAFF inhibits autophagy by activating Ca(2+)-CaMKII-dependent Akt/mTOR signaling pathways in normal and neoplastic B lymphocytes, thereby promoting cell proliferation and survival. Excess human-soluble BAFF (hsBAFF) inhibits autophagy and is accompanied by a decrease in LC3-II in normal and B lymphoid (Raji) cells. The knockdown of LC3 not only enhanced the inhibition of autophagy by hsBAFF but also attenuated the activation of hsBAFF in the Akt/mTOR pathway, thereby reducing hsBAFF-induced B cell proliferation/viability. In addition, it was also found that the inhibition of autophagy by hsBAFF is Akt/mTOR dependent. Akt inhibitor X, mTORC1 inhibitor rapamycin, mTORC1/2 inhibitor PP242, dominant negative Akt expression or mTOR knockdown attenuated hsBAFF-induced phosphorylation of ULK1, decreased LC3-II levels and increased cell proliferation/viability. At the same time, using BTATA/AM to chelate intracellular-free Ca(2+) ([Ca(2+)) i) or using EGTA or 2-APB to prevent [Ca(2+)] i elevation can significantly block hsBAFF-induced Akt/mTOR activation,

phosphorylation of ULK1 and reduction of LC3-II, and increased cell proliferation/viability. Similar results were observed during this process using KN93 to inhibit CaMKII or using CaMKII shRNA to reduce CaMKII expression (Dong et al. 2019).

## 7.3 The Bidirectional Role of Ca(2+) in the Regulation of Autophagy

The two sections above discuss two possibilities for Ca(2+) regulation of autophagy. One view is that Ca(2+) and IP3R act as inhibitors of autophagy, while another view focuses on the promotion of autophagy by Ca(2+), in which IP3R is also involved. These models may represent different Ca(2+) signal patterns, and which mode works depending on the state of the cell. The Ca(2+) signal may play an opposite role in cells under normal and stress conditions, thereby exerting different regulatory effects on the enhanced autophagy activity under basal and stress responses. Thus, on the one hand, spontaneous Ca(2+) signaling in healthy cells may inhibit basal autophagy through the mitochondrial pathway; on the other hand, stress conditions may promote Ca(2+) release to increase Ca(2+) concentration in the cytoplasm and promote autophagy through cytoplasmic molecules.

In healthy cells, IP3R releases basic Ca(2+) in response to basal levels of IP3, which are taken up by mitochondria and stimulate mitochondria to produce ATP via Ca(2+)-dependent enzymatic reactions. This can be considered as a positive, "healthy" signal. Blocking IP3R or inhibiting IP3 production would abolish this signal, leading to a decrease in ATP production, and further activation of the AMPK pathway to induce autophagy due to an increased AMP/ATP ratio (Decuypere et al. 2011a). In addition, IP3Rs also inhibited the separation of beclin1 from Bcl-2 and does not depend on its Ca(2+) release activity. Another positive feedback loop may exist between the Ca(2+) concentration near the cell membrane and the activity of calpain producing IP3, cAMP and PLCε. Blocking this pathway or blocking the L-calcium channel also abnormally reduces Ca(2+) transport to the mitochondria. Therefore, this basic Ca(2+) signal may be a quality control system for cell status. The presence of Ca(2+) spontaneous flow to the mitochondria suggests that the cells are healthy and do not require activation of stress pathways such as autophagy, and that lowering this Ca(2+) signal will result in activation of autophagy (Decuypere et al. 2011a). However, when the Ca(2+) signal continues to decrease or even completely disappear, the cells will eventually activate endoplasmic reticulum stress-induced apoptosis.

When the cells are under stress, the Ca(2+) signal may change, causing an increase in [Ca(2+)]cyt. This has been clarified in the role of stress conditions such as thapsigargin or calcium channel vectors in inducing Ca(2+) release into the cytoplasm. But the role of stress factors other than calcium mobilization, such as starvation or rapamycin, remains to be elucidated. Elevation of [Ca(2+)]cyt activates a variety of autophagy-promoting proteins, most notably CaMKKβ, which in turn increases

autophagy, a process that at least partially proceeds through the mTOR-dependent pathway (Decuypere et al. 2011a). In this respect, it is interesting to study which step in the autophagy is regulated by Ca(2+). However, the release of Ca(2+) into the cytoplasm can also cause an increase in mitochondrial Ca(2+) uptake, thereby promoting apoptosis. This dual result was also demonstrated by the ability of anti-apoptotic and anti-autophagy Bcl-2 to reduce Ca(2+) signaling. A challenge for future research will be to separate these two different Ca(2+)-dependent pathways to promote autophagy without interfering with the apoptotic pathway (Decuypere et al. 2011a).

The mechanism by which the Ca(2+) signaling is converted from anti-autophagy to autophagy is unclear. However, it is now clear that IP3R and its accessory proteins (such as Bcl-2 and beclin1) play a central role in the processing and alteration of Ca(2+) signaling in basal and stress states (Decuypere et al. 2011a). For example, JNK and DAPK kinases are known to regulate the phosphorylation status of Bcl-2 and beclin1 under starvation, thereby affecting the Bcl-2–beclin1 protein complex. Similarly, Bcl-2 phosphorylation affects its ability to reduce [Ca(2+)]ER because the hyperphosphorylated form of Bcl-2 is much weaker than non-phosphorylated in reducing the ability of [Ca(2+)] ER (Decuypere et al. 2011a). However, the role of Bcl-2 and beclin1 phosphorylation in its IP3R binding capacity and its ability to regulate IP3R Ca(2+) flux remains to be further investigated.

The regulation of autophagy by Ca(2+) signaling shows significant two-sidedness, depending on the different states of cells. On the one hand, in healthy cells, spontaneous Ca(2+) signaling may inhibit basal autophagy via the mitochondrial pathway. On the other hand, stress conditions can promote Ca(2+) release and thus increase [Ca(2+)]cyt, thereby promoting autophagy. In conclusion, Ca(2+) may exhibit opposite effects in cells under normal and stress conditions, and may exhibit different regulatory effects on basal autophagy and activation of autophagy under stress.

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# Chapter 8
# Endoplasmic Reticulum Stress and Autophagy


**Zhihao Qi and Linxi Chen**

**Abstract** In 1945, K. R. Porter et al. observed mouse embryonic fibroblasts (MEFs) and found that the cytoplasmic part of the cell had an unreported reticular structure, so it was named endoplasmic reticulum (ER). The major functions of the endoplasmic reticulum are: synthesis of intracellular proteins and the modification and processing of proteins. It is an important organelle in eukaryotic cells. It is a three-dimensional network structure in which complex and closed intracellular tubular intimal systems are intertwined. When cells are subjected to various strong stimulating factors such as nutrient deficiencies, $Ca^{2+}$ metabolic imbalance, toxin stimulation, and sustained oxidative stress stimulation, the cell homeostasis will be broken. In order to survive, a series of cell self-protection event will be initiated including the endoplasmic reticulum stress (ERS). The UPR can further promote the expression of the proteins which can help the misfolded and unfolded proteins restore to its normal structure through the activation of PERK, IRE1, and ATF6. However, the co-working of UPR and the ubiquitin-proteasome system still cannot make the endoplasmic reticulum restoring to its normal state, when the stimuli persist or are too strong. The damaged endoplasmic reticulum can be partially engulfed by the autophagic vesicles for degradation when the ERS persists. The degraded endoplasmic reticulum fragments can be reassembled into a new endoplasmic reticulum to restore the normal state of it. Hence, it seems that the autophagy has become the last mean to restore the homeostasis of endoplasmic reticulum.

**Keywords** Endoplasmic reticulum stress · Autophagy · Unfolded protein response · The endoplasmic reticulum-associated degradation

Z. Qi · L. Chen (✉)
Learning Key Laboratory for Pharmacoproteomics, Hunan Province Cooperative Innovation Center for Molecular Target New Drug Study, Institute of Pharmacy and Pharmacology, University of South China, Hengyang 421001, China
e-mail: lxchen6@126.com



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## 8.1 The Structure and Function of Endoplasmic Reticulum

The major functions of the endoplasmic reticulum are: synthesis of intracellular proteins and the modification and processing of proteins. It is an important organelle in eukaryotic cells. It is a three-dimensional network structure in which complex and closed intracellular tubular intimal systems are intertwined. The endoplasmic reticulum is a highly dynamic intracellular organelle, mainly in terms of quantity, type, and morphology. Interestingly, when the same cell undergoes different cell life activities or is in different physiological states, the structure and function of the endoplasmic reticulum will not remain the same. In 1945, K. R. Porter et al. observed mouse embryonic fibroblasts (MEFs) and found that the cytoplasmic part of the cell had an unreported reticular structure, so it was named endoplasmic reticulum (ER). Although it was found in later studies that the endoplasmic reticulum is mainly composed of one small vesicle and also exists in other parts of the cell, it is still customary to extend the name of endoplasmic reticulum.

According to the difference in structure and function, the endoplasmic reticulum can be divided into rough endoplasmic reticulum (rER) and smooth endoplasmic reticulum (sER). There is a large amount of ribosome attachment on the rough endoplasmic reticulum, which is mainly responsible for protein synthesis, while there is no ribosome on the smooth endoplasmic reticulum or only a small amount of ribosome attachment, which is mainly responsible for lipid synthesis. The endoplasmic reticulum is not only a substance synthesis plant in cells but also capable of glycosylation, acylation, hydroxylation of proteins, and the promotion of disulfide bond formation between proteins in the endoplasmic reticulum. Glycosylation is divided into N-linked glycosylation and O-linked glycosylation, the former mainly occurs in the endoplasmic reticulum, and the latter mainly occurs in the Golgi apparatus. GRP78 (glucose-regulated protein 78), also known as Bip (heavy-chain binding protein), is a molecular chaperone localized on the endoplasmic reticulum membrane by promoting the degradation and refolding of the unfolded proteins and incorrectly folded proteins accumulated in the endoplasmic reticulum restoring the homeostasis of endoplasmic reticulum. In addition, the sER is involved in the biotransformation process of hepatocytes, converting fat-soluble poisons into water-soluble and eliminating it from the body. In some cells of the body, the sER is highly specialized, such as in the cardiomyocytes and skeletal muscle cells called sarcoplasmic reticulum, in which a large amount of $Ca^{2+}$ is stored to regulate the muscle contraction (Ellgaard and Helenius 2003; Yan and Lennarz 2005).

## 8.2 The Endoplasmic Reticulum Associated Degradation

The homeostasis of endoplasmic reticulum is essential for the survival of the cells. To achieve this condition, the integrity of protein folding and maturation in the endoplasmic reticulum must be ensured. However, there is no eternal endoplasmic reticulum homeostasis which will eventually be broken by some specific physiological or pathological factors, causing a large amount of misfolded proteins accumulated in the endoplasmic reticulum. The endoplasmic reticulum has two ways to cope with the misfolded proteins accumulated in the ER lumen: one is the unfolded protein response and other is the endoplasmic reticulum associated degradation (ERAD) which mediates the transportation of misfolded proteins back to the cytosol and the degradation in the ubiquitin-proteasome system (Olzmann et al. 2013).

ERAD, a multistep process comprising recognition, transportation, and ubiquitination of ER misfolded proteins for cytosolic proteasomal degradation, was discovered by Ardythe McCracken and Jeffrey Brodsky at University of Pittsburgh in 1996. The misfolded proteins and unfolded proteins can be transport back to the cytosol by the protein sel-1 homolog 1 (SEL1L) for the ubiquitin-associated proteasomal degradation through a putative ER membrane channel HRD1 (an E3-ubiquitin ligase aka SYVN1), which is termed as the SEL1L–HRD1 ERAD. Ablation of SEL1L or HRD1 causes the embryonic lethal of mice in days 11–14, while the adult mice ablated the SEL1L or HRD1 die young within about 3 weeks, and mice knocked out of the SEL1L or HRD1 in adipocytes show lipodystrophy and postprandial hyperlipidemia. Deletion of SEL1L in mice AVP neurons cause polyuria and polydipsia, whereas in POMC neurons cause hyperphagia and obesity (Francisco et al. 2010; Kim et al. 2018).

## 8.3 The Endoplasmic Reticulum Stress

When cells are subjected to various strong stimulating factors (nutrient deficiencies, $Ca^{2+}$ metabolic imbalance, toxin stimulation, sustained oxidative stress stimulation), cell homeostasis will be broken, and in order to survive, a series of cell self-protection event will be initiated, including the endoplasmic reticulum stress (ERS). ERS will initiate three endoplasmic reticulum-related reactions, including unfolded protein response (UPR), endoplasmic reticulum overload response (EOR), and sterol regulation reaction to help clear the unfolded and incorrectly folded proteins accumulated in the endoplasmic reticulum to restore the homeostasis of ER. On the one hand, the ERS-induced UPR can promote the refolding and degradation of proteins by the molecular chaperone GRP78/Bip; on the other hand, it can also reduce the production of protein, finally restoring the homeostasis of the endoplasmic reticulum. Recently, it has been reported in the literature that ERS can restore the normal function of the endoplasmic reticulum or even restore the normal function of the whole cell through autophagy. However, autophagy is a ‘double-edged’ sword for the cells. On the one

hand, it can protect cells. For example, when cells are starved, cells can decompose intracellular substances through autophagy to provide cells with the necessary materials for survival and promote cell survival. On the other hand, when the stimulating factors inside and outside the cell persist, the level of autophagy rises, which in turn aggravates cell damage and eventually causes cell death. This cell death caused by excessive autophagy is called type II programmed cell death (Kapoor and Sanyal 2009).

### *8.3.1 The Endoplasmic Reticulum Stress Activate UPR*

The unfolded protein response (UPR) is a protective mechanism initiated by cells in order to restore the homeostasis of the endoplasmic reticulum when ERS occurs. The UPR can activate some transmembrane proteins located on the endoplasmic reticulum and then initiate a series of intracellular downstream signaling to promote expression of proteins, such as the chaperone GRP78/Bip or to reduce protein synthesis in the endoplasmic reticulum, and eventually restoring the normal state of endoplasmic reticulum. ERS can cause autophagy in cells by inducing the UPR, making the destroyed endoplasmic reticulum engulfed by the autophagic vesicles and restoring the homeostasis of endoplasmic reticulum. Three major branches of UPR are mediated by some transmembrane proteins located on the endoplasmic reticulum, including protein kinase R-like ER kinase (PERK), inositol requiring kinase 1 (IRE1), and activate transcription factor 6 (ATF6). PERK, IRE1, and ATF6 bind to GRP78/Bip when ERS does not happen, leaving it in an inactive state. The abnormally accumulated misfolded and unfolded proteins in the endoplasmic reticulum can compete with the PERK, IRE1, and ATF6 for the binding site of GRP78/Bip, activating the PERK, IRE1, and ATF6 through this competition, when ERS happens. Thus PERK, IRE1, and ATF6 are activated, which initiates the downstream signal transduction, affects the expression of certain genes in the nucleus, increases the synthesis of chaperones, increases the degradation of abnormally accumulated proteins, and finally reduces the synthesis of proteins, leading to the homeostasis of endoplasmic reticulum (Hetz 2012; Liu et al. 2016).

#### 8.3.1.1 PERK

The N-terminal domain of PERK is bound with GRP78/Bip, so it cannot be activated, when the ERS does not happen. The dimerization and the cross-phosphorylation happen in PERK, causing it no longer bind to GRP78/Bip and leading to the activation of PERK, when ERS occurs. The eukaryotic initiation factor 2α (eIF2α) is the downstream signal factor of PERK. PERK can phosphorylate the eIF2α at Ser-51, and the phosphorylated eIF2α can alleviate the pressure of protein accumulation by inhibiting the level of protein synthesis in the endoplasmic reticulum, thereby achieving the goal of restoring the normal function of endoplasmic reticulum.

#### 8.3.1.2 IRE1

The endoplasmic reticulum side domain of IRE1 has serine/threonine kinase activity and its cytoplasmic side domain also has RNA endonuclease activity. The abnormally accumulated misfolded and unfolded proteins, which can compete with the GRP78/Bip for binding to IRE1, is the cause for the release of GRP78/Bip from IRE1 when ERS happens and for the cross-phosphorylation and dimerization of IRE1, thus finally leading to the activation of its RNA endonuclease activity. The activated IRE1 can cleave the mRNA of certain proteins. For example, IRE1 can cleave the precursor of the X-box binding protein-1 (XBP-1) mRNA, which can bind to the promoter region of the endoplasmic reticulum stress element (ERSE) upregulating the expression of proteins involved in UPR, increasing the expression of proteins that help misfolded and unfolded proteins restore their normal morphology, and reducing the protein synthesis level in the endoplasmic reticulum restoring the homeostasis of it.

#### 8.3.1.3 ATF6

ATF6 is a transcription factor localized on the endoplasmic reticulum membrane. When ERS happens, ATF is separated from GRP78/Bip, while unlike the PERK and IRE1, ATF needs to be translocated to the Golgi with the help of coat protein complex II (COPII) vesicles, which can then be cleaved by the Site-1 protease (S1P) and Site-2 protease (S2P) in order to finish the activation of it. The ATF6 can directly promote the expression of proteins involved in the UPR without the activation of downstream signal transduction; for instance, the molecular chaperone GRP78/Bip can also directly promote the expression of the XBP-1, which is the transcription factor of ERSE.

## 8.4 The Endoplasmic Reticulum Stress and Autophagy

The abnormal accumulation of misfolded and unfolded proteins in the endoplasmic reticulum can cause the ERS, when a cell is subjected to strong intracellular or extracellular stress. The UPR can further promote the expression of the proteins which can help the misfolded and unfolded proteins restore to its normal structure through the activation of PERK, IRE1, and ATF6. It has been reported that the ubiquitin-proteasome system is also involved in the degradation of misfolded and unfolded proteins to further help the UPR restore the endoplasmic reticulum to its normal state, when the ERS-activated UPR is still insufficient to eliminate the misfolded and unfolded proteins accumulated in the endoplasmic reticulum. However, the co-working of UPR and the ubiquitin-proteasome system still cannot make the endoplasmic reticulum restore to its normal state, when the stimuli persist or are too strong. Hence, it seems that the autophagy has become the last mean to restore the homeostasis of endoplasmic reticulum. The damaged endoplasmic reticulum can

be partially engulfed by the autophagic vesicles for degradation when the ERS persists. The degraded endoplasmic reticulum fragments can be reassembled into a new endoplasmic reticulum to restore to the normal state.

### *8.4.1 The Endoplasmic Reticulum Stress Activates Autophagy Through UPR*

The PERK, IRE1, and ATF6 are the three different signal transduction pathways of UPR, which can not only promote the expression of proteins helping the misfolded and unfolded protein restore to its normal structure but can also further alleviate the ERS through the activation of cell autophagy, eventually restoring the homeostasis of the endoplasmic reticulum. It is noted that the three signal pathways induced by the ERS are all involved in the activation of cell autophagy but depends on the different cell type and cell living environment. The ERS-induced autophagy has a pro-survival effect of cell; hence it may be an important reason for the bad treatment effect of anti-tumor drugs. Indeed, it has been reported that inhibiting the autophagy of tumor cells will increase the c-Myc-dependent cell apoptosis through the pharmacological inhibitor and RNA interfering technology, but the c-Myc-dependent effect of promoting the cell apoptosis will be dramatically decreased when inhibiting the ERS-induced cell autophagy (Fujita et al. 2007; Yorimitsu et al. 2006).

PERK can promote the expression of autophagy-related gene Atg12 and LC3 through the phosphorylation of eIFα. Kouroku et al. (2007) found that gene knockout of PERK can inhibit the cell autophagy activated by the ERS-induced PERK-eIF2α cell signal transduction pathway, dramatically decreasing the numbers of autophagosome in cytoplasm in mouse embryonic cancer cells and embryonic fibroblasts (MEFs), and the above results indicate that the PERK-eIF2α cell signal transduction pathway is required for the ERS-induced cell autophagy. In addition, it is reported that the eIF2α can not only be phosphorylated by the PERK but also by the PKR (double-stranded RNA-activated protein kinase), GCN2 kinase (general control nonderepressible 2 GCN2), and (heme-regulated inhibitor HRI), which can activate the cell autophagy through the phosphorylation of eIF2α in the case of viral infection, nutrient depletion, and heme depletion. Moreover, it was found that the phosphorylated eIF2α, also involved in the amino acid deficiency, mimicked the formation of cell autophagy, although specific mechanism for it still remains unknown, at least indicating that the phosphorylated eIF2α plays a critical role in the ERS-induced cell autophagy (Liu et al. 2010; Talloczy et al. 2002).

The expression of transcriptional factor C/EBP-β will increase when ERS happens, which then further controls the expression of the death-associated protein kinase 1 (DAPK1). DAPK1 can phosphorylate Beclin-1 and free it from the negative regulator of autophagy Bcl-2, finally leading to the formation of cell autophagy. Interestingly, some researchers think that the real cell autophagy signal transduction pathway induced by ERS is IRE1 rather than PERK and ATF6. They found that the

IRE1 is required for the formation and aggregation of the autophagosomes rather than PERK and ATF6 in IRE1 or ATF6-deficient mouse MEFs and PERK-deficient mouse embryonic cells through the treatment of tunicamycin and thapsigargin.

In addition, they also found that the IRE1 can form a complex with tumor necrosis factor receptor-associated factor-2 (TRAF-2), which can phosphorylate the apoptosis signal-regulating kinase 1 (ASK1), and then the phosphorylated ASK1 further phosphorylates the JNK, which can promote the activation of the Bcl-2, eventually leading to cell autophagy. In their further experiments they found that the thapsigargin can inhibit the formation of autophagosomes in TRAF-2 KO MEFs through the inhibition of LC3. The same result is obtained by using the inhibitor of JNK, and more importantly the ERS still exists. Hence, the ERS-induced cell autophagy is mediated by the IRE1–TRAF2–JNK pathway (Kouroku et al. 2007; Liu et al. 2010; Talloczy et al. 2002; Ogata et al. 2006).

### *8.4.2 The Endoplasmic Reticulum Stress Activates Autophagy Through Releasing of the $Ca^{2+}$ into the Cytoplasm*

The ERS can not only activate the cell autophagy through the UPR but also promote the release of a lot of $Ca^{2+}$ from the endoplasmic reticulum into the cytoplasm, which can also induce the cell autophagy. Both DAPK1 and Calpain are $Ca^{2+}$-related protease and are located in the cytoplasm. It has been reported that the DAPK1 and Calpain can be activated by the cytoplasmic $Ca^{2+}$ upregulating the level of cell autophagy, but the specific mechanism remains unclear. It is reported that many agonists of autophagy still cannot induce the formation of autophagosome in Calpain KO MEFs and human osteosarcoma cells, causing there is no obvious change in the degradation of the misfolded proteins in the endoplasmic reticulum. It is noted that the cell autophagy cannot be activated by the use of rapamycin (inhibitor of mTOR) in the Calpain KO cells, although a lot of degradation of LC3-I and LC3-II emerged in the cytosol (Gozuacik et al. 2008; Hyrskyluoto et al. 2012; Madden et al. 2007).

IP3 (inositol-1,4,5-triphosphate) is an endogenous ligand of IP3R (inositol-1,4,5-triphosphate receptor) which can promote the releasing of $Ca^{2+}$ from the endoplasmic reticulum into the cytosol. It is reported that the inhibition of IP3R with its siRNA can increase the activity of autophagy rather than decrease it. Indeed, the calmodulin-dependent kinase kinase-β (CaMKK-β) can activate the AMPK (AMP-activated protein kinase), and then the activated AMPK can inhibit the mammalian target of rapamycin (mTOR), causing the inhibition of cell autophagy when ERS induces the release of $Ca^{2+}$ from the endoplasmic reticulum to the cytosol (Bhutia et al. 2011; Demarchi et al. 2006; Ganley et al. 2011). These above results indicate that the activation of cell autophagy, which is caused by the activation of IP3R, primarily relies on the AMPK–mTOR pathway. However, there are also some reports that the production of IP3 can be promoted by the intracellular ATP, which then

promotes the cell autophagy. The ATP-dependent cell autophagy is mediated by the $Ca^{2+}$–AMPK–mTOR pathway, and this reaction is slow and may take up to 2 days. Interestingly, the cell autophagy can be produced within 2 hours of using the pharmacological inhibitors of IP3R, the activity of mTOR was not inhibited, and there was no significant change in the cytoplasmic $Ca^{2+}$ content. The above data indicate that the activation or inhibition of IP3R caused by ERS can lead to the occurrence of autophagy, which is likely to be caused by IP3R through different signal transduction pathways, but how ERS can cause autophagy through IP3R and the mechanism remain unclear, hence further research is needed.

### *8.4.3 The Endoplasmic Reticulum Stress Inhibits the Bcl-2 Causing the Autophagy of Endoplasmic Reticulum*

ERS-induced autophagy is not significantly different from traditional autophagy and is also mediated by various autophagic factors. Beclin-1, also known as BECN1, and also called ATG6 in yeast, plays a key role in the formation of autophagosomes during autophagy. From a certain point of view, the content of Beclin-1 in the cell determines the degree of autophagy, and Beclin-1 is also a marker protein for autophagy formation. B-cell lymphoma-2 (Bcl-2) is mainly found in the endoplasmic reticulum, nuclear membrane, mitochondria, and its function is mainly to inhibit the cell apoptosis. It is reported in the literature that Bcl-2 has both promoting and inhibiting effects in the development of autophagy, but the specific mechanism is still unclear, and it may be associated with different subcellular localization and post-transcriptional modifications of Bcl-2. ERS initiates UPR, and UPR further inhibits Bcl-2 and ultimately causes autophagy by activating CHOP. For the inhibition of autophagy after activation of Bcl-2, it seems that there are two views in the academic world, the first one is Bcl-2 that can inhibit the interaction of Beclin-1, PI3 K and P150, and inhibit the formation of autophagy by blocking the formation of autophagosome membrane, and the second one is Bcl-2 that can inhibit $Ca^{2+}$-dependent autophagy by inhibiting the release of $Ca^{2+}$ induced by IP3R. However, it has also been reported in the literature that Bcl-2 can also inhibit autophagy caused by the endoplasmic reticulum IP3R inhibitor Xestospogin B, and autophagy induced in this way does not depend on $Ca^{2+}$. Therefore, whether Bcl-2-induced inhibition of autophagy requires $Ca^{2+}$ involvement or other mechanisms involved requires further research to elucidate (Cheng and Yang 2011; Kandala and Srivastava 2012; Marquez and Xu 2012; Pattingre et al. 2005) (Fig. 8.1).

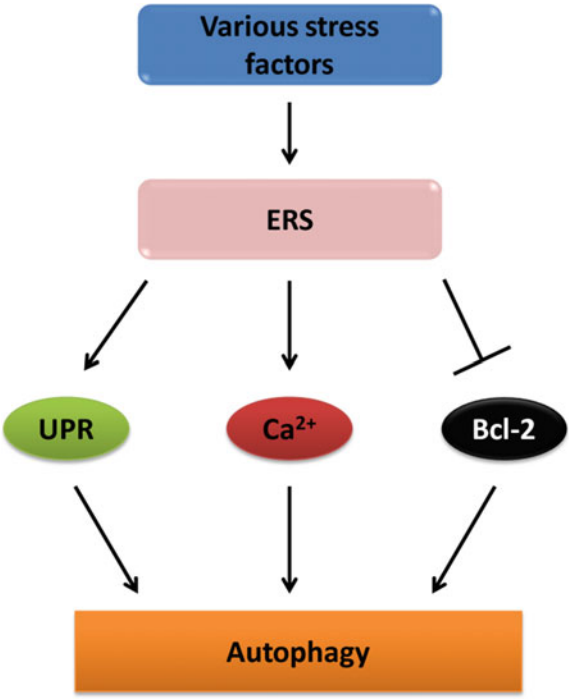


**Fig. 8.1** Illustration of the endoplasmic reticulum stress induced cell autophagy

## 8.5 Conclusion

The relationship between ERS and autophagy has only become a research hotspot in the research field in recent years. How does ERS cause autophagy in cells? There are still many unresolved issues. When cells are stimulated by various strong stimulating factors inside or outside the cell, the ERS occurs. ERS activates UPR to eliminate misfolded and unfolded proteins accumulated in the endoplasmic reticulum, and the ubiquitin-proteasome system is also involved in the degradation of abnormal accumulation proteins in the endoplasmic reticulum. It is worth noting that UPR and ubiquitin-proteasome cannot remove the misfolded and unfolded proteins from the endoplasmic reticulum in time when the stimulating factors persist. ERS will stimulate the production of autophagy to reduce the degree of swelling of the endoplasmic reticulum, relieving the pressure of protein accumulation in the endoplasmic reticulum, finally restoring the normal state of the endoplasmic reticulum and keeping the cells alive. However, when ERS causes excessive autophagy, it will lead to cell death. The 3-methyladenine (3-MA) was used to inhibit autophagy in MEFs following Atg5 KO and was found to reduce the ERS-induced cell death. The above indicates that if

we want to treat diseases caused by abnormal accumulation of proteins in the endoplasmic reticulum or cytoplasm by enhancing autophagy, more in-depth studies are needed to clarify whether autophagy is a pro-survival effect or a death-promoting effect on cells.

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# Chapter 9
# Oxidative Stress and Autophagy


**Qi Gao**

**Abstract** Living in a complex environment, humans are always faced with various external stimuli and internal changes including oxidative stress and tissue damage. To adapt to these stimuli, maintain physiological stability, and ensure survival, cells in the body initiate a series of interactive and regulatory response pathways. For example, increased reactive oxygen species in the body can induce autophagy through a variety of signalling pathways. This section will focus on ROS-mediated regulation of autophagy through PI3K/Akt, AMPK, JNK, ERK, ATG4, and other pathways.

**Keywords** ROS · Autophagy · PI3K/Akt · JNK

In 1990, Sohal and Allen et al. first proposed the concept of oxidative stress (OS) (Sohal and Allen 1990), that is, an increase in free radicals in tissues or cells or a decrease in scavenging ability that leads to disorder of the physiological oxidation system and antioxidant system, leading to oxidative damage processes caused by the accumulation of free radicals in the body. Under normal physiological conditions, the body's antioxidant system will remove ROS in a timely manner to maintain the balance between oxidation and antioxidant activity in the body. However, when the body is stimulated by various stressors or infected by pathogens, the ROS produced in the body can exceed the antioxidant defence ability of cells, causing the redox state to become imbalanced. Excessive ROS in tissues or cells can induce oxidative stress and lead to oxidative damage such as DNA hydroxylation, protein degeneration and tissue damage. To prevent further oxidative damage, organisms can activate a series of defence responses, such as enhancing the activity of antioxidant enzymes and initiating lysosomal degradation pathways. ROS produced during oxidative stress can induce autophagy through various mechanisms (Filomeni et al. 2015). Autophagy is a 'self-eating' phenomenon that widely exists in eukaryotic cells. This phenomenon is one of the important repair pathways by which cells can continue to survive by recycling nutrients under stress conditions through degradation of long-lived proteins and damaged organelles (Codogno and Meijer 2005). Autophagy can occur when

Q. Gao (✉)
Department of Clinical Laboratory, Shandong Provincial Hospital, Shandong University, Jinan 250014, Shandong, China
e-mail: 23147511@qq.com



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cells are under conditions such as starvation, hypoxia, endoplasmic reticulum stress and radiation. Autophagy can remove mitochondria, endoplasmic reticulum components, peroxisomes and proteins damaged by oxidative stress and thereby delay cell death. Thus, there is a close relationship between oxidative stress and autophagy (Scherz-Shouval et al. 2007a, b; Wu and Cederbaum 2013; Wang et al. 2015). This section mainly explains the oxidative stress system and its regulation of autophagy.

## 9.1 Oxidative Stress System

### *9.1.1 Types, Sources and Functions of ROS*

Reactive oxygen species (ROS) are a class of single-electron oxygen reduction products in organisms that are generated by leakage of the respiratory chain and by the consumption of approximately 2% of oxygen before the electrons can be transferred to the terminal oxidase. In other words, ROS are a class of oxygen-containing substances that are directly or indirectly transformed from molecular oxygen and have more active chemical reactivity than molecular oxygen. A complete set of oxidation-antioxidant systems exists in organisms. Under normal circumstances, ROS can be maintained in a stable range and can play active roles in anti-inflammatory and antibacterial activities. When the balance is broken and ROS levels continuously increase, they lead to the occurrence of disease.

#### 9.1.1.1 Types of ROS

Studies have shown that excessive reactive oxygen species (ROS) are direct triggers of oxidative stress. ROS are mainly divided into the following categories: (1) one-electron reduction products of oxygen such as the superoxide anion ($O_2^-$), the hydroperoxyl radical ($HO_2^-$) and the hydroxyl radical (•OH); (2) the double-electron reduction product of oxygen, hydrogen peroxide ($H_2O_2$); (3) paraffin peroxide homolysis products such as the alkoxy radical (RO•) and the alkyl peroxide base (ROO•); and (4) excited oxygen, singlet oxygen and carbonyl compounds. In addition, nitric oxide (NO), a small and very unstable gaseous free radical molecule, can also be regarded as a kind of ROS; NO and $O_2^-$ can react to produce $ONOO^-$•. ROS, which are products of normal redox reactions in the body, are involved in sterilization, detoxification and regulation of various metabolic pathways (Nauseef 2008; Yang 2002).

#### 9.1.1.2 The Sources of ROS

Oxygen has a unique molecular structure that easily accepts electrons to form ROS. The body is rich in oxygen and has the ability to produce large amounts of ROS. Depending on their sources, ROS can be broadly classified as exogenous or endogenous. Exogenous physical factors or compounds such as smoking, ionization, light, thermal shock, hyperbaric oxygen, drugs and environmental pollution, can produce ROS.

Normal Metabolism

Normal metabolism in the body can produce reactive oxygen species. Mitochondria are important sources of reactive oxygen species. Reactive oxygen species such as superoxide anionic free radicals ($O_2^-$), hydrogen peroxide ($H_2O_2$), hydroxyl free radicals (•OH) and singlet oxygen are all by-products of aerobic metabolism. In most cells, more than 90% of oxygen is consumed in mitochondria, and 2% of this oxygen is converted into oxygen-free radicals in the inner mitochondrial membrane and matrix. Reactive oxygen species in the body are not always harmful. For example, when the cell membranes of phagocytes are stimulated, large numbers of reactive oxygen species are produced through the respiratory burst mechanism. ROS are the main mediators with which phagocytes to play roles in phagocytosis and killing. However, under pathological conditions, the production and clearance of ROS become imbalanced, which often leads to ROS-mediated damage to the human body.

Radiation

It has long been recognized that radiation can produce reactive oxygen species in organisms. Water accounts for approximately 60% of the bodyweight of humans. The initial effects of radiation include decomposition of water, production of molecules such as H·OH·, and destruction of nucleic acids, proteins, and other macromolecules in cells; eventually radiation leads to disease. T. Herrling et al. found through electron spin resonance (ESR) that in vitro skin cells can generate oxygen-free radicals under the action of ultraviolet radiation and that the effects of this radiation are related to the intensity of the radiation and the penetration of the radiation into the skin.

Chemical Factors

Many chemical drugs, such as anticancer agents, antibiotics, pesticides, anaesthetics and aromatic hydrocarbons, can induce the production of reactive oxygen species. Hyperbaric oxygen can also induce the production of reactive oxygen species. M. Chavko and A. L. Harabin tested lipid and protein peroxidation in rats

raised at 5 atmospheres of pressure and found that reduced glutathione showed a downward trend, indicating the presence of oxidative damage. In addition, transition metal ions are important for the formation of reactive oxygen species, and the ability of these ions to remove electrons is the basis for the formation and expansion of many highly toxic reactive oxygen species. The most typical example is the iron-catalysed Fenton-type reaction, in which hydrogen peroxide generates highly active hydroxyl radicals in the presence of the transition metal iron, resulting in a greater toxic effect than that of hydrogen peroxide alone (Xie 2009).

#### 9.1.1.3 The Function of ROS

Oxygen is an indispensable gas in the context of biological processes. Organisms in environments short of oxygen will begin to suffocate and will even die. Therefore, since Joseph Priestley of England discovered oxygen in the early 1770s, it has been regarded as a beneficial gas for the human body. However, regardless of atmospheric oxygen or dissolved oxygen in the water has higher oxidation potential, in the presence of the metal iron, physiological components are constantly subjected to oxygen corrosion and 'rust'; of course, this corrosion is different from that of iron itself, but similar corrosion is reflected in the human body on the cellular level. In particular, the deterioration of various organs in the human body with increasing age is due to the natural progression of this type of corrosion, or 'rust'. In other words, oxygen can damage our bodies at the cellular level, and this damage is mainly caused by ROS. The specific ways in which oxygen causes physiological damage are discussed in the following sections.

##### Oxidative Damage to Nucleic Acids

One type of oxidative damage to DNA involves base modification. Hydroxyl radicals can add 5,6-double bonds to thymine to form thymine radicals. Changes in bases can lead to the destruction of many biochemical and protein synthesis processes under the control of specific DNA groups. The second type of damage is bond breakage. Free radicals can capture hydrogen atoms from the pentose in DNA, causing the formation of free radicals with unpaired electrons at the C4 position, which then break the chain at the β-position. $O_2$ can also decompose nucleotides, especially guanosine, in guanosine:adenosine:cytidine:uridine ratio of 26:13:8:1. After oxidative damage occurs, DNA may be broken, mutated or altered with regard to its thermal stability, thus severely affecting the normal transcription and translation processes of genetic information.

## Oxidative Damage to Proteins

The effects of reactive oxygen species on proteins include modification of amino acids, breakage of peptide chains, formation of cross-linked polymers of proteins and alteration of structure and immunogenicity.

**Modification of amino acids**
Amino acids, which play key roles in protein molecules, are particularly sensitive to free radical damage, especially aromatic amino acids and sulphur-containing amino acids. Different free radicals have specific effects on specific side chains of amino acids; for example, superoxide anion-mediated oxidation of methionine produces methionine sulfoxide, and that of cysteine produces cysteine sulfinate. Hydroxyl radicals remove hydrogen atoms from aliphatic amino acid α positions. Intermediate products such as alkyl radicals and peroxide radicals can oxidize tryptophan into kynurenine, N-methyl kynurenine and 5-hydroxytryptophan.

**Breakage of peptide chains**
Reactive oxygen species can break peptides in two ways: one way is by hydrolysis of peptides, and the other is by breakage of peptides directly at α-carbon atoms. The fracture mode depends on the types, concentrations and reaction rates of reactive oxygen species and protein. Hydrolysis of peptide bonds usually occurs at proline. The mechanism is that reactive oxygen species attack proline to introduce carbonyl groups and generate α-pyrrolidone. After hydrolysis, the peptide bonds are broken at the adjacent amino acids, and α-pyrrolidone becomes a new N-terminal, which can be further hydrolysed into glutamine. Peptides are also broken directly by the attack of reactive oxygen species on alpha-carbon atoms to form alpha-carbon peroxide groups, which are converted into amino peptides; these peptides are hydrolysed by weak acids into amino acids and dicarboxyl compounds.

**Formation of cross-linked protein polymers**
Multiple mechanisms can lead to protein cross-linking and polymerization. Protein molecules can be bound by two tyrosines, and cysteine can be oxidized to form disulfide bonds, both of these changes can form protein cross-links. Cross-linking can be divided into two forms: intramolecular cross-linking and intermolecular cross-linking. The numbers of tyrosines and cysteines in protein molecules can determine the form of cross-linking that occurs. In addition, malondialdehyde (MDA) produced by lipid peroxidation reacts with protein amino acid residues to produce enamine, which can also cause protein cross-linking. In vivo, the product of automatic alpha-carbonylaldehyde oxidization by monosaccharides can cross-link with proteins to deactivate enzymes, reduce membrane deformation, and lead to cell senescence and death.

**Alteration of protein configurations**
After oxidation, a protein is thermodynamically unstable; part of the tertiary structure opens, and the protein loses its original structure. When SOD is oxidized by $H_2O_2$ and ascorbate-Fe(III), ultraviolet absorption is enhanced and endogenous fluorescence is weakened, indicating that the enzyme molecules, originally arranged in a tight,

orderly manner, tend to become loose and disordered upon oxidation. In spin labelling studies, low concentrations of ascorbic acid-Fe(III) and $H_2O_2$ have been detected to affect the association of subunits of SOD molecules or their surrounding structures.

**Alteration of immunogenicity**
In a study on the effects of $H_2O_2$ alone or of a $H_2O_2$, $Cu^{2+}$ and ascorbic acid-Fe(III) system on bovine erythrocyte copper/zinc superoxide dismutase (SOD), human serum albumin (HSA) and human IgG, the results showed that response enhancement between SOD, HSA and IgG with those antibodies, suggesting that in certain autoimmune diseases, active oxygen may participate in the formation of antigen–antibody complexes.

Damage to Biological Membranes

Free radical damage to biological membranes affects cell membranes and organelle membranes made of polyunsaturated fatty acids, causing lipid peroxidation. Lipid peroxidation intermediate products such as lipid-free radicals (L·), lipid-oxygen-free radicals (LO·) and lipid-peroxyl-free radicals (LOO·) can react with hydrogen atoms in membrane proteins, generating free radicals in proteins and causing protein aggregation and cross-linking. In addition, lipid peroxide carbonyl products (such as malondialdehyde) can also attack the amino groups of membrane protein molecules, leading to intramolecular and intermolecular cross-linking of proteins. On the other hand, free radicals can also directly covalently bind to enzymes or receptors on the membrane. Oxidative damage affects many enzymes and receptors, ion channels embedded in the membrane system, altering the spatial configuration, destroying the integrity of the membrane, decreasing membrane fluidity, increasing membrane brittleness, hampering material and information exchange between the inside and outside of cells or organelles, affecting membrane function and antigen specificity and causing extensive damage and pathological changes. HO· is mostly produced in organelles, especially in mitochondria, causing damage to the mitochondrial membrane and causing energy metabolism disorder in cells and throughout the body.

ROS are strong oxidants that can kill a wide range of microorganisms including bacteria, spore, viruses and fungi, with a killing speed 600- to 3000-fold faster than that of chlorine. In recent years, due to the development of science and technology, there have been new developments in ROS applications for disinfection such as water, air, surface, food and vegetable disinfection, and ROS are now used in industry and agriculture (Xie 2009).

### *9.1.2 ROS Imbalance and Oxidative Stress*

The production of ROS in vivo is a dynamic process that is related to many in vitro and in vivo factors. ROS production directly determines whether oxidative stress occurs.

Oxidative stress is a negative effect produced by free radicals in vivo and is considered an important factor leading to ageing and disease. Low levels of ROS are involved in cellular signal regulation. ROS oxidize cysteine residues of target molecules and can participate in a variety of cellular regulatory processes as second messengers. ROS target molecules include kinases, phosphatases, redox-sensitive transcription factors, cell cycle regulatory proteins and membrane lipids. ROS-mediated oxidative modification of target proteins, peptide bond breakage and cross-linking can affect protein localization and interactions and enzyme activity in cells.

Nuclear factor κB (NF-κB) is a key cell survival factor mediated by ROS. $H_2O_2$ can induce NF-κB-inducing kinase(NIK)activation. NIK activates IKK, which catalyses the phosphorylation and degradation of I-κB (NF-κB inhibitory kinase), thereby activating NF-κB and translocation into the nucleus to exert transcriptional regulatory activity. $H_2O_2$ can also activate tyrosine kinase (Syk), and Syk can also catalyse the phosphorylation and degradation of I-κB. Activation of NF-κB eventually leads to increased expression of the anti-apoptotic protein Bcl-2 and the apoptotic inhibitor IAP, which play roles in maintaining cell survival.

ROS are also involved in hypoxia signal regulation. Hypoxia can occur under physiological and pathological conditions such as development, tumour progression, ischaemia and other conditions that are accompanied by whole-body or tissue hypoxia. Hypoxic conditions induce the expression of a series of genes to enable adaptation to the hypoxic environment for survival. The most critical of these proteins is the transcription factor HIF-lα. Under normal oxygen conditions, prolyl hydroxylase (PHD) catalyses HIF-lα rapid hydroxylation, which leads to proteasome degradation. Under hypoxic conditions, PHD activity was inhibited and stable HIF-lα enter the nucleus for transcriptional regulation. ROS are involved in the inhibition of anoxic conditions for PHD: Under hypoxia, $H_2O_2$ produced by ROS in the electron transport chain of mitochondria can inhibit PHD activity and stabilize HIF-lα. Hypoxia signal activation increases glycolysis and promotes angiogenesis and cell survival. Other mechanisms by which ROS regulate cell survival include killing invading microorganisms, activating heat shock factors and inducing PTEN inactivation (Huang and Klionsky 2007; Hussain et al. 2013).

Excessive production of ROS in cells can induce oxidative stress and lead to cell death through apoptosis, necrosis and autophagy. Mitochondrial permeability transition (PT) pore opening is induced by high levels of ROS. The opening of the PT pore is a key event for the occurrence of endogenous apoptosis. Two components of the PT pore complex, the enzyme adenine nucleotide translocator (ANT) located on the inner membrane and the voltage-dependent anion channel (VDAC) located on the outer membrane, are very sensitive to ROS-induced oxidative damage, and damage to these components can lead to mitochondrial membrane rupture and the release of apoptotic proteins. ROS-induced apoptotic cell death occurs under a variety of pathological conditions including stroke, inflammation and ischaemia. Many apoptosis-inducing agents, such as arsenic trioxide and buthionine sulfoximine, deplete the antioxidant enzymes in cells, and glutathione superoxidase can also induce the accumulation of large amounts of intracellular ROS, leading to cell death. Compared with apoptosis, necrosis is a passive form of cell death that often

occurs in cases of acute cell dysfunction and ATP depletion. Swelling and rupture of necrotic cells can cause the release of cellular contents into the extracellular space, causing inflammation and damage to surrounding tissues. In many cases, apoptosis and necrosis may occur sequentially or simultaneously in the same tissue. The transformation from apoptosis to necrotic cell death requires not only decreases in intracellular ATP but also increases in intracellular ROS (Takai et al. 2010).

In conclusion, low levels of ROS are involved in the regulation of cell survival signals, while excessive ROS lead to oxidative stress and cell death. The process of ROS-mediated oxidative stress-induced autophagy activation has attracted increasing attention and needs to be further explored.

## 9.2 Mechanisms of Autophagy Regulation by ROS

### *9.2.1 The Signalling Pathway of Autophagy Regulation by ROS*

Autophagy can be divided into six stages: induction, vesicular nucleation and extension, substrate recognition, autophagosome formation, autophagosome and lysosome fusion, and substrate degradation. Many factors induce autophagy, in this process, more than 30 autophagy-related genes (Atgs) play roles, but autophagosome formation is mainly mediated by the following four protein complexes: (1) the ULK1 complex (ULK1-Atg101-FIP200-Atg13), (2) the Type III phosphatidylinositol 3 kinase (PI3K) complex (Beclin1-VPS34-Atg14), (3) the Atg12-Atg5-Atg16 ubiquitination complex and (4) the LC3-II-PE ubiquitination complex (Yang and Klionsky 2010).

ROS are direct triggers of oxidative stress, and approximately 90% of ROS are derived from the respiratory chain of the inner mitochondrial membrane. Numerous studies have shown that ROS derived from mitochondria are the main inducers of autophagy under oxidative stress. ROS can induce autophagy by mediating various signalling pathways in the process of autophagosome formation. In the autophagy induction stage, ROS can induce autophagy by regulating mTOR. mTOR is a key negative regulator of autophagy whose activity is regulated by multiple signalling pathways, such as the PI3K-serine/threonine-protein kinase (Akt) and AMPK pathways. Studies have found that excessive ROS can activate autophagy by inhibiting PI3K-Akt-mTOR (Portal-Núñez et al. 2016). Perfusion of sevoflurane in isolated hearts of male Dutch pigs produces ROS that induce autophagy by activating AMPK to inhibit the mTOR signalling pathway (Shiomi et al. 2014). In the process of autophagosome formation, ROS, mainly by inhibiting the activity of Atg4, regulate autophagy, and ROS-mediated Atg4 deactivation causes LC3-II accumulation, inducing autophagy. A previous study found that under starvation conditions, cells produce large amounts of ROS; these ROS, particularly $H_2O_2$, inhibit LC3-II after Atg4 oxidation to ensure that autophagy body extension. ROS can promote ubiquitinated materials to be degraded; these materials combine with P62 and LC3-II for

targeting to autophagic bodies and subsequent degradation (Scherz-Shouval et al. 2007a, b).

In addition, ROS can also regulate autophagy through the mitogen-activated protein kinase (MAPK) signalling pathway. The MAPK pathway is composed of a group of Akt molecules that are activated successively in a cascading manner and plays important roles in cell proliferation, differentiation, stress adaptation and apoptosis. The MAPK pathway mainly includes c-Jun amino-terminal kinase (JNK), p38 kinase and extracellular signal-regulated kinase (ERK). One study found that the MAPK pathway can adjust the activity of the transcription factors activator protein 1 (AP-1), forkhead box transcription factor O (FoxO), and nuclear factor kappa B (NF-kappa B), which regulate autophagy-related gene expression and affect autophagy; in addition, ROS can arise through MAPK-mediated activation of autophagy (Sui et al. 2014). A previous experiment revealed that ROS induce autophagy of cultured mouse mesenchymal stem cells (MSCs) through the JNK signalling pathway. The p38 signalling pathway is involved in the ROS-activated autophagosome/lysosomal fusion stage associated with the gene expression of Atg7 and E3 during protein ubiquitination, and this process is dependent on the activation of FoxO transcription. Arsenite induces autophagy by ROS activation of the extracellular regulatory protein kinase ERK1/2 pathway (Liu et al. 2015; McClung et al. 2010; Huang et al. 2015). Below, we will introduce the relationship between ROS and autophagy regulation through PI3K/Akt, AMPK, ERK-JNK and ATG4.

### *9.2.2 ROS-Mediated Regulation of Autophagy Through the PI3K/Akt Signalling Pathway*

The phosphatidylinositol 3-kinase (PI3K) protein family is involved in the regulation of cell proliferation, differentiation, apoptosis and glucose transport. Increased PI3K activity is often associated with a variety of cancers. PI3K phosphorylates the third carbon atom of the phosphatidylinositol (PI) ring. The proportion of PI in cell membrane components is smaller than the combined proportions of phosphatidylcholine, phosphatidylethanolamine and phosphatidylserine. However, the PI content is greater in brain cell membranes than in other types of cell membranes. There are 5 phosphorylation sites on the inositol ring of PI, and multiple kinases can phosphorylate the fourth and fifth sites on the PI inositol ring; therefore, phosphorylation usually occurs at both of these sites, especially in the medial plasma membrane. In general, PI-4,5-bisphosphate (PIP2) produces diacylglycerol (DAG) and inositol-1,4,5-triphosphate under the action of phosphatase C. PI3K transfers a phosphate group to site 3, and the product has an important effect on cell function. For example, monophosphorylated PI-3-phosphate stimulates cell migration, whereas unphosphorylated PI-3-phosphate does not. While PI-3,4-diphosphate promotes cell proliferation and increases resistance to apoptosis, its precursor molecule PI-4-phosphate does not. PIP2 is converted to PI-3,4,5-triphosphate, which regulates cell adhesion, growth and survival.

PI3Ks can be divided into three categories: class I PI3Ks, class II PI3Ks and class III PI3Ks. The three classes vary in structure and function. The most widely studied PI3K class is class I, which includes heterodimers composed of a regulatory subunit and a catalytic subunit. The regulatory subunit contains the SH2 and SH3 domains and interacts with target proteins containing corresponding binding sites. The subunit commonly known as p85 was the first known subunit, but there are six known regulatory subunits ranging in size from 50 to 110 kDa. There are 4 types of catalytic subunits, namely, p110 α, β, γ, and δ; γ and δ are limited to leukocytes, while the rest are widely distributed in various cells.

Many proteins contain a Pleckstrin homology (PH) domain and thus bind to PI-3,4-P2 or PI-3,4,5-P3. These interactions control the timing and location of the binding of the protein to the membrane and in this way regulate the activity of the protein. This interaction between proteins and lipids may also lead to changes in protein structures and functions. PI3K activation results in the production of a second messenger, PIP3, on the plasma membrane. PIP3 binds to the PH domain-containing intracellular signalling proteins AKT and PDK1, prompting PDK1 to phosphorylate AKT at Ser308 and resulting in AKT activation. Other substrates of PDK1 include PKC (protein kinase C), S6K (p70S6) and SGK (serum/glucocorticoid regulated kinase). AKT is a serine/threonine kinase of the AGC family, also known as protein kinase B (PKB), that mainly has three domains: a PH domain, a catalytic domain and a regulatory domain. The regulatory domain is the main downstream effector of PI3K. AKT can be divided into three subtypes (AKT1, AKT2, and AKT3 or PKBα, PKBβ and PKBγ). The three subtypes have different functions, but some overlap.

ROS can induce autophagy by regulating mTOR. mTOR is a key negative regulator of autophagy whose activity is influenced by multiple signalling pathways such as the AMPK and PI3K serine/threonine-protein kinase (Akt) pathways. Studies have found that excessive ROS can activate autophagy by inhibiting PI3K-Akt-mTOR. In mammals, mTOR, a member of the phosphatidylinositol-3-kinase related kinase (PIKK) family, is a relatively evolutionarily conserved serine/threonine-protein kinase that plays a crucial central regulatory role in cell growth and proliferation and in cancer cell metabolism. In mammalian cells, the mTOR kinase complex has two distinct forms with different structures and functions. In one form, mTORCl, mTOR is bound to the protein raptor; this complex is extremely sensitive to rapamycin. In the other, mTORC2, mTOR is bound with Rictor; unlike mTORC1, mTORC2 is insensitive to rapamycin. mTORCl mainly mediates the regulation of cell growth, apoptosis and autophagy, while mTORC2 is mainly involved in cell survival and cytoskeleton recombination. Therefore, mTORCl plays a major role in the regulation of autophagy, and the activation of mTORCl plays a negative role in the regulation of autophagy (Inoki et al. 2002). A variety of growth factors and signal transduction complexes, including fibroblast growth factor (FGF), vascular endothelial growth factor (VEGF), human growth factor (HGF), Angiopoietin I (Ang1) and insulin, initiate PI3K activation. A corresponding receptor on the cell membrane activates type I phosphatidylinositol triphosphate kinase (PIP3); in this process, PIP is phosphorylated to form PIP3, which then binds to the intracellular signalling protein AKT and activates AKT through synergistic action with phosphoinositol-dependent protein kinase I.

Akt, a downstream effector of PI3K, interacts with PIP2 and PIP3 produced by class I PI3Ks to activate mTOR and inhibit autophagy.

Genomic mutations or deletions in PTEN (phosphatase and tensin homologue deleted on chromosome 10) are present in a wide range of human tumours. PTEN is a PIP3 phosphatase that, unlike PI3K, can transform PIP3 into PI-4,5-P2 by dephosphorylation. PTEN reduces Akt activation and blocks all downstream signalling events regulated by Akt. By producing PIP2, PTEN does much more than just block Akt. PIP2 acts as a substrate for phospholipase C (PLC), producing DAG and IP3 as second messengers, raising intracellular calcium levels and activating protein kinase C (PKC). Membrane-bound PIP2 also mediates the activity of a variety of ion channels, including calcium, potassium and sodium channels. PIP2 is also involved in the formation of membrane vesicles and in the interaction between the cytoskeleton and membrane. PIP2 also affects the activity of several enzymes involved in lipid metabolism including phospholipase D and ceramide 1-phosphate. By producing PIP2 from PIP, PTEN affects a wide range of cellular function and cellular pathways. PTEN plays an important role in weakening PIP3 signal transduction and increasing PIP2 levels. PTEN also plays a positive role in autophagy regulation by dephosphorylating PIP3 to release the inhibitory effect of class I PI3Ks on autophagy (Errafiy et al. 2013). Unlike the PI3Ks in the first two classes, class III PI3Ks upregulate autophagy.

Beclin 1, a homologous gene of ATG6 in yeast, is a tumour suppressor gene that is a positive regulator of autophagy and can promote the formation of autophagic vesicles. Bcl-2 is an anti-apoptotic protein that plays a role in downregulating autophagy by binding to Beclin 1's BH3 domain. Vps34 is a class III PI3K that activates autophagy. In mammalian cells, Beclin 1, Bcl-2 and class III PI3K/hVps34 complexes jointly constitute an important signalling pathway that can regulate autophagy. When stress occurs, the Beclin 1-bcl-2 complex dissociates, and the dissociated Beclin 1, in turn, binds to a class III PI3K to enhance its activity and induce autophagy. In addition, studies have shown that death-associated protein kinase (DAPK) can also upregulate autophagy by binding to Beclin 1's BH3 domain.

### *9.2.3 ROS-Mediated Regulation of Autophagy Through the AMPK Signalling Pathway*

AMP-activated protein kinase (AMPK) plays an important role in maintaining cellular homeostasis. AMPK is a highly conserved kinase that maintains the homeostasis of energy metabolism by coordinating multiple metabolic pathways. In mammals, AMPK in heterologous trimer form exists in a variety of organizations including α catalytic subunits and β and γ regulatory subunits. The N-terminus of the α subunit is the core area necessary for catalysis, containing a typical serine and threonine (Ser/Thr) protein kinase in catalytic area. The C-terminus of α subunit is mainly responsible for active control and for connection with the β and γ subunits. The α

subunits can be phosphorylated in multiple sites, and phosphorylation and dephosphorylation of Thr172 can precisely adjust AMPK activity. The β subunits can embed α and γ subunits or anchor them in the KIS and ASC domains. The γ subunit has four serial repeat cystathionine β-synthase (CBS) domains that together constitute the Bateman domain in the form of structural modules, which comprise the AMP binding site.

The activation of AMPK is a potentially potent factor in the regulation of autophagy and the prevention of disease. Therefore, AMPK agonists and inhibitors have also been the focus of research in recent years. Studies have shown that the activation of AMPK in hypoxia requires the induction of HIF. In addition, AMPK can also regulate the survival of cells under hypoxic stress through autophagy. AMPK activation has been reported to regulate energy balance at the levels of multiple organs by targeting several important substrates. Autophagy is a protective mechanism that allows cells to survive a variety of stressors. AMPK can promote autophagy by inhibiting the mTORC1 pathway, especially in the case of nutrient deprivation. Under hypotrophic conditions, AMPK is activated and phosphorylates TSC2, inhibiting mTORC1. AMPK directly phosphorylates Ser722 and Ser792 residual of mTOR associated protein Raptor to regulate the mTOR pathway. Recent studies have demonstrated that in mouse embryonic fibroblasts, AMPK can directly induce autophagy by directly phosphorylating ULK1 at Ser555 and Ser792 or at Ser317 and Ser777 in the case of glucose deprivation.

The growth and proliferation of cells are strictly regulated by the growth factor signalling pathway to prevent the occurrence of tumours. Cell growth and division, however, occur only when sufficient energy and nutrients are present. TOR plays a key role in balancing various metabolic processes, such as cell growth, proliferation and protein synthesis. Numerous studies have shown that TOR, an important regulator of growth factors and a nutrient-sensing kinase, regulates autophagy together with AMPK, an energy-sensing kinase.

The mammalian homologous protein mTOR can bind with multiple ligands to form two complexes with significant functional differences, which are called mTOR complexes 1 and 2. Under low-energy conditions in cells, AMPK is activated, leading to autophagy induction. However, mTORC1 activity depends on different positive signals, such as high energy levels, normal oxygen levels, normal amino acid levels and growth factor levels, which lead to autophagy inhibition. Initially, it was found that activated AMPK inhibited the activity of mTORC1 mainly through phosphorylation and activation of the mTORC1 negative regulator TSC2, thereby preventing cell growth and promoting autophagy. However, TSC2-deficient cells can still respond to reduced energy levels. Subsequent studies have confirmed that raptor in mTORC1 is a direct substrate for AMPK phosphorylation. This phosphorylation produces an anchor site for 14-3-3 protein (dephosphorylation of mTORC1) anchor site, which is necessary for AMPK-mediated inhibition of mTORC1. In general, AMPK alleviates the mTORC1-mediated inhibition of autophagy induction through at least two different pathways when the intracellular energy state is abnormal (Gwinn et al. 2008).

Tp53 plays a dual role in autophagy regulation. Tp53 in different locations in cells plays different roles in autophagy regulation. Tp53 promotes autophagy when localized in the nucleus. Nuclear Tp53 inhibits mTOR and promotes autophagy by activating AMPK and TSC2. Tp53 inhibits autophagy when localized in the cytoplasm. Tp53 plays this role in three main ways: by activating mTOR, by inhibiting AMPK, and by exerting direct action. In TP53-negative colon cancer cells, the levels of autophagy are continuously upregulated, while restoration of the Tp53 gene decreases autophagy levels.

### *9.2.4 ROS-Mediated Regulation of Autophagy Through the ERK and JNK Signalling Pathways*

Mitogen-activated protein kinase (MAPK) is a widely existing regulatory serine/threonine-protein kinase in vertebrates. The MAPK pathway is an important transmitter of cell signals from the cell surface to the nucleus. The mitogen-activated protein kinase (MAPK) family is a group of serine/threonine-protein kinases that can be activated by different extracellular stimuli such as cytokines, neurotransmitters, hormones, cellular stress and cell adhesion. MAPK is so named because it is expressed when cultured cells are stimulated by mitogen, a growth factor. MAPK is expressed in all eukaryotic cells. The basic component of the MAPK pathway is a conserved set of three kinases from yeast to humans, including MAPK kinase kinase (MAP kinase kinase kinase, MKKK), MAPK kinase (MAP kinase kinase, MKK) and MAPK. These three kinases can be activated in turn and jointly regulate cell growth, differentiation, adaptation to environmental stress, inflammation and other important cellular physiological and pathological processes. The mitogen-activated protein kinase (MAP kinase, MAPK) chain is an important pathway in eukaryotic signal transmission networks and plays a key role in gene expression regulation and cytoplasmic functional activities. The MAPK chain is composed of three protein kinases, MAP3K, MAP2K and MAPK, which in turn phosphorylate upstream signals to affect the downstream response molecules. The MAPKs belongs to the CMGC (CDK/MAPK/GSK3/CLK) kinase group. The closest relatives of MAPKs are the cyclin-dependent kinases (CDKs). Fourteen MKKKs, seven MKKs and 12 MAPKs have been identified in mammalian cells. Analyses have shown that these kinases belong to different subgroups. The MAPKs can be divided into four subgroups: extracellular signal-regulated protein kinase 1/2 (ERK 1/2), p38, c-jun amino-terminal kinase/stress-activated protein kinase (JNK/SAPK) and ERK5. The pathways involving these subgroups are named after them. For example, the MAPK pathway that uses JNK is called the JNK pathway, and the MAPK pathway that uses ERK is called the ERK pathway.

MAPK signal transduction pathways are activated by a variety of stimuli such as growth factors, cytokines, gamma rays, osmotic pressure and shear stress as fluid flows across the cell surface. MAPK cascade activation is at the centre of a variety of

signalling pathways, and MAPK is an important molecule that receives signals transmitted by the transformation of membrane receptors and brings these signals into the nucleus. MAPK plays a key role in many cell proliferation-related signalling pathways. In unstimulated cells, MAPK is at rest. When cells are stimulated by growth factors or other factors, MAPK is activated by the activation signals of MKK and MKKK, which present as progressive phosphorylation. In mammals, ERK is widely found in various tissues and is involved in the regulation of cell proliferation and differentiation. Many growth factor receptors and nutrition-related factor receptors require ERK activation to complete the signal transduction process. The JNK family includes key molecules of cellular signal transduction induced by various stressors that are involved in cellular responses to radiation, osmotic pressure, temperature changes and other stressors. P38 mediates inflammation and apoptosis and has thus become a target for the development of anti-inflammatory drugs.

#### 9.2.4.1 ROS-Mediated Regulation of Autophagy Through the JNK Signalling Pathway

ROS can activate JNK in a variety of ways. (1) Apoptosis signal-regulated kinase 1 (ASK1) acts as a bridge in the ROS-mediated activation of the JNK pathway. In the process of MAPK signal transmission, ASK1 activates the upstream MAPKKK of the JNK pathway through phosphorylation of MKK4 and MKK7, which can be activated by ROS and other signals. (2) The Src family kinases (SFKs) currently include LYN, FYN, LCK, HCK, FGR, BLK, YRK, YES and Src. Src is widely found in tissues and regulates cell growth, development, differentiation and death by interacting with important molecules in its signal transduction pathway. In the ROS-mediated JNK signalling pathway, loss of Src severely inhibits the activation of JNK. The Src pathway is one of the ROS-activated JNK signalling pathways. (3) GSTπ is another important intermediate molecule for ROS-mediated JNK activation. A recent study found that two kinds of GSTπ monomers can inhibit phosphorylation of ATF2 through direct interaction with JNK substrate activated transcription factor 2 (activating transcription factor 2, ATF2), thereby inhibiting the activation of JNK1 or JNK2. $H_2O_2$ can cause oligomerization of GSTπ, which is originally bound to the carboxyl terminal of JNK, and separate the GST-JNK complex. In this way, the inhibited JNK activity can be recovered. (4) Mixed-pedigree kinase 3 (MLK3) is a member of the MLK subfamily of the MAPKKK family and is a serine/threonine-protein kinase. MLK3 activates the serine/threonine-protein kinase of the MAPK pathway through phosphorylation, mediates the activation of the downstream signalling pathway and is an important link connecting ROS and JNK. Van denBerg et al. found that increased ROS levels induced by oxidative stress can activate the small G protein RALA through the c-Jun amino-terminal interacting protein 1 (JIP1) scaffold complex and then regulate the phosphorylation of JNK. (5) The receptor-interacting protein (RIP)-TRAF2 complex pathway is another important pathway by which ROS activate JNK. RIP and TRAF2 are important signalling molecules that activate the nuclear transcription factor NF-kB and trigger a signalling cascade

leading to apoptosis after TNF binds to TNF receptor 1. Recent studies have shown that RIP and TRAF2 can bind to each other in the lipid raft region on target cell membranes to form RIP-TRAF2 signal complexes, which directly activate the JNK pathway under the induction of ROS. (6) Inhibition of the activity of MAPK phosphatases (MKPs) is another effective way in which ROS activate JNK. Kamata et al. showed that intracellular $H_2O_2$ can inhibit the activity of MKPs by oxidizing the cysteine residues of MKPs; in addition, the oxidized MKPs are rapidly degraded by the ubiquitin-proteasome pathway, thereby eliminating MKP-mediated inhibition of JNK and leading to continuous activation of the JNK pathway. Hou et al. further confirmed that ROS-mediated MKP inactivation is the reason for the continuous activation of the JNK pathway.

Autophagy is a process in which cells rely on lysosomal pathways to degrade cytoplasmic proteins and organelles, and it is an important way for cells to remove damaged proteins and organelles. Autophagy is usually enhanced under conditions of starvation, oxidative damage and endoplasmic reticulum stress. Therefore, autophagy is a self-protective mechanism of cells that is induced and regulated by a variety of factors. The ROS-JNK pathway is an important pathway that induces and regulates autophagy.

### Autophagy Is Activated by Bcl-2 Phosphorylation

Studies have shown that JNK1, when activated by ROS, can directly phosphorylate the protein Bcl-2 to dissociate Bcl-2 from Beclin 1, a key autophagy protein; in addition, Beclin 1 can form a Beclin 1-Vps34-PI3K multi-protein complex after activation, thus activating autophagy. Autophagy is inhibited upon JNK1 inhibition or the exogenous introduction of Bcl-2 proteins with phosphorylation site mutations. However, structural activation of JNK1 induces multi-site phosphorylation of Bcl-2, leading to autophagy activation. Although Bcl-2 and Bcl-xl have similar structures and phosphorylation sites, whether Beclin 1 and Bcl-xl can be regulated by JNK1-mediated phosphorylation remains unclear.

### Autophagy Is Activated by Upregulation of ATG7

Wong et al. showed that the ROS-JNK signalling pathway can also directly upregulate the key autophagy genes ATG7 and ATG5 in a Beclin 1-independent way to activate autophagy. This activation was found only in tumour cells, not in benign tissues or normal cells (Wong et al. 2010).

### Autophagy Is Inhibited Indirectly by Activation of Apoptosis Signals

Apoptosis and autophagy are two important functions of cells, and research on the interaction between apoptosis and autophagy is a hot topic at present. Apoptosis

can inhibit the occurrence of autophagy in a variety of ways, whereas autophagy can also inhibit apoptosis. In the process of studying the mechanisms of apoptosis and autophagy, researchers have found that signalling molecules (such as JNK and Akt) are frequently involved in both signalling pathways, suggesting that apoptosis and autophagy are necessarily related. ROS activate JNK through the bispecific JNKKs (including MKK4 and MKK7), and activated JNK, in turn, can promote the expression of pro-apoptotic proteins such as p53, Bax, FasL and TNF through the transcription factor AP-1. Highly expressed pro-apoptotic proteins, such as Bax and Bak, act on mitochondria, which can promote the release of cytochrome C into the cytoplasm. Cytochrome C binds to caspase-9 and eventually acts on caspase-3, thereby activating caspase-3. Activated caspases play very important roles by lysing autophagy-related proteins; the lysed autophagy-related proteins can enter mitochondria, promote the release of cytochrome C and further promote the occurrence of apoptosis. Studies have shown that Beclin 1 can be cleaved by caspase-3 to produce a c-terminal Beclin 1 fragment, which can enter mitochondria to promote the release of cytochrome C, inhibit autophagy and induce apoptosis. In addition, Fasl and TNF ligands and death receptors expressed on cell membranes (Fas and TNFR) form death-induced signalling complexes (DISCs). DISCs promote precursor caspase-8 cleavage, generating activated caspase-8. On the one hand, activated caspase-8 can activate downstream caspase-mediated apoptosis and initiate the apoptosis signalling pathway. On the other hand, caspase-8 can induce the binding of c-flip, v-flip and other substances to ATG-3, thereby inhibiting the binding of ATG-3 and LC3 and inhibiting the occurrence of autophagy. When mouse L929 fibrosarcoma cells were treated with the broad-spectrum caspase-8 inhibitor zVAD, it was found that zVAD could promote the autophagic death of these cells, which indicated that the autophagy pathway was activated after the apoptosis signal was inhibited. Further studies have shown that inhibition of caspase-8 may be closely related to the production of ROS and the activation of JNK (Zhu et al. 2010).

### ROS-JNK Pathway-Mediated Regulation of Autophagy Is Dependent on Intracellular ROS Levels

The ROS-JNK pathway can mediate both apoptosis and autophagy, and the key lies in intracellular ROS levels. Moderate levels of ROS can lead to transient activation of JNK signalling, and the Beclin 1 pathway can induce increases in autophagy levels; but such effects are not enough to cause apoptosis. However, ROS above a certain level lead to continuous activation of JNK and induce apoptosis mediated by mitochondrial pathways. Recent studies have shown that treating multiple myeloma cells with oridonin can induce high levels of intracellular ROS production followed by increased apoptosis and autophagy inhibition. In contrast, low levels of ROS can promote autophagy and inhibit apoptosis. At low ROS levels (i.e. under basal oxidative stress conditions), JNK can mediate the occurrence of autophagy by activating Atg7 in cells. Studies have shown that under basal oxidative stress conditions,

knockout of the autophagy-related gene Atg7 can significantly reduce the $H_2O_2$-mediated autophagy response induced by drugs. Further studies have suggested that ROS-dependent JNK and ERK activation are both important upstream regulatory mechanisms that mediate increases in $H_2O_2$ and promote increases of autophagy levels.

These results suggest that the ROS-JNK pathway directly or indirectly activates or regulates the complex mechanism of autophagy in mammalian cells and that the levels of ROS directly affect this process. The exact effects of the ROS-JNK pathway on autophagy and apoptosis are not clear at present, but recent studies suggest that these effects may be closely related to ROS-mediated activation of JNK.

#### 9.2.4.2 ROS-Mediated Regulation of Autophagy Through the ERK Signalling Pathway

ERKs are members of the mitogen-activated protein kinase family. ERKs are involved in cell differentiation and activity and can be enhanced by epidermal growth factor (EGF) and platelet-derived growth factor (PDGF receptor). ROS can also activate these receptors leading to phosphorylation and activation of ERK. Preston et al. found that physiological concentrations of $H_2O_2$ can activate ERK and promote cell proliferation, and other researchers have also found that ROS can activate ERK. In contrast, in 2013, Wang et al. found that antioxidant substances can inhibit the proliferation of HeLa cells, accompanied by the weakening of ERK activity and increases in $H_2O_2$ with can increase the phosphorylation of ERK and then activate it.

ERK and JNK are downstream effector molecules of autophagy induced by ROS, and their mechanisms are diverse and complex. Kangmao Huang et al. found that honokiol can induce autophagy in osteosarcoma cells through the ROS-ERK pathway. Other studies have shown that ROS can regulate the Ras/Raf/ERK signalling pathway and further regulate the expression of downstream AP-1 binding genes.

### *9.2.5 Oxidation of ATG-4 by ROS Regulates Autophagy*

The kinase mTOR is an important regulatory molecule that induces autophagy. Activation of mTOR (through Akt and MAPK signal transduction) inhibits autophagy, while negative regulation of mTOR (through AMPK and p53 signal transduction) promotes autophagy. Three related serine/threonine kinases, UNC-51-kinases 1, 2 and 3 (ULK1, ULK2 and UKL3, respectively), have the same function as yeast Atg1 and can serve as downstream molecules of the mTOR complex. ULK1 and ULK2 form a large complex together with the mammalian homologue of mAtg13, an autophagy-related (Atg) gene product, and the skeletal protein FIP200, which is directly homologous to yeast Atg17. Class III PI3K complexes including hVps34, Beclin-1 (a mammalian homologue of yeast Atg6), p150 (a mammalian homologue of

yeast Vps15) and Atg14-like protein (Atg14L or Barkor) or UV resistance-associated genes (UVRAGs) are necessary for autophagy induction. Atg genes control the formation of autophagosomes through Atg12-Atg5 and LC3-II (Atg8-II) complexes. Atg12 binds to Atg5 via a ubiquitin-like reaction involving Atg7 and Atg10 (corresponding to E1- and E2-like enzymes, respectively). Atg12-Atg5 conjugates to form a large complex with Atg16 through non-covalent interactions. LC3/Atg8 is spliced by the Atg4 protease to produce cytoplasmic LC3-I. LC3-I also binds to phosphatidylethanolamine (PE) by a ubiquitin-like reaction involving Atg7 and Atg3 (corresponding to E1- and E2-like enzymes, respectively). The lipid form of LC3, also known as LC3-II, attaches to the membranes of autophagosomes.

Binding and dissociation of Atg4 are both necessary for normal autophagy. At least four Atg4 mammalian homologues have been reported based on sequence homology with the yeast *Saccharomyces cerevisiae* (Sc) Atg4. Two of the homologues, HsAtg4A and HsAtg4B, have been shown to cleave Atg8: HsAtg4A cleaves mainly GATE-16, whereas HsAtg4B cleaves three homologues (GATE-16, GABARAP and LC3), with the highest efficiency for LC3. Autophagy-related gene 4 (ATG4) is a cysteine protease that plays an important role in the formation of autophagic bodies. This protease is regulated by the redox of a disulfide bond between Cys338 and Cys394 and can be restored by thioredoxin (Trx) very effectively, indicating that this redox enzyme plays an important role in the regulation of ATG4. Autophagy activity mediated by rapamycin in thioredoxin mutant cells is significantly higher than that in wild-type cells. In addition, in vivo studies have shown that Cys338 and Cys394 are necessary for the formation of autophagosomes, because mutations in these cysteines lead to aggregation of ATG8 in autophagosomes. Therefore, changes in ATG4 activity can be mediated by intracellular redox processes, thereby regulating the formation of autophagosomes. Another study found that under starvation conditions, PI3K III and Beclin 1, together with other signalling molecules in cells, produce large amounts of ROS, especially $H_2O_2$. After oxidation of Atg4 by $H_2O_2$, LC3-II delipidation is inhibited; however, the processing of the C-terminus of LC3 by Atg4 is not affected, ensuring the formation of autophagosomes. In addition to affecting the activity of ATG4, ROS induce Beclin l expression and thereby promote autophagy activation. In contrast, autophagy activation promotes the production of catalase through selective autophagic degradation, leading to ROS accumulation and the formation of a positive feedback regulatory loop between autophagy and ROS. How does $H_2O_2$ regulate Atg4? Atg4 is a cysteine protease containing several conserved cysteine residues. There are two catalytic sites on HsAtg4A, Cys77 and Cys81. Ruth scherz-shouval et al. found that these two catalytic sites are key factors in the redox regulation of HsAtg4A. In vitro, mutation of these sites significantly affected the sensitivity of the protein to $H_2O_2$ and inhibited the formation of GATE-16-labelled autophagosomes in cells. A similar mutation in HsAtg4B, C78S, produced a similar effect. The research team also found that $H_2O_2$ can directly inactivate Atg4 in vitro and that binding of $H_2O_2$ to Cys77 or Cys81 can reversibly form sulfonic acid to shield Cys77 or lead to disulfide binding of Cys77 and Cys81 through oxidation, thereby protecting Cys77.

## 9.3 Conclusion

Oxidative stress is one of the important causes of disease. Autophagy is necessary for cells to overcome starvation and oxidative stress conditions. In recent years, researchers have explored how to alleviate the effects of oxidative stress from the perspective of nutrition. The elucidation of the mechanisms of autophagy provides a new direction for the study of oxidative stress. Oxidative stress can activate autophagy, which can clear the damage caused by oxidative stress, delay cell death and maintain intracellular homeostasis. Determining how to alleviate oxidative stress by regulating autophagy will provide new ideas for studies on the mechanisms of antioxidant substances.

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# Chapter 10
# Non-coding RNAs and Autophagy


**Honghong Yao, Bing Han, Yuan Zhang, Ling Shen and Rongrong Huang**

**Abstract** Autophagy is an important metabolic pathway of cells. Cells degrade harmful intracellular components with the aid of autophagy to maintain a healthy state. In recent decades, the study of non-coding RNA in the regulation of autophagy has been a hot area. Mounting evidence indicates that many ncRNAs are involved in the dynamic process of autophagy, and further studies were undertaken to dissect the detailed cellular and molecular mechanisms underlying this process. In this chapter, we mainly summarized the regulation of different non-coding RNAs in autophagy as well as the detailed mechanisms. Based on these findings, we also discussed the roles of non-coding RNAs in the diagnosis, treatment, and prognosis of diseases with an emphasis on their use as potential biomarkers and therapeutic targets for different diseases.

**Keywords** ncRNAs · Autophagy · miRNAs · lncRNAs · circRNAs

## Abbreviation

| | |
|---|---|
| 3-MA | 3-methyladenine |
| ADAR1 | Adenosine to inosine acting on RNA enzyme 1 |
| ANXA2 | Annexin A2 |
| APP | Amyloid precursor protein |
| ceRNA | Competitive endogenous RNA |
| circRNA | Circular RNA |
| ciRNAs | Circular intronic RNAs |
| CSD | Cold shock domain |
| DFCP1 | Double FYVE-containing protein 1 |
| EcircRNAs | Exonic circRNAs |
| EIcircRNAs | Exonic-intronic circRNAs |

H. H. Yoa (✉) · B. Han · Y. Zhang · L. Shen · R. R. Huang
Department of Pharmacology, School of Medicine, Southeast University, Nanjing 210009, Jiangsu, China
e-mail: yaohh@seu.edu.cn



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| | |
|---|---|
| eIF4A | Eukaryotic initiation factor 4A |
| gRNA | Guide RNA |
| lncRNA | Long non-coding RNA |
| miRNA | Micro RNA |
| mRNA | Message RNA |
| mt tRNAs | Mitochondrial tRNAs |
| ncRNA | Noncoding RNA |
| NMD | Nonsense-mediated mRNA decay |
| PI3P | Phosphatidylinositol 3-phosphate |
| piRNA | Piwi-interacting |
| Plekhm1 | Pleckstrin homology domain-containing protein family M member 1 |
| pre-miRNA | Precursormicro RNA |
| pri-mRNA | Primary micro RNA |
| PS1 | Presenilin 1 |
| RBP | RNA-binding protein |
| RES | Ribsome entrysite |
| RISC | RNA-induced silencing complex |
| ROS | Reactive oxygen species |
| rRNA | Ribosomal RNA |
| siRNA | Small interfering |
| snoRNA | Small nucleolar RNA |
| snRNA | Small nuclear RNA |
| SR | Serine/argine |
| TIPARP | TCDD inducible poly[ADP-ribose] polymerase |
| tRNA | Transfer RNA |
| ULK | Unc-51-like kinase |
| UTR | Untranslation region |
| Vps | Vesicular protein sorting |

## 10.1 MicroRNAs and Autophagy

### *10.1.1 MicroRNAs (miRNAs)*

MicroRNAs (miRNAs) are endogenous short noncoding RNAs (ncRNAs) of 20–25 nucleotides that mediate gene expression. They are highly conserved among species and are not only expressed in almost all eukaryotes but also in several viruses, suggesting their powerful biological functions.

#### 10.1.1.1 The Biogenesis of miRNAs

In the nucleus, the miRNA genes are transcribed into polyadenylated primary miRNAs (pri-miRNAs) by RNA polymerase. Pri-miRNAs can be up to several thousand nt long and contain 22–25 nt long sequences of mature miRNA. A nuclear protein complex participates in the processing of pri-miRNA to single hairpins termed precursor miRNA (pre-miRNA). The complex comprises the RNase III enzyme Drosha, DGCR8 and several factors such as p68 and p72. Subsequently, pre-miRNA enters the cytoplasm through a direct interaction with Exp5. In the cytoplasm, the dicer enzyme cuts the pre-miRNA to a mature length, and the molecule remains double-stranded. Finally, one of the two strands will be translated into AGO family proteins and then assembled into the RNA-induced silencing complex (RISC) to participate in gene silencing.

#### 10.1.1.2 The Function of miRNAs

Although microRNAs were identified in 1993, their role in gene regulation was recognized approximately 10 years later. Studies have shown that assembly into an RNA-induced silencing complex (RISC), which mainly includes AGO and GW182, is required for miRNA function. AGO proteins are highly conserved, and small RBPs (RNA-binding proteins) are composed of four domains: the N-terminal domain, the PAZ domain, the MID domain and the PIWI domain. The MID and PIWI domains can bind to the 5′ end of miRNA, while the PAZ domain can bind to the 3′ end. The mammalian genome encodes four AGO proteins (Ago1–4), but only Ago2 can degrade messenger RNA (mRNA) that is completely complementary to the full miRNA sequence. GW183, as a binding protein of AGO, can bind to AGO through the GW domain at the N-terminus, whereas the silencing domain of the C-terminus of GW183 can be used as a platform to recruit various auxiliary proteins, such as PABP and CCR4-NOT, and then repress translation from the mRNA. The central role of miRNAs in this process is to bind to the complementary sequences at the 3′-UTR of the mRNA through the seed region, which contains 2–8 nucleotides at the 5′-terminal, and then identify the corresponding RNA to regulate gene expression at the posttranscriptional level through RISC. A single miRNA can target multiple genes to regulate their expression, and multiple miRNAs can also regulate the same gene to precisely regulate the expression of a single gene. miRNAs have been confirmed to be involved in many biological processes, such as cell development, proliferation, differentiation, and apoptosis, and they play an extremely important role in the regulation of autophagy.

### *10.1.2 MiRNA-Mediated Regulation of Autophagy*

Studies have shown that miRNAs are involved in various stages of autophagy including autophagic induction, vesicle nucleation, vesicle elongation, vesicle retrieval and fusion, and they are also involved in the regulation of upstream signaling pathways that can affect autophagy induction (Fig. 10.1).

#### 10.1.2.1 Autophagic Induction

In higher mammals, autophagy is mainly activated by ULK (unc-51-like kinase) complexes, which contain ULK 1/2, ATG13, FIP200 and ATG101. Among them, ULK1/2 is the core protein that initiates autophagy. When cellular metabolism is disrupted by starvation and oxidative stress, mTOR activity is inhibited, AMPK kinase is activated, and ULK1/2 is rapidly activated to phosphorylate downstream substrates such as ATG13, Beclin1, and VPS34. ATG13 can further promote the activation of ULK1/2 and mediate the phosphorylation of FIP200. As a scaffold protein, FIP200 is involved in the assembly of ATG proteins. ATG101, as a hydrophilic protein, can stabilize ATG13 and protect it from proteasomal degradation. Therefore, all components of the ULK complex have indispensable functions. miRNA can affect the formation of the ULK complex by regulating the expression of various components of ULK complex proteins.

In the C2C12 myoblast cell line, miR-20a and miR-106b, which belong to the miR-17 family, may participate in regulating leucine deprivation-induced autophagy by inhibiting ULK1 expression (Wu et al. 2012). Other miRNAs belonging to the miR-17 family, such as miR-20b, miR-106a, miR-93, and miR-17-5p, were also confirmed

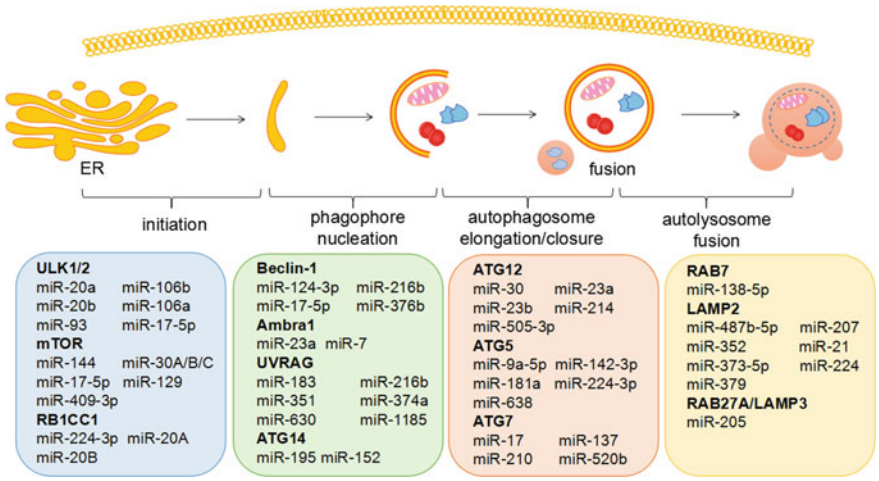


**Fig. 10.1** MicroRNAs involved in autophagy regulation

to inhibit the expression of ULK1 and inhibit autophagy. This demonstrated the powerful role of the miR-17 family in regulating the induction phase of autophagy. In addition, miR-489, miR-142-5p, and miR-25 have been reported to affect autophagy by targeting ULK1. However, miR-26a and miR-26b can target and regulate ULK1 and ULK2, respectively. In addition, ULK2 is regulated by miR-885-3p, suggesting that miR-885-3p might contribute to the regulation of squamous cell carcinoma cell autophagy and/or apoptosis upon cisplatin exposure. Furthermore, ATG13 was reported to be regulated by miR-133a-3p, and FIP200 was regulated by miR-20a, miR-20b, miR-224-3p, and miR-309-3p. Due to the negative regulation of autophagy by mTOR, the miR-99 family can indirectly promote autophagy by inhibiting the IGF-1R/Akt/mTOR signaling pathway, while miR-100 can directly target the 3′-UTR of mTOR mRNA to inhibit mTOR and thus activate autophagy. All of the miRNAs described above affect autophagy at the stage of autophagic induction.

#### 10.1.2.2 Vesicle Nucleation

During vesicle nucleation, the double membrane-bound vesicles of autophagosomes are formed by collecting proteins and liposomes. Vesicle nucleation is induced by the PI3K complex, which is mainly composed of PI3KC3 (hVPS34), Beclin-1, p150, and ATG14L. In addition, several proteins, such as Ambra1, Bif-1, UVRAG and Rubicon, can bind and regulate the PI3K complex. Many miRNAs are involved in the regulation of vesicle nucleation, and previous studies mainly focused on miRNA regulation of Beclin-1 expression. Thus far, multiple miRNAs, including members of the miR-30 family, miR-124-3p, miR-216b, miR-17-5p, and miR-376b, were found to affect Beclin-1 expression and autophagy by targeting the 3′-UTR of Beclin-1 mRNA. miR-23a can target Ambra1 and inhibit the autophagy of fibroblasts during UV-induced photoaging (Zhang et al. 2016). In addition to the direct regulation of Beclin-1, miRNAs can indirectly regulate Beclin-1 to affect autophagy. As a protein with a BH3 domain, Beclin-1 can bind bcl-2/bcl-xl, and PUMA, which also has a BH3 domain, can release Beclin-1 through the competitive binding of bcl-2/bcl-xl to induce autophagy. Studies have shown that miR-143 can inhibit the methylphenidate-induced autophagy of microglia by targeting PUMA, which illustrates the multiple regulatory effects of miRNAs on autophagy. Ambra1 was also targeted by miR-7, leading to the miR-7-mediated promotion of lung cancer cell proliferation by inhibiting autophagy. UVRAG was found to be regulated by the miR-125 family, including miR-183, miR-216b, miR-351, miR-374a, miR-630, and miR-1185. All of these studies demonstrated the powerful regulatory role of miRNAs in the vesicle nucleation stage.

#### 10.1.2.3 Vesicle Elongation

During vesicle elongation, two ubiquitylation-like conjugation pathways are involved. The first pathway is based on ATG12, which can be activated by the

ubiquitin-activating enzyme ATG7 and bind with ATG5 under the influence of the ubiquitin ligase ATG10, finally forming the ATG12-ATG5-ATG16L complex with the addition of ATG16L. This pathway mainly participates in the dilation of the autophagosome. The second pathway involves the conjugation of LC3 to a lipid molecule. LC3 should be cleaved at its carboxy terminus by ATG4 to generate the cytosolic free LC3-I form in which the glycine residue is exposed. After activation by ATG7 and the action of ATG10, LC3-I binds to phosphatidylethanolamine and is modified to LC3-II on the surface of the autophagosome membrane.

Due to the large number of proteins participating in this stage, many of the corresponding miRNAs involved in the regulation of these proteins have been identified. For example, ATG12 is regulated by a variety of miRNAs, such as the miR-30 family members miR-23a, miR-23b, miR-214, miR-505-3p, etc. The miR-30 family is also involved in the regulation of ATG5 by targeting the 3′-UTR of ATG5 mRNA. However, this does not mean that the members of the miR-30 family can regulate autophagy. A recent investigation indicated that ATG5 also has non-autophagic functions. After LPS stimulation in human brain microvascular endothelial cells, miR-30d was downregulated, which subsequently upregulated the expression of ATG5. Instead of activating the autophagic pathway, these biological events promoted the transformation of endothelial cells into mesenchymal cells (Yang et al. 2018). ATG5 was also regulated by miR-9a-5p, miR-142-3p, miR-181a, miR-224-3p, miR-638, etc. Inside the cell, miR-142-3p can also simultaneously regulate the expression of ATG16L1 and exert important effects on autophagy during vesicle elongation. The miR-17 family members miR-106a and miR-106b have two binding sites on the mRNA 3′-UTR of the ATG16L1 gene; however, only miR-106b inhibits starvation-induced autophagy by inhibiting the expression of ATG16L1, whereas the miR-106a regulation of autophagy does not appear to be through ATG16L1 and instead may be via other mechanisms. The miR-17 family can also participate in the regulation of ATG7 expression and inhibit autophagy including miR-137, miR-210, and miR-520b. The 3′-UTR of ATG10 mRNA contains sites targeted by miR-4458, miR-4667-5p and miR-4668-5p, which indicates that they can simultaneously regulate ATG10 expression, thereby inhibiting the autophagy-mediated elimination of *Burkholderia pseudomallei* in human lung epithelial cells. ATG4D is regulated by miR-101 and plays a negative regulatory role in vesicle elongation by regulating the binding of RAB5AATG12 and ATG5. The above results showed the important role of miRNAs in vesicle elongation.

#### 10.1.2.4 Vesicle Retrieval

Vesicle retrieval is the stage of recycling some components such as surface receptors in autophagosomes. The mechanism, however, is not yet clear. ATG9, a transmembrane protein, is necessary for this stage. ATG9 continuously shuttles between the autophagosome precursor structure and other structures or organelles (such as the endoplasmic reticulum, Golgi apparatus, and mitochondria). This process also requires ATG2 and ATG18. Unfortunately, research on miRNA regulation at this

stage is relatively limited. Until recently, only miR-34a was known to directly target the ATG9A mRNA 3′-UTR and inhibit ATG9A expression and autophagy. These results demonstrated that a miR-34a mutation can prolong the life of *Caenorhabditis elegans* by enhancing autophagy (Jurong et al. 2013). Recent studies have also suggested that microRNA-130a inhibits autophagy by activating ATG2B and DICER1 and inducing the killing of chronic lymphocytic leukemia cells. These studies suggest the importance of miRNAs in the vesicle retrieval stage.

#### 10.1.2.5 Lysosomal Fusion

During lysosomal fusion, the engulfed cargo is degraded after the fusion of autophagosomes and lysosomes. In mammals, RAB7 is mainly involved in this process, and LAMP1 and LAMP2 also play important roles. Although RAB27A and LAMP3 have not been thoroughly studied, some research suggests that they are related to the vesicle fusion of autophagosomes. A recent study found that miR-138-5p can indirectly regulate RAB7 by targeting SIRT1, thereby inhibiting autophagy in the vesicle fusion phase in pancreatic cancer. miR-487b-5p can promote lung cancer cell proliferation by targeting LAMP2 to inhibit autophagy. In the process of ischemic stroke, miR-207 and miR-352 can affect autophagy by directly targeting LAMP2. LAMP2 is also regulated by miR-21, allowing miR-21 to inhibit molecular chaperone-induced autophagy in a Parkinson's disease model. miR-224, miR-373-5p, and miR-379 can target LAMP2 and regulate its expression. Furthermore, miR-205 can inhibit autophagy by regulating RAB27A and LAMP3, thereby affecting the sensitivity of prostate cancer cells to cisplatin.

In addition, studies have shown that certain miRNAs can target multiple proteins at various stages of autophagy. For example, miR-33a-5p and miR-33a-3p have been reported to directly target key autophagy effectors, such as ATG5, ATG12, LC3B and LAMP1, and to inhibit AMPK-dependent autophagic activation and lysosomal gene transcription by targeting FOXO3 and TFEB, which in turn affect autophagy in many ways (Ouimet et al. 2016). miRNAs may also affect autophagy through indirect regulation. miR-378 was reported to activate Akt by targeting PDK1, which indirectly activated mTORC1, inhibited FoxO1 and FoxO3, and ultimately promoted autophagy in skeletal muscle, which provides a potential therapeutic target to treat myopathies.

## 10.2 Long Noncoding RNAs and Autophagy

### *10.2.1 Long Non-coding RNAs (lncRNAs)*

lncRNAs are nonprotein-coding RNA transcripts that are longer than 200 nucleotides and have a similar structure to messenger RNA (mRNA) with a 5′-cap and poly (A)

tail. In recent years, with the development of second-generation sequencing technology, lncRNA and its functions have gradually attracted the interest of researchers. Growing evidence shows that lncRNAs are broadly involved in the regulation of human disease such as cancer, autoimmunity, cardiovascular disease, and neural disease. However, the current research on lncRNA is still the tip of the iceberg, and there are still many unknowns awaiting further excavation.

#### 10.2.1.1 The Biological Characteristics of lncRNAs

lncRNAs exhibit highly spatially and temporally restricted expression patterns in the process of tissue differentiation and development. lncRNAs can be transcribed from various genomic regions such as enhancers, promoters, introns, antisense coding, or intergenic regions of genes. Specifically, lncRNAs show a relatively lower expression level but much more tissue-specific pattern than protein-coding genes, suggesting the specific regulatory role of lncRNAs and potential specific targets for disease treatment. Based on the relative position of the lncRNA coding sequence and protein-coding gene, lncRNAs can be divided into five groups: intergenic, intronic, bidirectional, sense and antisense lncRNAs.

lncRNA expression is controlled by both transcriptional and epigenetic factors. Activating histone marks correlate well with the expression of lncRNA genes, which is similar to protein-coding genes. However, lncRNA genes were found to contain more methylation around the TSS (transcriptional start site) than protein-coding genes regardless of their expression status, suggesting that the epigenetic regulation of lncRNAs at the level of DNA methylation is markedly dissimilar from that of protein-coding genes. To date, multiple transcription factors have been found to transactivate the expression of lncRNAs, including Nanog, Sox2, Oct4, TP53, and ZNF143. Transcribed lncRNAs are further subject to posttranscriptional processing, such as 5′ capping, polyadenylation, alternative splicing, and RNA editing.

Although lncRNAs are present in both the nucleus and the cytoplasm, the fact that many lncRNAs are enriched in the nucleus highlights a potential role for lncRNAs as epigenetic regulators within the nucleus. The biophysical analysis showed that lncRNAs can be folded into a functional secondary structure that binds to DNA/RNA or protein and participates in physiological and pathological processes. Multiple lines of evidence demonstrate that lncRNAs exert their functions at the level of epigenetics via interactions with RNA-binding proteins (RBPs) at specific DNA regions, such as promoters or enhancers. Nevertheless, the role of lncRNAs present in the cytoplasm cannot be ignored. ceRNA (competing endogenous RNA) is the common mechanism for lncRNAs present in the cytoplasm to be involved in physiological activities. When thrombus occurs, WTAPP1 lncRNA inhibits the regulation of miRNA on the downstream target gene MMP-1 by adsorbing miR-3 and miR-120-5p, resulting in the activation of the PI3K/AKT/mTOR pathway and the regulation of endothelial progenitor cell autophagy.

In addition, some lncRNAs have highly conserved sequences and structures among different species and may be involved in the conservation of biological functions between different species. The fact that nonconserved RNAs are present in specific species and tissues highlights their involvement in the species-specific regulation of biological activities.

#### 10.2.1.2 The Biological Functions of lncRNAs

In contrast to the intensively studied miRNAs, lncRNAs are larger and thus have a complex secondary structure. The complicated structure endows lncRNAs with the ability to bind protein, RNA, and DNA and thus with several regulatory capacities. In addition to these transcriptional and epigenetic regulatory activities, lncRNAs have been found to be important players in posttranscriptional regulation, such as functioning as mRNA editors, mRNA splicing regulators, and small ncRNA reservoirs. Breakthroughs over the past few years have revealed numerous examples of lncRNAs that regulate the progression from DNA to RNA to protein. Some recent excellent reviews have summarized the mechanisms by which lncRNA regulates chromatin structure and transcriptional control.

lncRNAs can regulate gene expression at the epigenetic level, including dose compensation, chromatin modification, and genomic imprinting. The most typical role of lncRNAs involved in dose compensation is participation in the balance of the expression of X-linked genes. The Xist lncRNA is transcribed from the XIST gene on an X chromosome. When an X chromosome is inactivated, the expression of the originally suppressed lncRNA Xist is upregulated (Yan et al. 2018). Chromatin modification refers to the process of adding and removing chemical groups to components of chromatin, such as DNA and histones. As a molecular scaffold, the lncRNA HOTAIR can bind to the histone modification complex to promote histone H3K4 demethylation and regulate the expression of target genes. Genomic imprinting, also known as genetic imprinting, is the biological process of labeling the information of its parental origin on a gene or genome by certain modifications. Gene expression levels and whether a gene is silent depend on whether the gene came from the male or female parent and the chromosome on which it is located The lncRNA H19 is one of the earliest discovered lncRNAs and is also an imprinted gene located on human chromosome 11p15, which is important for allele-specific expression on the imprinted gene cluster.

lncRNAs can regulate gene expression at the transcriptional level. Transcriptional regulation is an important part of eukaryotic gene expression and the most important method to regulate gene expression. lncRNAs are also regulated in a variety of ways at the transcription level such as by competing for transcription factors or recruiting protein complexes to influence gene expression. The lncRNA MUF is highly expressed in liver cancer tissues and activates the Wnt/β-catenin signaling pathway by binding to annexin A2 (ANXA2), leading to epithelial-mesenchymal transformation. However, MUF lncRNA can also act as an endogenous sponge of

miR-34a to remove the miR-34a-mediated inhibition of the downstream target gene Snail1 and promote epithelial-mesenchymal transformation (Yan et al. 2017).

lncRNAs can regulate gene expression at the posttranscriptional level and mainly have three functions: splicing, translation, and degradation of mRNA. After transcription, mRNA needs further processing to become mature. Precursor mRNA (pre-mRNA) is the original transcription product including exon and intron sequences and noncoding sequences of a gene. Therefore, RNA exon splicing and intron removal are required for posttranscriptional processing. The lncRNA MALAT1 can regulate the distribution and activity of serine/arginine (SR) splicing factors leading to variable splicing of pre-mRNA. Further analysis found that the lncRNA MALAT1 regulated alternative splicing via SR protein phosphorylation, including the SRSF1, SRSF2, and SRSF3 proteins. Eukaryotic initiation factor 4A (eIF4A) is an RNA helicase. Bc1 lncRNA activates its ATPase to mediate the translation of eIF4A by blocking the double-stranded cleavage activity of eIF4A. lncRNA can be used as a miRNA sponge to block the binding of miRNAs to mRNA 3′-UTRs and affect the degradation of mRNA. Moreover, lncRNAs regulate mRNA stability through the nonsense-mediated mRNA degradation (NMD) pathway.

## *10.2.2 Regulation of Autophagy by lncRNAs*

### 10.2.2.1 lncRNAs Related to Autophagic Initiation

As described in Chaps. 3 and 4 of this book, AMPK and mTORC1 promote autophagic initiation to maintain cellular homeostasis under conditions of hypoxia, ischemia, and malnutrition. Recent studies have found that many lncRNAs regulate autophagy by directly or indirectly affecting the AMPK and mTORC1 molecules. The downregulation of H9 lncRNA in diabetic rats relieves the transcriptional repression of DIRAS3, resulting in the inactivation of the PI3K/AKT/mTOR pathway and autophagic activation. Additionally, the downregulation of H9 lncRNA can increase Beclin 1 and ATG7 expression, which indicates the role of H9 lncRNA in mediating autophagy. In contrast, the exogenous overexpression of H19 lncRNA induces autophagic cell death in cerebral ischemia and reperfusion (I/R) injury. H19 lncRNA induces autophagy by inhibiting DUSP5 expression, a mitogen-activated protein kinase phosphatase. The downregulation of DUSP5 leads to the phosphorylation of ERK1/2, which induces autophagic initiation. The opposite effects of H19 lncRNA on autophagy in diabetic cardiomyopathy and cerebral I/R injury indicate that conditional gene interference targeting H19 lncRNA may be an efficient therapeutic approach to different pathological processes (Fig. 10.2).

lncRNAs are good mediators of AMPK. AMPK promotes its activation by binding to NBR2 lncRNA. Intriguingly, the expression of NBR2 lncRNA can also be induced by the increased activation of AMPK under energy stress. Healthy tissue depends on the combination of AMPK and lncRNA NBR2 to resist energy stress and tumors. NBR2 lncRNA expression is reduced in human cancers, and loss of its

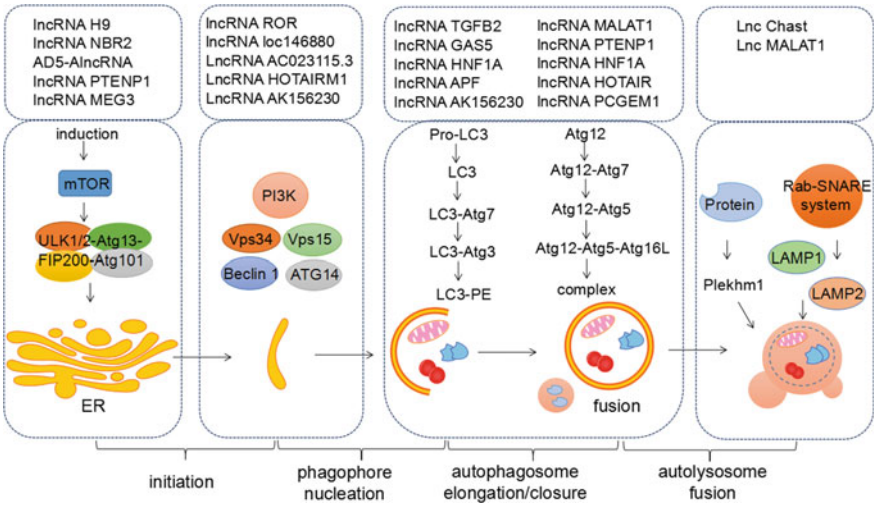


**Fig. 10.2** Long non-coding RNA involved in autophagy regulation

function is correlated with poor prognosis for cancer patients. lncRNAs commonly bind proteins to mediate the activity of proteins and participate in physiological functions. However, identifying the mechanisms of this effect is still difficult: does it occur through changing the conformation of the protein or by altering its affinity for other regulatory factors?

In addition to H9 lncRNA and NBR2 lncRNA, many lncRNAs are involved in autophagic initiation such as AD5-AlncRNA, PTENP1 lncRNA, and MEG3 lncRNA. Among them, AD5-A lncRNA is particularly interesting because it is an artificial lncRNA synthesized in vitro. miR-21, miR-216, and miR-27 are key molecules leading to sorafenib resistance, which is a new type of multitargeted oral drug for clinical tumor treatment. The overexpression of Ad5-AlncRNA can sequester these miRNAs from binding to the 3′-UTR of the target mRNA, repressing AKT/mTOR activity, promoting autophagic activation and subsequently reversing sorafenib resistance. Therefore, synthetic lncRNA is similar to the endogenous lncRNA and can regulate autophagy (Tang et al. 2016).

#### 10.2.2.2 lncRNAs Related to Phagophore Nucleation

Autophagosomes are the core elements of autophagy. The rough endoplasmic reticulum or Golgi membrane encapsulating broken organelles or abnormally folded proteins in the cytoplasm can turn into phospholipid bilayer vesicles. Phagophore nucleation is the first step for cells to recruit proteins and for lipids to form autophagosome membranes. Mammal phagophore nucleation is mainly regulated by the class III PI3K complex, which mainly comprises Vps34, Vps15, Beclin 1, and ATG14

to generate phosphatidylinositol 3-phosphate (PI3P). PI3P recruits double FYVE-containing protein 1 (DFCP1) and other ATG proteins to promote the formation of the omegasome. Previous studies have confirmed that lncRNAs regulate Beclin 1 and affect vesicle nucleation. The lncRNA ROR reversed gemcitabine and tamoxifen resistance in breast cancer by upregulating Beclin 1 expression. Respiratory exposure to a PM2.5 environment-induced reactive oxygen species (ROS). A high level of ROS caused the upregulation of the lncRNA loc146880, which downregulated Beclin 1 mRNA levels, leading to autophagy and lung cell migration in the lungs. Although the ROR and loc146880 lncRNAs are known to mediate vesicle nucleation by Beclin 1, the relationship between other lncRNAs and Beclin 1 or the Vsp family needs to be further studied.

#### 10.2.2.3 lncRNAs Related to Autophagosome Elongation

The two unique ubiquitin-like conjugation systems have crucial roles in the elongation and closure of the isolated membrane. Driven by ATG7 (E1-like enzyme) and ATG10 (E2-like enzyme), ATG12 conjugates to ATG5 and then interacts with ATG16 (mammal ortholog is ATG16L) to form the ATG12-ATG5-ATG16 complex. Subsequently, the ATG12-ATG5-ATG16 complex, ATG7 and ATG3 (E2-like enzyme) jointly transform LC3 from its cytosolic soluble isoform (LC3-I) to its membrane-anchored isoform (LC3-II).

Many proteins are involved in the autophagy elongation process, which also involves many lncRNAs. A study revealed that the lncRNA TGFB2 can be upregulated by vascular endothelial cell (VEC) inflammation and functions as a sponge for miR-3960, miR-4488, and miR-4459, thereby increasing the expression levels of their targets such as ATG13, ceramide synthase 1 (CERS1), and La ribonucleoprotein domain family member 1 (LARP1). In addition, overexpression of the lncRNA TGFB2 increases ATG3, ATG7, and P62 expression, probably through upregulating LARP1 by sponging miR-4459, an RNA-binding protein related to transcript stability and mRNA translation. Moreover, when lncRNA TGFB2 prevents the miR-3960-mediated repression of CERS1, the production of C18-ceramide is increased to induce mitophagy by directly interacting with LC3-II-containing autophagolysosomes upon Drp1-dependent mitochondrial fission. Given that autophagy and inflammation have an intricate relationship, intervening in the expression of the TGFB2 lncRNA could be a possible treatment strategy for infectious and autoimmune diseases.

The lncRNA GAS5 has been reported to inhibit autophagy and enhance cisplatin sensitivity in NSCLC cells. In contrast to its deletion in several species of tumors, GAS5 lncRNA is upregulated in osteoarthritis (OA), repressing autophagy and stimulating the apoptosis of OA chondrocytes, which is a key determinant responsible for cartilage degradation and thus OA pathogenesis. The increased expression of GAS5 lncRNA in OA represses autophagy possibly through downregulating Beclin 1, ATG3, ATG5, ATG7, ATG12, and LC3B expression. GAS5 lncRNA-mediated repression of autophagy is beneficial for enhancing drug sensitivity, but it also results

in the occurrence of OA. Therefore, different models of interference with GAS5 may be essential for distinct therapeutic purposes.

The lncRNA HNF1A-AS1 can sequester miR-30b from binding to its target ATG5 and thereby provoke autophagy in HCC. In addition, Beclin 1 and ATG12 were defined as targets of miR-30b in a previous study. The TGFB2, GAS5, and HNF1A-AS1 lncRNAs promote autophagic progression by regulating key proteins in the process of autophagosome elongation.

#### 10.2.2.4 lncRNAs Related to Autolysosome Fusion

The final step of autophagic flux is the fusion of the autophagosome and the lysosome to form an autolysosome, where the autophagic cargo is degraded. The core molecules in this stage include the Rab-SNARE system and the lysosome membrane proteins LAMP1 and LAMP2. In addition, adaptor proteins are necessary to link endocytic and autophagic pathways to the lysosome. Pleckstrin homology domain-containing protein family M member 1 (Plekhm1) is one of these adaptor proteins that contain an LC3-interacting region, which mediates the fusion of endosomes and autophagosomes with lysosomes. The lncRNA Chast can suppress autophagy by downregulating Plekhm1 and possibly ATG5 expression to induce cardiomyocyte hypertrophy. The lncRNA MALAT1 represses autolysosome fusion via the downregulation of LAMP1 and LAMP2, leading to autophagic inhibition (Yang et al. 2017).

### *10.2.3 lncRNAs Regulated by Autophagy*

Growing evidence suggests that lncRNAs play key roles in autophagy and that it is particularly important to investigate whether lncRNAs can be regulated by autophagy. Previous studies have shown that autophagy can degrade several types of RNAs and associated ribonucleoprotein complexes, which implies that lncRNAs may be degraded in autolysosomes (Frankel et al. 2017). The lncRNA PVT1 is the sole lncRNA reported to be regulated by autophagy thus far. lncRNA PVT1 is upregulated in diabetes, and autophagic repression decreases its transcriptional level. PVT1 lncRNA is probably not degraded by autophagy, as it is downregulated when autophagy is repressed. Thus, further extensive investigations are needed to demonstrate what determinants participate in this process.

## 10.3 Circular RNAs and Autophagy

### *10.3.1 Circular RNAs*

Protein-coding genes and their transcripts are the most studied sequences in eukaryotic cells. However, protein-coding genes and RNAs comprise only a small fraction of genomes and transcriptomes. Indeed, the vast majority of sequences in the human genome do not encode proteins, and non-coding RNAs account for almost 95% of the total RNA transcribed from eukaryotic genomes. circRNAs were first identified in 1976 in an electron microscopy-based study of RNA viruses and have since been found in humans, mice, rats, fungi, and other organisms. Unlike linear RNA molecules, circRNAs are closed circular molecules with a covalently closed-loop structure that lack 5′-3′ polarity or a polyadenylated tail. As a large proportion of the non-coding RNA family, circRNAs have drawn intense interest over the last few years. Non-coding RNAs have been increasingly shown to function in gene regulation and contribute to the development of many human disorders.

#### 10.3.1.1 circRNA Biogenesis

Although circRNAs are derived from precursor mRNAs (pre-mRNAs), their biogenesis remains elusive. circRNAs differ from other RNAs in their remarkable continuous closed-loop structure, which is covalently linked by free 3′ and 5′ ends. This closed-loop structure is also called a "back-splicing" structure.

circRNAs can mainly be classified into three categories: exonic circular RNAs (ecircRNAs), circular intronic RNAs (ciRNAs), and exon-intron circular RNAs (EIciRNAs). Several models have been proposed to explain the possible formation of ecircRNAs, such as lariat-driven circularization and intron pairing-driven circularization models. In a lariat-driven circularization event, RNA is folded during pre-RNA synthesis, making multiple exons close to each other and "jump" to form a ring-shaped RNA intermediate, and then, circRNA is generated. The "intron pair-driven cyclization" model suggests that reverse complementary ALU sequences exist in introns downstream of exons, and their pairing mediates reverse splicing to form circRNA.

The synthesis of circRNA is regulated by many factors. Many RNA-binding proteins (RBPs) are involved in the biosynthesis of circRNA, such as Muscle blind protein (MBL protein), Quaking protein (QKI protein), and adenosine to inosine acting RNA enzyme 1 (ADAR1), which can inhibit circRNA synthesis (Han et al. 2018a).

#### 10.3.1.2 circRNA Function

Many of the functions of circRNAs have been elucidated over the past few years. For example, circRNAs can act as gene expression regulators via different regulatory modes, regulate transcription and alternative splicing, interact with RBPs, serve as miRNA sponges and function in translation.

circRNAs Serve as miRNA Sponges or Competing Endogenous RNAs

circRNAs are mainly distributed in the cytoplasm, which has increased interest in the role of circRNAs in posttranscriptional regulation. In 2013, it was confirmed for the first time that circRNAs may be used as a sponge or competitive endogenous RNA to regulate the expression of target genes of microRNAs. The competitive endogenous RNA (ceRNA) hypothesis holds that there are RNA-binding sites on microRNAs, while there are also RNA-binding sites on circRNAs. By competing with microRNAs, circRNAs can indirectly regulate the translation of RNA. Earlier, the ceRNA hypothesis mainly involved RNAs, including RNA, transcribed pseudogenes and lncRNA. The ceRNA hypothesis also includes circRNA. For example, ciRS-7/CDR1 circRNA contains more than 70 conservative binding sites of microRNA-7. CiRS-7/CDR1 circRNA can regulate the activity of microRNA-7 by binding with microRNA-7 and affect the expression of target genes of microRNA-7 (Qu et al. 2018).

circRNA Interactions with RBPs

Some RNA can bind to RNA-binding proteins or act as storage/isolation proteins, thus participating in protein substructure localization. For example, circMbl, derived from muscleblind (MBL/MBNL1), contains multiple MBL binding sites. When the MBL protein is overexpressed, circMbl can absorb excess protein. CircMbl can regulate the MBL protein level in this way.

circRNAs Regulate Transcription or Splicing

circRNAs can influence their parent genes through cis or trans actions. circRNAs can directly participate in gene expression regulation by regulating linear RNA transcription and variable splicing. For example, in the exon hopping model, precursor RNAs can also be alternatively spliced to form mature linear RNA while cyclizing to form ecircRNAs. Thus, the synthesis of ecircRNAs will competitively hinder the synthesis of homologous linear RNA, but it may also increase the expression of the circRNA itself or its corresponding linear RNA. In addition, the cyclization and nonlinear splicing of pre-RNA containing translation initiation sites indicates

that the formation of circRNA reduces the formation of RNA and the translation of downstream proteins. This effect is called an "RNA trap" (Meng et al. 2017).

Role in Translation

In addition to these functions, circRNA has been found to have translational functions. Because circRNA lacks some common features of coding RNA such as the m7GPPPN cap structure at the 5′ end and the polyadenylation tail structure at the 3′ end, it is considered a non-coding RNA and cannot encode the protein. There are many internal ribosome entry sites (RESs) in circRNAs suggesting that circRNAs may have the ability to encode proteins. Circ-ZNF609 was the first circRNA found to directly translate proteins that participate in muscle development (Legnini et al. 2017).

### *10.3.2 circRNAs and Autophagy Regulation*

Depending on their unique 3-dimensional covalent structure, circRNAs effectively capture or sequester RNAs or proteins and release them in subcellular locations to mediate autophagy regulation. circRNA may be cleaved by autophagic degradation and regulate autophagy accordingly.

Astrocytes, the most abundant cell type in the central nervous system (CNS), play critical roles in regulating and maintaining CNS homeostasis in normal physiological situations. In pathological conditions, astrocytes become activated and are characterized by abnormal morphology with reactive astrogliosis. Astrocyte activation plays a detrimental role in various neurological pathologies, including stroke, Parkinson's disease, Alzheimer's disease, and drug abuse. Astrocyte activation is associated with neuronal damage, as there is a close relationship between methamphetamine-induced degeneration of dopaminergic neurons in the striatum and concomitant reactive astrogliosis. circHIPK2 inhibits the activity of endogenous microRNA-124 by blocking the activity of microRNA-124, which leads to the upregulation of sigma-1 receptor expression and finally affects the activation of astrocytes by co-regulating autophagy and endoplasmic reticulum stress (Huang et al. 2017). In stroke, circHECTD1 acts as an endogenous microRNA-142 sponge, inhibiting the activity of microRNA-142, thereby inhibiting the expression of TCDD-induced ADP-ribose polymerase (TIPARP) and astrocyte autophagy (Han et al. 2018b). In addition, circRNA participates in the regulation of peripheral nerve injury through autophagy. The expression of circRNA-2837 was significantly downregulated in a rat sciatic nerve injury model. The downregulation of circRNA-2837 alleviated sciatic nerve injury by inducing autophagy. Mechanistically, the knockdown of circRNA-2837 may protect neurons against neurological injury by acting as a sponge for members of the miR-34 family (Zhou et al. 2018).

## 10.4 Other Non-coding RNAs and Autophagy

Non-coding RNAs (ncRNA) include many types of RNAs that do not encode proteins. Based on their function, length and structure, ncRNAs are divided into microRNAs (miRNAs or miRs; 19–23 bp), long noncoding RNAs (lnRNAs; >200 bp and linear), circular RNAs (circRNAs; >200 bp and circular), transfer RNAs (tRNA; 74–95 bp), ribosomal RNAs (rRNAs; 121–5000 bp), small nuclear RNAs (snRNAs; 100–300 bp), small nucleolar RNAs (snoRNAs; 100–300 bp), guide RNAs (gRNAs; 55–70 bp), piwi-interacting RNAs (piRNAs; 24–30 bp), and small interfering RNAs (siRNAs; 21–25 bp).

Over the past few decades, increasing evidence has indicated that ncRNAs, ranging from miRNAs to lncRNAs and even circRNAs, mediate the transcriptional and posttranscriptional regulation of autophagy-related genes by participating in autophagy regulatory networks. This book provides a detailed introduction to the regulatory roles of miRNAs, lncRNAs, and circRNAs in autophagy. Because the relationship between other ncRNAs and autophagy is less studied, this section will briefly introduce recent research progress.

### *10.4.1 Transfer RNAs and Autophagy*

#### 10.4.1.1 Transfer RNA

In 1953, Watson and Crick first announced the three-dimensional structure of the DNA double helix and changed people's understanding of life. Crick thus proposed a central rule to explain the process of genetic information transmission: genetic information is transmitted exclusively to RNA through DNA and then from RNA to protein in the process of transcription and translation. However, in his hypothesis, a key "conjugator", which is a key molecule that can specifically connect nucleic acid and protein sequences, was not found. Shortly afterward, this key "conjugator" molecule, named transfer RNA (tRNA), was discovered along with an important family of enzymes, amino acid-tRNA synthase, which specifically catalyzes the coupling of amino acids with their transporting RNA carriers. tRNAs are essential adapter molecules in translation that carry specific amino acids and, by complimentary codon–anticodon base pairing, ensure the incorporation of the correct amino acid sequence in the nascent polypeptide.

#### 10.4.1.2 The Relationship Between tRNAs and Autophagy

Current studies on the relationship between tRNA and autophagy are limited. Recent studies have shown that the interaction between tRNA and autophagy plays a key

role in the stability and translation of RNA, the regulation of translation efficiency and aging.

The cytoplasmic localization, stability, and translation of messenger RNA (messenger RNA) are influenced by its binding proteins. These binding proteins mainly include cold shock domain (CSD) structural proteins, nuclear heterogeneous ribonucleoproteins and serine/arginine-rich proteins. Recent studies have shown that the most abundant RNA-binding proteins in the human body, including the CSD-containing Y-box-binding protein 1 (YBX1), the closely related YBX3 protein and other RNA-binding proteins, such as SRSF1, SRSF2, SRSF3, and hnRNP A1 and H, are specifically and closely related to mitochondrial tRNAs (mt tRNAs). Although the function of mt tRNA in the cytoplasm is not yet clear, the dynamic interaction between mt tRNA and RNA-binding proteins may affect the stability or translation of cytoplasmic RNA. Y-box-binding proteins play a wide range of biological roles in the process of nuclear DNA replication, DNA repair, and transcription, as well as in the process of mRNA processing through their interaction with nucleic acids. YBX1 and YBX3, together with many hnRNP proteins and serine/arginine-rich proteins, can bind to newly synthesized precursor messengers, thereby regulating the splicing and polyadenylation of precursor messengers, promoting the transfer of messengers to the cytoplasm and controlling the translation, stabilization, and localization of cytoplasmic messengers. Recent studies have shown that tRNA and autophagy are involved in these processes. Autophagy induces the release of mt RNA into the cytoplasm and promotes the abovementioned processes. Although the biological function of mt tRNAs in the cytoplasm is still uncertain, the dynamic interaction between mt tRNAs and RNA-binding proteins plays an important role in the stability and translation of RNA (Jady et al. 2018).

Aging is a complex biological process that all organisms must undergo, but its exact molecular mechanism is not yet clear. Because of its short life span, conservative aging pathway, and easy genetic and environmental control, single-cell eukaryotic budding yeast has become the main aging research model. Furthermore, budding yeast is the only system in which the number of times a cell divides, also known as replicative lifespan, can be accurately determined. Protein synthesis is a key factor affecting cell growth, replication, and survival, which is widely regulated by external and internal factors. Translation efficiency is closely related to cell life span. In old cells of budding yeast, the phosphorylation level of eIF2a mediated by stress kinase Gcn2 increased, which reduced the overall translation efficiency of yeast cells, while the expression of the downstream transcription activator Gcn4 did not decrease significantly. Overexpression of tRNA in young yeast cells can activate the stress kinase Gcn2 and prolong cell life through a Gcn4-dependent pathway. In addition, overexpression of Gcn4 can prolong cell life in an autophagy-dependent manner without altering the overall translation efficiency; that is, autophagy-mediated by overexpression of tRNA can prolong cell life (Hu et al. 2018).

## *10.4.2 Ribosomal RNAs and Autophagy*

### 10.4.2.1 Ribosomal RNA

Ribosomal RNA (rRNA) is the most abundant type of RNA, accounting for approximately 82% of the total RNA. It binds to proteins to form ribosomes, whose function is to synthesize amino acids into peptide chains under the guidance of RNA. When rRNA exists alone, it does not perform its function. rRNA binds to various proteins to form ribosomes and serves as an 'assembly machine' for protein biosynthesis.

### 10.4.2.2 The Relationship Between rRNAs and Autophagy

There are few studies on the relationship between rRNA and autophagy. A few studies have shown that autophagy-dependent rRNA degradation plays an important role in maintaining nucleotide stability and animal development. During autophagy, protein aggregates and organelles are transferred to lysosomes for degradation, and the degradation products are reused by cells to maintain intracellular stability. Many cell components, such as organelles, can be used as substrates for autophagy. Ribosomes have long been observed in autophagy by electron microscopy as markers of cytoplasmic degradation. Except for selective autophagy, mature ribosomes in yeasts can also be selectively phagocytosed and scavenged under long-term nitrogen deficiency. In this process, ribosome subunits are likely to be independent degradation targets that involve ubiquitination and deubiquitination. Ribosomes contain approximately 50% of cellular proteins and 80% of total RNA. In the process of autophagy-mediated degradation, these proteins and RNA molecules are degraded in lysosomes, and their degradation products can be used as the main source of amino acids and nucleotides in nutrient deficiency. Therefore, autophagic degradation of ribosomes is essential for the survival of yeast cells under nutritional deficiency. RNST-2, a ribonucleic acid endonuclease of the T2 family of nematodes, is a key enzyme for the degradation of rRNA in lysosomes. Recent studies have shown that RNST-2 deficiency can induce the aggregation of rRNA and protein in lysosomes, which indicates that RNST-2 mediates the autophagic degradation of rRNA in lysosomes. Lack of RNST-2 can cause developmental defects of embryos and larvae of nematodes and shorten the lifespan of nematodes. In addition, the absence of RNST-2 and pyrimidine nucleotides at the same time would lead to the death of nematode embryos. Supplementation of uridine or cytidine could inhibit the death of embryos (Liu et al. 2018b).

In addition, the process of anabolism and catabolism of intracellular substances is strictly regulated by the energy supply of cells. Energy stress can inhibit the biosynthesis of rRNA and induce autophagy. However, the relationship between rRNA biosynthesis and autophagy remains unclear. Nucleoprotein NAT10 plays a key role in the biosynthesis of rRNA and the transformation between autophagy. Under physiological conditions, NAT10 acetylation activates rRNA biosynthesis and

inhibits autophagy. In conditions with an adequate energy supply, NAT10 binds to the autophagy regulator Che-1 K288 and acetylates it, inhibiting the transcriptional activation of the Che-1-mediated downstream genes Redd 1 and Deptor. Under energy stress, NAT10 deacetylates under the action of Sirt 1, inhibiting the biosynthesis of rRNA activated by NAT10. In addition, deacetylation of NAT10 renders its inhibition of Che-1-mediated autophagy ineffective. These results suggest that the acetylation of NAT10 is of great significance for the conversion of metabolic reactions to synthesis and catabolism and provide a new mechanism for the regulation of rRNA biosynthesis and autophagy by nucleoproteins (Liu et al. 2018a).

## *10.4.3 Small Nuclear RNAs and Autophagy*

### 10.4.3.1 Small Nuclear RNA

Small nuclear RNA, also known as intranuclear small RNA, is involved in the processing of RNA in the nucleus of eukaryotic cells. snRNA binds with many proteins to form small ribonucleoproteins, which participate in the splicing of pro-messenger RNA and process it into mRNA.

### 10.4.3.2 The Relationship Between snRNAs and Autophagy

In some aging-related diseases, mutations in pathogenic genes not only cause the occurrence of positive symptoms but also change the monoclonal conformation of the proteins or metabolites encoded by them, which leads to the increase and accumulation of potentially toxic proteins with an anti-hydrolysis ability. The lysosomal system is the most important protein hydrolysis system in mammals and is responsible for the treatment of abnormal proteins. In the lysosomal system, abnormal proteins are decomposed by two main pathways: (1) the autophagic lysosome system is mainly responsible for the degradation of proteins with a long half-life and is also the only way to degrade organelles and protein aggregates or inclusions; (2) the transfer of membrane components and extracellular substances to lysosomes under specific targeted signals. Many chemicals can regulate the level of autophagy at different stages of autophagy. Rapamycin can increase autophagy by inhibiting mTOR, and 3-methyladenine (3-MA) can inhibit autophagic formation by blocking PI3K. Genetic analysis of neurodegenerative diseases shows that there is a close relationship between neurodegenerative diseases and lysosomal network function. During the development of AD, a series of pathological changes take place in the network of the neuronal lysosome system, including increased lysosome biosynthesis and inhibition of lysosome ingestion, which ultimately lead to the destruction of the lysosome clearance mechanism. In AD patients, there are many autophagic vacuoles (AVs) and autophagosomes in the brain. This suggests that autophagy is impaired in AD patients because AVs are rare in the brains of healthy people. In

addition, AVs mainly accumulate in amyloid precursor protein (APP), and Aβ peptides are produced by APP during autophagy. In normal cells, these Aβ peptides are degraded in lysosomes immediately after they are produced. Presenilin 1 (PS1) is a component of gamma-secretase and an essential part of lysosomal acidification. Recent studies have shown that PS1 can increase the expression of U1 snRNA and cause adverse changes in the expression of APP, the production of Aβ peptides and cell apoptosis. Overexpression of U1 snRNA can significantly activate autophagy, leading to dysfunction of the autophagic lysosomal system and exacerbating AD symptoms (Cheng et al. 2018).

## 10.5 Summary and Prospect

In the past decade, the study of ncRNA-mediated autophagic regulation has been a research hotspot in the field of biology. As research progresses, many miRNAs that can regulate autophagy have been discovered, and the regulatory mechanisms of miRNAs on autophagy are becoming increasingly clear. miRNAs regulate the autophagic process mainly at the posttranscriptional level by affecting the expression of autophagy-related genes. However, the miRNAs currently involved in the regulation of autophagy are likely to only be the tip of the iceberg, and more autophagy-related miRNAs will be discovered in the near future. With the increasing development of research, the regulatory mechanism of miRNA on autophagy will likely become clearer, which will provide a new strategy for the clinical treatment of various diseases related to autophagy. Current studies indicate that most lncRNAs regulate autophagy-related protein expression by a ceRNA mechanism. In addition to chromatin and histone remodeling, transcriptional regulation and protein–protein interactions, lncRNAs have more complex functions associated with autophagic regulation to be elucidated. Due to the high spatial and temporal specificity and tissue specificity, lncRNAs may be used as biomarkers for autophagy-related diseases and may be used to develop potential therapeutic measures. There are still few studies on circRNAs and autophagy. However, given the unique biological characteristics of circRNAs, circRNAs also have the potential to become an autophagy-related research tool.

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# Chapter 11
# Epigenetic Regulation of Autophagy


**Li-Fang Hu**

**Abstract** Epigenetics refers to reversible and hereditary changes in gene expression without alterations in DNA sequences, such as DNA methylation, histone modification and chromatin remodelling. It was first proposed by Waddington in the book *Introduction to Modern Genetics* in 1939. Autophagy includes at least four processes: autophagy induction, autophagosome formation, autophagosome fusion with lysosomes and lysosomal degradation of cytoplasmic components. The whole process is complex and dynamic, and involves at least 30 autophagy-related proteins. This degradative machinery is regulated by multiple signal molecules. Autophagy was once considered to be a cytoplasmic event; however, in recent years, emerging evidence suggests that nuclear components (transcription factors, histone modification, microRNAs, etc.) also play an important role in autophagy regulation (Baek and Kim 2017). Among them, epigenetic regulation of autophagy has gained much attention. The epigenetic machinery can not only modify autophagy-related genes but also affect some signal molecule genes that regulate autophagy, thus impacting their transcription and subsequent autophagy. This chapter focuses on the role and recent progress in autophagy regulation by DNA methylation and histone modifications. The role of non-coding RNAs such as microRNA in autophagy regulation will be covered in other chapters.

**Keywords** Epigenetic · Autophagy · DNA methylation · Histone acetylation · Histone methylation

## Abbreviations

| | |
|---|---|
| CBP | CREB binding protein |
| DAPK | Death-associated protein kinase |
| DNMT | DNA methyltransferase |
| HAT | Histone acetyltransferase |

L.-F. Hu (✉)
Institute of Neuroscience, Soochow University, Suzhou, China
e-mail: hulifang@suda.edu.cn



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HDAC Histone deacetylase
HMT Histone methyltransferase
JMJD6 JmjC domain-containing protein 6
KAT Lysine acetyltransferase
KDM Histone or lysine demethylase
LAMP2 Lysosomal-associated membrane protein 2
5-mC 5-methylcytosine
MeCP2 Methyl-CpG-binding protein 2
NOR1 Nitro domain containing protein 1
NSCLC Non-small cell lung cancer
PAD4 Peptide arginine deiminase 4

## 11.1 Autophagy Regulation by DNA Methylation

### *11.1.1 Introduction to DNA Methylation*

DNA methylation refers to the methylation of the fifth carbon atom of cytosine into 5-methylcytosine (5-methylcytosine, 5-mC), which is catalysed by DNA methyltransferase (DNMT) using S-adenosylmethionine as a methyl donor. This modification mainly occurs in the CpG dinucleotide sequence. In most regions of the genome, the appearance frequency of CpG sequences is low. However, in some specific regions, such as the gene promoter, CpG dinucleotides are arranged in high frequency series and called CpG islands. DNA methylation usually results in gene silencing because 5-mC blocks DNA binding to transcription factor complexes. Conversely, DNA demethylation often activates gene transcription. DNA methylation is the earliest identified and most characterized machinery of epigenetic modifications. Notably, the methylated CpG island can be recognized by methyl-CpG-binding protein 2 (MeCP2), which further recruits histone deacetylase (HDAC) and histone methyltransferase (HMT) and other histone-modifying enzymes to alter chromatin structure and subsequently modulate gene transcription. Therefore, it is noteworthy that DNA methylation often interacts with histone modification and thus synergistically regulates gene transcription.

### *11.1.2 DNA Methylation of Autophagy-Related Genes*

To date, many autophagy-related genes have been found to be methylated and silenced, thus inhibiting the process of autophagy and autophagic flow in some pathological situations. Here are some examples.

#### 11.1.2.1 ULK Kinase/ATG1

In yeast, autophagy-related protein (Atg) 1, along with Atg13 and Atg17, comprises the ULK1 kinase complex, which plays a critical role in autophagy initiation. The yeast Atg1 has two homologous proteins in mammals, ULK1 and ULK2, collectively known as ULK kinase. Currently, most studies focus on the regulation of ATG1/ULK1 by mTOR and AMPK signalling pathways. In a nutrient-rich environment, activated mTOR phosphorylates and inhibits ULK1 activity. During nutrient deprivation, mTOR activity is inhibited, and thus, its inhibition of ULK1 is released, which activates ULK1 kinase and initiates autophagy activation. Energy depletion, such as glucose deprivation, induces AMPK activation and phosphorylates the ULK1 Ser317 and Ser777 sites, thereby inducing autophagy. Mounting evidence suggests that in addition to modulation by mTOR and AMPK, DNA methylation also exerts an effect on *ULK* transcription and thus autophagy induction. It was reported that ULK1 and ULK2 expressions were significantly decreased in gliomas and that the promoter region of ULK2 was hypermethylated, whereas the ULK1 promoter was not affected. ULK2 activation inhibited astrocyte transformation and glioma growth by enhancing autophagy activity. This implies that the alteration of *ULK2* DNA methylation influences the autophagy activity of glioma cells and thus participates in the formation of glioma.

#### 11.1.2.2 Beclin1/ATG6

Beclin1 (yeast Atg6 homologous protein) is a multifunctional protein in autophagy. It not only forms a complex by binding to Atg14L and Barkor and regulates autophagy initiation but also interacts with other proteins, such as Vps34 and Bcl-2, to regulate autophagosome maturation. Li et al. found that the *Beclin1* mRNA level was decreased in 14 out of 20 breast cancer patients, and its protein expression was also lowered in 13 out of 20 patients. Downregulation of *Beclin1* transcription was attributed not only to the decrease in gene copy number but also to the abnormal hypermethylation of the CpG enrichment region in the promoter and intron 2 of the *Beclin1* gene. When breast cancer cells were treated with methyltransferase inhibitors, Beclin1 expression was upregulated, accompanied by increased autophagic activity and inhibition of tumour growth, suggesting that DNA methylation of *Beclin1* inhibited its transcription and autophagy.

#### 11.1.2.3 LC3/Atg8

LC3 is a homologue of the yeast autophagy-related gene *Atg8*. There are many subtypes of LC3 in mammalian species, namely, LC3A, LC3B and LC3C. The amino acid sequences of LC3A and LC3B are highly consistent. Both of these proteins participate in autophagosome formation and are commonly used markers of autophagy. During autophagy, LC3-I covalently binds with phosphatidylethanolamine to form

LC3-II under the action of ATG7 and the ATG12–ATG5–ATG16L complex. LC3-II binds to the membrane of autophagosomes. Therefore, the LC3-II/I ratio is a biomarker of autophagosome formation in experimental studies. Previous studies reported that *LC3A* was silenced due to DNA hypermethylation in a variety of cancer cells, and the DNMT inhibitors could increase *LC3A* transcription and inhibit the growth of cancer cells. This suggests that hypermethylation of *LC3A* may inhibit autophagy and promote tumourigenesis. Another study also reported that the DNA methylation inhibitor deoxyazacytidine increased LC3-II levels and autophagy activity in human chronic myeloid leukaemia K-562 and MEG-01 cell lines. All the data indicate that DNA methylation is involved in the regulation of autophagy.

#### 11.1.2.4 LAMP2

Recent evidence suggests that methylation modification of the *lysosomal-associated membrane protein 2 (LAMP2)* gene causes autophagy deficiency, which may be involved in the pathogenesis of Danon disease, an X-linked fatal cardiomyopathy. By generating patient-specific induced pluripotent stem cells (iPSCs) and differentiating them into cardiomyocytes (iPSCs-CMs), the researchers reproduced the histological features and autophagy failure of Danon disease. Administration of DNMT inhibitors reactivated the silent *LAMP2* alleles in these iPSCs and alleviated the autophagic failure, suggesting that silencing of the *LAMP2* gene caused by DNA methylation was responsible for the autophagic failure and was related to the development of Danon disease (Ng et al. 2016).

### *11.1.3 DNA Methylation of Autophagy Regulatory Molecules*

Apart from the autophagy-related genes mentioned above, DNA methylation can also modify the genes that encode autophagy regulatory signal molecules. Abnormal methylation in some tumour-related genes has been reported to be involved in autophagy regulation.

#### 11.1.3.1 Nitro Domain-Containing Protein 1 (NOR1)

*Nitro domain-containing protein 1 (NOR1)* is a putative tumour suppressor gene. Li et al. found that the *NOR1* promoter was hypermethylated, which was associated with the downregulation of NOR1 expression in nasopharyngeal carcinoma cells compared with that in normal tissues. DNMT inhibitors reversed the downregulation of NOR1 expression and clone formation of nasopharyngeal carcinoma cells. Meanwhile, the levels of LC3-II/LC3-I and autophagy activity as well as cell survival were suppressed. These results suggest that hypermethylation of the *NOR1* promoter enhances the autophagy and survival of nasopharyngeal carcinoma cells. However,

the exact molecular mechanism by which NOR1 regulates autophagy has yet to be elucidated.

#### 11.1.3.2 Death-Associated Protein Kinase (DAPK)

*DAPK* is a newly identified tumour suppressor gene that is extensively involved in cell proliferation, apoptosis, autophagy and other pathological processes. Hypermethylation of CpG islands in the *DAPK* promoter, resulting in the silencing of this gene, has been reported in a variety of cancer cells. It correlates with the formation and metastasis of tumours. DAPK interacts with several autophagy-regulated proteins, such as mTOR, Beclin1 and PI3K, thus playing a positive or negative role in autophagy regulation in different conditions. For instance, it was found that following arsenic treatment, the CpG islands in the *DAPK* promoter were hypermethylated and the transcription of *DAPK* was suppressed, while the number of autophagosomes and Beclin1 expression were increased in SV-HUC-1 cells. These changes were reversed by the DNMT inhibitor Aza (Chai et al. 2007). These findings suggest that DNA hypermethylation of the *DAPK* gene is involved in autophagy regulation. However, a recent study found no significant correlation between the transcription of the *DAPK* gene and its DNA methylation status by comparing the association among the degree of DAPK promoter methylation and its mRNA and protein expression in tumour and adjacent non-tumour tissues from 15 breast cancer patients and matched controls (Streckmann et al. 2018). The difference may be caused by the small sample size of this study. Meanwhile, these results also indicate that the relevance of DNA methylation of the DAPK gene in tumourigenesis has yet to be investigated further.

#### 11.1.3.3 SOX1

SOX1 belongs to the superfamily of SRY (sex-determining region Y)-box containing transcription factors. It can inhibit Wnt/β-catenin signalling by directly binding to β-catenin, leading to its degradation and function loss. Several studies reported that the SOX1 promoter was hypermethylated in hepatocellular, nasopharyngeal, oesophageal and non-small cell lung cancer (NSCLC) cells, associated with lower SOX1 expression in these tumour cells. Moreover, long-term exposure to cisplatin can also induce methylation of the SOX1 promoter in ovarian cancer cells and participate in cisplatin resistance. A further study demonstrated that the promoter of *SOX1* was abnormally hypermethylated, associated with the suppression of *SOX1* transcription in NSCLC cells, especially in cisplatin-resistant NSCLC cells. Moreover, silencing *SOX1* promoted cisplatin-induced autophagy in NSCLC cells, indicating that DNA hypermethylation of the *SOX1* gene participated in autophagy regulation in NSCLC cells.

### 11.1.4 Autophagy Regulation by DNA Demethylation

DNA methylation is a dynamic process, and methylated DNA can also be demethylated. It is believed that there are two means of DNA demethylation: active and passive demethylation. Active DNA demethylation is mainly mediated by enzymes that convert 5-mC to unmethylated cytosine. Passive DNA demethylation occurs along with DNA replication, during which the methylated DNA is eliminated due to the semi-reserved mode of DNA replication. As such, methylated CpG is "diluted" with the progress of DNA replication, resulting in passive DNA demethylation. Although active DNA demethylation widely exists in many cells, its molecular mechanism remains controversial. Ten–eleven translocation (TET) proteins, including TET1, TET2 and TET3, are α-ketoglutarate- and $Fe^{2+}$-dependent dioxygenases that catalyse the hydroxylation of 5mC to 5-hmC. The identification of the TET family reveals a new pathway involved in DNA demethylation. It has emerged as one of the hotspots in epigenetic research in the past decade. A recent study found that *TET1* knockout downregulated autophagy in glioma U251 cells, while its overexpression upregulated autophagy, indicating that TET1 plays a role in cancer development by regulating autophagy activity (Fu et al. 2017). However, whether the exact mechanism of autophagy regulation by TET1 is related to demethylation of autophagy-related genes remains to be further studied. Another study reported that TET2 was downregulated during the pathogenesis of atherosclerosis in an ApoE knockout mouse model. The downregulation of TET2 promotes methylation of the *Beclin1* promoter, leading to autophagy flux impairment in endothelial cells (Peng et al. 2016). Therefore, these data strongly suggest that DNA demethylation affects autophagy by regulating the transcription of autophagy-related genes. However, research on the relationship between DNA demethylation and autophagy has only begun. At present, little is known about this topic, which deserves further study.

### 11.1.5 Conclusion

In summary, the study on autophagy regulation by DNA methylation is still in its infancy. To date, only a few autophagy-related genes and signalling molecules have been reported to undergo DNA methylation and thus affect autophagy activity. Most of the current information regarding autophagy regulation by DNA methylation comes from cancer-related studies. The impact of DNA methylation on autophagy may be tissue- or cell-specific. Whether alterations in DNA methylation contribute to other autophagy-related diseases, such as infection and neurodegeneration, remains to be explored further. In addition, current knowledge on DNA methylation and autophagy comes from the methylation alteration analysis of some genes during DNMT inhibition by pharmacological tools. However, these DNMT inhibitors have non-specific effects. Thus, this still needs to be confirmed with other technical or experimental evidence. The identification of the DNA demethylase TET family offers

a new avenue for exploring the correlation between DNA methylation and autophagy in the future.

## 11.2 Autophagy Regulation by Histone Modifications

### *11.2.1 Introduction to Histone Modifications*

Histones are the chief protein components of chromatin, acting as spools around which DNA winds. The structural unit of the histone-DNA complex is called the nucleosome. Five major subtypes of histones exist: H1, H2A, H2B, H3 and H4. Histones H2A, H2B, H3 and H4 are known as the core histones, while H1 is known as the linker between the two nucleosomes. The nucleosome core is formed by two H2A–H2B dimers and an H3–H4 tetramer. The H3 and H4 histones have long tails at the N terminus, where lysine and arginine residues are enriched and often protrude from the nucleosome. The tails can undergo post-translational modifications, including methylation, acetylation, phosphorylation, ubiquitination, SUMOylation and ADP-ribosylation, which often results in changes in chromatin structure and leads to activating or silencing of gene transcription. Post-translational modification of histones provides a readable marker for the binding of other proteins to DNA, which produces synergistic or antagonistic effects to regulate gene transcription and expression. This modification plays a similar role to the DNA codon, so it is also called the "histone code". Notably, different histone modifications often affect one another. Histone acetylation often interacts with other epigenetic modifications and finely regulates gene transcription. Compelling evidence suggests that histone modifications not only affect autophagy induction but also play a role in the maintenance of long-term autophagy flux during prolonged exposure to autophagic stimuli, which is closely related to the pathogenesis of tumours and neurodegenerative diseases. Histone modification is a research hotspot of epigenetic regulation of autophagy and appears to be the fastest-growing branch of autophagy research in recent years (Fullgrabe et al. 2014a; Shin et al. 2016b).

### *11.2.2 Autophagy Regulation by Histone Acetylation*

Histone acetylation is a histone modification that mainly occurs at the N-terminal conserved lysine residues of H3 and H4. It is coordinately regulated by histone acetyltransferase (HAT) and histone deacetylase (HDAC). As its name implies, HATs transfer the acetyl groups from acetyl coenzyme A to specific lysine residues at the N-terminal of the histone. They are also known as lysine acetyltransferases (KATs), which mainly include four groups: GCN5-related acetyltransferases (GNAT), MYST-related acetyltransferases, p300/CBP acetylases (KAT3B/KA), and

T3A and Rtt109 (KAT11). As a superfamily, 18 subtypes of HDACs have been identified thus far. They can be divided into four categories: type I includes HDAC1–3 and HDAC8, type II includes HDAC4–7 and HDAC9–10, type III includes SIRT1–7 and type IV includes HDAC11. Among them, types I, II and IV are zinc-dependent, while type III catalyses deacetylation with $NAD^+$ as a cofactor. The distribution of HDACs is subcellular specific and may change under different conditions. For example, SIRT1 is mainly expressed in the nucleus under normal conditions, but it can translocate into the cytosol by a variety of stimuli. In the cytosol, SIRT1 catalyses the deacetylation of several autophagy-related proteins and their regulators, such as ATG5, ATG7, LC3, FOXO and E2F1. Interestingly, SIRT1 deacetylates non-histone proteins in the cytoplasm and often activates autophagy, whereas it induces histone deacetylation that inhibits autophagy. In addition to SIRT1, other histone deacetylases, such as HDAC6, also affect the acetylation of non-histones and thus regulate autophagy. Deacetylation of non-histones is one of the hotspots in the field of autophagy-related research.

Relative to non-histone modification, histone acetylation has not received much attention until recently. In 2009, Madeo et al. first proposed the concept that histone modifications regulate autophagy. In ageing yeasts, they found that spermidine-induced autophagy relied on HAT inhibition, which caused global hypoacetylation of histone H3 and inhibited the transcription of many genes. Interestingly, the transcription of some autophagy-related genes (ATG) remained unaffected, and autophagy was still activated during this process. They believe that selective transcriptional activation of *ATG* is critical for yeast to save "resources" under starvation. In fact, some earlier studies by other groups already suggested that histone acetylation could affect autophagy. In 2004, Shao et al. found that the HDAC inhibitors butyrate and SAHA induced autophagic death in several human cancer cell lines. Notably, HDAC inhibitors also deacetylate non-histone proteins in the cytoplasm. Moreover, autophagy was commonly considered to be a cytoplasmic event, and nuclear events were not recognized as a regulator of this process. Thus, researchers focused on the acetylation of non-histone proteins in the cytoplasm when interpreting the results at that time. In recent years, compelling studies have demonstrated an important role of histone acetylation in sustained autophagy in response to long-term nutritional deprivation or stress (Fullgrabe et al. 2014b). The autophagy-related acetylations of histone 4 at position 16 lysine (H4K16ac) and histone 3 at position 56 lysine (H3K56ac) are the most studied.

#### 11.2.2.1 H4K16ac

Unlike most histone modifications, the acetylation of H4K16 not only affects the nucleosome level but also has an impact on the chromatin structure; thus, it plays a central role in chromatin remodelling and gene transcription. In humans, hMOF/KAT8 and SIRT1 act as a pair of molecular switches to coordinate the acetylation of H4K16, thereby regulating autophagic activity in cells. Specifically,

hMOF catalyses the acetylation modification of H4K16 and upregulates the expression of autophagy-related genes, whereas SIRT1 activation produces antagonistic effects, deacetylating H4K16 and inhibiting basal autophagy under normal conditions. Autophagy activation induced by several stimuli in different cell lines is related to a global reduction of H4K16 acetylation. hMOF/KAT8 is an autophagy substrate. Autophagy-induced degradation of hMOF and the consequent decrease in H4K16 acetylation inhibits the transcription of autophagy-related genes and the persistence of autophagy, which forms a negative feedback preventing autophagic death. It was observed that the autophagy-inducer rapamycin significantly increased cell death in normal somatic cells, HeLa cells and the U1810 cell line, and this was attenuated by blockade of H4K16 deacetylation using a SIRT1-specific inhibitor or hMOF overexpression (Fullgrabe et al. 2013). This suggests that the degree of H4K16 acetylation not only regulates autophagy but also plays a role in cell death or survival decisions. Notably, SIRT1-induced non-histone deacetylation often results in autophagy activation. Conversely, histone deacetylation by nuclear SIRT1 leads to autophagy inhibition. In addition, histone acetylation is often associated with other histone modifications and participates in autophagy regulation. For instance, H4K16ac often cooperates with H3K9me2 to inhibit autophagic flow. In addition, H4K16ac is tightly connected to H4K20me3. During autophagy, the decrease in H4K16ac and the increase in H4K20me3 often collectively inhibit gene expression. Thus, these results suggest that different histone modifications often interact with each other to fine-tune the transcription of autophagy-related genes and autophagy flux. In other words, the alteration of autophagy activity may be the consequence of a variety of histone modification changes. Therefore, when studying the impact of histone modification on autophagy, researchers cannot neglect the potential effect of other histone modifications on autophagy when focusing on one specific modification.

#### 11.2.2.2 H3K56ac

H3K56 is located in the entry and exit of the nucleosome. Acetylation of H3K56 interrupts the interaction between the histone and DNA and inhibits transcription. Researchers observed an inhibition by rapamycin on H3K56 acetylation in yeast and revealed for the first time a positive regulation of the TOR signalling pathway on H3K56 acetylation. The acetylation of human H3K56 is regulated by EP300/KAT3B/P300 and KAT2A/GCN5. It has been reported that EP300 knockout activates autophagy, while EP300 overexpression inhibits starvation-induced autophagy. However, it remains to be determined whether EP300 affects autophagy merely via the regulation of H3K56 acetylation because EP300 is also known to regulate the acetylation of multiple autophagy-related proteins, including ATG5, ATG7, MAP1LC3 and ATG12. Moreover, the deacetylase of H3K56 is still controversial. Deacetylases, including HDAC1, HDAC2, SIRT1, SIRT2 and SIRT3, have been reported to catalyse the deacetylation of H3K56. It is noteworthy that although

these deacetylases regulate autophagy, the role of non-histone deacetylation cannot be ruled out.

It is noteworthy that intracellular HAT and HDAC cooperatively maintain a dynamic balance between histone and non-histone acetylation and deacetylation (Fig. 11.1). The dysregulation of HAT and/or HDAC functions caused by some pathological factors results in changes in the degree of protein acetylation and upregulation or downregulation of some autophagy-related genes or their regulatory molecules and thus leads to the development of various diseases such as tumours and neurodegenerative disorders. Mounting evidence suggests that HDAC inhibitors promote autophagy by increasing the acetylation of non-histone proteins in the cytoplasm (described in other chapters). Therefore, HDAC inhibitors have become a new direction of drug research and development for these diseases. Currently, researchers have demonstrated that changes in histone acetylation are often correlated with alterations in

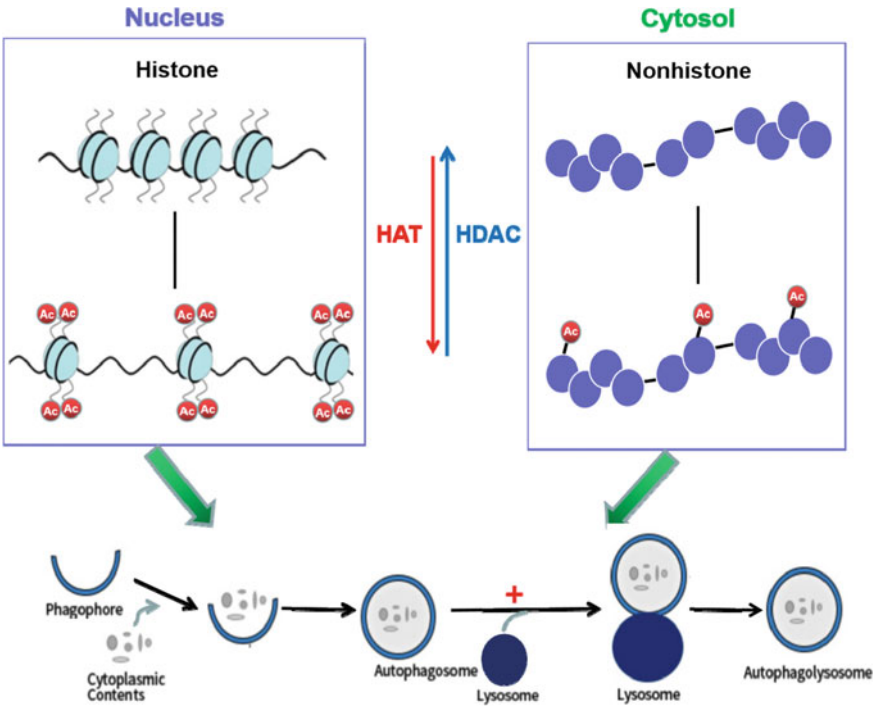


**Fig. 11.1** Autophagy regulation by histone acetyltransferase (HAT) and histone deacetylase (HDAC)-mediated histone and non-histone acetylation. HATs and HDACs co-regulate and maintain the dynamic balance between histone and non-histone acetylation and deacetylation. This plays a critical role in the different processes of autophagy by promoting or suppressing the expression of autophagy-related genes, their regulatory molecules or affecting the protein–protein interaction. Specifically, the deacetylation of non-histone proteins in the cytoplasm often activates autophagy, while deacetylation of histones in the nucleus inhibits autophagy. Thus, the impact of histone and non-histone acetylation on autophagy activity is inconsistent

non-histone acetylation in the cytoplasm. However, several studies show an opposite effect exerted by histone and non-histone acetylation on autophagy activity. Hence, it is highly suggested to use other techniques, such as nucleus/cytoplasm separation, to clarify the net contribution of histone or non-histone acetylation changes in autophagy in addition to their application with pharmacological tools.

### *11.2.3 Autophagy Regulation by Histone Methylation*

In addition to acetylation, histone methylation is also involved in autophagy regulation. Histone methylation is catalysed by histone methyltransferase (HMT) and usually occurs on lysine (K) and arginine (R) residues. Among them, K4, K9, K27, K36, K79, R2, R17 and R26 of histone H3, and R3 and K20 of H4 can be methylated. Lysine can be monomethylated, dimethylated and trimethylated, while arginine can only be monomethylated and dimethylated. This greatly increases the complexity of histone methylations. Moreover, similar to that of histone acetylation, the process of histone methylation is reversible, and the demethylation of lysine and arginine residues is mediated by specific histone demethylases. Histone arginine demethylases mainly include peptide arginine deimise 4 (PAD4) and JmjC domain-containing protein 6 (JMJD6). Histone lysine demethylases include LSD1, JHDM1, JHDM2 and JMJD2.

The effect of histone lysine methylation on gene transcription depends not only on the modified position and methylation degree but also on the gene region where methylation occurs. For example, methylation of heterochromatin H3K9 often leads to gene silencing, while H3K9 methylation in gene coding regions activates gene transcription. H3K4 methylation is often concentrated in the promoter, serving as a marker of gene transcription activation. Methylation of H3K27 is related to gene transcription inhibition. Histone demethylation has complex effects on gene transcription. For example, the regulation of gene expression by lysine demethylase LSD1 depends on its specific substrate. In summary, histone methylation and demethylation are dynamic and reversible complex processes catalysed by specific enzymes that participate in biological processes, such as gene transcription and autophagy (Fig. 11.2). Here, we list a brief review of histone methylation modifications involved in autophagy.

#### 11.2.3.1 H3K4me3

Dimethylation and trimethylation of histone H3 at lysine 4 (H3K4me2/me3) are involved in transcription activation, and the highest H3K4me3 level occurs near the transcription start point of highly expressed genes. H3K4 is methylated by SET1 and the mixed lineage leukaemia family of HMTs (MLL1) and demethylated by KDM1A/LSD1 and KDM5/JARID1. H4K16ac and H3K4me3 often reside in the same nucleosome units in human cells, and this is consistent with the interaction

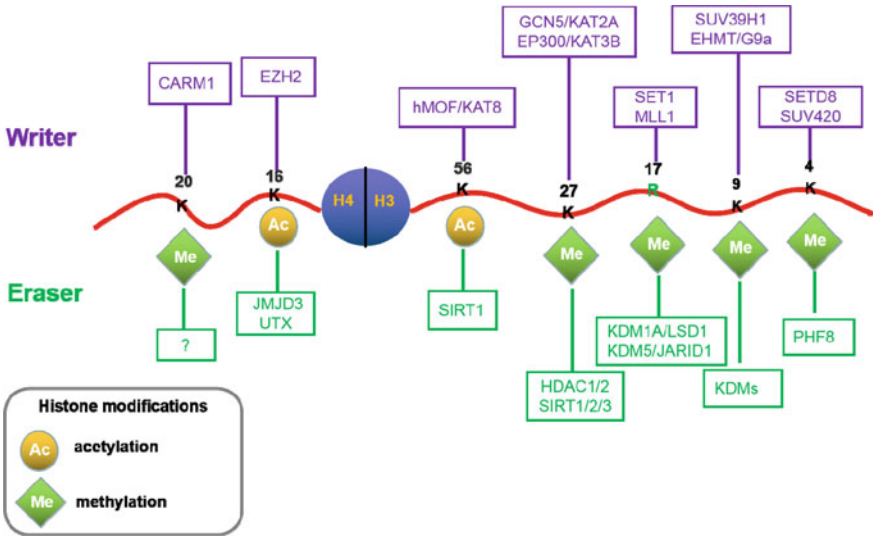


**Fig. 11.2** Histone modifications involved in autophagy regulation. Covalent modification of histone is catalysed or removed by specific enzymes. Accordingly, the enzymes that promote and inhibit histone modification can be called writers and erasers, respectively. Histone modifications involved in autophagy regulation mainly occur on H3 and H4. In contrast to acetylation, histone methylation occurs not only on lysine (K) but also on arginine (R) residues. Ac, acetylation; Me, methylation

between their corresponding catalytic enzymes KAT8 and KMT2A/MLL1. Similar to the change of H4K16ac, autophagy often results in a decrease in H3K4me3 and leads to global transcription inhibition in multiple cell lines from yeast to human. This may be a conservative mechanism of energy savings in response to persistent starvation. WNT/β-catenin signal activation was reported to inhibit *SQSTM1/p62* transcription and autophagy activity, which is related to the increase in H3K4me3 (Petherick et al. 2013). During autophagy, WNT dissociates from the promoter region of *SQSTM1* and is degraded, which in turn results in a decrease in H3K4me3. Consequently, this weakens the inhibition by WNT on *SQSTM1* transcription and thus increases *SQSTM1* transcription and autophagy activity. This process was verified in mixed lineage leukaemia and other cancer cells (Sierra et al. 2006; Wend et al. 2013).

#### 11.2.3.2 H3K9me3/H3K9me2

H3K9 can not only be mono-, di- or even tri-methylated by histone methylases such as SUV39H1 and EHMT2/G9a but can also be acetylated under the action of histone acetylases such as KAT2A and KAT2B/PCAF. Interestingly, the deacetylation of H3K9 is required for the increase in H3K9 dimethylation. H3K9 methylation is usually involved in gene silencing. It was reported that EHMT/G9a binds to the promoter of several autophagy-related genes, such as LC3B and WIPI1, resulting in

H3K9 methylation and silencing of these genes in normal conditions (de Narvajas et al. 2013). During nutrient deprivation-induced autophagy, EHMT2 is released from the promoters of these autophagy-related genes, resulting in subsequent demethylation and acetylation modifications of H3K9 and thus promoting the transcription and expression of autophagy-related proteins. EHMT2 inhibitors were found to increase BNIP3 and LC3 levels. When autophagy is induced by rapamycin along with a persistent inhibition of EHMT2 by specific inhibitors, autophagy appears to be overstimulated, resulting in autophagic death in cells. Therefore, methylation of H3K9 not only regulates autophagy but also has a neglectable impact on cell survival. Notably, several members of the histone lysine demethylase family can catalyse the demethylation of H3K9. For example, KDM2B can demethylate H3K9 and induce autophagy.

#### 11.2.3.3 H4K20me3

Methylation modification of H4K20 is involved in gene silencing, and H4K20me3 is often distributed in some constitutively expressed heterochromatin regions. H4K20 methylation can be catalysed by several enzymes, including SETD8 and SUV420. Specifically, SETD8 catalyses the monomethylation of H4K20 (H4K20me1), which can be further methylated to H4K20me2 and H4K20me3 by SUV420. PHF8 is identified as an H4K20 demethylase. Serum deprivation-induced autophagy increased the level of H4K20me3, which was accompanied by the alteration of H4K16 acetylation. Studies have shown that the deacetylation of H4K16ac and the increase in H4K20me3 antagonistically regulated 20–30% of human genome expression. The mechanism is related to RNA polymerase II pause (Kapoor-Vazirani et al. 2011). Therefore, the deacetylation of H4K16ac and the methylation of H4K20 during autophagy may work together to fine-tune the pause of RNA polymerase II and repress the transcription of some autophagy-unrelated genes.

#### 11.2.3.4 H3R17me2

H3R17 (H3R17me2) dimethylation was reported to enhance the transcription of several autophagy-related genes and transcription factor EB (TFEB), a key transcription factor in autophagy. H3R17me2 is mainly mediated by arginine methyltransferase 1 (CARM1). Under nutrient-rich conditions, the stability of CARM1 is regulated by the E3 ubiquitin ligase SKP2 in the nucleus, not in the cytoplasm. Nutritional starvation and glucose deprivation result in AMP-activated protein kinase (AMPK)-dependent phosphorylation of FOXO3a in the nucleus, which in turn transcriptionally suppresses SKP2. This inhibition led to an increase in CARM1 protein, followed by an increase in histone H3R17me2. Genome-wide analysis showed that this process played a role in transcriptional activation of autophagy-related and lysosome

genes (Shin et al. 2016a). This study revealed a new mechanism for the AMPK–SKP2–CARM1 signalling axis in regulating histone modification and autophagy during prolonged nutrition deprivation.

#### 11.2.3.5 Other Histone Methylations

In addition to the methylation modification of the sites mentioned above, EZH2-mediated H3K27me3 also affects autophagy activity. The expression of EZH2 was increased in human rectal colon cancer tissues. *EZH2* knockout or its inhibitor reduced H3K27me3 and activated autophagy, which may be of relevance for the treatment of rectal colon cancer (Wei et al. 2015). However, a recent study found that EZH2-mediated H3K27me3 and DNMT1-induced DNA methylation synergistically inhibited the expression of cystic fibrosis transmembrane conductance regulator (CFTR), which caused autophagy activation and aggravated homocysteine-induced liver injury in mice (Yang et al. 2018). Therefore, EZH2-induced H3K27me3 may have specific effects on autophagy under different pathological conditions. This also confirms the complexity and diversity of histone methylation during autophagy, which should not be ignored in the research.

### *11.2.4 Autophagy Regulation by Other Histone Post-translational Modifications*

Over the past decade, a large number of studies have revealed interactions between histone methylation and acetylation and autophagy. However, the roles and mechanisms of other covalent modifications of histones, such as phosphorylation, SUMOylation, ubiquitination and ADP ribosylation, in autophagy are still in infancy. A recent study reported a role for H2BK120 monoubiquitination (H2Bub1) in autophagy, which identified a novel epigenetic mechanism. H2Bub1 is critical for maintaining lower autophagy activity under basal conditions. However, when the cells are exposed to nutrient deprivation or starvation, the expression of deubiquitinase USP44 is upregulated, resulting in a decrease in H2Bub1, which may lead to global changes in the expression of genes, especially those autophagy-related genes. These alterations in gene expression eventually contribute to autophagy activation after starvation. It was also observed that this process is accompanied by changes in hMOF expression and the H4K16ac degree (Chen et al. 2017). Notably, other studies show that histone methylation, such as H3K4 and H3K79 methylation, can also be affected by H2Bub1. Therefore, different histone modifications are tightly connected, and the exact role of this interaction in autophagy and autophagy-related diseases needs to be further explored.

## 11.3 Conclusion and Prospective

In conclusion, the current knowledge regarding the role of DNA methylation and histone post-translational modifications in autophagy has only revealed the tip of the iceberg of autophagy regulation by the epigenetic machinery. In addition to DNA methylation and histone post-translational modification, great progress has been made in autophagy regulation by non-coding RNA in recent years. This topic is elaborated in other chapters. There are interactions among various epigenetic regulations. Specifically, DNA methylation affects histone acetylation, and different histone modifications, including methylation, acetylation, and even ubiquitination, interact with each other. All of these factors determine the complexity of epigenetic regulation on autophagy. Special attention should be paid to the changes in non-histone acetylation in the cytoplasm, which is an important factor for autophagy regulation. Therefore, when studying the impact of compounds targeting acetylation-modifying enzymes on autophagy, we should comprehensively analyse the changes in both histone and non-histone acetylation with multiple approaches. Finally, epigenetic modification appears to be a dynamic and reversible event in the nucleus, and DNA methylation could have an effect on histone acetylation and other modifications, and vice versa. In addition, the HDACs show potential effects on non-histones in the cytoplasm. Thus, all of these factors contribute to the complexity of epigenetic regulation of autophagy. Therefore, it will be of great significance to strengthen the study on autophagy regulation by epigenetic modifications, which can help to further understand the molecular mechanism of autophagy regulation and its role in the pathophysiological processes of tumours, neurodegenerative diseases and other disorders.

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# Chapter 12
# Protein Modification and Autophagy Activation


**Rui Wang and Guanghui Wang**

**Abstract** Protein modification refers to the chemical modification of proteins after their biosynthesis, which is also called posttranslational modification (PTM). PTM causes changes in protein properties and functions. PTM includes an attachment of addition of functional groups, such as methylation, acetylation, glycosylation and phosphorylation; a covalent coupling of small peptides or proteins, such as ubiquitination and SUMOylation; or chemical changes in amino acids, such as citrullination (conversion of arginine to citrulline). Protein modification plays an important role in cellular processes. Since a protein can be modified in different ways, such as acetylation, methylation and phosphorylation, the functions of proteins are different under different modification states. Moreover, the same modification at different sites may have completely different effects on protein function. For example, phosphorylation at some sites in a protein may lead to a functional activation, while phosphorylation at other sites may cause an inhibition of the functions. Thus, different modifications, combinations and sites changes lead to different functional regulations of a protein, resulting in different effects in the cells. In autophagy, PTMs are widely involved in the regulation of autophagy, including ubiquitination, phosphorylation and acetylation. Ubiquitination is the covalent conjugation of ubiquitin to the substrates through a series of enzymes. Phosphorylation refers to an attachment of a phosphoryl group into a protein, primarily on serine, threonine and tyrosine, which is catalyzed by the kinases. Phosphorylation, a common modification, regulates protein function and localization. Phosphorylation in autophagy regulates the activity of autophagy-associated proteins and the initiation and progression of autophagy by regulating signaling pathways. Acetylation means the addition of acetyl groups onto lysine or N-terminal segment of target proteins through acetyltransferases. Acetylation and deacetylation are both involved in the regulation of autophagy initiation and selective autophagy by controlling the acetylation level of important proteins in the autophagy process. In this chapter, we will focus on the regulation of ubiquitination and phosphorylation in autophagy.

R. Wang · G. Wang (✉)
Laboratory of Molecular Neuropathology, Jiangsu Key Laboratory of Neuropsychiatric Diseases & Department of Pharmacology, College of Pharmaceutical Sciences, Soochow University, Suzhou 215123, China
e-mail: wanggh@suda.edu.cn



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## Abbreviation

| | |
|---|---|
| ALS | Autophagy-lysosome system |
| AMPK | AMP-regulated protein kinase |
| CC | Coiled-coil domain |
| CTD | C-terminal domain |
| Cvt | Cytoplasm-to-vacuole target |
| E1 | Ubiquitin-activating enzyme |
| E2 | Ubiquitin-conjugating enzyme |
| E3 | Ubiquitin ligase |
| HDAC6 | Histone deacetylase 6 |
| LIR | LC3-interacting region |
| MAPK | Mitogen-activated protein kinase |
| NBR1 | Neighbor of breast cancer 1 |
| OPTN | Optineurin |
| PAS | Pre-autophagosomal structure |
| PE | Phosphatidylethanolamine |
| PI | PtdIns |
| PI3KC3 | PI3K class III complex |
| PI3P | PtdIns3P |
| PKC | Protein kinase C |
| PP | Protein phosphatase |
| PS | Proline/serine-rich |
| TORC1 | Target of rapamycin complex 1 |
| TSC2 | Tuberous sclerosis complex 2 |
| Ub | Ubiquitin |
| UBA | Ubiquitin-associated |
| ULK | Unc-51-like kinase |
| UPS | Ubiquitin-proteasome system |
| ZZ | Zinc finger |

## 12.1 Ubiquitin-Proteasome System and Autophagy

In cells, there are several pathways for protein degradation: (1) ubiquitin-proteasome system (UPS); (2) autophagy-lysosome system (ALS); and (3) protein hydrolysis. Since the hydrolyzed fragments of a protein after protein hydrolysis are often degraded by the UPS, major pathways regulating protein degradation are UPS and

ALS. Under normal condition, protein degradation is mainly accomplished by UPS. UPS is specific for short-lived protein degradation, which specifically recognizes and degrades proteins through ubiquitination by E3 ligase, regulating protein homeostasis. Autophagy is primarily responsible for the degradation of long-lived proteins and misfolded or aggregated proteins, as well as degradation of damaged organelles. Although autophagy has not been considered to have significant selectivity for protein degradation, recent studies have shown that autophagy requires ubiquitination for the degradation of some organelles and abnormal proteins, and thus there is a selective mechanism in autophagy.

### *12.1.1 Ubiquitination and Autophagy*

Ubiquitination has long been recognized as a specific marker for protein degradation by the proteasome. Recently, the covalent linking of ubiquitin chains with substrates has been recognized to be important in selective autophagy, which is closely related to the discoveries of autophagy receptors and adaptor proteins, as well as the studies of misfolded protein degradation in neurodegenerative diseases. Misfolded proteins appear in many cellular physiological and pathological processes, such as heat shock; or conformational changes of proteins caused by genetic mutations, and abnormal modifications of proteins. Under normal condition, many new synthetic proteins are misfolded. These proteins are not only prone to aggregation but also interfere with normal cellular functions. Although molecular chaperones include heat shock proteins that promote the degradation of abnormal proteins, many misfolded proteins form aggregates within the cell and cannot be degraded by the proteasome due to their large size. In neurodegenerative diseases, misfolded proteins often form aggregates and are ubiquitinated. For example, the nuclear inclusions in Huntington's disease contain a large number of polyubiquitinated mutant huntingtin. The polyubiquitin chains on these proteins can be recognized by autophagy adaptors, driving them to be degraded through autophagy (Fig. 12.1). Therefore, autophagy plays an important role in the degradation of misfolded proteins and aggregates (Rape 2018).

#### 12.1.1.1 Ubiquitination of Substrate

Both UPS and autophagy pathways use ubiquitin chains as a marker for substrate recognition. Ubiquitin (Ub) is a highly stable 76 amino acid protein conserved in all eukaryotes. This high evolutionary conservation plays an important role in the recognition by proteins that bind to ubiquitin through their interacting domains and in the homeostasis in the regulation of ubiquitinated substrates. Substrate conjugated with a single ubiquitin is called monoubiquitination, which regulates protein function and transport. Conjugation with multiple ubiquitins is called polyubiquitination. Polyubiquitination can occur through any lysine residue on ubiquitin (e.g., K3, K6, K11, K27, K29, K48 or K63) and can generate different types of ubiquitin chains (e.g.,

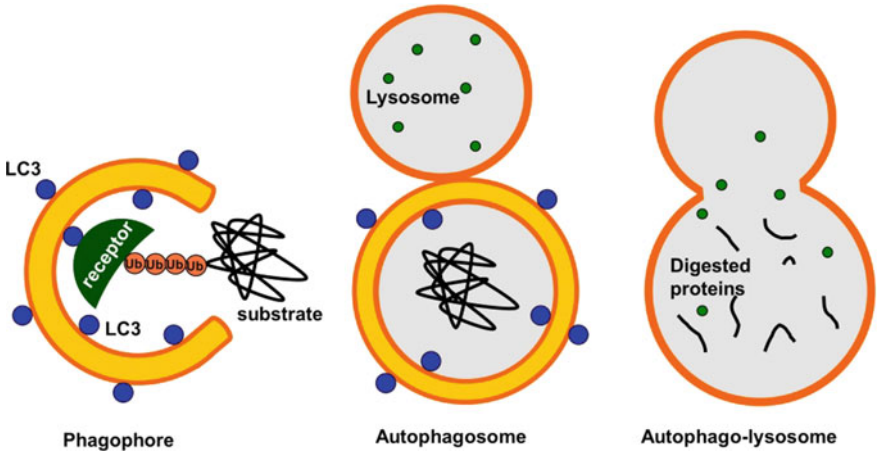


**Fig. 12.1** During autophagy, the ATG8-binding motif (AIM) or LC3-interacting regions (LIRs) on receptor proteins can interact with Atg8 or LC3 that is coupled to PE. The UBA (ubiquitin associating domain) on receptor proteins interacts with polyubiquitin chains that are conjugated to the autophagic substrates. Thus, autophagic receptor proteins function as scaffold proteins that connect ubiquitinated substrates and phagophore-anchored LC3, to drive the formation of autophagasome that will be fused to lysosomes

K11-linked, K48-linked or K63-linked ubiquitin chains). It is generally believed that ubiquitin-binding proteins recognize and preferentially bind to ubiquitins that are conjugated to proteins. The domains closely related to the UPS and autophagy are: (1) ubiquitin-associating (UBA) domain that is presented in the proteasome-associated proteins (such as Rad23) and in autophagy receptors (such as p62 and NBR1); (2) ubiquitin-interacting motif (UIM) that is presented in the proteasome protein S5a/Rpn10/Pus1; (3) a zinc finger ubiquitin-binding domain (ZnF UBP) that is presented in histone deacetylase 6 (HDAC6), or a ubiquitin-binding zinc finger (UBZ) domain that is presented in the autophagy receptor NDP52.

Ubiquitination of a substrate involves three processes: ubiquitin activation, ubiquitin conjugation and ubiquitin ligation. First, ubiquitin-activating enzyme (E1) activates C-terminal cysteine of ubiquitin and forms a thioester bond with ubiquitin. Secondly, ubiquitin is transferred from E1 to ubiquitin-conjugating enzyme (E2), and then binds to ubiquitin ligase (E3) by E2. Finally, the ubiquitin covalently links to the substrate protein by E3, which creates an isopeptide bond between a lysine of the target substrate and the C-terminal glycine of ubiquitin. The next ubiquitin can be ligated to last ubiquitin that has been conjugated to substrate, thus forms polyubiquitin chains. Since ubiquitin itself forms a long chain linking to the substrate, the ubiquitin chain formed by the linking of different lysine sites of ubiquitin (such as K48 and K63 sites) often determines the functions as well as the fate of substrate. During autophagy, the autophagy adaptor recognizes the ubiquitinated substrate and anchors it to the autophagic vesicle membrane, thereby allowing the substrate to be

engulfed by autophagosomes and to be degraded by lysosomes. Therefore, ubiquitination is very important for substrates to be recognized by the autophagy system. It is also considered that ubiquitination is important in selective autophagy (Kwon and Ciechanover 2017).

#### 12.1.1.2 Autophagy Receptors

The autophagy receptors are mainly as follows: TOLLIP/Cue5, p62, NBR1, OPTN and NDP52 (Fig. 12.2).

The CUE domain targeting (CUET) proteins are early identified autophagy receptors involved in the clearance of cytotoxic aggregates. This family of proteins includes Cue5 in yeast and its mammalian homolog TOLLIP, which binds to ubiquitin through its CUE domain. However, in TOLLIP and Cue5, except CUE structure, their structures are obviously different. In addition to a CUE domain, TOLLIP has a TOM1 (target of myb1 homolog) binding domain and a phospholipid-bound $Ca^{2+}$-dependent membrane targeting module (C2). In addition, the C terminus of Cue5 carries an

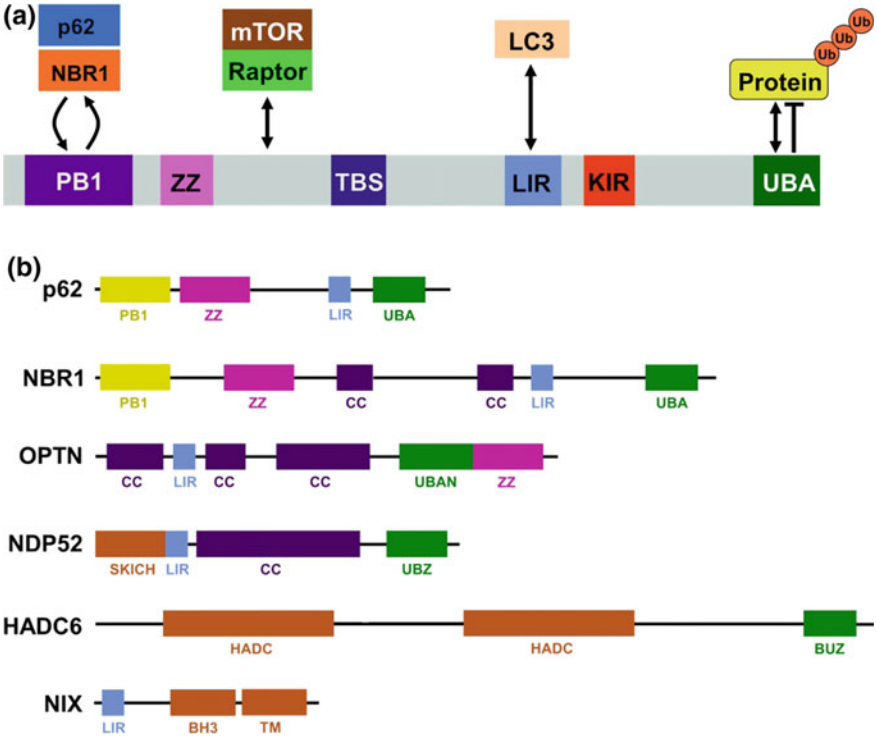


**Fig. 12.2** Diagram of the structures of p62, NBR1, OPTN and NDP52. **a** Domains for protein interaction or functional regulation on p62. **b** Diagram of the structures of p62, NBR1, OPTN and NDP52

ATG8-binding motif (AIM), while TOLLIP contains two functional LC3-interacting regions (LIRs) located within the C2 domain. The CUE domains of Cue5 and TOLLIP have no preference for interactions with K48- or K63-linked Ub chains, thus they may not distinguish different Ub linkage. The CUE domain of TOLLIP has similar affinity to free Ub like p62 does, but its affinity to K48- and K63-linked polyubiquitin chains is stronger than p62.

p62 is a multifunctional protein with multiple domains in its structure (Fig. 12.2). p62 was first identified to be associated with tumors. The PB1 (Phox and Bpem1) domain at the N terminus of p62 binds to PKCζ and PKCι, thereby regulating NFκB pathway. In tumorigenesis, p62 activates different signaling pathways under different environmental conditions, and can be either a tumor-promoting factor or a tumor suppressor. In ductal pancreatic cancer cells, p62 binds to TRAF6 to activate Ras, thereby further activating NFκB to increase p62 transcription. The newly synthesized p62, in turn, binds to TRAF6 to amplify the Ras signal and promote tumorigenesis. However, phosphorylation of p62 at T269 and S272 can inhibit tumor growth and invasion. Recent studies have found that a motif of p62 between the zinc finger (ZZ) and the TRAF6-binding domain (TBD) binds to the mTOR regulatory factor Raptor, and affects the lysosomal localization of mTOR complex 1 (mTORC1) on lysosomal membrane and promotes Rag GTPase-mediated mTOR activation; thus has an important role in both tumorigenesis and autophagy.

p62 functions in autophagy as a receptor protein that mediates substrate degradation. Johansen group first reported in 2007 that p62 binds to LC3 and mediates the degradation of ubiquitinated aggregates by autophagy. The exposed glycine residue at the C-terminal of LC3 couples to PE to form LC3-II, and LC3-II is then tightly anchored to the membrane of phagophore. Eleven amino acids at residues 332–343 of p62 form a LIR bound to LC3-II, and thus connect p62 to phagophore via LC3. There is a ubiquitin-associating domain (UBA) at the C terminus (amino acids 386–440) of p62. UBA recognizes ubiquitin and binds to polyubiquitinated proteins. Thus, p62 binds to LC3 and polyubiquitinated proteins, thereby functioning as an autophagic receptor to deliver its bound ubiquitinated protein to autophagosomes. In the recognition of polyubiquitin chains, the binding ability of UBA to K63-linked ubiquitin chain is stronger than K48-linked chains, and the binding ability of UBA to ubiquitin chain can be significantly increased after phosphorylation of p62 at S403 on UBA by casein kinase 2 (CK2) (Lee and Weihl 2017).

Another receptor similar to p62 is NBR1, although the two proteins differ in protein sequences. NBR1 has many similarities in structure to p62. NBR1 has a LIR bound to LC3 and a UBA domain bound to ubiquitin chain. They have functional similarities in autophagy regulation. p62 and NBR1 form homodimers or heterodimers for the regulation of autophagy. p62 can form dimmers by self-binding through its PB domain (Phox and Bpem1 domain) (Fig. 12.2), while NBR1 can form dimmers through its coiled-up coil domain (CC) bound to PB domain of p62 (Kirkin et al. 2009).

The other two autophagy receptors are OPTN and NDP52, both of which carry LC3 and ubiquitin-binding domains. These proteins are associated with the immune process, which mediates clearance of bacteria by recognizing ubiquitinated bacteria.

Interestingly, both are involved in the TANK-binding kinase 1 (TBK1) signaling pathway. TBK1 is a serine/threonine kinase, a member of the IKK family that has antibacterial effects. TBK1 phosphorylates OPTN at serine adjacent to LIR, which enhances the binding of OPTN to LC3, thereby facilitating the clearance of ubiquitinated bacteria.

#### 12.1.1.3 Autophagy Receptors and Substrate Recognition

Microtubule-associated protein light chain 3 (LC3), a homolog of Atg8 in mammalian cells, is similar to Atg8 and is involved in autophagy activation. LC3 exposes glycine at the C terminus to form LC3-I after the process of Atg4 protease. Then, under the action of the ubiquitin-like system composed of Atg7 and Atg3, the exposed glycine can be conjugated to phosphatidylethanolamine (PE) to form LC3-II that binds to phagophore to mediate autophagy. There is no Atg8 homolog in yeast, but in mammalian cells, the homologs of Atg8 include LC3, γ-aminobutyrate receptor-associated protein (GABARAP) and γ-aminobutyrate receptor-associated protein-like protein (GABARAP-like protein). In the presence of autophagy receptors, such as the mitochondrial autophagic receptors FUNDC1 and NIX, LC3-II can bind directly to them, leading to an induction of mitophagy. However, in most cases, LC3 binds to receptors and mediates autophagic degradation of proteins with polyubiquitin chains recognized by autophagy receptors, p62, NBR1, histone deacetylase 6 (HDAC6) and OPTN. All these receptors have the LC3-interacting region (LIR) and UBA domain. These receptors are similar to scaffolds that can bind both LC3 and polyubiquitin chains through these two domains, thereby promoting autophagy activation and mediating substrate degradation (Fig. 12.2). Cell and animal experiments have also shown that autophagy receptors are involved in the autophagic degradation of neurodegenerative disease proteins (Lu et al. 2014). Autophagy receptors involved in aggregate clearance include p62, NBR1, OPTN and other receptors that can form oligomers and bind directly or indirectly to members of the ATG8/LC3/GABARAP family. For example, p62 is polymerized into helical filaments through its PB1 domain, and even assembled into hetero-oligomers with other proteins (such as huntingtin proteins) to facilitate autophagy degradation of aggregates. NBR1 can form oligomers through its CC1 domain, or form hetero-oligomers with p62 through its PBQ domain interacting with PB1 domain on p62. The oligomerization of p62 results in high affinity of Ub and LC3/GABARAP, significantly increasing the binding of p62 and LC3 to ubiquitin chains on aggregates, which transports substrates to autophagosomes. However, an absence of PB1 domain of p62 can inhibit the recruitment of p62 to autophagosomes (Lee and Weihl 2017).

#### 12.1.1.4 HDAC6

Histone deacetylases (HDACs) were first discovered due to their deacetylation of histones in the nucleus, and later they were also found to act on cytosolic proteins.

Tubulin is the first confirmed acetylated protein whose acetylation state is regulated by the deacetylases HDAC6 and SIRT2, and lysine acetyltransferase (KAT9), forming a reversible process of acetylation and deacetylation. The stability and function of microtubule are regulated by reversible acetylation of α-tubulin. In the absence of nutrients, acetylation of α-tubulin at L40 enhances MAPK/JNK phosphorylation. Activation of the MAPK/JNK signals promotes the dissociation of Beclin-1 and Bcl-2, and free Beclin-1 initiates the formation of phagophore. The acetylation of tubulin is also critical for the transport of autophagosomes and the fusion of autophagosome with lysosomes. HDAC6 does not have a LIR, but has a region that binds to ubiquitin chains and a domain that binds to motor proteins (kinesin family member 1, KIF1 and dynein). In the process of autophagy, HDAC6 drives autophagic degradation of substrates through its interaction with ubiquitinated proteins and motor proteins. Since motor proteins are recruited on microtubule after tubulin acetylation, HDAC6 interacts with motor proteins to drive autophagic cargoes to move along the acetylated microtubules toward the lysosomal-enriched centrosomes, thereby promoting the fusion of autophagosomes and lysosomes. Loss of function mutant motor protein will disturb autophagic clearance of intracellular aggregates since autophagosomes cannot fuse to lysosomes, leading to an accumulation of LC3-II in cells (Calderilla-Barbosa et al. 2014).

### *12.1.2 The Relationship Between the Ubiquitin-Proteasomal System and Autophagy*

Autophagy and the UPS have been considered to be independent and parallel systems previously. The UPS degrades short-lived proteins and autophagy degrades long-lived proteins. However, recent studies have shown that they interact under many conditions and can share the same substrates, such as the UPS substrate IκB kinase (IKK), which is also found to be degraded by autophagy. Inhibition of autophagy also impairs the function of the proteasome, causing an increase in the level of proteasomal substrates (such as p53). The expression of p62 after autophagy inhibition will increase. Although it does not affect the proteasome activity, it increases the binding to the ubiquitinated substrates that are shared by the proteasome, affecting the normal delivery of these substrates to the proteasome, leading to the accumulation of the UPS substrates. The evidence showing the association between UPS and autophagy comes from studies in Atg5 or Atg7 conditionally knockout mice. These mice developed neurodegenerative phenotypes and appeared ubiquitin-positive aggregates in the neurons. Since the UPS activity was not significantly changed, it suggests that the ubiquitinated proteins are autophagic substrates, thus defects in autophagy cause abnormal accumulation of these ubiquitinated proteins. We will focus on the effects of the UPS on autophagy in the following content.

#### 12.1.2.1 Effects of Ubiquitin Proteasome System on Autophagy

Studies have shown that UPS has a great impact on autophagy. In mitophagy, parkin, an E3 ligase, provides a signal that can be recognized by receptors for the ubiquitination of mitochondrial proteins (substrates), thereby mediating the activation of selective mitophagy (see "Autophagy and mitochondrial renewal and quality control" section), which provides clear evidence for an involvement of ubiquitin in autophagy. In cells, the administration of the proteasome inhibitors will cause an increase and accumulation of ubiquitinated proteins that can be transported to form aggresome near the nucleus. In neurodegenerative diseases, the accumulation of ubiquitinated proteins that form aggregates can be partially degraded by autophagy. Therefore, the clearance of ubiquitinated proteins by autophagy can be considered as a compensatory mechanism for the proteasomal dysfunction.

HDAC6 and p62 are proteins that link autophagy and the UPS. HDAC6 recognizes ubiquitinated proteins, transports them to autophagosomes and transports autophagosomes by its binding to motor proteins. In *Drosophila* and mouse models, loss of HDAC6 function can cause an increase of ubiquitinated aggregates, indicating that HDAC6-mediated autophagy degrades ubiquitinated aggregates. Overexpression of HDAC6 in *Drosophila* can alleviate the functional damage of the UPS caused by polyglutamine-induced neurotoxicity. This protective process of HDAC6 depends on autophagy activation. In neurodegenerative diseases, many ubiquitinated pathogenic proteins are ubiquitinated with K63-linked ubiquitin chains. These K63-linked ubiquitinated proteins are often substrates of autophagy, but not degraded by the proteasomes. As a protein involved in ubiquitination, p62 plays an important role in mediating both autophagy and proteasomal degradation pathways.

The K48-linked polyubiquitin chains are abundant in cells. When the proteasome is inhibited, K48-linked ubiquitin chains increase rapidly, indicating that they are substrates of the proteasome. In ubiquitin chain recognition, the K48-linked polyubiquitin chains usually drive the substrate protein to be degraded by the proteasome, while the monoubiquitination and K63-linked polyubiquitin chains play more important roles in autophagy. Studies of the UBA domains on p62 and NBR1 indicate that these receptors preferentially bind to the K63-linked polyubiquitin chains. The binding capacity of the UBA domain of NBR1 to K63-linked two ubiquitins is approximately 60-fold stronger than that of monoubiquitin. The UBA domain of p62 has similar effect, preferably binding to the K63-linked polyubiquitin chains, but still has a certain affinity for the K48-linked polyubiquitin chains. Thus, it suggests that the substrate proteins labeled with the K63-linked polyubiquitin chains are more easily recognized by the autophagic receptors and degraded by autophagy.

Monomers and dimers of receptors containing ubiquitin-like (UbL) domains (either with or without AIM) in soluble states are effectively degraded by the proteasome, but are not degraded after aggregation. In contrast, oligomerization of AIM-containing receptor promotes autophagy-dependent degradation of intracellular aggregated proteins, but blocks the proteasomal degradation of soluble proteins. Therefore, the receptors binding to the proteasomes or AIM on ATG8 decide the degradation pathway after the substrate ubiquitination. The oligomerization of the

receptor or its binding to ATG8 is important for substrate selectively degraded by autophagy.

The UPS and autophagy system are closely related and interacted with each other. The UPS is directly involved in the regulation of autophagy, such as the promotion of autophagy through ubiquitination of autophagy substrates. In addition, the functional changes of UPS will affect the autophagy activity (Peng et al. 2017).

#### 12.1.2.2 Ubiquitin-Like System of ATG and Autophagy Activation

It is well known that in the process of ubiquitination, by steps, ubiquitin-activating enzyme E1, the ubiquitin-conjugating enzyme E2 and ubiquitin ligase E3 finally catalyzes the formation of an isopeptide bond between the lysine substrate and the glycine at the C terminus of ubiquitin to direct ubiquitin to the substrate.

In the autophagy activation, coupling with PE is a key step for Atg8 or LC3-II bound to phagophore. Interestingly, although Atg8 or LC3-II has no amino acid sequence or structure similar to ubiquitin, the core Atgs constitute a ubiquitin-like system, which can conjugate Atg8 or LC3-II to PE in a way similar to the activation and coupling of ubiquitin, to connect Atg8 or LC3 with phagophore. In this system, Atg8 or LC3-II is equivalent to a ubiquitin, so that they are also called ubiquitin-like proteins, while other Atg or Atg complexes act like E1, E2 and E3 enzymes in ubiquitination system.

Unlike the ubiquitination system, in autophagy activation, two parallel pathways mediate Atg8 or LC3-II to conjugation PE (Fig. 12.3). One consists of the ubiquitin-like protein Atg12, the E1-like protein Atg7, the E2-like protein Atg10 and the substrate-like Atg5. Through Atg7 and Atg10, the glycine of ubiquitin-like protein Atg12 can bind to the lysine of Atg5 via an isopeptide bond to form an Atg5–Atg12 complex, which can further bind to Atg16 to form a large complex with molecular weight close to 350 kDa. The Atg5–Atg12–Atg16 complex is thought to have E3-like activity and can conjugate Atg8 or LC3-II to PE. Although the Atg5–Atg12–Atg16 complex has E3-like activity on this pathway, there are no E3-like proteins involved in entire activation process. Another pathway consists of the ubiquitin-like protein Atg8 or LC3, the E1-like protein Atg7, the E2-like protein Atg3, the Atg5–Atg12–Atg16 complex and PE. The ubiquitin-like protein Atg8 or LC3 is first hydrolyzed by protease Atg4 to expose its C-terminal glycine, followed by the action of E1-like Atg7 and the action of E2-like Atg3, and finally the Atg8 or LC3 were coupled to PE by the E3-like Atg5–Atg12–Atg16 complex. Unlike the formation of a chain by ubiquitin and proteins (substrate or ubiquitin chains) catalyzed by the ubiquitination system, the Atg5–Atg12–Atg16 complex in the autophagy system ultimately catalyzes the coupling of proteins (Atg8 or LC3) and lipids (PE). Since PE is a component of membrane of phagophore, this system enables Atg8 or LC3 to bind to phagophore through PE, thus activating autophagy (Kaufmann et al. 2014).

Therefore, the autophagy activation system is similar to a system of ubiquitin activation. The components in autophagy have the functions similar to ubiquitin,

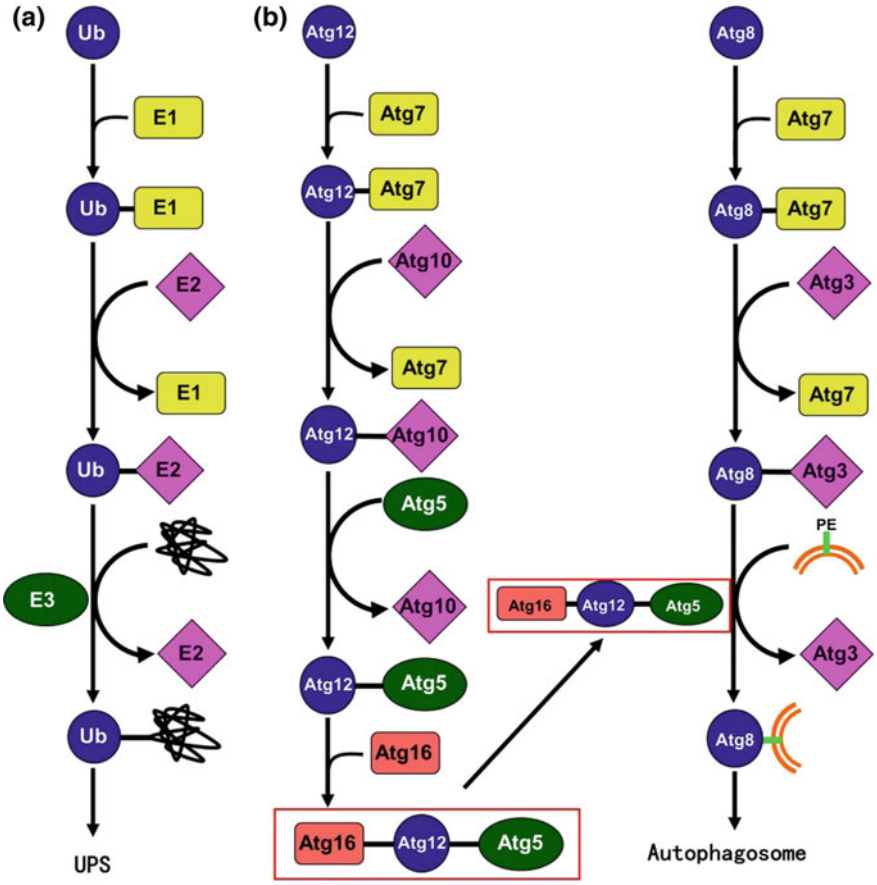


**Fig. 12.3** Ubiquitin-like system of ATG in autophagy. **a** In the process of ubiquitination, by steps, ubiquitin-activating enzyme E1, the ubiquitin-conjugating enzyme E2 and ubiquitin ligase E3 finally catalyzes the formation of an isopeptide bond between the lysine of the substrate and the glycine at the C-terminus of ubiquitin to direct ubiquitin to the substrate. **b** In autophagy, two parallel pathways mediate Atg8 or LC3-II to conjugation PE. One consists of the ubiquitin-like protein Atg12, the E1-like protein Atg7, the E2-like protein Atg10 and the substrate-like Atg5. The Atg5–Atg12–Atg16 complex is thought to have E3-like activity and can conjugate Atg8 or LC3-II to PE. Another pathway consists of the ubiquitin-like protein Atg8 or LC3, the E1-like protein Atg7, the E2-like protein Atg3, the Atg5–Atg12–Atg16 complex and PE. After multiple catalytic steps, the Atg8 or LC3 was finally coupled to PE by the E3-like Atg5–Atg12–Atg16 complex. Since PE is a component of membrane of phagophore, this system enables Atg8 or LC3 to bind to phagophore through PE, thus activating autophagy

ubiquitin enzyme and substrates that play a role in autophagy activation through a series of reactions.

## 12.2 Protein Phosphorylation and Autophagy

At the normal physiological level, autophagy is mainly responsible for removing aged or damaged organelles and proteins. Although the molecular mechanisms of autophagy are well studied, the cell signaling pathways, particularly how kinases regulate autophagy, remain to be explored. Many different kinases are involved in the regulation of different processes in autophagy.

Protein phosphorylation is a common posttranslational modification of proteins. It transfers a phosphate group at the γ position of ATP or GTP to a threonine, serine or tyrosine residue on substrate, which is catalyzed by a protein kinase. The phosphorylation causes a change of protein surface charge, leading to a conformation change, thereby influencing protein activity. Phosphorylation, as one of the important covalent modification in cells, plays an extremely important role in cell signaling, which is controlled by the synergistic action of protein kinase and phosphatase (Farre and Subramani 2016). Phosphorylation is closely related to the initiation and progression of autophagy.

### *12.2.1 Kinase Complex and Autophagy Regulation*

During autophagy process, monitoring of the internal and external environment of cells and rapid transduction of signals from various signaling pathways are required. The reversible phosphorylation of proteins can facilitate cells to adjust the functions. The process of autophagy undergoes following steps: initiation, recognition, selection, vesicle formation, autophagosome-lysosomal fusion and degradation, and finally the release of degraded products to cytosol. Many autophagy-related proteins such as Atg1, TOR (target of rapamycin), AMPK (AMP-dependent protein kinase) and PI3K (class III phosphatidylinositol-3 kinase) and autophagy-regulation proteins such as MAPK (mitogen-activated protein kinase) and PKC (protein kinase C) are protein kinases and participate in the regulation of the autophagy process by phosphorylating the substrates. Thus, protein kinases play an important role in autophagy regulation.

#### 12.2.1.1 ULK1 Complex

*Atg1* is the first identified autophagy-related gene that encodes a serine/threonine protein kinase, the only protein kinase in the yeast Atg protein family. Atg1 is essential for Cvt (cytoplasm-to-vacuole target) pathway in yeast. In the induction of autophagy

in yeast, Atg1 interacts with Atg13, Atg17, Atg29 and Atg31 to form a complex, which in turn recruits other Atg family proteins to the pre-autophagosomal structure (PAS). The binding of Atg1 to Atg17 is dynamic, and this process is regulated by Atg13 (Gatica et al. 2018).

The binding between Atg1 and Atg13 is regulated by the TOR signaling pathway. TOR, as a target protein of rapamycin, itself has serine/threonine protein kinase activity. In nutrient-rich condition, TORC1 (target of rapamycin complex 1) inhibits the interactions of Atg13 with Atg1 by phosphorylating several serine residues on Atg13, which inhibits the complex formation of Atg1–Atg13–Atg17 to decrease autophagy initiation. In contrast, TORC1 activity was inhibited under starvation conditions or the administration of rapamycin, thus leading to Atg13 dephosphorylation. Dephosphorylated Atg13 and Atg1 exhibit a high affinity to form the Atg1–Atg13–Atg17 complex, which activates the kinase activity of Atg1 to initiate the formation of phagophore, ultimately leading to the initiation of autophagy. In this process, four serine residues of the Atg13 protein are phosphorylated by TORC1, including S437, S438, S646 and S649. Thus, the phosphorylation state of Atg13 controls its binding to Atg1.

In *Drosophila*, activated Atg1 phosphorylates Atg13, Atg17 and itself. Autophosphorylation of Atg1 at T226 and S230 promotes autophagy initiation and the formation of the Atg1–Atg13–Atg17 complex, thereby recruiting other proteins for PAS formation.

In vertebrates, there are at least five Atg1 homologs, Unc-51-like kinases 1-4 (ULK1-4) and ATK36. Among them, ULK1 and ULK2 have significant functional features similar to Atg1, which a catalytic domain and a non-catalytic domain at the N terminus, a proline-rich serine-rich region and a C-terminal domain (CTD). Similar to Atg1 in yeast, ULK1 has kinase activity and mediates phosphorylation of Atg13 and FIP200. ULK1 and ULK2 bind to mammalian ATG13 via their conserved C-terminal domain to form the ULK–ATG13–FIP200–ATG101 complex. The function of ULK1 and ULK2 can compensate each other. In the process of non-selective autophagy, the loss of function of one kinase can be compensated by another kinase. However, the compensatory effect of ULK2 on ULK1 has cell-type specificities and depends on specific autophagy types.

The ULK–ATG13–FIP200–ATG101 complex plays an important role in the formation of autophagy isolation membranes, which is an early event in the initiation of autophagy. ULK1 and ULK2 are serine/threonine kinases in the complex whose kinase activity is critical for the recruitment of Vps34 to phagophore. Recruitment of Vps34 complex that contains Vps15, Beclin-1 and ATG14 to phagophore phosphorylates phosphoinositide (PtdIns, PI) to produce phosphoinositide 3-phosphate (PtdIns3P, PI3P). PI3P recruits phospholipid-binding proteins to initiate autophagy. In addition, the ULK complex is also extremely important for the recruitment of ATG16L–ATG12–ATG5 oligomers to phagophore. In nutrient-deficient condition, the formation of the ULK1 complex recruits the Atg5–Atg12–Atg16 complex to phagophore by a direct interaction between FIP200 and ATG16L. The Atg5–Atg12–Atg16 complex has E3-like activity and conjugates Atg8 or LC3-II to PE (Fig. 12.3) (Nazio and Cecconi 2017).

In addition, ULK1 plays a role in mitophagy. In ULK1 knockout mice, mitochondrial clearance during the maturation of red blood cells is delayed.

#### 12.2.1.2 mTOR Complex

mTOR is a serine/threonine kinase, a member of the phosphoinositide kinase-related kinase family. It was first identified as a substrate for rapamycin. As a sensor for the level of nutrition and energy, mTOR has crucial role in regulating the initiation of autophagy. mTOR can form two complexes, including rapamycin-sensitive mTORC1 (containing mTOR, Raptor, mLST8 and PRAS40) and rapamycin-insensitive mTORC2 (containing mTOR, Rictor, mLST8 and Sin1). These two complexes are different in protein composition and function. Downstream of mTORC1 mainly includes p70S6K (70 kDa 40S ribosomal protein S6 kinase) and 4E-BP1 (eukaryotic translation initiation factor 4E binding protein 1). Activated mTORC1 phosphorylates p70S6K and 4E-BP1 (phosphorylated 4E-BP1 abolishing the translation inhibition of eIF4E) and initiates protein synthesis. However, mTORC2 regulates autophagy mainly by regulating kinases such as Akt and PKC (protein kinase C) (Saxton and Sabatini 2017).

The kinase activity of mTORC1 depends on the nutritional status of the body, and it regulates autophagy by phosphorylating proteins required in the formation of phagophore. In yeast, TOR regulates the phosphorylation of Atg13, which affects the initiation of autophagy by controlling the affinity of Atg1 to Atg13. In mammals, the interaction between mTORC1 and the ULK–Atg13–FIP200 complex depends on a direct binding of the subunit Raptor in mTORC1 to ULK1/2 in the ULK–Atg13–FIP200 complex. In nutrition-rich status, activated mTOR inhibits the activity of ULK1/2 kinase by phosphorylating ULK1/2 and Atg13, thereby inhibiting the formation of phagophore, which is different from the process in yeast in which TOR only phosphorylates Atg13 (Fig. 12.4). There are 16 phosphorylation sites in ULK1, among which T180 is the self-phosphorylation site of ULK1. Upon starvation or rapamycin administration, mTORC1 dissociates from the ULK1/2–Atg13–FIP200 complex, leading to dephosphorylation of ULK1/2 and Atg13 at the sites that are phosphorylated by mTOR. The phosphorylation level of ULK1 at S638 and S758 can be reduced by more than ten times. Dephosphorylation of ULK1/2 activates its autophosphorylation, which leads to the ULK1/2–Atg13–FIP200 complex moving to PAS to activate autophagy. Dephosphorylated Atg13 binds to ULK1 via a FIP200-independent pathway, but binds to ULK2 via a FIP200-dependent pathway, respectively. This process contributes to the stabilization of ULK1/2 and activates its kinase activity to phosphorylate Atg13 and FIP200 (Fig. 12.4).

In addition, ULK1 promotes phosphorylation of Raptor at S69, T706, S792, S855, S859, S863 and S877 in vivo. The phosphorylation of Raptor inhibits the activity of mTORC1 by affecting the binding ability of the Raptor to the substrates. Thus, ULK1-mediated phosphorylation of Raptor is thought to be a feedback regulation of the mTOR activity.

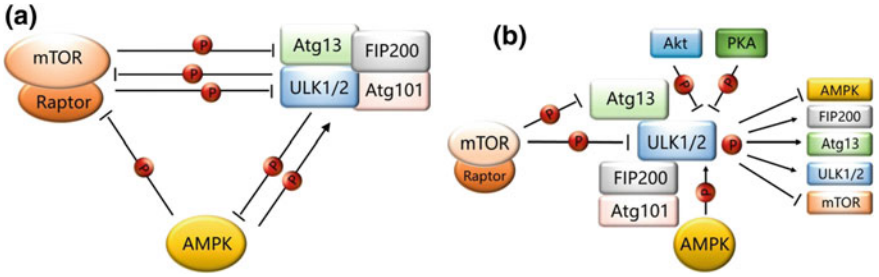


**Fig. 12.4** ULK1/2–Atg13–FIP200–Atg101 complex. **a** ULK complex, mTORC1 and AMPK form a loop to regulate autophagy. **b** Diagram displays the phosphorylation of ULK complex, mTORC1 and AMPK. The activities of ULK1/2–Atg13–FIP200–Atg101 complex and mTORC are controlled by each other. AMPK and ULK are also regulated through their interactions. The ULK complex is important for autophagy regulation as it received multiple upstream kinase signals and transfers to downstream targets by phosphorylating its substrates

mTORC2 negatively regulates autophagy via Akt. mTORC2 activates Akt by phosphorylating Akt at S473, leading to phosphorylation and inactivation of FoxO3 (forkhead box protein O3). Phosphorylation of FoxO3 by Akt results in an exposure of two 14-3-3 protein binding sites, leading to FoxO3 binding to 14-3-3 protein. The interactions between FoxO3 and 14-3-3 protein cause a conformational change of FoxO3, which leads to an exposure of the NES (nuclear export signal), transporting FoxO3 out of the nucleus by the nuclear export protein. In addition, the binding of FoxO3 to 14-3-3 protein blocks the NLS region of the FoxO3, causing its retention in the cytoplasm and loss of transcription activity, which decreases the expression of autophagy-related genes, such as *LC3* and *Bnip3*.

The activity of mTORC1 depends on its localization to Rab7- and LAMP2 (lysosomal-associated membrane protein 2)-positive vesicles. Therefore, lysosomes play a key role in regulating mTORC1 activity. The recruitment of mTORC1 to lysosomes and retention of mTORC1 on lysosomes are conducted by a small GTPases namely Rag GTPase, a regulator complex that is located on late endosomes/lysosomes, MAPK (mitogen-activated protein kinase) and mTOR activator factor 1. Rag GTPase is an atypical member of the small GTPase Ras superfamily. It stably anchors to the lysosomal membrane by directly binding to Raptor, which is a protein in the regulator complex resident on the lysosome. Raptor binds to mTOR, targeting mTORC1 to lysosomes and activating them. Mammals have four Rag proteins that form heterodimers: Rag A or Rag B (highly homologous) and Rag C or Rag D (highly similar in protein sequences). The formation of the heterodimeric complex Rag A/B-Rag C/D enhances its stability and induces activation of mTORC1. The binding of mTORC1 to Rag GTPases is highly dependent on the binding state of guanine nucleotides in the heterodimers. The activated conformation of heterodimer that is composed of Rag A/B·GTP and Rag C/D·GDP can bind to Raptor and pull mTORC1 to the lysosomal surface through Raptor. Enrichment of amino acids and glucose promotes the accumulation of Rag A/B·GTP-Rag C/D·GDP. However, in the absence of amino acids, Rag A/B·GTP in the Rag heterodimer is converted to

Rag A/B·GDP, which is an inactivated conformation, leading to a detachment of Raptor and mTORC1 from lysosomes. The detachment of mTORC1 from lysosomes leads to a decrease of its activity, which promotes the formation of aPAS for autophagy initiation by the ATG1/ULK1 complex. In addition, the decreased activity of mTORC1 reduces the phosphorylation of its downstream substrate TFEB, induces TFEB nuclear translocation, and transactivates the expressions of the autophagy-lysosomal gene (Munson and Ganley 2015).

#### 12.2.1.3 PI3K Class III Complex

Phosphoinositide 3-kinase (PI3K) is a member of the phospholipase kinase family. It has phosphoinositide kinase activity and serine/threonine kinase activity, and can phosphorylate phosphoinositide to generate phosphoinositide 3-phosphate. PI3KC3 involves multiple steps of autophagy and is an important protein-regulating autophagy (Hurley and Young 2017; Stjepanovic et al. 2017). In yeast, the only PI3K is Vps34 that functions in autophagy and vacuolar protein sorting (VPS). Vps34 forms a stable complex with the myristoylated serine/threonine protein kinase Vps15 on the membrane, and its kinase activity is regulated by Vps15. Cyclin-dependent kinase 1 (Cdk1) and Cdk5 can phosphorylate Vps34 at T159, thereby affecting the binding of Vps34 to Beclin-1, leading to a decrease of Vps34 activity. In addition, Cdk5 also phosphorylates Vps34 at T668. T668 is located in the catalytic domain of Vps34, which influences Vps34 kinase activity. Therefore, the phosphorylation of these two sites on Vps34 regulates their kinase activity, controls the production of PI3P and ultimately affects the formation of autophagosomes. Cdk1 plays an important role in the process of mitosis, which may explain why autophagy is strictly controlled by mitosis. In addition, Cdk5 is an important kinase in central nervous system and has been shown to play a role in Alzheimer's disease. Abnormal activation of CDK5 may cause neurodegeneration through negative regulation of autophagy (Fig. 12.5).

Protein kinase D (PKD) also phosphorylates Vps34 at multiple sites, including the T668 in its enzymatic active catalytic domain. This phosphorylation increases Vps34 activity, leading to the production of PI3P and the formation of autophagosomes.

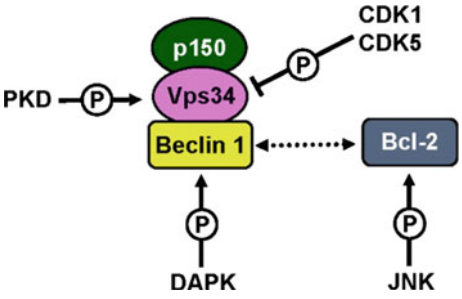


**Fig. 12.5** Diagram for the regulation of PI3K class III complex. PI3KC3 kinase activity is regulated by PKD, DAPK, CDK1 and CDK5. The interactions of Beclin-1 or Bcl-2 with Beclin-1 in PI3KC3 is regulated by their phosphorylation status

In mammals, PI3KC3 is a homologous protein of yeast Vps34 and is closely related to the formation of autophagosomes. It promotes the formation of autophagosomes by phosphorylating PI3 to produce PI3P and recruits proteins containing FYVE or PX sequences in the cytosol. PI3KC3 can also form a complex with Beclin-1 to participate in the formation of autophagosomes. The mammalian PI3K class III complex contains hvps34, hvps15 (P150) and Beclin-1 (Atg6), forming three major subcomplexes including Beclin-1–Atg14L–PI3K III, Beclin-1–UVRAG–Rubicon–PI3K III and Beclin-1–UVRAG–PI3K III.

Atg14L (also known as Barkor) is a homologous of yeast Atg14 in mammals, which can interact with both Beclin-1 and hVps34. The Vps34–Vps15–Beclin-1 complex containing Atg14L can induce the formation of bilayer membrane structure, which is essential for the formation of early autophagosomes (Pyo et al. 2018).

## *12.2.2 Key Regulators of Autophagy and Phosphorylation*

### 12.2.2.1 Beclin-1

Beclin-1 is a mammalian homolog of the yeast Atg6. It is a tumor-suppressor gene, and its heterozygous deletion can cause malignant transformation of cells. Beclin-1 interacts with proteins including Bcl-2 family proteins, Ambra1, nPIST, VMP1, Rab5, FYVE-CENT, estrogen receptor, MyD88/TRIF, SLAM, Survivin, PINK1 and HMGB1. Interaction between Beclin-1 and antiapoptotic members of the Bcl-2 family, such as Bcl-2 and Bcl-xl, is an important determining factor for autophagy and apoptosis. Bcl-2 protein regulates autophagy in two ways, one by direct interaction with Beclin-1; and another, by indirect regulation through Ambra1.

Beclin-1 is involved in the formation of two important PI3K class III complexes: PI3K class III complex I and PI3K class III complex II. Beclin-1 binds to PIK3C3/Vps34 and the regulatory subunit Vps15/PIK3R4 to form a highly regulated complex, promoting the production of PI3P. PI3K class III complex I consisting of Vps34, Beclin-1, Atg14 and membrane-anchored Vps15 is a complex required for the local production of PI3P that promotes autophagy initiation. Together with ULK1 complex, PI3K class III complex I induces the formation of PAS. The UVRAG (UV-radiation resistance-related gene) can compete with Atg14 on binding to Beclin-1, forming PI3K class III complex II that is composed of UVRAG, Beclin-1, Vps34 and Vps15. PI3K class III complex II mainly acts on vesicle sorting, transport of autophagosomes and autophagosome fusion with lysosomes, but does not affect autophagy initiation.

The interactions between the Bcl-2 family proteins and Beclin-1 may be transient, allowing a flexible and dynamic regulation of autophagy induction. Phosphorylation of either Bcl-2 family proteins or Beclin-1 can block their interactions. Early studies showed that DAPK can phosphorylate Beclin-1 at T119, a very important site in the BH3 domain of Beclin-1, which promotes Bcl-xl dissociation from Beclin-1 and induces autophagy. The c-Jun N-terminal kinase 1 (JNK1) phosphorylates Bcl-2 at

T69, S70 and S87 sites under the stimulation of cell starvation. Phosphorylation of these sites results in the release of Beclin-1 from the Bcl-2 and induces autophagy activation (Fig. 12.5).

Phosphorylation of Beclin-1 at S15 by ULK1 activates the PI3K class III complex I to produce PI3P, thereby initiating autophagy. ULK1/2-mediated phosphorylation of Beclin-1 at S15 also targets the PI3K class III complex II containing UVRAG, which promotes autophagosome maturation. Interestingly, phosphorylation of Beclin-1 at S15 also promotes the interactions between Beclin-1 and E3 ligase parkin, which induces parkin translocation to mitochondria, and promotes mitophagy. In addition to S15, ULK1/2 phosphorylates Beclin-1 at S30 to positively regulate the formation of autophagosomes.

The cellular energy sensor AMPK also directly phosphorylates Beclin-1 to participate in autophagy regulation. AMPK-mediated phosphorylation of Beclin-1 at S93 and S96 is required for the activation of Vps34–BECN1–ATG14 complexes under glucose starvation. T388 of Beclin-1 is another site for AMPK phosphorylation. Phosphorylation of T388 not only enhances the binding of Beclin-1 to the Vps34–ATG14–Vps15 complex, but also reduces the formation of the Beclin-1–Bcl-2 complex, thereby promoting autophagy initiation.

In addition to the serine/threonine kinase, tyrosine phosphorylation of Beclin-1 inhibits its role in autophagy. FAK/PTK2 (focal adhesion kinase/protein tyrosine kinase-2)-mediated phosphorylation of Beclin-1 at Y233 inhibits Beclin-1 interaction with ATG14 and inhibits autophagy. Activation of epidermal growth factor receptor induces the phosphorylation of Beclin-1 at Y229, Y233 and Y352, which inhibits autophagy and enhances tumorigenesis in a non-small-cell lung cancer transplantation model (Menon and Dhamija 2018).

#### 12.2.2.2 TFEB

The transcription factor EB belongs to the micropthalmia-transcription factor E (MiT-TFE) family, which includes four members: MITF, TFEB, TFE3 and TFEC. All of them belong to the helix–loop–helix (HLH) leucine zipper transcription factors, with high sequence similarity. Similar to other HLH family proteins, this family protein binds to DNA palindrome sequences (CACGTG) by homo or heterodimers, which are known as targeting E-box to activate transcription. A slight difference is that the MiT-TFE family proteins recognize more than the GTCACGTGAC sequence, that is, the CLEAR motif. TFEB and MiT-TFE family proteins can transactivate many genes encoding autophagosomal and lysosomal proteins, promoting the production of autophagosomes and lysosomes.

The localization of TFEB in cell is largely dependent on phosphorylation of specific serine residues. mTOR kinase phosphorylates specific serine residues of TFEB, which plays a major role in the regulation of TFEB subcellular localization. mTOR-mediated phosphorylation of TFEB is nutritionally dependent, suggesting that mTOR complex mediates phosphorylation of TFEB. Three serine sites (S122, S142 and S211) of TFEB can be phosphorylated by the mTOR complex. A conversion

of S142 or S211 to alanine (S142A, S211A) in TFEB leads to form a constitutive nuclear localization TFEB, showing a cellular effect similar to mTOR inhibitor-treated cells. In vitro kinase phosphorylation experiments demonstrate that S122 can be directly phosphorylated by mTORC1. In cells, phosphorylation of S211 induces the binding of TFEB to 14-3-3 protein, which allows TFEB to be retained to the cytosol by 14-3-3 protein, thereby inhibiting TFEB nuclear translocation. Phosphorylation of serine S142 and S211 also promotes the binding of the E3 ubiquitin ligase STUB1 to TFEB, thereby inducing a degradation of TFEB by the proteasome. Therefore, the phosphorylation of TFEB by mTOR complex not only affects its subcellular localization but also regulates its stability, thereby regulating TFEB function.

TFEB is involved in the regulation of lysosomal and autophagosomal biogenesis, gene transcriptions of lysosomal hydrolase proteins, lysosomal membrane proteins and autophagy proteins, which responds to lysosomal stress and senses cellular nutrient conditions. Phosphorylation-regulated TFEB nuclear translocation plays an important role in its activation. Inhibition of mTOR activity reduces TFEB phosphorylation and facilitates TFEB translocation into the nucleus, effectively activating gene transcriptions for lysosomal proteins and autophagy-related proteins. Dephosphorylation of TFEB by calcineurin and protein phosphatase 2 (PP2A) is also involved in the activation of TFEB. Activated TFEB promotes autophagy-lysosomal gene transcriptions, and regulates lysosomal biosynthesis, autophagy, lysosomal hydrolysis activity and lysosomal localization (Xia et al. 2016).

#### 12.2.2.3 AMPK

AMP-regulated protein kinase (AMPK) is a classical energy sensor that is activated at low energy levels. AMPK is a heterotrimeric kinase composed of α, β and γ subunits. In addition, each subunit has multiple subtypes, such as α-1-2, β-1-2 and γ-1-3. AMPK kinase activity depends on phosphorylation of α-subunit at T172, and adjusting the β- and γ-subunits. The γ-subunit binds to ATP, ADP and AMP to sense the energy state in cells.

The β-subunit of AMPK contains a domain that binds to carbohydrates, the exact function of which is unknown. It is speculated that this domain contributes to the subcellular localization of AMPK or helps the kinase localize to glycogen-associated substrates. In mammalian cells, AMPK-dependent autophagy is affected by inhibition of mTOR. AMPK regulates mTOR activity by direct phosphorylation of the upstream regulators tuberous sclerosis complex 2 (TSC2) and the mTORC1 subunit Raptor. Under starvation condition, AMPK senses the decrease of intracellular ATP/AMP ratio and activates TSC2 by phosphorylating it directly, which inhibits mTORC1, leading to upregulation of autophagy. In addition, AMPK also directly phosphorylates the mTORC1 subunit Raptor at S722 and S792, thus induces a binding of Raptor to 14-3-3 protein, thereby inhibiting mTORC1 and activating autophagy.

Recent studies have shown that AMPK can bind to and phosphorylate ULK1, including the S758, S317, S778, S467, S556, T575 and S638 sites. In addition, phosphorylation of ULK1 at S758 by mTORC1 will affect the binding of AMPK to ULK1.

Furthermore, all three subunits of AMPK can be phosphorylated by ULK1 and ULK2, so the activity of AMPK is negatively regulated by ULK1 and ULK2. The phosphorylation sites of AMPK α1-subunit include S360/T368, S397 and S486/T488; phosphorylation sites of β2-subunit include S38, T39, S68 and S173; phosphorylation sites of γ1-subunit include S260/T262 and S269. Thus, ULK1-mediated phosphorylation of the AMPK subunits indicates that this is a regulatory feedback pathway and is involved in the negative regulation of autophagy in cells (Russell et al. 2014).

#### 12.2.2.4 Other Kinases

Under nutrient-rich conditions, the yeast membrane protein Atg11 is a specific factor in the cytoplasm-to-vacuole target (Cvt) pathway. Atg9 binds to Atg11 and mediates its localization to PAS. During autophagy, Atg9 localization to PAS is independent on Atg11 but requires physical interactions with Atg17 and the presence of Atg1. Although Atg1 kinase activity is not essential for this process, the kinase activity of Atg1 is required to regulate the balance of assembly and dissociation of Atg9 on PAS. In mammals, with starvation induction, mAtg9 requires ULK1 and Atg13 to transport from the Golgi trans-tubular network (TGN) to autophagosomes.

There are eight Atg8 homologous genes in mammals, in which LC3 is well studied. The C-terminal G120 site was cleaved within 6 min after LC3 protein synthesis to generate a cytosolic LC3-I with a molecular weight of 18 kDa. LC3-I is then converted to LC3-II by binding to PE. LC3-PE is localized on the membrane and can be detected throughout the autophagy process. LC3 is phosphorylated at S12 by PKA. In autophagy induction, the S12 site exhibits a dephosphorylation status. It is therefore stated that the phosphorylation of LC3 at S12 regulates the integrating process of LC3 onto autophagosomes, however, the yeast LC3 homolog, Atg8, has no PKA phosphorylation site, which suggests that it is a mammalian-specific site. In addition, the T6 and T29 of LC3 are also phosphorylated by PKC, but no significant effects on autophagy.

LC3 not only participates in the extension of phagophore but also in the recognition of target proteins. Casein kinase 2 (CK2) directly phosphorylates p62 at S403, resulting in an increase in the affinity of the ubiquitin-binding domain of p62 to the ubiquitin chain. In addition, it has been found that the antibacterial TANK-binding kinase 1 (TBK1) plays an important role in the Salmonella autophagy (autophagy regulation of intracellular pathogens). This process depends on the direct binding of the autophagy receptors OPTN to TBK1, which promotes the binding of OPTN to LC3. During this process, TBK1-induced phosphorylation of OPTN enhances the affinity of OPTN to LC3, thereby promoting autophagic clearance of Salmonella.

### 12.2.3 Other Protein Kinases and Protein Phosphatases

cAMP-dependent protein kinase A (PKA) is an important regulatory kinase for cAMP. It inhibits autophagy by phosphorylating Atg1 and Atg13 in yeast. For Atg1, the PKA-dependent phosphorylation sites are S508 and S515; for Atg13, they are S344, S437 and S581. In mammalian cells, PKA directly phosphorylates ULK1 at S1043, resulting in inactivation of the ULK1.

Akt is the major downstream signaling molecule of PI3K and is also a serine/threonine kinase. Akt is phosphorylated at T308 by phosphatidylinositol-dependent kinase-1 (PDK1), and can also be phosphorylated at S473 by mTORC2. Phosphorylation of these sites activates Akt. The activated Akt inhibits mTORC1 activity by phosphorylation of TSC2 at S939 and T1662, to induce autophagy. In addition, Akt also phosphorylates ULK1 at S774 to regulate autophagy.

Mitogen-activated protein kinases (MAPKs) are a family of serine/threonine kinases that respond to a variety of extracellular stimuli. Once MAPKs are activated, the downstream phosphorylation-dependent kinase can be activated by a cascade reaction. MAPK members include ERK, p38 and JNK. An ERK inhibitor PD98059 can inhibit phosphorylation of MAPK-dependent GAIP protein, thereby inhibiting starvation-induced autophagy. In addition, activation of ERK can also result in inhibition of mTORC1 or mTORC2, thereby increasing the expression of Beclin-1 and ultimately leading to autophagy activation. Overexpression of ERK2 in glioblastoma not only induces autophagy but also induces mitophagy. JNK can promote stability and activate p53 by phosphorylating p53, thereby promoting autophagy by upregulation of autophagy-related genes such as AMPK and Bnip3. In addition, starvation stimulation promotes dissociation of Beclin-1 and Bcl-2 and induces autophagy activation through phosphorylation of Bcl-2 at T69, S70 and S87 by JNK1 (Barutcu et al. 2018).

In the regulation of autophagy, phosphatases also play important roles. Protein phosphatase dephosphorylates the phosphorylated protein and negatively regulates signal transduction. Inhibition of serine/threonine protein phosphatase in rat hepatocytes results in severe inhibition of autophagy. Protein phosphatase (PP2A) and PP1A inhibitor okadaic acid can effectively inhibit autophagy at very low doses, demonstrating that PP2A and PP1 play an important role in maintaining autophagy. PP2A can also promote the dephosphorylation of Gln3, resulting in translocation of Gln3 to the nucleus, which induces transcriptional expressions of various autophagy-related genes, such as Atg8 and Atg14. In yeast, TORC1 inhibits the activity of PP2A by phosphorylation of Tap42, thereby inhibition of autophagy. Other protein phosphatases also play an important role in autophagy regulation, such as phosphatase PP2C that dephosphorylates AMPK.

Protein phosphorylation is closely related to all the steps in autophagy. The kinases, functioning in combination with protein phosphatases, regulate the entire process of autophagy.

## 12.3 Conclusions

Protein modification is involved in the whole process of autophagy, including the initiation, activation and regulation of autophagy, as well as the regulation of recognition and degradation of autophagic substrates by the autophagy system. Many of these processes are closely related to diseases. Studies on the relationship between protein modification and autophagy are of help for uncovering the mechanism of autophagy and for understanding its association with diseases and the discoveries of therapeutic targets.

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# Chapter 13
# Other Molecular Mechanisms Regulating Autophagy


**Nan Zhang and Ying Zhao**

**Abstract** Autophagy is a catabolic process in eukaryotic cells that delivers cytoplasmic components and organelles to the lysosomes for digestion. It is thought that various environmental signaling pathways are somehow integrated with autophagy signaling, such as the lack of nutrients (amino acids or glucose or others), changes in pH or osmotic pressure. Autophagy has been seen as an adaptive response to stress. In the extreme cases of starvation, the breakdown of cellular components promotes survival by maintaining cellular energy levels. A series of studies have found that the signaling pathway regulating autophagy is very complex. In addition to 40 autophagy-related genes (ATG) involved in the formation of autophagosomes, there are many other transcription factors that participated in the regulation of autophagy. This chapter focuses on the role of FoxO, NFκB, E2F and TFEB in autophagy.

**Keywords** Autophagy · FoxO · NFκB · E2F · TFEB

## 13.1 Regulation of Autophagy by FoxO Family Members

### *13.1.1 FoxO Transcription Factors*

The FoxO family proteins are widely expressed in heart, vascular endothelium, adipocyte, liver, brain, skeletal muscle and other organs and tissues. Members of the mammalian FoxO family, FoxO1, FoxO3, FoxO4 and FoxO6, have emerged as important targets for many kinds of diseases, as they can modulate processes associated with angiogenesis, stem cell proliferation, cardiovascular injury, tumorigenesis and vascular cell longevity. Molecules of FoxO proteins consist of several domains: a highly conserved forkhead DNA binding domain (DBD), a nuclear localization signal (NLS) located just downstream of DBD, a nuclear export sequence (NES) and a C-terminal transactivation domain. Post-transcriptional modification sites such as

N. Zhang · Y. Zhao (✉)
Department of Biochemistry and Molecular Biology, School of Basic Medical Sciences, Peking University Health Science Center, Beijing 100191, China
e-mail: zhaoying0812@bjmu.edu.cn



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acetylation sites and phosphorylation sites in FoxO have important effects on its DNA binding activity and subcellular localization.

FoxO transcription factors function mostly as transcriptional activators, and their activities can be inhibited by insulin and growth factor signaling. In the presence of insulin and insulin-like growth factor (IGF), the PI3K-Akt signaling pathway is activated and kinases such as Akt and SGK (serum and glucocorticoid-induced kinase) directly phosphorylate FoxO factors at three conserved residues, resulting in FoxO binding to cytoplasmic protein 14-3-3 and repression of transcriptional activity. In the absence of insulin or growth factor signal, or during starvation, FoxO factors translocate to the nucleus where they activate expression of some stress response or metabolic genes. They can also be phosphorylated at diverse residues by a number of different stress-responsive kinases, including AMPK, JNK, MST1, as well as ERK and p38 MAPK.

In addition to being phosphorylated, FoxO family members can also be acetylated/deacetylated, ubiquitinated and methylated on arginine and lysine in response to oxidative stress or changes in nutrient status (Eijkelenboom and Burgering 2013). The activity of FoxO proteins can also be affected by its ubiquitination or acetylation. For example, lysine methyltransferase SETD7 (SET domain containing 7) methylates FoxO3 at K270, leading to the inhibition of its DNA-binding activity. An oncogenic subunit of the SKP1-cullin1-F-box (SCF) E3 ubiquitin ligase complex, SKP2, ubiquitinates FoxO and promotes its degradation. FoxO proteins can also be acetylated by histone acetyltransferase such as p300, CREB binding protein (CBP) and CBP-related factors. Acetylated FoxO proteins may translocate to the cell nucleus but have diminished activity, since acetylation of lysine residues on FoxO proteins has been shown to limit the ability of FoxO proteins to bind to DNA. Besides, the NAD-dependent deacetylase sirtuin-1 (SIRT1) can also affect cell survival by regulating FoxO activity.

FoxO family members regulate autophagy through a variety of mechanisms in different cell types, and they usually activate autophagy due to the family's transcriptional activity.

### *13.1.2 The Role of FoxO3 in Regulating Autophagy*

Function of FoxO in autophagy was first proposed in a mouse muscle atrophy model. Neurological diseases, nutrition disorders, accelerated protein degradation and other pathological conditions lead to muscle atrophy. As a major regulator of muscle atrophy, FoxO3 activates protein degradation by inducing autophagy in muscle cells (Sanchez et al. 2014). FoxO3 is translocated into the nucleus in response to starvation, and then induces expression of a number of autophagy genes involved in various stages of the process, including *LC3b*, *Gabarapl1*, *PI3kIII*, *Ulk2*, *Atg12 l*, *Beclin1*, *Atg4b* and *Bnip3* to promote autophagy. Among them, gain and loss of function in experiments identified Bnip3, a BH3-only mitochondrial protein, as a key player downstream of FoxO3 in muscle. It is important that FoxO3 binds to Bnip3 promoter

and directly activates *Bnip3* gene expression. Bnip3 activates autophagy by promoting autophagosome formation and LC3 lipidation. Some other proteins in skeletal muscle can also regulate autophagy through FoxO3. For example, SIRT1 can inhibit muscle cell atrophy and stimulate muscle growth through inhibition of autophagy by blocking the activity of FoxO3.

In addition to regulating skeletal muscle atrophy, FoxO3 has also been shown to play a role in cardiomyocyte autophagy (Ferdous et al. 2010). Under starvation conditions, endogenous FoxO3 localizes to the nucleus and directly binds to the promoter of *Gabarapl1* and *Atg12*, promotes autophagosome formation and reduces cardiomyocyte cell size. Besides, FoxO3 can directly regulate autophagy by inducing myocyte-specific ubiquitin ligases atrogin-1 and MuRF1. Moreover, FoxO proteins are selectively expressed in nervous system and have diverse biological functions. For example, FoxO3 may be involved in a number of physiological and pathological processes, including cerebral endothelial vascular cell survival, mouse cerebellar granule neurons injury by oxidative stress, neonatal hypoxic-ischemic and hippocampal neuronal injury. FoxO3 is important for the control of autophagy in neuronal cells in Parkinson's disease (PD) patients (Santo and Paik 2018). High level of α-synuclein is an important risk factor for Parkinson's disease. In human dopaminergic neurons model based on overexpression of α-synuclein, mild FoxO3 activity protects nigral neurons against the accumulation of α-synuclein through promoting autophagosome formation, and then prevents death of substantia nigral neurons. In Huntington's disease (HD) cell model and in the brain tissue of HD patients, increased expression and nuclear localization of FoxO3 have also been observed but the functional meaning remains unclear.

Autophagy has dual roles in cancer. It inhibits cell carcinogenesis by preventing the accumulation of toxic or carcinogenic damaged proteins and organelles, but also supports tumor growth under low nutrient conditions. FoxO has been suggested to function as a tumor suppressor and its activity is associated with a reduced risk of cancer (Chiacchiera and Simone 2010). Several studies have shown that FoxO3 may play an important anti-tumor role. FoxO3 overexpression inhibits tumor growth in vitro and tumor size in vivo in breast cancer, and the cytoplasmic location of FoxO3 correlates with low survival rate in patients with breast cancer. Invasive human breast cancer usually exhibits high expression of interferon regulatory factor-4 binding protein (IBP), which directly activates mTORC2 and upregulates phosphorylation of Akt S473 and FoxO3 T32, and leads to the translocation of FoxO3 from the nucleus to the cytoplasm, thereby inhibiting the transcription of autophagy-related genes, downregulating autophagy levels, and promoting breast cancer proliferation. In colorectal and ovarian cancer cells, FoxO3-dependent transcriptional program is activated in response to decreased glycolysis caused by inhibition of the p38α/HIF1α pathway, first leading to autophagy and cell cycle arrest as an attempt to retain energy and increase ATP levels, but then leading to autophagic cell death in conditions of persistent stress. Studies in leukemia, prostate cancer and glioblastoma also highlight the importance of FoxO3's anti-tumor activity. In these cells, FoxO3 induces transcription of autophagy-related genes *Atg7*, *Atg12*, *Beclin1*, *Gabarap* and *Gabarap2*, promotes the level of autophagy and inhibits tumor cell growth.

### *13.1.3 The Role of FoxO1 in Regulating Autophagy*

FoxO1 is involved in the regulation of autophagy in myocardium. Similar to FoxO3, FoxO1 also regulates cardiomyocyte cell size by affecting autophagy (van der Vos and Coffer 2011). Cellular stress, such as starvation or ischemia-reperfusion, leads to decreased Akt phosphorylation and increased SIRT1 protein levels. They both control the shuttling of FoxO1 into nucleus, leading to increased transcriptional activity and expression levels of *LC3* and *Gabarapl1*, ultimately resulting in upregulation of autophagy and reducing cardiomyocyte cell size. In addition, in cardiomyocytes, FoxO1 deacetylation by SIRT1 induces the expression of RAS-associated GTP-binding protein Rab7A, which mediates the fusion of mature autophagic vacuoles and lysosomes.

FoxO1 has also been found to drive apoptosis and autophagy for modulating neural cell survival, and affecting neurodegenerative diseases. The c-Jun N-terminal kinase (JNK) signal transduction pathway is implicated in the regulation of neuronal function, and JNK inhibits autophagy in neurons by a FoxO1-dependent manner. In JNK-deficient neurons, FoxO1 is phosphorylated at serine 246, localized in the nucleus and transcriptionally activates Bnip3 and Atg12. In normal neurons, Beclin1 binds to the Bcl-X complex, whereas in JNK-deficient neurons, this interaction is significantly attenuated. FoxO1-induced expression of Bnip3 that displaces the autophagic effector Beclin1 from inactive Bcl-X complexes also increases the expression of the autophagy-related genes *LC3b* and *Atg12*. In conclusion, JNK-deficient neurons are dependent on increased autophagy mediated by FoxO1/Bnip3/Beclin1 pathway for survival. In addition, FoxO1 interacts with Trib3 to regulate neuronal survival. Amyloid beta (Aβ) is produced by the proteolytic cleavage of the transmembrane protein amyloid precursor protein by enzyme complexes α, β and γ-secretases, and the accumulation of Aβ induces neuronal loss and cognitive impairments. Aβ upregulates the expression of *Trib3* gene in neurons, which directly activates Ulk1 to induce autophagy, and also activates FoxO1 by inhibiting Akt activity. At the same time, activated FoxO1 further enhances *Trib3* gene expression by binding to its promoter. In Aβ treated neuronal cells, the interaction between Trib3, Akt and FoxO1 can induce apoptosis and autophagy, ultimately leading to neuronal death. As FoxO activity is lost during aging, a decrease in autophagy may result in neuronal dysfunction and Aβ production. However, during development, inhibition of FoxO activity to block autophagy may be a necessary condition for cell survival. In *Drosophila*, the loss of FoxO leads to inhibition of autophagy and long-term survival of neuroblasts and neurogenesis in centers responsible for learning and memory. Besides, FoxO proteins have protective effects on neurons in some neurological diseases (Maiese 2016). In transgenic mice expressing mutant Huntington's protein (mHtt), XBP1 knockdown results in the accumulation of FoxO1 in nucleus. Enhanced autophagy helps facilitate the elimination of toxic mHtt protein, and then delay disease progression. In cell experiments, overexpression of wild-type FoxO1 also increases autophagy levels and promotes mHtt clearance.

FoxO1 also mediates starvation-induced autophagy through a transcription-independent pathway in cancer (Fig. 13.1). In FoxO1 low-expressing lung cancer cell H1299, it was found that cytoplasmic FoxO1 is involved in autophagy by over-expressing the FoxO1 plasmids with specific fragments deletion, and this process is independent of gene transcription. Under serum starvation or oxidative stress, acetylation of cytoplasmic FoxO1 is induced by dissociation from deacetylase SIRT2. Acetylated FoxO1 binds to and activates Atg7 to enhance autophagy. In addition, FoxO1 interacts with XBP1u to regulate autophagy. Phosphorylation of XB1u by ERK is required for its binding to FoxO1, which localizes FoxO1 to the 20S proteasome for degradation. Therefore, autophagy is inhibited. An inverse correlation between XBP1u and FoxO1 expression has been found in 229 cases of human colorectal cancer tissues. The relationship between XBP1u and FoxO1 expression is also highly correlated with p62 status in these cancer tissues. The expression level of FoxO1 is elevated in normal tissues and significantly decreased in colon cancer, which is negatively correlated with p62 expression level, indicating that FoxO1 may inhibit tumor growth by autophagy. Therefore, FoxO1 in cytoplasm may play a key role in connecting autophagy and tumor suppression.

The ability of FoxO transcription factors to induce autophagy has also been demonstrated in *Drosophila*. Myoblast-specific overexpression of dFoxO (*Drosophila* FoxO) induces autophagy by activating the transcription of 4E-BP, an important regulator of overall translation levels in cells, which delays the accumulation of protein aggregates and mitigates the loss of muscle functionality during aging.

In addition to the above-mentioned mechanisms, FoxO family proteins are involved in autophagy regulation of different cell types, such as hepatocytes, renal

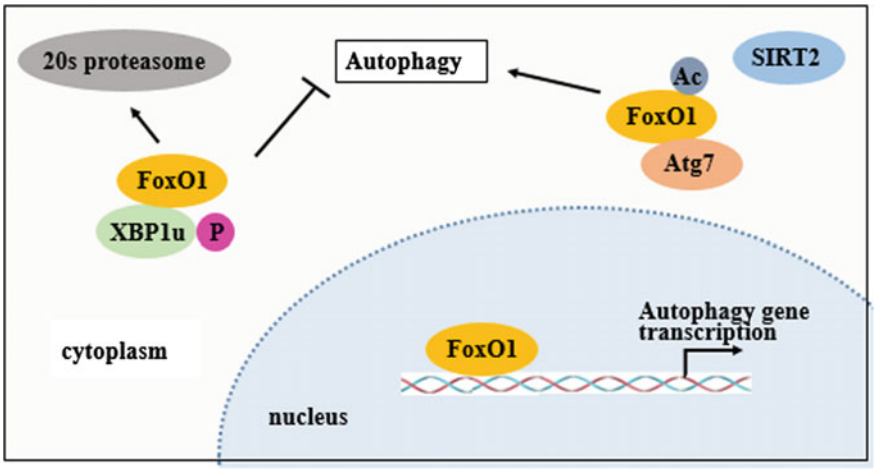


**Fig. 13.1** FoxO1 activates autophagy in transcription-dependent or transcription-independent manners. FoxO1 shuttle between the cytosol and nucleus, controlling autophagy through transcriptional regulation, or by directly binding to Atg7 and XBP1u

tubular cells and hematopoietic stem cells (Webb and Brunet 2014). For example, in liver-specific FoxO1/3/4 knockout mice, RNA levels of Atg5, Beclin1, LC3b and Atg14 reduced, autophagy is inhibited and can be much more prone to fatty liver and hypertriglyceridemia. The first-line anti-diabetic drug metformin can alleviate fatty liver by modulating SIRT1-FoxO mediated autophagy. In mouse primary renal tubular cells, FoxO3 also upregulates Bnip3 expression and promotes autophagy. In conclusion, activation of autophagy by FoxO is a ubiquitous phenomenon in differentiated cells.

## 13.2 NFκB and Autophagy

The transcription factor NFκB (nuclear factor kappa B) plays a key role in regulating the immune response to infection and cytokine production. NFκB regulates the expression of a broad range of genes involved in the development, cell proliferation, survival, differentiation and senescence. It also plays a pivotal role in regulating inflammation and the innate and adaptive immune responses. Dysregulation of NFκB pathway may lead to cancer, viral infections and a variety of inflammatory diseases.

NFκB is formed through the dimerization of five subunits, including members of the Rel protein family, namely RelA (p65), c-Rel, RelB, NFκB1 (p50 and its precursor p105) and NFκB2 (p52 and its precursor p100). Two pathways are involved in the activation of NFκB: the canonical and non-canonical pathway. The canonical or classical pathway is activated by a large range of stimulus, microbial, viral infections and proinflammatory cytokines. In this pathway, NFκB dimers, mostly p50/RelA and p50/c-Rel, are usually retained in the cytoplasm by their interaction with specific inhibitor IκB. Stimuli activate NFκB through IκB kinase (IKK)-dependent phosphorylation and subsequent proteasomal degradation of IκB proteins. The non-canonical or alternative pathway, triggered by a limited number of stimuli, plays a central role in the generation of lymphoid organs and in B-cell maturation and survival. In this pathway, NFκB dimers p52/RelB or p52/p52 are activated by the limited proteasomal degradation of the NFκB 2/p100 precursor. This proteolysis is initiated by the activation of IKKα homodimers by upstream NFκB inducing kinase (NIK). Unlike the stable mature IKK proteins, the mature NIK is rapidly degraded by the proteasome in a TNF receptor-associated factor 3 (TRAF3)-dependent manner.

The level of autophagy is controlled positively or negatively by NFκB through its inducer (Fig. 13.2). In Ewing's sarcoma, breast cancer and leukemia cells, TNF-mediated NFκB activation represses autophagy, both through ROS inhibition and mTOR activation. Autophagy is induced by ROS accumulation when NFκB is inactivated. In high-risk myelodysplastic syndromes and acute myeloid leukemia, NF-κB inhibition facilitates starvation-induced cell death of cancer cells. In Mantle cell lymphoma (MCL), highly expressed transglutaminase 2 (TG2) forms a complex with NFκB, promotes NFκB nuclear location and increases its activity. TG2, NFκB and its downstream interleukin-6 (IL-6) are all able to regulate autophagy. Under hypoxic conditions, hypoxia-inducible factor (HIF-1α) induces autophagy through NFκB. In

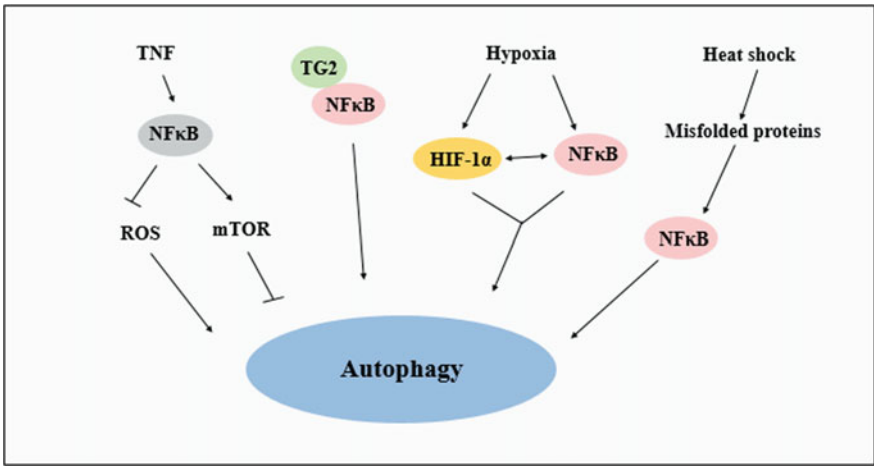


**Fig. 13.2** Autophagy is controlled positively or negatively by NFκB through its inducer. In Ewing's sarcoma, breast cancer, and leukemia cells, TNF-mediated NFκB activation represses autophagy through both ROS inhibition and mTOR activation. In MCL, highly expressed TG2 forms a complex with NFκB and promotes autophagy. Under hypoxic conditions, HIF-1α induces autophagy through NFκB. In response to heat shock stress, the IκB/NFκB complex loses its stability and activates NFκB. The misfolded protein accumulated during heat shock stress is degraded by autophagy and the cell survival rate increased

response to heat shock stress, the IκB/NFκB complex loses its stability and activates NFκB. The misfolded protein accumulated during heat shock stress is degraded by autophagy and the cell survival rate increased.

NFκB can also influence autophagy by regulating the expression and activity of autophagy-related genes (Trocoli and Djavaheri-Mergny 2011). NFκB and its family member p65/RelA recognize and bind to the promoter of Beclin1, promote Beclin1 gene expression and enhance autophagy. Interestingly, the regulation of Beclin1 by NFκB also has the opposite effect due to upstream factors. Toll-like receptors (TLR) signals induced by NFκB translocate to the nucleus via TRAF6, transcriptionally activate NFκB downstream factor A20, a factor that inhibits the ubiquitination of Beclin1, and thereby limits autophagy. In addition, NFκB regulates autophagy by modulating Bnip3. Under normal conditions, p65 prevents E2F1 from binding to the Bnip3 promoter, thereby inhibiting transcription of Bnip3 and decreasing autophagy levels. NFκB activity is decreased in response to hypoxia, leading to an increase of Bnip3 expression levels and induction of autophagy. Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) can be used to treat breast cancer. TRAIL binds to intracellular receptors to induce cytotoxicity and triggers apoptosis preferentially in cancer cells, while in mammary epithelial cells, the upstream kinase of the IKK complex TAK1 (TGFβ activated kinase 1) activates autophagy through AMPK-mediated inhibition of mTORC1, and protects cells against TRAIL-induced cytotoxicity. Interleukin-1 (IL-1) can cause a similar phenomenon, and IL-1 also activates autophagy through a TAK1/AMPK-dependent mechanism. Besides, the

upstream activation factors of the IKK pathway, TAB2 and TAB3, inhibit autophagy by interaction with Beclin1.

In some cases, activation of NFκB requires autophagy (Criollo et al. 2012). In mouse embryonic fibroblasts (MEF), nutrient starvation, treatment by mTOR inhibitor rapamycin or p53 inhibitor PFT-α (cyclic pifithrin α) and other autophagy stimulus can activate IKK, further leading to the degradation of IκBα in a phosphorylation-dependent manner, and nuclear translocation of NFκB.

## 13.3 E2F and TFEB Are Involved in the Molecular Regulation of Autophagy

### *13.3.1 Bidirectional Regulation of Autophagy by the E2F Family*

The E2F family contains a series of transcription factors involved in the regulation of cell cycle and plays an important role in cell proliferation, DNA repair, differentiation and development. Members of the E2F family can act as both transcriptional activators and transcriptional repressors (Polager and Ginsberg 2008).

E2F1 is an important transcriptional activator that upregulates the expression levels of *LC3*, *Ulk1*, *ATG5* and *DRAM* genes via binding to their promoters. However, when E2F1 forms a complex with retinoblastoma protein RB1, E2F1 inhibits transcription and has bidirectional role in autophagy regulation. In cancer cells, cytokine TGFβ induces autophagy via E2F1 activity inhibition by RB1. Silenced E2F1 leads to autophagy by promoting autophagic vacuole formation. While in autophagy induced by hypoxia, the RB1–E2F1 complex recognizes the promoter of Bnip3, attenuates the activation of Bnip3 by the hypoxia-inducible factor HIF-1α and inhibits Bnip3 activity to prevent autophagic cell death.

Another important transcription factor in the E2F family, E2F4, is involved in the regulation of autophagy in placenta and trophoblast cells in preeclampsia. Preeclampsia is a severe pregnancy disorder characterized by increased oxidative stress, trophoblast cell death and increased level of autophagy. In preeclampsia, E2F4 protein level is elevated, and its binding to the N-acylsphingosine amidohydrolase 1 (ASAH1) promoter is enhanced, which inhibits ASAH1 gene expression. Repression of ASAH1 induces autophagy in trophoblast cells by regulating the balance between the Bcl2 family proteins BOK and MCL1.

### 13.3.2 TFEB Promotes Autophagy

The transcription factor EB (TFEB) is a member of the basic helix–loop–helix leucine-zipper family and was first recognized as a key transcription factor in the process of lysosome formation. Several studies have shown that TFEB can regulate the transcriptional levels of some autophagosome-related genes, such as *Atg9*, *Wipi1/2*, *LC3*, *p62*, vacuolar fusion-related genes, including *Vps11*, *Vps18* and vacuolar proton pump V-ATPase subunits. Thus, TFEB regulates autophagy levels by regulating the expression of various related genes throughout the process from autophagosome formation to degradation. When lysosomes are abnormal, TFEB has been found to translocate from cytoplasm to nucleus, regulates the expression of autophagy-related proteins to form new lysosomes, which ultimately help clear the substances by degradation.

Similar to FoxO1, the cellular localization and activity of TFEB are affected by its post-translational modification. The activity of TFEB is strictly controlled by environmental conditions through post-translational modification. Under normal conditions, it is highly phosphorylated by mTORC1, ERK2, Akt and other kinases, binds to cytoplasmic protein YWHA (14-3-3). Therefore, TFEB is located in cytoplasm with limited activity (Fig. 13.3). Following nutrient deprivation and subsequent mTORC1 inactivation, TFEB is dephosphorylated and rapidly translocated to the nucleus, and promotes a series of autophagy and lysosomal-related gene expression. Active TFEB also associates with late endosomal/lysosomal membranes through interaction with the LAMTOR/Rag/mTORC1 complex. In addition, detection of lysosomal amino

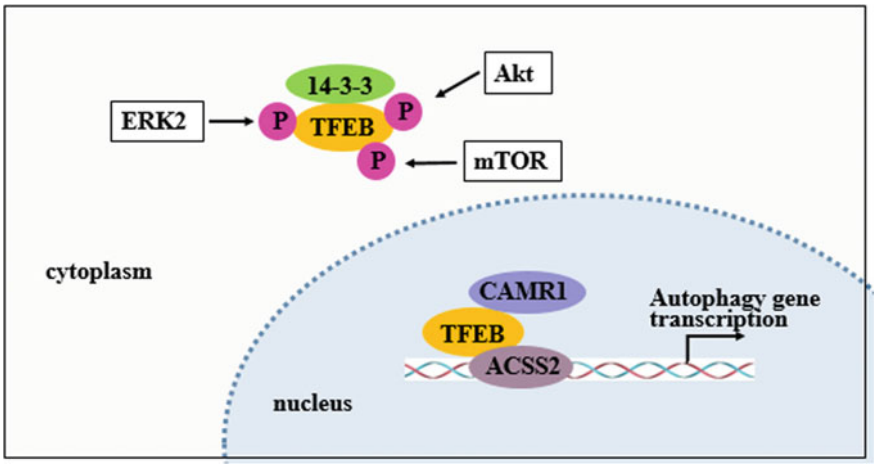


**Fig. 13.3** TFEB promotes autophagy. TFEB is phosphorylated by mTORC1, ERK2, and Akt and is sequestered in the cytoplasm via interaction with 14-3-3 under nutrient-rich conditions. Upon nutrient deprivation, TFEB is dephosphorylated and translocates to the nucleus where it interacts with CAMR1 and ACSS2 and activates autophagy and lysosome gene transcription

acids by the Rag GTPase complex and activation of mTORC1 are important for regulating starvation and stress-induced nuclear transfer of TFEB. Except for mTORC1 inactivation, TFEB nuclear translocation is also regulated by lysosomal calcium signaling. $Ca^{2+}$ is released from lysosomes, thereby establishing $Ca^{2+}$ microdomains near lysosomes, activated calcineurin (Cn) binds and dephosphorylates TFEB, thus promoting its nuclear translocation.

Transcriptional activation of autophagy genes by TFEB also involves AMPK-dependent changes in histone modifications (Sakamaki et al. 2018). In the absence of glucose or amino acid, TFEB is involved in the regulation of autophagy by AMPK–SKP2–CARM1 signaling pathway. Upon glucose deprivation, the AMP/ATP ratio increases and AMPK becomes active, leading to stabilization of arginine methyltransferase CARM1 by suppressing the expression of ubiquitin ligase S-phase kinase-associated protein 2 (SKP2) via FoxO3. CARM1 then interacts with TFEB and co-activates TFEB-mediated transcription. Interestingly, dimethylation at H3R17 by CARM1 is found to be necessary for autophagy. AMPK also phosphorylates acetyl-CoA synthetase 2 (ACSS2), which facilitates its nuclear translocation and interaction with TFEB. This leads to local production of acetyl donor acetyl-CoA and increased in histone H3 acetylation at TFEB target gene promoters for transcriptional activation.

Taken together, following nutrient deprivation, mTORC1 inactivation and AMPK activation cooperatively induce autophagy and lysosome genes by altering TFEB localization and the chromatin environment of its target genes.

Autophagy is involved in various aspects of cell physiology, while autophagy disorder is associated with a range of diseases. Autophagy process needs to be properly regulated to maintain the stability of intracellular environment. In addition to the above-mentioned molecular mechanisms, there are many other proteins involved in the regulation of autophagy, such as ZKSCAN3, SREBF2/SREBP2, Ume6 complex and Pho23 (Feng et al. 2015). These transcription factors can activate or inhibit autophagy in transcription-dependent or transcription-independent manners. As our understanding of autophagy regulation mechanisms continues to expand, there is likely to be more widespread use of therapeutic approaches to autophagy in clinical applications.

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# Chapter 14
# The Role of Nanomaterials in Autophagy


**Min Wei and Wei-Dong Le**

**Abstract** With the development of nanotechnology and the emergence of new nanomaterials, the effect of nanomaterials on autophagy regulation has attracted increasing attention. Nanomaterial-mediated autophagy regulation has potential applications in the diagnosis and treatment of autophagy-related diseases, such as cancer treatment, drug sensitization and neurodegenerative diseases. Different nanomaterials can regulate autophagy through different mechanisms because of their unique physical, chemical and biological properties. In this chapter, we will introduce the effects and mechanisms of autophagy mediated by nanomaterials and the applications of autophagy induced by different nanomaterials in the field of biomedicine.

**Keywords** Nanomaterials · Autophagy · Autophagy-modulating effect

## 14.1 Autophagy Regulation Mediated by Nanomaterials

Nanomaterials refer to materials with at least one dimension in the nanoscale (i.e., 1–100 nm) or consisting of basic units at the nanoscale (approximately 10–100 tightly aligned atoms). When the three dimensions of materials are all in the nanoscale range, the materials are considered nanoparticles. The fluctuation of electrons and the interaction between atoms will be affected by the size at the nanometer scale; therefore, when the particle size is reduced to a certain size on the nanometer scale, the particle usually shows better physical and chemical properties than conventional materials with the same composition.

The development of nanotechnology and the emergence of new nanomaterials have brought revolutionary achievements to biomedicine. Nanotechnology has been increasingly penetrating the life sciences. An increasing number of nanomaterials are widely used in research on drug delivery, bioimaging, biosensors, medical diagnoses and other biomedical fields. With the increasing application of nanotechnology in

M. Wei · W.-D. Le (✉)
Liaoning Provincial Center for Clinical Research on Neurological Diseases, The First Affiliated Hospital, Dalian Medical University, Dalian 116021, People's Republic of China
e-mail: wdle@sibs.ac.cn



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biomedicine, increasing research has been performed to explore the cellular processing of nanomaterials and underlying molecular mechanisms. Recently, autophagy induced by nanomaterials has attracted more attention. Many nanomaterials have been shown to induce autophagy both in vivo and in vitro, and some nanomaterials have made some progress in the field of biomedicine.

To date, many nanomaterials have been found to cause autophagy, including gold nanoparticles, graphene, carbon nanotubes, fullerene and its derivatives, silica, α-alumina, iron oxide, dendrimers and cationic liposomes. The characteristics of autophagy regulation by nanomaterials have potential applications in the diagnosis and treatment of autophagy-related diseases, such as cancer and neurodegenerative diseases.

### *14.1.1 Activating Autophagy Clearance*

Autophagy plays an important role in maintaining cell homeostasis and protecting cells from damage. When cells ingest nanoparticles, they are recognized as foreign bodies and activate the clearance mechanisms of the organism via triggering autophagy. At present, there are three types of autophagy: macroautophagy, microautophagy and chaperone-mediated autophagy. Nanoparticles usually induce macroautophagy, similar to the autophagic process by which cells remove invasive pathogens. When nanoparticles enter the cell through endocytosis, the nanoparticles in the cytoplasm are isolated and wrap into the membrane structure, forming a closed double-layer membrane structure called the autophagosome. Then, the autophagosomes fuse with lysosomes, forming autolysosomes, and the cargos are degraded by lysosomal enzymes. It is worth noting that the autophagic regulation effect induced by nanomaterials does not necessarily enhance the autophagic scavenging ability of cells. In contrast, when a large number of nanoparticles accumulate in the cells, they will damage the downstream pathway, blocking the autophagic flux and causing cell damage.

### *14.1.2 Blocking Autophagic Flux*

Autophagic flux induced by nanomaterials can be blocked by disrupting lysosome function. After endocytosis into cells, a large number of nanoparticles accumulate in lysosomes, resulting in enhanced permeability of lysosomal membranes, increased pH and inactivation of lysosomal enzymes. All these cellular and molecular changes prevent the fusion of lysosomes and autophagosomes, which leads to accumulated autophagosomes and blocked autophagic flux. A typical example of this cellular process is autophagy caused by gold nanoparticles. Initially, researchers observed an increase in autophagy in cells after gold nanoparticle treatment. They believe that gold nanoparticles could induce autophagy. However, further studies revealed that

the increase in autophagosomes is due to the blockage of autophagic flux rather than the induction of autophagy (Ma et al. 2011).

To better understand the relationship between nanomaterials and autophagy, it is particularly important to dynamically monitor changes in autophagic flux. At present, several methods can be used to detect the formation of autophagic bodies, including transmission electron microscopy, GFP-LC3 dots, LC3 immunofluorescence staining and LC3 western blotting (Klionsky et al. 2016). These methods are also widely used in the detection of autophagy induced by nanomaterials. However, these commonly used methods are relatively static observation methods, and autophagy is a multi-step, dynamic process. The increase of autophagosomes not only increases the ability of cells to degrade autophagic contents but also may be related to the blockage of autophagic flux. Both the increase in autophagic activity and the blockage of autophagic flux can lead to the accumulation of autophagosomes in cells. Therefore, autophagic flux analysis is often used to distinguish the two possibilities, including the use of autophagy-related drugs to detect LC3-I/II turnover, the GFP-LC3 cleavage test, GFP-mCherry-LC3B dual fluorescence analysis and detection of long-lived protein degradation (Klionsky et al. 2016).

## 14.2 Underlying Mechanisms of Autophagy Modulated by Nanomaterials

As novel autophagy regulators, nanomaterials affect autophagy through various mechanisms. Previous studies have shown that different nanomaterials have distinct autophagy mechanisms due to their different physical and chemical properties. The mechanisms for nanomaterial-modulated autophagy can be broadly categorized into three classes: oxidative stress, direct regulation of autophagic signaling pathways, such as Akt/mTOR, and alteration of the expression level of autophagy-related genes or proteins (Zheng et al. 2016). The possible pathways of nanomaterial-mediated autophagy are shown in Fig. 14.1.

Oxidative stress is considered to be one of the main causes of cytotoxicity caused by nanoparticles, which plays an important role in the regulation of autophagy (Stern et al. 2012). Reactive oxygen species (ROS) are chemical active molecules containing oxygen. They are natural byproducts of normal oxygen metabolism and play a key role in cell homeostasis. The main sources of ROS include mitochondria, endoplasmic reticulum, peroxisome and NADPH oxidase complexes (Wen et al. 2013). Mitochondria play an important role in the production of ROS in cells. On the one hand, nanomaterials can increase ROS production by interacting with mitochondria. However, metal or organic substances on the surface of nanomaterials can cause redox reactions. In addition, excited electrons on the surface of nanomaterials can also lead to an increase in ROS in cells. For example, gold-coated iron nanoparticles can release $Fe^{2+}$ ions and participate in ROS generation. ROS are involved in the

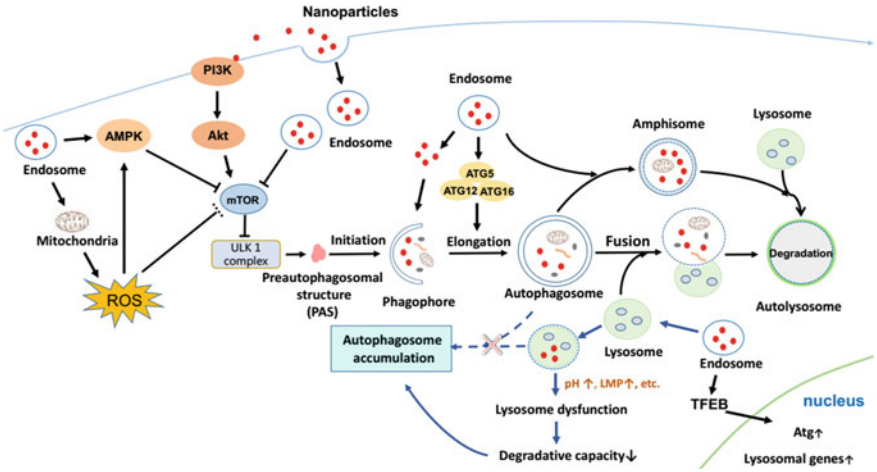


**Fig. 14.1** The possible pathways of nanomaterial-mediated autophagy

regulation of the mTOR signaling pathway, which activates or inhibits the activity of mTORC1 in a dose- and time-dependent manner and regulates autophagy.

The degradation of nanoparticles in lysosomes can also directly induce ROS. Lysosomes are considered to be the conventional targets of cytotoxicity and autophagy induced by nanoparticles (Stern et al. 2012). The accumulation of nanoparticles in lysosomes results in lysosome swelling and the release of cathepsin, which are accompanied by an increase in ROS levels and autophagy. In this process, autophagy is upregulated as a protective mechanism to compensate for the inadequacy of lysosomal degradation. At the same time, increasing lines of evidence show that the accumulation of nanoparticles in lysosomes can cause lysosome alkalization and lysosome damage, ultimately leading to the blockage of autophagic flux (Stern et al. 2012).

Nanoparticles can also interact directly with the mTOR signaling pathway. The changes in mTOR activity mediated by nanoparticles are associated with the regulation of the mTORC1 activators AKT and PI3K, as well as the mTORC1 inhibitors AMPK and TSC (Hulea et al. 2016). In the process of endocytosis, nanoparticles may affect the recruitment/activation of PI3K/AKT in the local area of the cell membrane, thus altering the ability of AKT to activate mTORC1 (Hulea et al. 2016). Because AKT can be activated by mTORC2, the change in AKT activity mediated by nanoparticles may be at least secondary to mTORC2 regulation in some cases. In addition, the interaction between nanoparticles and lysosomes may also affect lysosome recruitment and mTORC1 activation. Nanoparticles can also activate TFEB nuclear translocation and overexpression and enhance the transcription of autophagy-related genes (ATGs) and lysosome genes, thereby promoting the occurrence of autophagy (Zhang et al. 2018; Liu et al. 2018).

In conclusion, the mechanisms of autophagy regulation by various nanomaterials may be different due to their diverse physicochemical properties and biological functions; thus, the specific mechanisms need to be further clarified.

## 14.3 Biomedical Applications of Autophagy Modulated by Nanomaterials

Autophagy modulation by nanomaterials has potential applications in drug sensitization, abnormal protein aggregate removal and degradation, immunoregulation and cancer treatment. Table 14.1 summarizes the autophagic regulation of some nanomaterials. Next, we will select several nanoparticles that have been extensively studied to illustrate their relationship with autophagy regulation and their application in biomedicine.

**Table 14.1** Autophagy modulation by nanomaterials

| Nanoparticles | Cells | Labeled protein/signaling | Effect on autophagy modulation |
|---|---|---|---|
| Gold nanoparticles | NRK | LC3↑, p62↑ | Autophagy blockade and lysosomal dysfunction (Ma et al. 2011) |
| Fullerene C60 | HeLa; MEF; MCF-7 | Atg5 | Autophagy-mediated chemosensitization in cancer cells (Zhang et al. 2009) |
| Single-walled carbon nanotubes | Primary glia from CRND8 AD murine | mTOR-S6K | Autophagy induction and lysosomal activation (Xue et al. 2014) |
| Graphene oxide | HeLa; GFP-Htt(Q74)/PC12 | PtdIns3K and MEK/ERK1/2 | Activation of autophagy and elimination of ubiquitinated mutant Huntington protein (Jin et al. 2016) |
| Graphene oxide | Mouse embryonic stem cells | LC3↑, p62↑ | Autophagy blockade and lysosomal dysfunction (Wei et al. 2019) |

(continued)

**Table 14.1** (continued)

| Nanoparticles | Cells | Labeled protein/signaling | Effect on autophagy modulation |
|---|---|---|---|
| Graphene oxide QDs | GC-2; TM4 | LC3↑, p62↑ | Autophagy blockade and lysosomal dysfunction (Ji et al. 2016) |
| Silver nanoparticles | Primary MEF; Hela | PtdIns3K | Autophagy induction; inhibition of autophagy enhances the anti-cancer activity of silver nanoparticles (Lin et al. 2014) |
| Silver nanoparticles | THP-1 monocyte | LC3↑, p62↑ | Autophagy blockade and lysosomal dysfunction (Xu et al. 2015) |
| CdTe and CdTe/CdS/ZnS QDs | PC12; HEK293 | LC3↑ | Autophagy-sensitized cytotoxicity (Li et al. 2014) |
| PAMAM | A549 | Akt-TSC2-mTOR | Autophagic cell death (Li et al. 2009) |
| Silica nanoparticles | L-02 | EIF2AK3 and ATF6 UPR | Autophagy blockade and lysosomal dysfunction (Wang et al. 2018) |
| Copper oxide | HUVECs | LC3↑, p62↑ | Autophagy blockade and lysosomal dysfunction (Zhang et al. 2018) |
| Iron oxide | A549; IMR-90 | Akt-AMPK-mTOR | Selectively induce autophagy and kill cancer cells (Khan et al. 2012) |
| Titanium dioxide | HaCaT | LC3II, p62, NBR1, beclin1 and ATG5 | Autophagy induction at low dose; autophagy blockage at high dose (Lopes et al. 2016) |
| Bismuth nanoparticles | HEK293 | AMPK/mTOR | Autophagy induction; nephrotoxicity (Liu et al. 2018) |
| Tetrahedral DNA nanostructures | Chondrocytes | PI3K/AKT/mTOR | Enhanced cell autophagy (Shi et al. 2018) |

### *14.3.1 Carbon Nanomaterials*

Carbon nanomaterials are one of the earliest studied nanomaterials. Carbon nanomaterials, including carbon nanotubes, graphene, fullerene C60 and nanodiamond, are new materials that are widely studied and applied. Among them, the first three types of carbon nanomaterials are the most mature. These new carbon materials possess many excellent physical and chemical properties and are widely used in biosensors, gene and drug delivery and stem cell tracking. Studies have shown that carbon nanomaterials can induce autophagy in different cell types.

#### 14.3.1.1 Fullerene and Derivatives

Fullerenes (C60) are spherical or ellipsoidal conjugates composed of closed, hollow five- and six-membered rings. Unlike other nanomaterials, fullerenes and their derivatives have an antioxidant effect by scavenging free radicals and protecting cells from ROS damage. Fullerenes and their derivatives can also play an important role in autophagy regulation. For example, C60 fullerene-pentoxifylline nanoparticles were found to induce autophagy as a protective mechanism against β-amyloid peptide-induced cytotoxicity in neuro-2A cells (Lee et al. 2011). Therefore, this fullerene derivative has potential application value in drug design for amyloid-related diseases, such as Alzheimer's disease (AD). In addition, fullerene C60 and its derivatives have an autophagy-dependent chemosensitization effect on cancer cells. Studies have shown that fullerene C60 and its derivative C60 (Nd) nanoparticles can induce autophagy at very low concentrations in cells, and the autophagy effect of C60 (Nd) is more potent (Wei et al. 2010). By modulating autophagy at the nontoxic level, cancer cells are more sensitive to doxorubicin (DOX), and the killing effect of DOX is enhanced (Zhang et al. 2009). Notably, the method is also effective for drug-resistant cell lines. In summary, fullerene combined with traditional anticancer drugs (such as DOX) shows a synergetic effect on cancer cells by regulating autophagy, thereby improving the curative effect and reducing the potential side effects.

#### 14.3.1.2 Graphene Oxide

Graphene has attracted increasing attention because of its excellent physicochemical properties and low cost since its first discovery in 2004. Graphene, especially graphene oxide, is widely used in biomedical fields such as bioimaging, targeted drug delivery, cancer therapy and biological detection. As a precursor of graphene, graphene oxide has the advantages of simple synthesis, good biocompatibility and good water dispersion. It has been found that graphene oxide can induce autophagy in RAW264.7 macrophages in a concentration-dependent manner. Graphene oxide can activate autophagy in CT26 colon cancer cells, trigger autophagy and TLR-4/TLR-9 signaling cascades, which have anti-tumor effects (Chen et al. 2014). In

addition, the combination of graphene oxide and cisplatin (CDDP) can improve the chemosensitivity of cancer cells to anti-cancer drugs (Chen et al. 2015). In addition to initiating autophagy, graphene oxide may induce the opposite autophagy effect due to the different sizes of materials, synthesis methods, surface functional groups, and so on. Graphene oxide quantum dots (average diameter 3.28 + 1.16 nm) can inhibit lysosomal degradation by reducing the activity of cathepsin B in GC-2 and TM4 cells, thus blocking autophagic flux (Ji et al. 2016). Our latest research also shows that graphene oxide nanocolloids can inhibit the fusion of autophagosomes and lysosomes by increasing the pH of lysosomes and the permeability of lysosome membranes in mouse embryonic stem cells, ultimately blocking the autophagic flux (Wei et al. 2019). These different conclusions also illustrate the complexity of the modulation of autophagy by nanomaterials. We need to conduct a concrete analysis of each specific question when using nanomaterials to regulate autophagy.

#### 14.3.1.3 Carbon Nanotubes

Carbon nanotubes can be regarded as curled graphene sheets. According to the number of graphene sheets, carbon nanotubes can be divided into single-walled carbon nanotubes and multi-walled carbon nanotubes. In biomedical research, increasing attention has been paid to the biological function and toxicity of carbon nanotubes. It has been found that the effects of nanomaterials on autophagy vary depending on the surface properties of the nanomaterial. For example, carboxyl-modified (COOH–) single-walled carbon nanotubes induce autophagic death in A549 cells through the AKT-TSC2-mTOR pathway, while polyaminobenzene sulfonic acid (PABS) and polyethylene glycol (PEG) modified carbon nanotubes cannot induce autophagosome formation (Liu et al. 2011). By changing the surface groups of carbon nanotubes, autophagy can be regulated to a controllable level or even completely avoided. To better understand the role of the surface chemistry of carbon nanotubes in autophagy regulation, Yan et al. systematically studied the autophagy effect of 81 multi-walled carbon nanotubes modified by different combinations of chemically modified surface ligands (Wu et al. 2014). They found that multi-walled carbon nanotubes with different chemical compositions induced autophagy to different degrees by activating different signal pathways, which further demonstrated the flexibility and specificity of nanoparticles in regulating autophagy. Although nanoparticle-induced autophagy can cause nanotoxicity, proper regulation of autophagy may have therapeutic potential. In addition, functionalized single-walled carbon nanotubes can reverse the abnormal activation of mTOR signaling and lysosomal protein hydrolysis defects, thus helping to eliminate autophagic substrates (Xue et al. 2014). Lysosomal dysfunction has been recognized as a major cause of Alzheimer's disease, Parkinson's disease and other neurodegenerative diseases. These findings suggest that carbon nanotubes might serve as potential neuroprotective therapeutics for neurodegenerative diseases.

### *14.3.2 Metal and Metal Oxide Nanomaterials*

Metal and metal oxide nanomaterials, including gold nanoparticles, silver nanoparticles, titanium dioxide, iron oxide, alumina, zinc oxide and a variety of rare earth metal oxides, have been reported to regulate autophagy.

#### 14.3.2.1 Gold Nanoparticles

Gold nanoparticles, also known as colloidal gold, are biocompatible nanomaterials widely used in the fields of drug delivery, cancer treatment and cell imaging. Gold nanoparticles can induce oxidative stress and autophagy regulation in cells, and the levels of autophagy-related proteins LC3-II and ATG7 are upregulated. Further studies show that the accumulation of autophagosomes and the increased LC3B-II expression induced by gold nanoparticles are due to the blockade of autophagic flux (Ma et al. 2011). In fact, gold nanoparticles cause the accumulation of autophagosomes and autophagic substrate p62 by blocking the fusion of autophagosomes and lysosomes. The uptake of gold nanoparticles is particle-size-dependent. Compared with smaller (10 and 25 nm) particles, larger (50 nm) gold nanoparticles are more likely to be internalized into cells and lead to more autophagosome accumulation (Ma et al. 2011). The engulfed gold nanoparticles in lysosomes result in lysosome alkalization, which weakens the lysosome degradation ability and ultimately blocks autophagic flux. Nanoparticles can either induce autophagy or block autophagy, both leading to the upregulation of LC3-II and the accumulation of autophagosomes. Therefore, it is necessary to detect the autophagy process dynamically and comprehensively when nanomaterials are used as autophagy regulators.

#### 14.3.2.2 Metal Oxide Nanoparticles

Metal oxide is a binary compound composed of oxygen and another metal element, such as iron oxide, zinc oxide, alumina, titanium oxide or manganese oxide. These metal oxide nanoparticles have autophagy regulation characteristics to varying degrees. It was found that iron oxide nanoparticles could kill cancerous cells by inducing excessive autophagy in A549 lung cancer cells but had no obvious cytotoxicity to normal human lung fibroblasts (IMR-90) (Khan et al. 2012). Another study on the relationship between autophagy and photothermal therapy mediated by iron oxide nanoparticles in cancer showed that the photothermal effect of iron oxide nanoparticles can upregulate the autophagy level in cancerous MCF-7 cells, and inhibiting autophagy can enhance the killing effect of photothermal therapy by increasing cell apoptosis (Ren et al. 2018).

After exposure to human umbilical vein endothelial cells (HUVECs), copper oxide deposits in lysosomes destroyed lysosome function, resulting in impaired autophagic

flux and the accumulation of undegraded autophagosomes (Zhang et al. 2018). However, blocking the deposition of copper oxide in lysosomes can significantly reduce the death of HUVECs. Further studies show that inhibiting copper oxide nanoparticle deposition in lysosomes reduces the release of copper ions, which is considered to be a key factor involved in the toxicity of copper oxide (Zhang et al. 2018).

When titanium dioxide nanoparticles are exposed to human immortalized keratinocyte HaCaT at a noncytotoxic level, autophagy is induced at a low dose (0.16 μg/mL) but blocked at a higher dose (25 μg/mL), indicating that the concentration of nanoparticles has a great influence on autophagy regulation (Lopes et al. 2016). Therefore, special attention should be paid to the dosage of nanoparticles when using them to regulate autophagy for biological applications.

#### 14.3.2.3 Rare Earth Metal Oxide Nanoparticles

Rare earth metal oxides are one of the most important types of metal oxides. They have excellent application prospects in nano-labeling, diagnosis and treatment. Since neodymium oxide ($Nd_2O_3$) was found to induce autophagy in NCI-H460 non-small-cell lung cancer in 2005, several rare earth metal oxides, including samarium oxide ($Sm_2O_3$), europium oxide ($Eu_2O_3$), gadolinium oxide ($Gd_2O_3$) and terbium oxide ($Tb_2O_3$), have been found to induce autophagy (Yu et al. 2009). In addition, the nano-sized rare earth metal oxides, yttrium oxide ($Y_2O_3$), ytterbium oxide ($Yb_2O_3$) and lanthanum oxide ($La_2O_3$), were found to induce autophagy in HeLa cells (Zhang et al. 2010). Researchers note that the autophagy-inducing effect may be a universal biological effect of rare earth metal oxide nanoparticles.

### *14.3.3 Liposomes and Polymer Nanomaterials*

#### 14.3.3.1 Cationic Liposomes

Liposomes are spherically structured lipid bilayers that form when phospholipids are dispersed in water. Cationic liposomes, such as commercialized lipofectamine, are positively charged on the surface. They are widely used as gene/drug carriers due to their ability to transport DNA, RNA and macromolecule substances. Cationic liposomes can induce autophagy through a non-mTOR-dependent mechanism, and the transfection efficiency of cationic liposomes is also affected by the level of cell autophagy (Man et al. 2010). The autophagy regulation effect of cationic liposomes is helpful for improving the efficiency of gene and drug delivery. However, it is noteworthy that the "proton sponge effect" of cationic nanoparticles may lead to lysosome dysfunction, which may lead to the failure of lysosome recruitment and the activation of mTORC1, thus exerting a certain influence on autophagy (Xu et al. 2015).

#### 14.3.3.2 Poly(D,L-Lactide-Co-Glycolide)

Poly (lactic-co-glycolic acid) (PLGA) is a degradable organic compound that is randomly polymerized by lactic acid and glycolic acid. PLGA has been certified by the Food and Drug Administration of the United States (FDA) and has been formally included in the United States Pharmacopoeia as a pharmaceutical excipient. Studies show that PLGA nanoparticles can induce autophagy and are regulated by the class III PI3K complex. The combination of PLGA nanoparticles loaded with docetaxel (DTX) and the autophagy inhibitors 3-methyladenine (3-MA) or chloroquine can significantly enhance the anti-tumor effect of DTX, both in vivo and in vitro (Zhang et al. 2014). This is because PLGA nanoparticles can induce autophagy, degrading the loaded drug and thus reducing its efficacy. When combined with autophagy inhibitors, nanocarrier-induced autophagy was inhibited, and the pharmacodynamics were significantly improved. It is suggested that the relationship between nanocarriers and autophagy should be taken into account when designing nanodrugs to fully demonstrate the advantages of nanodrugs.

### *14.3.4 Quantum Dots*

Quantum dots (QDs) are a new type of nanomaterial with excellent fluorescence properties. They are usually between 1 and 10 nm in size with excellent optical properties, such as strong photostability, high fluorescence quantum yield and narrow excitation spectrum. QDs can be used to locate and trace intracellular target molecules and have been widely used in bioimaging and other related fields. However, the cytotoxicity of QDs has led to a bottleneck, restricting their clinical application, and has attracted extensive attention. Eleverstov et al. observed for the first time that QDs could induce autophagy in cells. They found that smaller $QD_{525nm}$ QDs could effectively induce significant autophagy in bone marrow mesenchymal stem cells, while $QD_{605nm}$ (twice the size of $QD_{525nm}$) could not induce autophagy in cells, indicating that the size of QDs played an important role in autophagy induction (Zabirnyk et al. 2007). In addition, the toxicity of CdSe (average particle size 5.1 nm) QDs is several times that of InGaP (average particle size 3.7 nm) QDs with similar sizes. Different element compositions may be the main reason for the difference in intracellular toxicity of QDs, and this toxicity is positively correlated with autophagy. Most QDs are composed of heavy metal ions (such as $Cd^{2+}$), which may lead to potential toxicity that hinders their practical application. Cadmium telluride (CdTe) QDs can enhance the sensitivity of cells to cadmium ions ($Cd^{2+}$) through autophagy (Li et al. 2014). Therefore, although CdTe QDs dissociate fewer cadmium ions (approximately a quarter) into cells than $CdCl_2$ solution treatment at the corresponding concentration, CdTe quantum dots are far more toxic to cells than $CdCl_2$. In addition, CdTe/CdS/ZnS quantum dots can protect differentiated PC12 cells from cell damage induced by 1-methyl-4-phenylpyridine ion (MPP+) and hinder the accumulation of α-synuclein induced

by MPP+ (Li et al. 2014). The autophagy-inducing activities of CdTe/CdS/ZnS QDs have a good protective effect on the cell model of PD in vitro.

## 14.4 Prospect

Diverse materials induce different autophagy phenomena. Even the underlying mechanisms of autophagy modulation by nanoparticles with the same nanomaterial but with different sizes, shapes and surface modifications are different, which provides various models for studying autophagy. Autophagy initiated by nanomaterials has two aspects: on the one hand, it can enhance the ability of cells to remove foreign bodies; on the other hand, excessive autophagy can also cause type II programmed cell death. Suppression of abnormal mTOR signaling by nanoparticles can be used in anticancer and neuroprotective therapies. At the same time, the potential toxicity of nanoparticles to normal cells should also be considered. In addition, due to the nondegradability of most nanomaterials, the accumulation of a large number of nanomaterials in cells or the toxicity of the materials themselves will cause damage to the corresponding organelles and eventually lead to the blockage of autophagic flux.

Although some progress has been made in the regulation of autophagy by nanomaterials, many challenges remain unsolved. The autophagy regulation effect of nanomaterials is influenced by many factors, such as material composition, particle size, shape, surface modification, dosage, treatment time, synthesis method and cell lines. Therefore, the results of autophagy regulation mediated by nanoparticles are variable under different situations. The degree and mechanism of autophagy induced by various nanomaterials still lack a relatively uniform standard. Furthermore, most of the studies on the regulation of autophagy by nanomaterials are carried out in vitro. It is difficult to truly reflect the effect of nanomaterials on autophagy modulation in organisms. At the same time, how to reduce the toxicity of nanomaterials in the process of autophagy and how to use nanomaterials to control the level of autophagy and apply it to clinical diagnosis and treatment of diseases are still large challenges. Although there are still many problems to be further elucidated, with the further study of the autophagy mechanism of nanomaterials, it is believed that autophagy modulation by nanomaterials will provide more possibilities for the development of biomedicine.

## 14.5 Summary

The autophagy regulation effect of nanomaterials not only has potential applications in the treatment of cancer and neurodegenerative diseases but also provides new choices for drug discovery and development. At the same time, nanomaterials also provide excellent models for autophagy research. In addition, different

molecular mechanisms of autophagy are induced by different nanomaterials, and related research is still in its infancy. Further research is needed to effectively regulate autophagy induced by nanoparticles and transform this autophagy regulation effect into a new method for disease treatment.

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# Chapter 15
# Structural Basis of Autophagy Regulatory Proteins


**Lifeng Pan, Jianping Liu and Ying Li**

**Abstract** Autophagy is an evolutionarily conserved lysosome-dependent intracellular degradation process that is essential for the maintenance of cellular homeostasis and adaptation to cellular stresses in eukaryotic cells. The most well-characterized type of autophagy, the macroautophagy, involves the progressive sequestration of cytoplasmic components into dedicated double-membraned vesicles called autophagosomes, which ultimately fuse with lysosomes to initiate the autophagic degradation of the sequestered cargo. In the past decade, our understanding of the molecular mechanism of macroautophagy has significantly evolved, with particular contributions from the biochemical and structural characterizations of autophagy-related proteins. In this chapter, we focus on some autophagy regulatory proteins involved in the macroautophagy pathway, summarize their currently known structures, and discuss their relevant molecular mechanisms from a perspective of structural biology.

**Keywords** Autophagy · Structural biology · Macroautophagy · Autophagy regulatory proteins

## 15.1 Introduction

Autophagy is a highly regulated and lysosome-dependent catabolic process of degrading cytosolic components, including bulk protein aggregates, dysfunctional organelles, and invading pathogens, for the maintenance of cellular homeostasis and/or adaptation in response to multiple cellular stresses in eukaryotic cells (Klionsky and Emr 2000). Autophagy plays a crucial role in many physiological processes, including growth, development, senescence, and immune defense, and autophagy dysfunction is associated with a large number of human diseases, such as cancer and neurodegenerative diseases (Levine and Kroemer 2019). Based on the different ways to deliver cargo to the lysosome, autophagy in mammals can be classified into three types: macroautophagy, microautophagy, and chaperone-mediated autophagy.

L. Pan (✉) · J. Liu · Y. Li
Shanghai Institute of Organic Chemistry, Chinese Academy of Sciences, Shanghai 200032, China
e-mail: panlf@sioc.ac.cn



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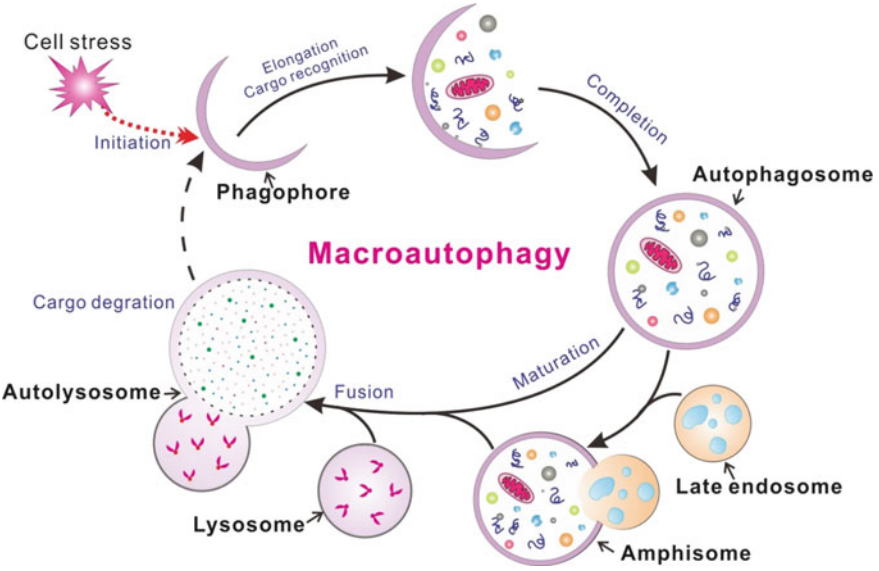


**Fig. 15.1** A schematic diagram showing the overall process of autophagy in mammalian cells

In this section, we just focus on the macroautophagy process; hereafter, all references to autophagy mentioned refer to macroautophagy. The autophagy process can be divided into the following main steps (Fig. 15.1): First, autophagy is initiated under some cellular stresses, such as nutrient deprivation, protein aggregation, and pathogen invasion, and the isolation membrane is formed (the initiation step). Next, the isolation membrane undergoes growth and expansion and finally closure to form the autophagosome; meanwhile, the targeted cargo is sequestered and encapsulated into the autophagosome (the expansion and closure step). Subsequently, some mature autophagosomes fuse with endosomes to form amphisomes (the maturation step). Finally, the mature autophagosomes and amphisomes fuse with lysosomes to generate autolysosomes, and the cargoes encapsulated in the autophagosome are then degraded by a series of lysosomal proteases and are recycled for reuse (the fusion and degradation step). A large number of proteins have been identified that participate in the different steps of the autophagy process through genetic and other relevant studies. For instance, the mTOR complex and AMPK kinase are involved in the regulation of the initiation step; the ATG1/ULK complex, the class III PI3K complex, the ATG8 and ATG12 ubiquitin-like conjugation systems, and autophagic phosphatidylinositol three-binding proteins participate in the formation of autophagosomes. FYCO1, KIF5B, and JIP1 are involved in the transport and maturation of autophagosomes; two autophagic SNARE complexes, the Synatxin17/VAMP8/SNAP29 SNARE complex and the YKT6/Synatxin7/SNAP29 SNARE complex, along with relevant tethering factors, are involved in the autophagosome–lysosome fusion process. In addition to the non-selective bulk autophagy process, recent studies have discovered that there is a substantial number of selective autophagy processes (Green and Levine 2014; Stolz

et al. 2014), such as the selective autophagy of aggregated proteins (aggrephagy), invading pathogens (xenophagy), dysfunctional mitochondria (mitophagy), peroxisomes (pexophagy), ribosomes (ribophagy), endoplasmic reticulum (ER) subdomains (reticulophagy), glycogen (glycophagy), stress granules (granulophagy), and ferritins (ferritinophagy). Previous structural studies of autophagy-related proteins have elucidated the working mechanisms of a large number of autophagic proteins and the pathogenesis of relevant disease-causing mutations to lay a strong foundation for further understanding the molecular mechanisms of the autophagy process. Owing to space limitations, in this chapter we will just summarize some recent advances in the structural biology of autophagy regulatory proteins that are related to bulk autophagy.

## 15.2 Structural Studies of Proteins Related to Autophagy Initiation

### *15.2.1 The mTOR Complex*

Mechanistic target of rapamycin (mTOR) is a crucial serine/threonine kinase in eukaryotes and functions as a master regulator of cell metabolism and growth. mTOR integrates a wide array of cues, such as nutrients, growth factors, and cellular energy levels, to determine the balance between anabolism and catabolism in a cell. As a kind of anabolic process, autophagy is tightly coupled to the mTOR pathway (Saxton and Sabatini 2017). By associating with different companion proteins, mTOR may form two distinct types of kinase complexes: mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2). mTORC1 consists of three core components, mTOR1, mLst8, and Raptor (Fig. 15.2). In addition, two inhibitory subunits, PRAS40 and DEPTOR, can also be found in mTORC1. Similar to mTORC1, the core of mTORC2 consists of mTOR1, mLst8, and Rictor instead of Raptor (Fig. 15.2). Additional regulators, DEPTOR, mSin1, and Protor1/2, can also associate with the core subunits of mTORC2 (Saxton and Sabatini 2017).

mTORC1 inhibits autophagy through the phosphorylation of several components of the autophagy-initiating UNC-5-like kinase (ULK) complex, such as ATG13, ULK1, and ULK2 (Kim and Guan 2015; Ganley et al. 2009). In mammalian cells, the phosphorylation of ULK1 at residue S758 by mTORC1 prevents the phosphorylation of ULK1 by AMPK, which can activate ULK1 (Kim et al. 2011). In yeast, mTORC1 phosphorylates the ATG13 protein and disrupts its binding to ATG1, which is the homologue of mammalian ULK1/2 (Kamada et al. 2000). mTORC1 can also inhibit ULK1 stability through phosphorylating the autophagy/Beclin-1 regulator (AMBRA1) whose un-phosphorylated form recruits E3-ligase TRAF6 to modify and stabilize ULK1 with a K63-linked ubiquitin chain (Nazio et al. 2013). In addition to the ULK complex, mTORC1 also regulates the VPS34 complex whose PI3K kinase activity is vital for the formation of autophagosomes (Kim et al. 2013). ATG14L

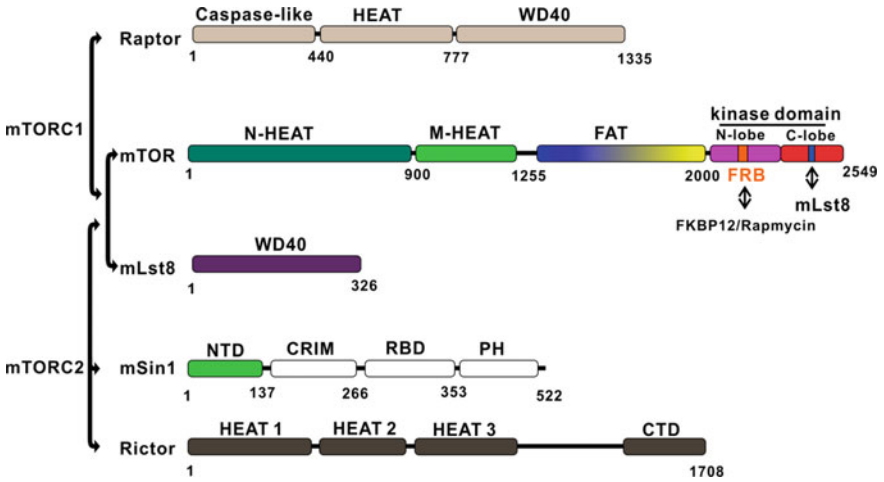


**Fig. 15.2** Domain organizations of the core subunits of human mTORC1 and mTORC2. The same color scheme is used in all structure figures. HEAT: Huntington: EF3A, ATM, TOR repeats; WD40: WD40 repeats; N-HEAT: N-terminal HEAT repeats; M-HEAT: middle HEAT repeats; FAT: Frap, ATM, TRRAP domain; FRB: FKBP12-rapamycin-binding domain; NTD: N-terminal domain; CRIM: conserved region in the middle; RBD: Ras-binding domain; PH: pleckstrin homology domain; CTD: C-terminal domain

and VPS34 form a complex dedicated to autophagy initiation. mTORC1 can phosphorylate ATG14L and inhibit the lipid kinase activity of VPS34 (Yuan et al. 2013). mTORC1 also regulates the expression levels of many autophagy genes through direct phosphorylation of the master transcription factor TFEB (Settembre et al. 2011). Upon phosphorylation of the S142 and S211 residues of TFEB by mTORC1, TFEB is sequestered in the cytoplasm and is unable to promote the transcription of many genes involved in the autophagy pathway (Settembre et al. 2012). In contrast to mTORC1, mTORC2 cannot directly regulate autophagy but may indirectly suppress autophagy through phosphorylating the T450 residue in AKT, which is the upstream regulator of mTORC1 (Oh et al. 2010). Given the importance of mTORC1/2 complexes, several groups have devoted great efforts in determining the structures of mTORC1 and mTORC2. Recently, the structures of both complexes have been successfully solved to a high resolution by the cryo-EM technique (Figs. 15.3a and 15.4a) (Yang et al. 2016, 2017; Chen et al. 2018). In the following, we will summarize the important findings from these structural studies.

mTOR belongs to the PI3K-related kinase family and has several unique insertions in its kinase domain, which function as regulatory elements and docking sites for partner proteins. In the crystal structure of an N-terminal-truncated fragment of mTOR in complex with mLst8, mLst8 binds to a two α-helices insertion in the C-lobe of the mTOR kinase domain (Fig. 15.3b) (Yang et al. 2013), while the FKBP12-rapamycin binding (FRB) domain, which is another unique insertion, protrudes from

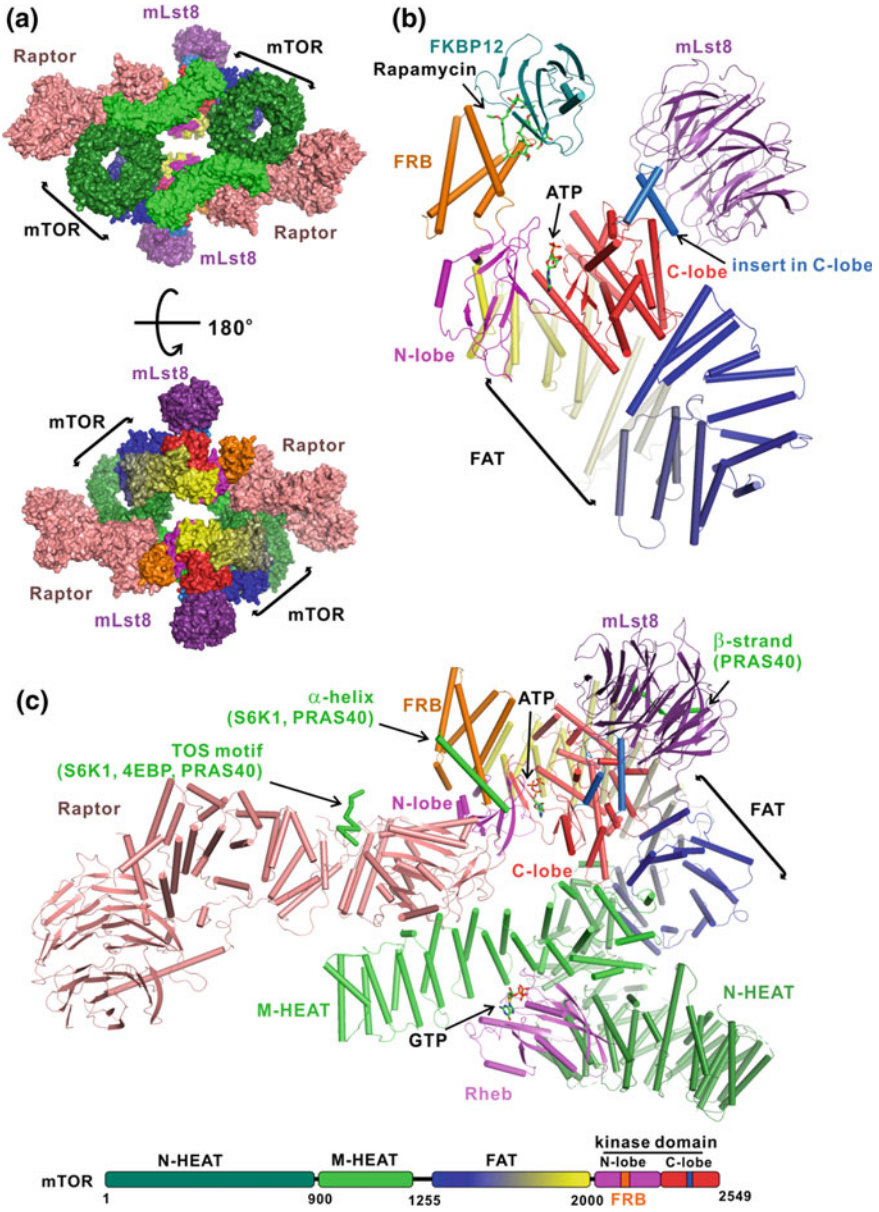


**Fig. 15.3** The structure of mTORC1. **a** A color-coded representation of the dimerized mTORC1 surface containing subunits mTOR, mLst8, and Raptor (PDB code: 6BCX). **b** A cartoon representation of the crystal structure of the complex between mLst8 and the FAT-kinase domain fragment of mTOR (PDB code: 4JSV). The structure of FKBP12-rapamycin (PDB code: 1NSG) is docked to the FRB domain by aligning the FRB domain in structures 4JSV and 1NSG. **c** A composite view of the cryo-EM structure of mTORC1 consisting of Raptor, full-length mTOR, mLst8, and the GTPase Rheb. Substrates S6K1 and 4EBP and the inhibitory protein PRAS40 use multiple short motifs to interact with binding sites in Raptor, the FRB domain of mTOR, and/or mLst8

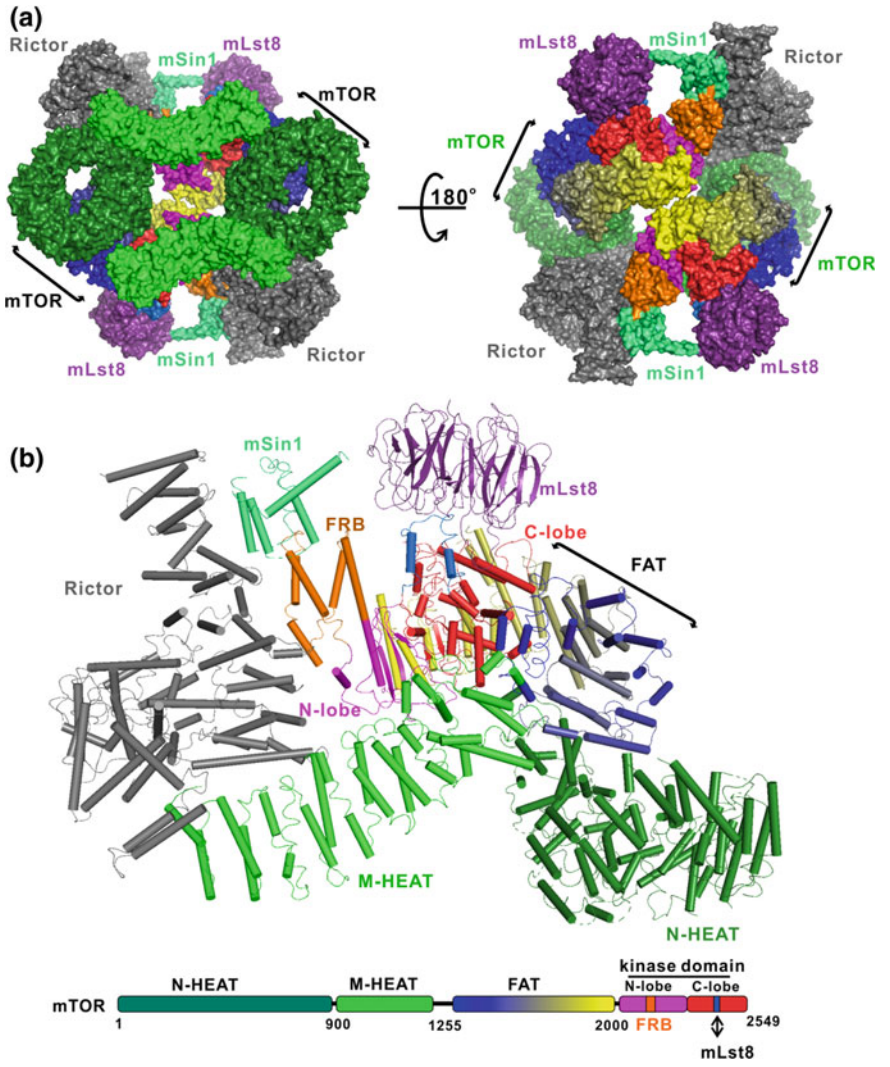


**Fig. 15.4** The structure of mTORC2. **a** A color-coded representation of the dimerized mTORC2 surface, containing subunits mTOR, mLst8, mSin1, and Rictor (PDB code: 5ZCS). **b** The cryo-EM structure of mTORC1, consisting of Rictor, the full-length mTOR, mLst8, and mSin1

the N-lobe of the kinase domain (Fig. 15.3b). Furthermore, docking of the FKBP12-rapamycin complex to the FRB domain of mTOR in the complex structure does not show any contact with other parts of mTOR (Fig. 15.3b). As a common signature, the FAT domain upstream of the kinase domain adopts a super-helical α-solenoid shape and wraps around the kinase domain, contacting both the N- and C-lobes (Fig. 15.3b). The whole mTORC1 complex, containing mTOR, Raptor, and mLst8, is a large complex with a size of ~1 M Dalton and is extremely challenging for X-ray crystallization. Fortunately, in the past few years, the rapid advancement of cryo-EM has paved the way for determining the atomic structures of very large protein complexes. The structure of mTORC1 was recently determined using the cryo-EM technique (Yang et al. 2016, 2017). Several groups revealed that mTORC1 forms a dimer and adopts a hollow "lozenge" shape mainly through dimerization between mTOR HEAT repeats, which can be further divided into N-HEAT and M-HEAT domains (Fig. 15.3a). The N-HEAT domain adopts a super-helical α-solenoid configuration and contacts the FAT domain beneath the N-lobe of the kinase domain, while the M-HEAT domain adopts an extended conformation beneath the C-lobe of the kinase domain and associates with the N-terminal caspase domain of Raptor to help Raptor localize between the M-HEAT and FRB of mTOR (Fig. 15.3a). The activity of mTORC1 can be promoted by the small GTPase Rheb. The structure of mTORC1 in complex with a small GTPase Rheb in GTP-bound form reveals how the binding of Rheb at the N-HEAT domain induces a large conformational change in the downstream FAT domain and frees the N-lobe of the kinase domain to adopt the active conformation (Fig. 15.3c). Many cancer-associated hyper-activating mutations are found in the FAT domain and lower the energy barriers for the N-lobe of the kinase domain transitioning to the active conformation (Yang et al. 2017). The structure of the substrate or inhibitory protein in complex with the FRB domain of mTOR, Raptor, and mLst8 clearly shows the mechanism for substrate recruitment and inhibition in the mTORC1 complex. The Raptor subunit in mTORC1 recognizes the TOR signaling sequence (TOS) motif in two key substrate proteins (S6K1 and 4EBP) with the same binding site (Fig. 15.3c). S6K1 is further recruited to mTOR by another short hydrophobic sequence through binding to the FKBP12-rapamycin docking site in the FRB domain (Yang et al. 2017). This observation intuitively shows that rapamycin inhibits mTORC1 through competition with FRB-dependent substrate recruitment. Notably, the substrate proteins use motifs that are distant from the phosphorylation sites to associate with mTORC1, thereby facilitating the flexible adjustment of the binding strength of the substrates. Meanwhile, this observation also fits well with the wide substrate spectrum of mTORC1. The inhibitory protein PRAS40 binds to the same sites in both Raptor and the FRB domain as that of the substrates S6K1 and 4EBP1 (Fig. 15.3c). In addition, PRAS40 also binds to the mLst8 subunit by forming a short β-strand. This unique mLst8-binding motif is important for PRAS40's inhibition of mTORC1, since its deletion significantly impairs the efficacy of PRAS40 (Fig. 15.3c) (Yang et al. 2017). It seems that PRAS40 depends on tethering itself to mLst8 to effectively compete against mTORC1 substrates bound to Raptor and the FRB domain.

Recently, the structure of mTORC2 was also determined by cryo-EM (Fig. 15.4a) (Chen et al. 2018). mTORC2 also forms a dimer and adopts a hollow "lozenge" shape similar to mTORC1 (Figs. 15.3a and 15.4a). Moreover, the core subunit of mTOR associates with mLst8 in the same mode as that in mTORC1 (Figs. 15.3a and 15.4a). Rictor makes a contact with the N-HEAT domain of mTOR in a region that overlaps with the Raptor binding surface in mTORC1 (Figs. 15.3c and 15.4b), which explains the mutually exclusive role of Raptor and Rictor in mTORC1 and mTORC2. Another part of Rictor associates with the small mSin1 protein and covers a large portion of the FRB domain of mTOR, thereby blocking the binding of FKBP12-rapamycin to the FRB domain (Figs. 15.3b and 15.4b). This binding mode clearly explains why rapamycin does not directly inhibit mTORC2. The structural studies of mTORC1/2 greatly benefit from the cryo-EM technique, which is capable of determining the structure of huge protein complexes. The exclusive binding mode of Raptor and Rictor differentiates in mTORC1 and mTORC2. The multiple binding ability of the FRB domain in mTOR to its binding partners explains the difference of rapamycin sensitivity in mTORC1 and mTORC2. The structures of complexes between mTORC1 and substrates or inhibitory proteins reveal that mTORC1 recruits substrates through multivalent bindings with several motifs in the substrate. The Rheb/mTORC1 complex shows the activation mechanism of mTORC1 through a long-distance allosteric effect induced by Rheb binding.

### *15.2.2 AMPK*

The main sensor of cellular energy levels in eukaryotic cells is a trimeric kinase complex called AMP-activated protein kinase (AMPK), which is conserved from single-celled yeast to humans. AMPK is activated by elevated AMP/ATP and ADP/ATP ratios under energy stress and restores ATP levels by inhibiting anabolism to reduce consumption and promoting catabolism to produce ATP (Garcia and Shaw 2017). As a cellular process for the supply of nutrients under energy stress, autophagy can be potently activated by AMPK through several means. For example, AMPK phosphorylates and activates ULK1 to initiate the autophagic cascade. AMPK also phosphorylates TSC2 in the TSC1/TSC2 complex, enhances the potency of this complex as a GAP factor for Rheb, disrupts its binding to mTORC1, and finally inhibits mTORC1 (Inoki et al. 2003). Therefore, AMPK can promote autophagy indirectly through the mTOR pathway when under stress. In addition to targeting ULK1 in the initiating step, AMPK can also phosphorylate the VPS34 and Beclin-1 proteins that control the growth of autophagosomes. Specifically, phosphorylation of VPS34 by AMPK shifts VPS34 from the non-autophagic complex to the pro-autophagic complex that contains Beclin-1 phosphorylated by AMPK (Kim et al. 2013). In addition, AMPK also phosphorylates and regulates the localization of ATG9, a transmembrane protein that participates in the early autophagosome (Mack et al. 2012). In the autophagy of mitochondria, AMPK can directly phosphorylate mitochondrial fission factor (MFF)

to facilitate the damaged mitochondrial fission into small fragments and clearance by autophagosomes (Toyama et al. 2016).

The trimeric AMPK complex consists of a catalytic subunit (α subunit) and two regulatory subunits (β and γ subunits) (Fig. 15.5a). In mammals, the α subunit has two isoforms, while the β and γ subunits are encoded by two and three isoforms, respectively. The isoforms have very similar protein sequences but different expression levels in different tissues and can form distinct AMPK complexes with a somewhat functional redundancy (Ross et al. 2016). The α subunit has a serine/threonine kinase domain in the N-terminus, an auto-inhibitory domain and a regulatory α-linker in the middle, followed by the C-terminal domain for binding to the β and γ subunits and a serine/threonine-rich region (ST-loop) in the final C terminus (Fig. 15.5a). The β subunit contains a carbohydrate-binding module (CBM) in the middle and a C-terminal domain that binds to the α and γ subunits (Fig. 15.5a). The γ subunit contains four cystathionine β-synthase (CBS) repeats (Fig. 15.5a) and forms four potential binding sites for AMP, ADP, or ATP, which confers AMPK with the ability to sense cellular energy levels (Calabrese et al. 2014; Xiao et al. 2011, 2013). The three-dimensional structure of the AMPK complex is organized around the α subunit. The CBM domain of the β subunit wraps a large surface of the N-lobe of the kinase domain, while the CTD of the β subunit is sandwiched between the γ subunit and the CTD of the α subunit (Fig. 15.5b). The AID domain and α-linker of the α subunit further wrap around the CTD of the β and γ subunits (Fig. 15.5b). The activation loop (the phosphorylation of a critical residue T172 in the activation loop is a prerequisite for the full activation of AMPK) in the kinase domain protrudes from the catalytic cleft and resides at the interface between the C-lobe of the kinase domain and the CTD of the β subunit (Fig. 15.5b). The architecture of AMPK enables two regulatory subunits to allosterically modulate the kinase activity, mainly through the regulation of phosphorylation and dephosphorylation of T172 in the activation loop. Mechanistically, AMP molecules bind to the CBS repeats in the γ subunit, induce binding between the α-linker of the α subunit and the γ subunit, and restrict the flexibility of the α-linker, resulting in a tighter association between the C-lobe of the kinase domain and the γ subunit, protecting T172 from dephosphorylation (Gowans et al. 2013). At the same time, the binding of the α-linker to the γ subunit also shifts the AID in the α subunit away from the kinase domain, releasing its inhibitory effect. The β subunit also modulates the kinase activity allosterically. Activating compounds, such as A769662, bind to the interface between the CBM domain of the β subunit and the N-lobe of the kinase domain. The α-helix is induced in the β subunit and interacts with the C-helix (which is important for ATP binding) in the kinase domain (Xiao et al. 2013). This interaction shifts the kinase domain toward an active conformation with a higher substrate-binding affinity and protects T172 from dephosphorylation. In vivo, glycogen associates with the CBM domain of the β subunit and inhibits the interaction between CBM and the kinase domain; therefore, it seems that glycogen inhibits AMPK through a similar allosteric mechanism (Li et al. 2015). As mentioned previously, the phosphorylation of T172 in the activation loop is a hallmark of AMPK activation. Several protein kinases, such as LKB1 and CAMKK2, are the main kinases responsible for the phosphorylation of T172 in response to energy

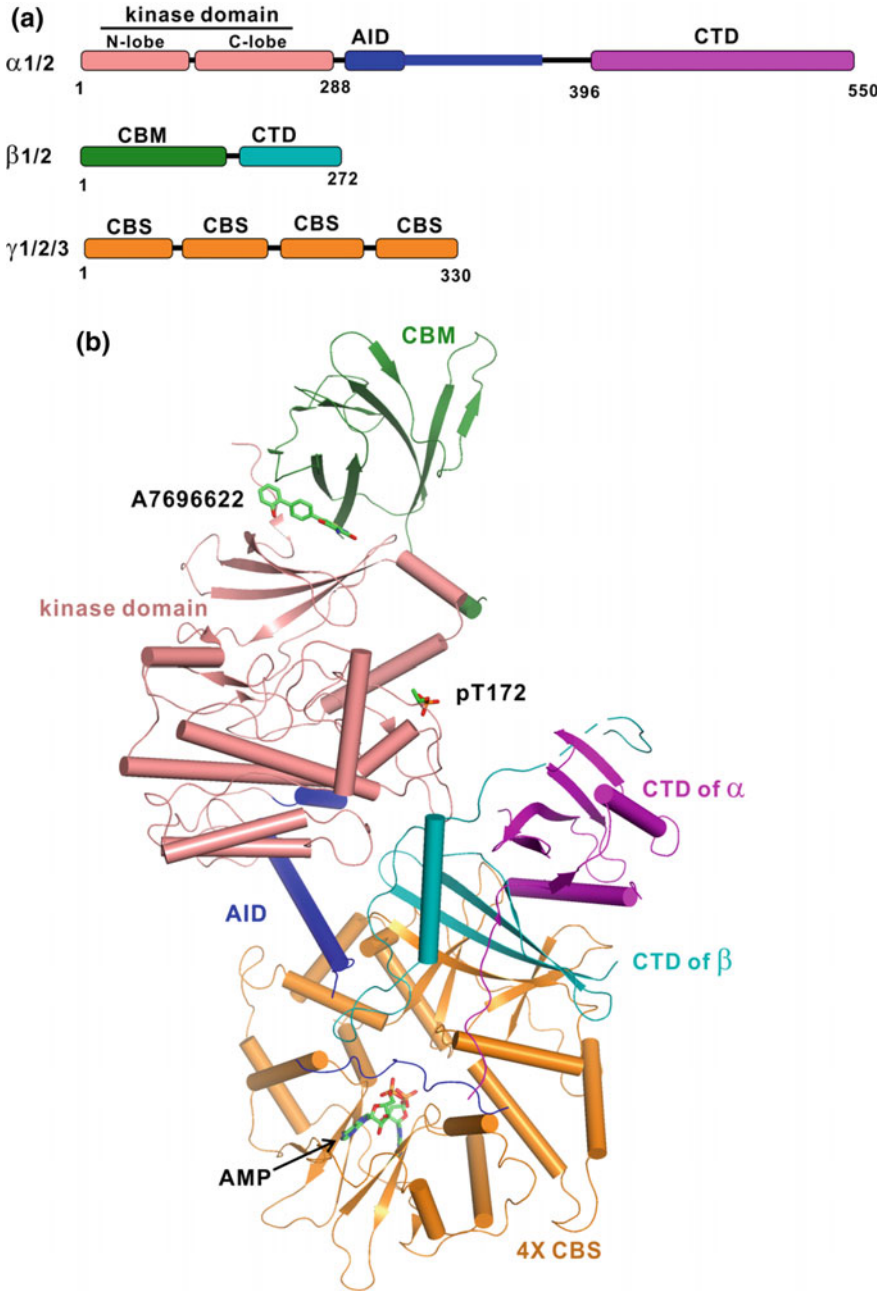


**Fig. 15.5** The structure of AMPK. **a** Domain architectures of α, β, and γ subunits in the AMPK complex. **b** The crystal structure of AMPK (PDB code: 4CFF). Domains are colored in the same scheme used in (**a**)

stress or the stimulation of hormones (Shaw et al. 2004; Hawley et al. 2005). In contrast, AMPK can be inhibited by the phosphorylation of several residues in the unstructured ST-loop, although the inhibition mechanism is still largely unknown. In addition to phosphorylation, several other types of post-translational modifications can also regulate the activity, localization or stability of AMPK, such as reactive oxygen species-mediated oxidation, myristoylation, or ubiquitination (Shao et al. 2014; Hardie 2014; Hardie et al. 2016). Structural studies of AMPK in complex with either AMP or activation compounds have revealed the activation mechanism of AMPK. These studies explain how two regulatory subunits sense the energy level through direct binding to AMP or glycogen, which either activates or inhibits the kinase domain through induced interactions between the α subunit and the regulatory subunit.

## 15.3 Structural Studies of Proteins Involved in the Formation of Autophagosomes

### *15.3.1 The ATG1/ULK Complex*

ATG1 kinase (ULK in mammals) is a vital regulatory protein in the initiation of autophagy and is the only core protein with a serine/threonine kinase activity in the autophagy pathway. In autophagy, ATG1/ULK associates with several other proteins to form a large protein complex to conduct its autophagic function and to regulate the initiation process of autophagy (Hurley and Young 2017; Mercer et al. 2018). In yeast, the ATG1 core complex consists of ATG1, ATG13, ATG17, ATG29, ATG31, and ATG11 (Fig. 15.6a). As a binding partner of ATG1, ATG13 is required for

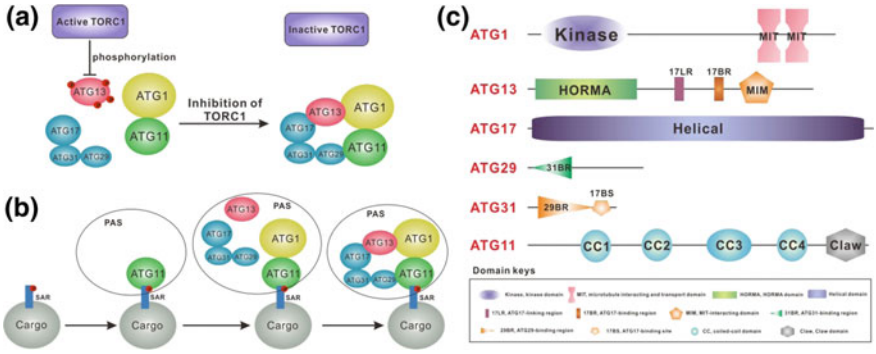


**Fig. 15.6** The components and organization of the ATG1 complex in yeast. **a** A schematic diagram showing the assembly mode of the ATG1 complex under the conditions of nutrient deprivation or TORC1 inhibition. **b** A schematic diagram showing the assembly mode of the ATG1 complex in selective autophagy. **c** The domain organizations of proteins in the ATG1 complex

ATG1 kinase activity and the progression of autophagy. ATG13 is heavily phosphorylated under nutrient-rich conditions but is rapidly dephosphorylated upon nutrient deprivation or the inhibition of target of rapamycin complex 1 (TORC1) (Farre and Subramani 2016). Then, dephosphorylated ATG13 can interact with ATG1 and ATG17 at the phagophore assembly site (PAS). Given that ATG17 is a part of the stable ATG17/ATG29/ATG31 sub-complex, under nutrient deprivation conditions or the inhibition of TORC1, an ATG1/ATG13/ATG17/ATG29/ATG31 quinary complex will form (Fig. 15.6a). This large protein complex can function as a scaffold to further recruit downstream autophagic proteins to the PAS, thereby initiating the formation of autophagosomes (Kamada et al. 2000; Farre and Subramani 2016). In the selective autophagy processes of yeast, after some intracellular autophagy stimulations, the selective autophagy receptors (SAR) bind to autophagic cargoes and become activated. Then, the activated SAR is recognized by ATG11, which in turn recruits ATG1. Next, ATG1 associates with ATG13 and the related ATG17/ATG29/ATG31 sub-complex to form a protein complex with a large molecular weight to link with SAR (Fig. 15.6b) (Farre and Subramani 2016). Thereafter, the activated ATG1 complex recruits additional autophagy proteins, leading to the recruitment of ATG8 and, ultimately, the expansion of the phagophore (Farre and Subramani 2016).

As a key component of the ATG1 complex, ATG1 mainly contains an N-terminal kinase domain and two C-terminal MIT (microtubule interacting and transport) domains, MIT1 and MIT2 (Fig. 15.6c). ATG13 mainly consists of an N-terminal HORMA, an ATG17-linking region (17LR) and an ATG17-binding region (17BR), both of which can interact with ATG17, and a C-terminal MIT-interacting motif (MIM) (Fig. 15.6c). ATG17 consists of a single helical domain (Fig. 15.6c), which can mediate the dimerization of ATG17. The adaptor protein ATG11 is a large protein and contains four coiled-coil domains and a C-terminal Claw domain (Fig. 15.6c), which recognizes the activated selective autophagy receptors. Previous biochemical and structural studies showed that the two MIT domains of ATG1 form a dimer in solution and can directly interact with the MIM domain of ATG13 to mediate ATG1/ATG13 complex formation (Fujioka et al. 2014; Ragusa et al. 2012). In the determined crystal structure of the monomeric ATG1/ATG13 complex (Fujioka et al. 2014), each of the two MIT domains of ATG1 fold into a similar antiparallel three-helix bundle, while the ATG1-binding MIM domain of ATG13 mainly contains two α-helices and a connecting linker, and in particular, the two α-helices of ATG13 MIM domain pack against two grooves located at opposite sides of ATG1 (Fig. 15.7a). In addition, the recently solved ATG13/ATG17/ATG29/ATG31 complex structure revealed that the whole complex adopts an S-shaped dimeric configuration (Yamamoto et al. 2016), and the structural core is assembled by two crescent-shaped ATG17 molecules (Fig. 15.7b). Specifically, two ATG17 molecules interact with each other in the C-terminal region to mediate dimer formation, and two ATG29/ATG31 complexes bind to the concave surface of each crescent-shaped ATG17 (Fig. 15.7b). Notably, only part of the ATG31 molecule in the ATG29/ATG31 complex directly contacts ATG17 (Fig. 15.7b). Meanwhile, through its 17BR and 17LR sites, ATG13 interacts with ATG17 (Fig. 15.7b). It is worth noting that an ATG13 molecule can use its 17BR and 17LR sites to simultaneously associate with two different ATG17

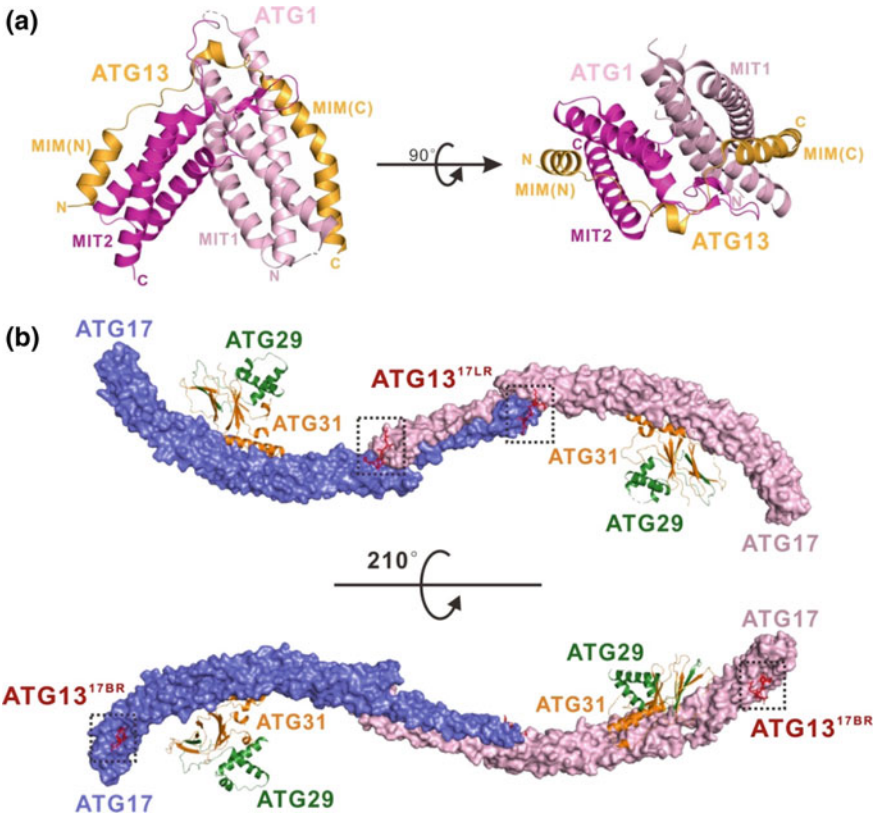


**Fig. 15.7** Currently known structures related to the ATG1 complex. **a** The overall structure of the ATG1/ATG13 complex (PDB ID: 4P1N). **b** The combined surface representation and the ribbon-stick model showing the overall crystal structure of the ATG13/ATG17/ATG31/ATG29 complex (PDB ID: 5JHF). In this representation, the ATG17 molecule is shown as a surface model, the ATG29/ATG31 complex as a cartoon model, and ATG13 as a stick model

dimers, thereby promoting the self-assembly of multimeric ATG1 complexes for PAS organization (Yamamoto et al. 2016). Unfortunately, the structure of ATG11 and how ATG11 interacts with the ATG13/ATG17/ATG29/ATG31 complex are still unknown.

In mammalian cells, the mammalian homologues of ATG1 are the uncoordinated-51-like kinases 1 and 2 (ULK1 and ULK2), and the ULK complex is composed of ULK1/2, ATG13, ATG101, and FIP200 (also named RB1CC1) (Fig. 15.8a). In mammals, ATG13 and FIP200 have some structural and functional similarities to the ATG13, ATG11, and ATG17 proteins in yeast, and both of these proteins can stabilize ULK1/2 to increase its kinase activity (Hurley and Young 2017; Mercer et al. 2018). ATG101 is a completely novel autophagy protein in mammals and has no homology in yeast; it helps maintain ULK1/2 basal phosphorylation and promotes its stabilization together with ATG13 (Hurley and Young 2017; Mercer et al. 2018).

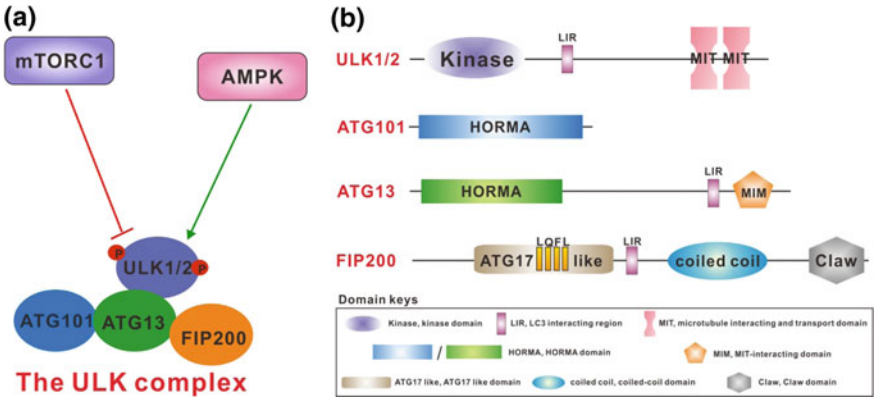


**Fig. 15.8** The components and organization of the ULK complex in mammals. **a** A schematic diagram showing the assembly of the ULK complex and its regulation by mTORC1 and AMPK. **b** The domain organizations of proteins in the ULK complex

In contrast to that of the ATG1 complex in yeast, the formation of the ULK complex is not regulated by nutrient status (Hosokawa et al. 2009). The ULK complex serves as a bridge to connect the upstream nutrient/energy sensors, mTOR1 and AMPK, with the downstream molecular machines for autophagosome formation (Fig. 15.8a). The AMPK and mTOR kinases have been found to mediate the phosphorylation of ULK1/2, and phosphorylated ULK1/2 has been considered as a key regulator of autophagy (Kim et al. 2011). For instance, mTOR1, through its Raptor subunit, can directly bind to ULK1/2 in a manner dependent on cellular amino acid availability and inhibits autophagy via phosphorylation of ULK1 at multiple sites (Kim et al. 2011; Hurley and Young 2017; Hosokawa et al. 2009).

Like ATG1 in yeast, ULK1/2 contains an N-terminal kinase domain, a LIR motif, and two C-terminal MIT domains (Fig. 15.8b). ATG13 in mammals has a similar domain organization as that of ATG13 in yeast and contains an N-terminal HORMA domain followed by an LIR motif, along with a C-terminal MIM domain that can bind to the MIT domains of ULK1/2 (Fig. 15.8b). ATG101 contains only one HORMA domain (Fig. 15.8b). There is no apparent ATG17 homologue in mammals, but FIP200 (a focal adhesion kinase family-interacting protein of 200 kDa) could serve as a functional counterpart. FIP200 is a large scaffold protein, and detailed sequence analyses suggest that FIP200 could be a hybrid molecule of ATG17 and ATG11. FIP200 contains an N-terminal ATG17-like domain, an LIR motif, a coiled-coil region, and a C-terminal Claw domain that is also found in the C-terminal of ATG11 (Fig. 15.8b). So far, the atomic structure of the entire ULK complex has not been solved. However, previous structural studies have revealed that the HORMA domain of ATG13 can directly interact with the HORMA domain of ATG101 (Suzuki et al. 2015; Qi et al. 2015), forming a 1:1 heterodimer (Fig. 15.9a). Furthermore, the HORMA domain of ATG101 has a highly conserved motif containing Trp110 and Phe112, which is called the WF-finger (Fig. 15.9a). In the ATG101/ATG13 complex

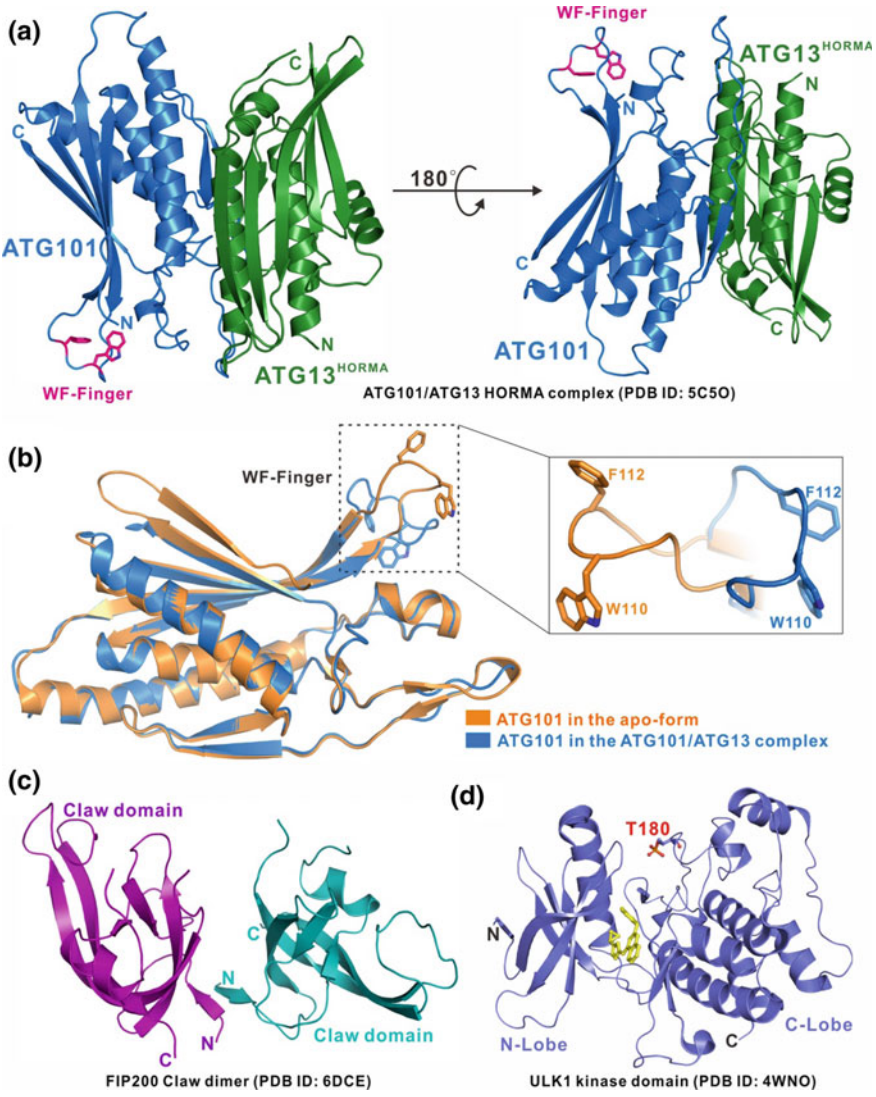


**Fig. 15.9** Determined structures related to the ULK complex. **a** The overall structure of ATG101 in complex with the ATG13 HORMA domain (PDB ID: 5C50). In this drawing, ATG101 is colored in blue and ATG13 HORMA in green. The unique WF-finger is shown in the stick mode and colored in pink. **b** Structural comparison of the WF-finger conformations in the apo-form (PDB ID: 4WZG) (orange) and in the ATG101/ATG13 complex (blue). **c** The overall structure of the dimeric Claw domain of FIP200 (PDB ID: 6DCE). **d** The ribbon-stick model showing the overall structure of the ULK1 kinase domain bound to an inhibitor (PDB ID: 4WNO). In this representation, the bound inhibitor and the phosphorylated T180 residue are shown in the stick model

structure, the WF-finger has a distinct conformation compared with that in the free ATG101 structure (Fig. 15.9b). In particular, in the apo-form structure of human ATG101 (Michel et al. 2015), the WF-finger can exist in an out conformation, while the WF-finger is folded back onto the core HORMA domain in a closed conformation in the complex structure (Fig. 15.9b) (Suzuki et al. 2015). The WF-finger of ATG101 was demonstrated to be a functionally important motif responsible for recruiting downstream factors to the autophagosome formation site in mammals (Suzuki et al. 2015). However, the downstream effectors of the ATG101 WF-finger remain unknown. Recently, Sascha Martens and colleagues determined the crystal structure of the FIP200 Claw domain (Turco et al. 2019), which adopts an oligonucleotide/oligosaccharide binding fold and is assembled by a six-stranded β-sheet together with a short α-helix (Fig. 15.9c). However, due to the lack of complex structures, the detailed binding modes of the Claw domain with autophagy receptors are unknown. Furthermore, the determined crystal structure of the human ULK1 kinase domain revealed that it adopts a canonical kinase domain fold (Lazarus et al. 2015), consisting of an N-lobe and a C-lobe linked by a long connecting loop (Fig. 15.9d). The crystal structure also showed a phosphothreonine in the activation loop at Thr180, which is induced by an auto-phosphorylation event (Fig. 15.9d). Given the crucial role of ULK1 in autophagy, many diseases, including cancer, neurodegenerative diseases, and Crohn's disease, can be attributed to the impairment of the autophagy regulation function (Lee et al. 2013). Especially in cancer, ULK1 has become a potential therapeutic target (Chen et al. 2017). Small-molecule agonists targeting the autophagy promoter ULK1 can initiate the process of autophagy, leading to the inhibition and even death of tumor cells. Therefore, the development of ULK1 agonists has become a hot topic of current research.

### *15.3.2 The Class III PI3K Complex*

PI3Ks are a family of related intracellular signal-transducing kinases that have the ability to phosphorylate phosphoinositides on the 3′ position of the inositol ring (Whitman et al. 1988). PI3Ks can be divided into three classes based on different structures and substrate preferences (Fritsch and Downward 2013). The class III PI3K complex, PI3KC3, consisting of a catalytic subunit Vps34 and several regulatory subunits, can phosphorylate phosphatidylinositol (PI) to generate phosphatidylinositol 3-phosphate (PI3P), is functional downstream of the ULK complex (the ATG1 complex in yeast), and is essential for autophagy initiation (Fig. 15.10a, b) (Hurley and Young 2017). As yet, two types of PI3KC3 complexes have been identified: PI3KC3-C1 and PI3KC3-C2. These complexes have three identical components, namely, Vps34, Vps15 (P150), and Beclin-1 (Vps30 or ATG6 in yeast); however, they differ in the fourth component: ATG14L (ATG14 in yeast) for PI3KC3-C1 or UVRAG (Vps38 in yeast) for PI3KC3-C2 (Fig. 15.10c) (Hurley and Young 2017; Backer 2016). PI3KC3-C1 works in the early stage of autophagy and is important for autophagy initiation. In contrast, PI3KC3-C2 primarily functions in the later stages

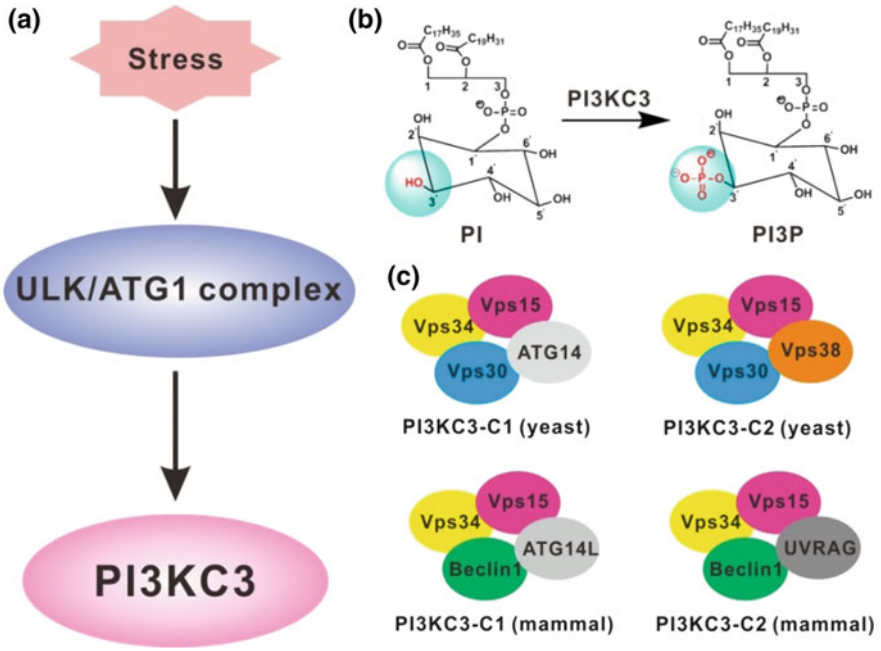


**Fig. 15.10** The PI3KC3 complex in the autophagy pathway. **a** A schematic diagram showing the signals transduced from the ULK/ATG1 complex to the downstream PI3KC3 complex in autophagy. **b** The enzymatic reaction catalyzed by PI3KC3. **c** A schematic cartoon diagram showing the assemblies of PI3KC3-C1 and PI3KC3-C2 in yeast and mammals

of autophagy, such as in autophagosome–lysosome fusion and autolysosomal tubulation (Kim et al. 2015a; Munson et al. 2015), and additionally, PI3KC3-C2 plays a non-autophagic role in endosome trafficking and multivesicular body formation (Backer 2008).

In yeast, Vps34 contains an N-terminal lipid-binding C2 domain, a helical domain, and a C-terminal kinase domain (Fig. 15.11a). Vps15 contains an N-terminal kinase domain that can bind the kinase domain of Vps34, a helical domain, and a C-terminal WD40-repeat domain (Fig. 15.11a). Vps30 has an N-terminal domain, two coiled-coil domains, and a BARA domain (Fig. 15.11a). Vps38 contains a C2 domain, a coiled-coil domain, and a BARA2 domain (Fig. 15.11a) that can interact with the BARA domain of Vps30. ATG14 only has a coiled-coil domain (Fig. 15.11a). The determined crystal structure of PI3KC3-C2 in yeast shows that PI3KC3-C2 displays a 1:1:1:1 molar ratio of four subunits and adopts an overall Y-shaped architecture, consisting of two long arms and a short hook-like base (Fig. 15.11b) (Rostislavleva et al. 2015). Specifically, the base of PI3KC3-C2 is formed by the NTD and CC1 domains of Vps30 and by the CC1 domain of Vps38 (Fig. 15.11b). One of the arms is assembled by Vps15 and Vps34, and the other arm includes domains from all four subunits (Fig. 15.11b). The C2 domain of Vps34 is located at the heart of the complex and interacts with all of the subunits (Fig. 15.11b). Vps34 and Vps15 interact with

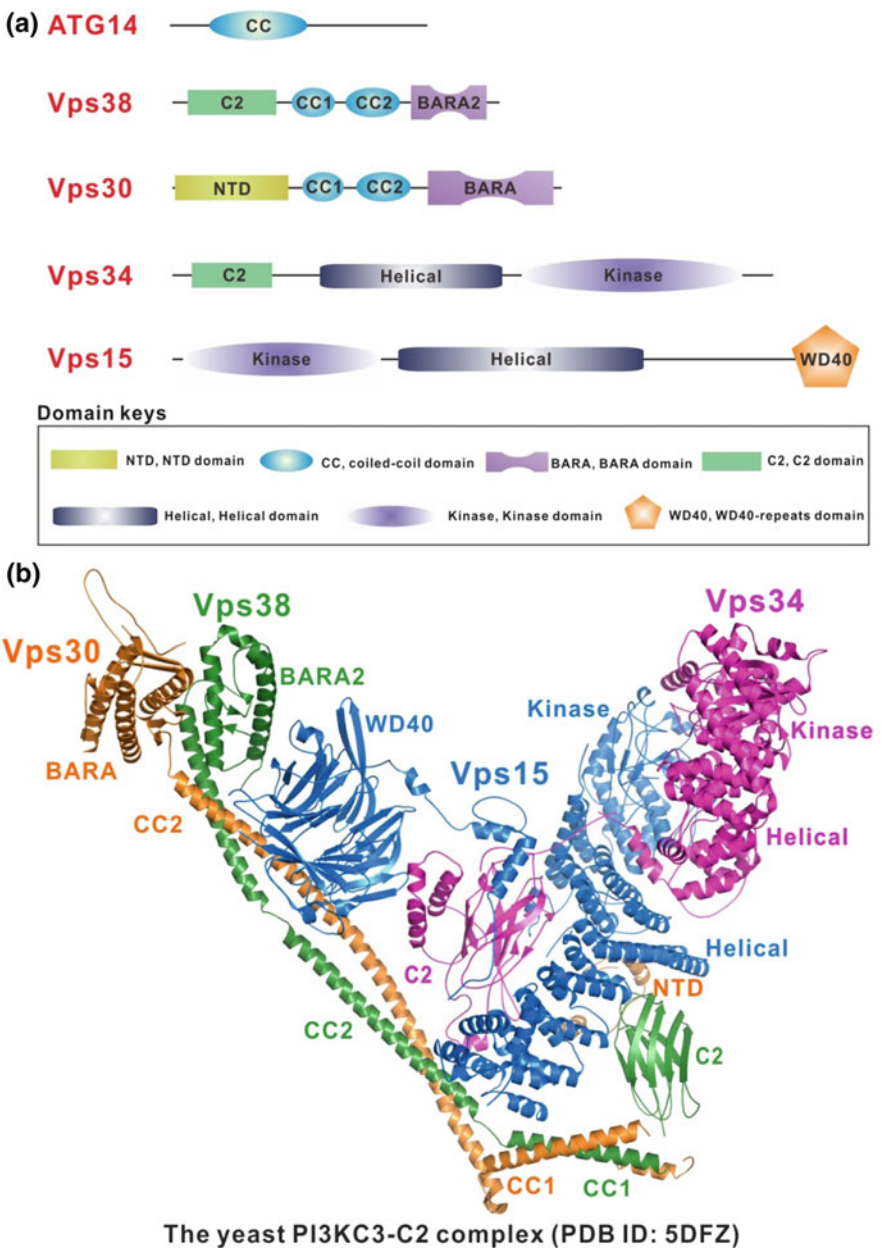


**Fig. 15.11** The related components and currently known structures of the yeast PI3KC3 complexes. **a** The domain organizations of proteins involved in the yeast PI3KC3 complexes. **b** The overall crystal structure of yeast PI3KC3-C2 (PDB ID: 5DFZ). In this drawing, Vps34 is colored in magenta, Vps15 in blue, Vps38 in green, and Vps30 in orange

each other in an antiparallel fashion via their kinase domains (Fig. 15.11b). Vps30 and Vps38 show similar architectures, but they show differences in their N-terminal regions, where Vps38 has a C2 domain and Vps30 has an NTD domain. In the C-terminal, Vps30 has a BARA domain that binds side-by-side to the BARA2 domain of Vps38 (Fig. 15.11b). The tips of the two arms, the kinase domain of Vps34 and the Vps15 N-terminal region of one arm, as well as the BARA domains of Vps30/Beclin-1 of the other arm, are likely responsible for the membrane interaction of PI3KC3-C2 (Rostislavleva et al. 2015).

In mammals, Vps34 contains an N-terminal lipid-binding C2 domain, a helical domain, and a C-terminal kinase domain (Fig. 15.12a). P150 (Vps15) contains an N-terminal kinase domain, followed by a HEAT domain and a C-terminal WD40-repeat domain (Fig. 15.12a). Beclin-1 has a coiled-coil domain and a BARA domain (Fig. 15.12a). UVRAG mainly contains a C2 domain, followed by a coiled-coil domain (Fig. 15.12a). ATG14L has a coiled-coil domain and a C-terminal BATs domain that can bind to the membrane (Fig. 15.12a). Recently, the cryo-EM structures of human PI3KC3-C1 and PI3KC3-C2 have been reconstructed at sub-nanometer resolutions (Ma et al. 2017). Human PI3KC3-C1 and PI3KC3-C2 share a common L-shaped overall architecture with two arms, but they have distinct features (Fig. 15.12b, c). Interestingly, biochemical and structural characterizations revealed that the ATG14L BATs domain and the C-terminal region of Vps34 directly bind to the membrane, and the BATs domain of ATG14L is responsible for the membrane anchoring of PI3KC3-C1, whereas the C-terminal region of Vps34 determines the orientation of PI3KC3-C1 on the membrane (Ma et al. 2017). Owing to the lack of the BATs domain in ATG14L, PI3KC3-C2 binds much more weakly than PI3KC3-C1 to PI-containing membrane structures (Ma et al. 2017). However, due to the lower resolution of these cryo-EM structures, the precise molecular features that underlie the functional differences between the PI3KC3-C1 and PI3KC3-C2 complexes are still unknown.

### *15.3.3 The ATG8 and ATG12 Ub-like Conjugation Systems*

ATG8 and ATG12 are two ubiquitin-like (UBL) family members specifically involved in the autophagy pathway. The recruitment of ATG8 family proteins to the forming phagophore membrane is an essential event in autophagy and is mediated by two ubiquitin-like (UBL) cascades that are highly conserved in eukaryotic cells (Geng and Klionsky 2008). The ATG8 family proteins in mammals can be divided into two main subfamilies, LC3 and GABARAP, and include seven members known as MAP1LC3A (LC3A), MAP1LC3B (LC3B), MAP1LC3B2 (LC3B2), MAP1LC3C (LC3C), GABARAP, GABARAPL1, and GABARAPL2. Despite the lack of similarity in amino acid sequence, the crystal structures of ATG8 family proteins and ATG12 reveal a conserved ubiquitin-like structural core (Fig. 15.13a–c). In addition, the conjugations of ATG8 family proteins with phosphatidylethanolamine (PE) and ATG12 with ATG5 are both mediated by an enzymatic cascade that is similar to the

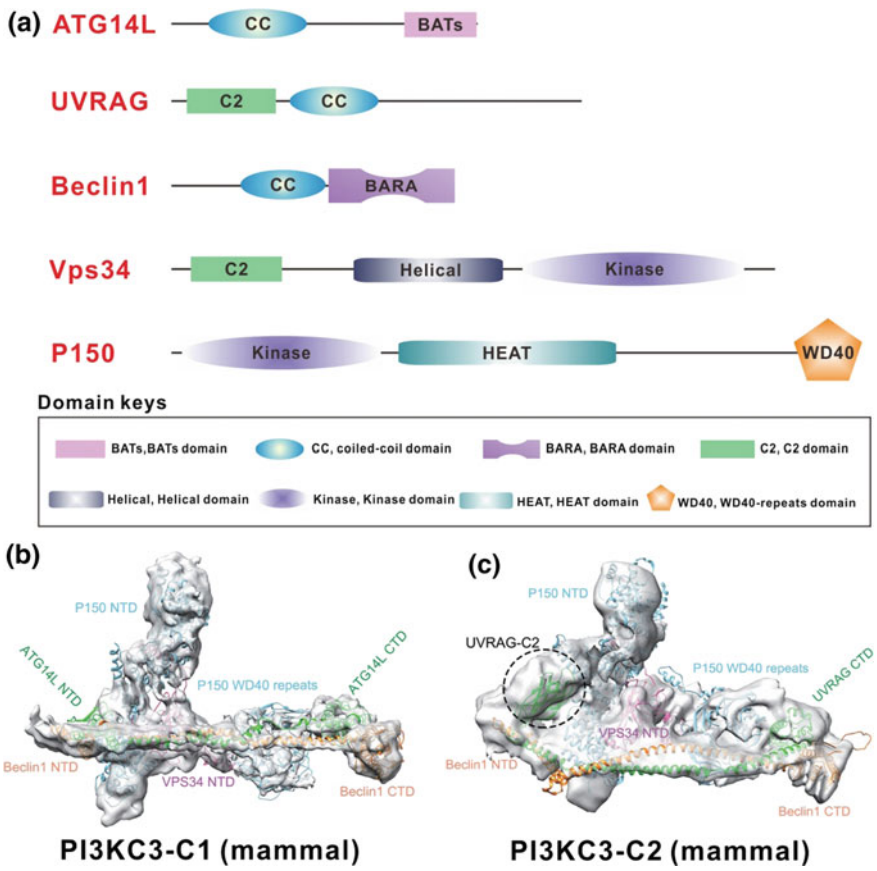


**Fig. 15.12** The components and the cryo-EM structures of the PI3KC3 complexes in mammals. **a** The domain organizations of proteins involved in the PI3KC3 complexes in mammals. **b** The EM volume of PI3KC3-C1 docked with the built atomic model. In this drawing, the Beclin-1, ATG14L, Vps34, and P150 protein subunits are colored in orange, light green, hot pink, and cyan, respectively. **c** The EM volume of PI3KC3-C2 docked with the hypothetical atomic model of PI3KC3-C2 (the C-terminal region of Vps34 is excluded), which was directly extracted from the structure of yeast PI3KC3-C2 (PDB ID: 5DFZ). The (**b**) and (**c**) panels are adapted and modified from a recent study (Ma et al. 2017)

ubiquitin system (Fig. 15.14). Specifically, the extreme C-terminal region of an ATG8 family protein is first removed by proteolytic processing mediated by the ATG4 family proteases to expose a glycine residue (Fig. 15.14c). This exposed glycine forms a thioester bond with a cysteine residue in the E1-like ATG7, followed by conjugation to ATG3, an E2-like enzyme (Fig. 15.14c). The ATG12–ATG5/ATG16L1 complex, having an E3-like activity, is formed in the second ubiquitin-like cascade involving ATG12, which is covalently linked to ATG5 by the E1-like ATG7 and the E2-like ATG10 (Fig. 15.14b). Through the interaction between ATG16L1 and WIPI2b, the

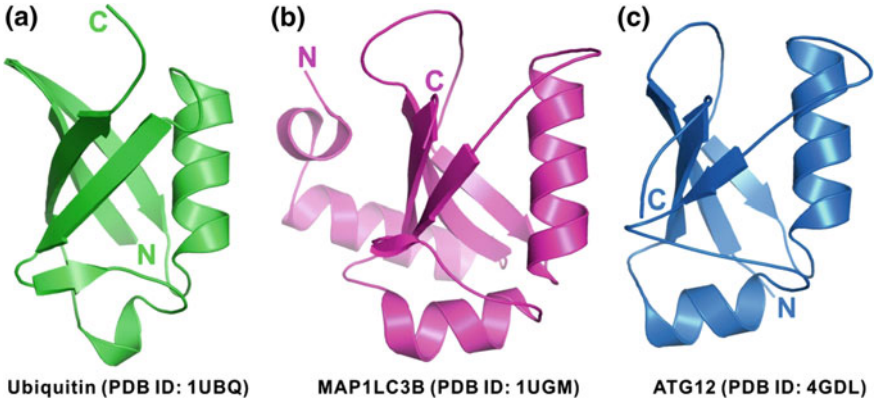


**Fig. 15.13** Structural comparisons of ubiquitin, the ATG8 family member MAP1LC3B, and ATG12. **a**–**c** Ribbon diagrams showing the overall structures of ubiquitin (PDB ID: 1UBQ) (**a**), MAP1LC3B (PDB ID: 1UGM) (**b**), and ATG12 (PDB ID: 4GDL) (**c**). In this drawing, the three structures are shown in the same orientation

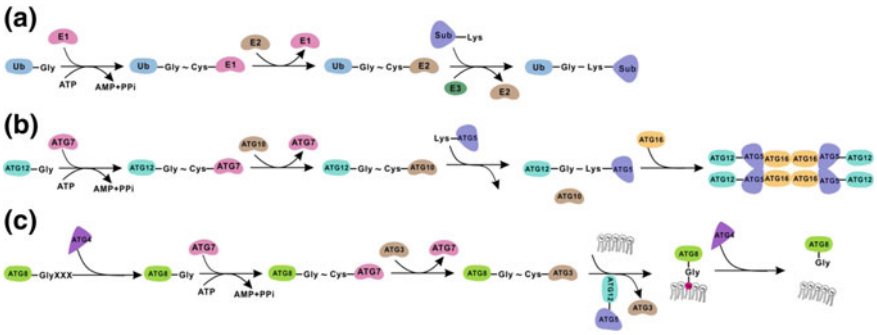


**Fig. 15.14** The conjugations of ATG8 with PE, and ATG12 with ATG5, are both mediated by ubiquitin-like enzymatic cascade. **a** A schematic diagram showing the conjugation process in the ubiquitin system. **b** A schematic diagram showing the conjugation process of ATG12 with ATG5. **c** A schematic diagram showing the conjugation process of ATG8 with PE. PE, phosphatidylethanolamine. PPi, pyrophosphate. Ub, ubiquitin. Sub, substrate

ATG12–ATG5/ATG16L1 complex is recruited to the phagophore where it can further recruit the ATG3–ATG8 conjugate mediated by the specific interaction between ATG12 and ATG3. Ultimately, with the help of the ATG12–ATG5/ATG16L1 complex, the C-terminal glycine residue of ATG8 conjugated with ATG3 is covalently linked to the amine head group of PE in the phagophore membrane, leading to the formation of the ATG8-PE adduct (Fig. 15.14c). The ATG8-PE adducts are incorporated into the growing phagophore membrane and decorate both the outer and inner membranes of phagophores. The importance of ATG7, ATG3, ATG10 and other components of the ATG8 and ATG12 ligation pathways is reflected by the numerous pathologies observed in animals lacking these proteins (Geng and Klionsky 2008).

Therefore, major efforts have been devoted to characterizing the structures and functions of these proteins. In the following, we will summarize some important structural findings from these studies.

#### 15.3.3.1 The Cleavage of ATG8 Family Proteins by ATG4

The cleavage of a stretch of extreme C-terminal residues of an ATG8 family protein is mediated by the ATG4 family proteases, which have four homologues in mammals (ATG4A-D), with ATG4B performing the primary role in autophagy (Fernandez and Lopez-Otin 2015). Structural studies of ATG4B revealed that it comprised a classical papain-like domain and a unique short-finger domain that is specific to the ATG4 family proteases (Fig. 15.15a) (Sugawara et al. 2005). The papain-like domain of ATG4B contains a catalytic triad formed by the critical C74, D278, and H280 residues

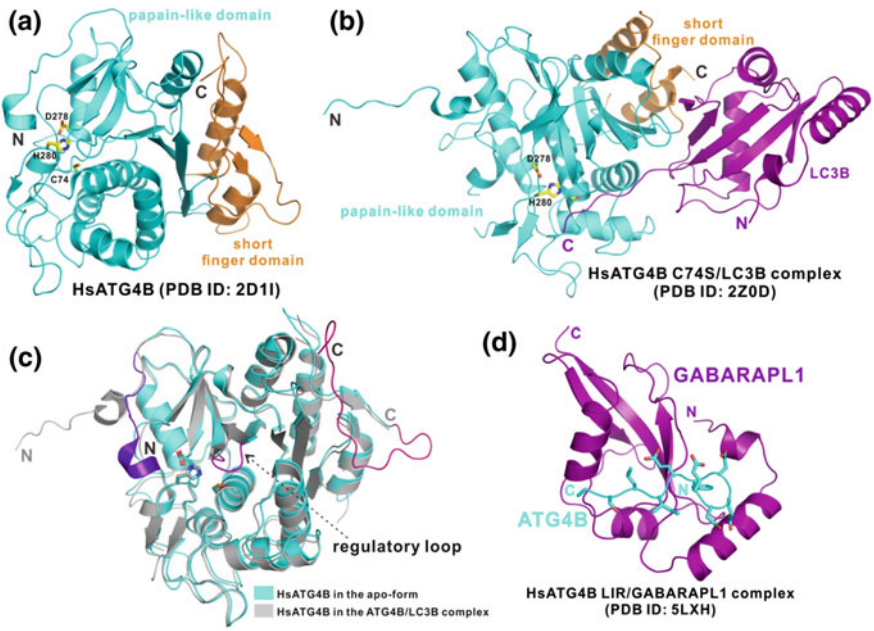


**Fig. 15.15** Determined structures related to human ATG4B. **a** The overall structure of human ATG4B (PDB ID: 2D1I). In this drawing, the papain-like domain of ATG4B is colored in cyan, and the short finger domain of ATG4B is in orange. The side chains of the critical C74, D278, and H280 residues forming a catalytic triad are shown in the stick mode. **b** The ribbon-stick model showing the overall structure of human ATG4B C74S mutant in complex with LC3B (PDB ID: 2Z0D). **c** Structural comparison of the conformations of human ATG4B in the apo-form (PDB ID: 2D1I) (cyan) and in the ATG4B/LC3B complex (PDB ID: 2Z0D) (gray). **d** The ribbon-stick model showing the overall structure of GABARAPL1 in complex with the C-terminal LIR motif of ATG4B (PDB ID: 5LXH). In this presentation, the bound LIR motif of ATG4B is shown in the stick model

(Fig. 15.15a), which are strictly conserved among ATG4 homologues. Overall, the ATG4 family proteins have high structural similarities to papain family cysteine proteases and deubiquitinating enzymes (DUBs), and their catalytic mechanism is similar to that of papain (Sugawara et al. 2005). In the determined complex structure of ATG4B and LC3B (Satoo et al. 2009), LC3B mainly consists of three segments: the N-terminal segment consisting of two α-helices, the central ubiquitin-like core, and a C-terminal tail (Fig. 15.15b). The ubiquitin core of LC3B is bound to the interface between the two sub-domains of ATG4B, and the C-terminal tail of LC3B inserts into the active site of ATG4B located in the central region of the papain-like domain (Fig. 15.15b). The binding sites of LC3B for ATG4B are localized in the C-terminal tail and its surrounding region, while the N-terminal segment and the canonical LIR-binding pocket have no interaction with ATG4B (Fig. 15.15b). Comparing the structures of free ATG4B and the ATG4B/LC3B complex reveals that the regulatory loop, along with the N-terminal and C-terminal tails of ATG4B, undergoes large conformational changes in the complex structure (Fig. 15.15c). In particular, the regulatory loop masking the entrance of the active site of free ATG4B is lifted up, and the C-terminal region is dislodged from the LC3-binding site, thereby generating a groove along which the LC3B tail enters the active site (Fig. 15.15c). Meanwhile, the N-terminal tail masking the exit of the active site of free ATG4B is detached from the enzyme core to expose a large flat surface (Fig. 15.15c), which might enable ATG4B to access the membrane-bound LC3B-PE molecules. Interestingly, a recent structural study showed that the C-terminal region of ATG4B contains a canonical LIR motif (Fig. 15.15d), which was required for the efficient cleavage of ATG8 family proteins and the stabilization of the unlipidated forms of GABARAP and GABARAPL1 in cells (Rasmussen et al. 2017).

#### 15.3.3.2 The Recognition and Activation of ATG8 and ATG12 by ATG7

As an E1-like enzyme in autophagy, ATG7 activates and forms a thioester bond with two ubiquitin-like proteins, ATG8 and ATG12, in an ATP-dependent manner. Extensive structural studies revealed that ATG7 mainly comprised two globular domains, a unique N-terminal domain (NTD) that does not share significant homology with other E1 s, and a C-terminal domain (CTD), which comprised an adenylation domain (AD) and an extreme C-terminal ATG7-specific domain (ECTD) (Fig. 15.16a, b) (Noda et al. 2011; Hong et al. 2011). In contrast to canonical E1s, ATG7 lacks a separate catalytic cysteine domain; instead, ATG7 contains a catalytic Cys residue within the adenylation domain (Fig. 15.16a). The CTD is responsible for the dimer formation of ATG7 (Fig. 15.16b) and can specifically bind and activate ATG8 and ATG12. In particular, according to the crystal structure of the ATG7 CTD/ATG8 complex, the distance between the carboxyl group of the extreme C-terminal Gly residue of ATG8 and the bound α-phosphate group of ATP is short enough to permit adenylation without large conformational changes (Fig. 15.16c). After adenylation of ATG8, the catalytic Cys507 residue of ATG7 attacks the adenylate to form ATG7–ATG8 thioester intermediates. However, the extreme C-terminal Gly residue of ATG8 is

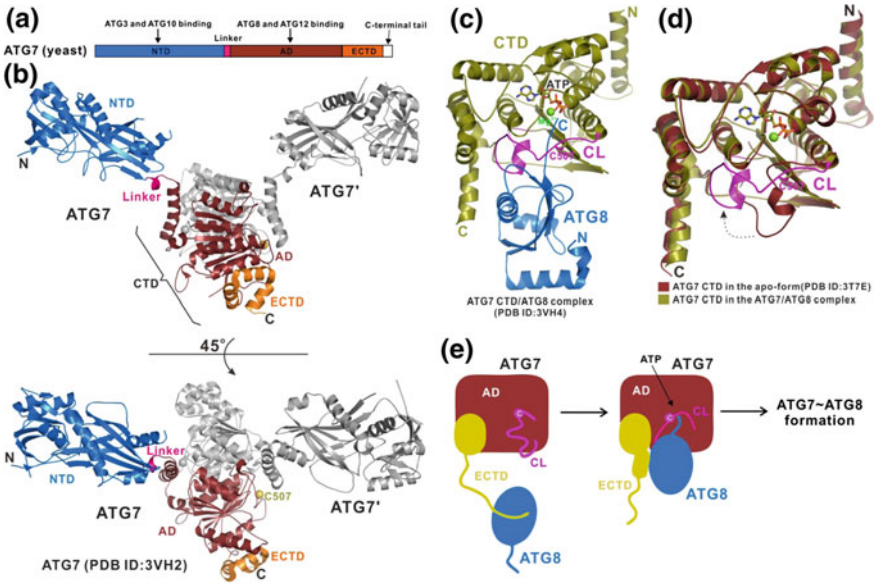


**Fig. 15.16** Structural basis for the recognition and activation of ATG8 by ATG7. **a** The domain organization of ATG7. **b** The overall structure of yeast ATG7 dimer (PDB ID: 3VH2). In this drawing, the NTD domain of one ATG7 molecule in the homodimer is colored in blue, the short linker is in pink, the AD domain is in brown, and the ECTD domain of ATG7 is in orange. The side chain of the catalytic C507 residue located in the AD domain of ATG7 is shown in the stick-sphere mode. **c** The ribbon-stick model showing the overall structure of the CTD domain of yeast ATG7 in complex with ATG8 (PDB ID: 3VH4). The bound ATP molecule and the catalytic C507 residue are shown in the stick model, and the CL of ATG7 is colored in magenta. **d** Structural comparison of the conformations of the ATG7 CTD domain in the apo-form (PDB ID: 3T7E) (brown) and in the ATG7/ATG8 complex (PDB ID: 3VH4) (olive). **e** A schematic diagram showing how ATG8 is recognized and activated by ATG7

far away from the catalytic Cys507 residue of ATG7, which is impossible for the thioester bond formation reaction between them (Fig. 15.16c). Therefore, a large conformational change in the unique ATG7 crossover loop (CL), where Cys507 is located, is likely required for the formation of the ATG7–ATG8 thioester intermediate. Notably, ATG7 CL has two distinct conformations in the structures of free ATG7 CTD and ATG7 CTD in complex with ATG8 (Fig. 15.16d), suggesting that the conformation of ATG7 CL is intrinsically variable, which may be sufficient for positioning the catalytic Cys507 residue in proximity to the activated C terminus of ATG8. In addition, the C-terminal loop of ATG7 ECTD contains an LIR motif. Biochemical and structural studies demonstrate that ATG8 is initially recognized by the C-terminal tail of ECTD and is then transferred to an AD, where the extreme C-terminal Gly residue of ATG8 is attacked by the catalytic cysteine residue to form a thioester bond (Fig. 15.16e) (Noda et al. 2011). Unfortunately, due to the lack of any available structural information for the ATG7/ATG12 complex, the detailed mechanism for the recognition and activation of ATG12 by ATG7 remains unknown.

#### 15.3.3.3 The Recognition and Loading of ATG3 and ATG10 by ATG7

The NTD of ATG7 can specifically recognize two distinct autophagic E2-like proteins, ATG3 and ATG10. Recent structural studies revealed that ATG3 alone adopts a hammer-like architecture, mainly consisting of a "handle" and a "head" (Fig. 15.17a) (Kaiser et al. 2012; Yamaguchi et al. 2012; Yamada et al. 2007). Specifically, the "head" region resembles a canonical E2 core domain, while the "handle" region (HR), consisting of a long helix and a loop (Fig. 15.17a), is a structural segment unique to ATG3 and contains an LIR/AIM motif, which can specifically interact with ATG8. In addition, at the interface between the "head" and the "handle" moieties, ATG3 has a unique flexible region (FR) that contains a short helix and mediates the binding of ATG7 (Fig. 15.17a). In contrast, ATG10 lacks the unique FR and the LIR/AIM motif in its primary structure. The structure of ATG10 shows that it forms an elongated shape and resembles a canonical E2 core domain (Fig. 15.17b) (Hong et al. 2012). Notably, although ATG3 and ATG10 have different C-terminal structures, their catalytic domains share similar structural features. Both contain an N-terminal helix followed by a backside four-stranded antiparallel β-sheet, a cysteine loop, and a long central helix. Interestingly, unlike ATG3, ATG10 has a C-terminal extension of the fourth β-strand, which folds into a β-hairpin and forms an additional strand that is incorporated into an extended β-sheet on the backside of the

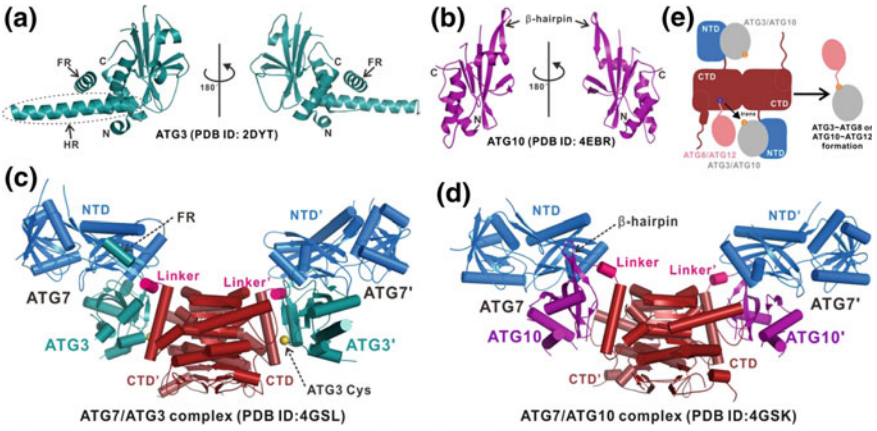


**Fig. 15.17** Structural basis for the recognition and loading of ATG3 and ATG10 by ATG7. **a** The overall structure of yeast ATG3 (PDB ID: 2DYT). The "handle" region (HR) and the unique flexible region (FR) of ATG7 are further highlighted. **b** The overall structure of yeast ATG10 (PDB ID: 4EBR). The β-hairpin region of ATG7 is indicated. **c** The overall structure of yeast ATG7 in complex with ATG3 (PDB ID: 4GSL). In this drawing, the NTD domain of ATG7 is colored in blue, the short linker is in pink, and the CTD domain is in brown. The side chain of the catalytic Cys residue of ATG3 is shown in the stick-sphere mode. **d** The overall structure of yeast ATG7 in complex with ATG10 (PDB ID: 4GSK). In this drawing, the NTD domain of ATG7 is colored in blue, the short linker is in pink, and the CTD domain is in brown. The bound ATG10 is shown in purple. **e** A schematic diagram showing how the activated ATG8 or ATG12 is transferred to ATG3 or ATG10 in a *trans* manner

E2 core domain (Fig. 15.17b). The determined structures of ATG7 in complex with ATG3 and ATG10 reveal that ATG7 uses common multipart interfaces to recruit ATG3 and ATG10. These include the "shoulder" portion of the ATG7 NTD "wing", which interacts with the unique FR of ATG3 and the β-hairpin of ATG10, the "underwing" portion of the NTD, which binds to structural segments along the length of the backsides of both ATG3 and ATG10, and the junction region between NTD and CTD of ATG7, cradling the edge of both ATG3 and ATG10 (Fig. 15.17c, d). Strikingly, in the ATG7 and ATG3 complex structure, much of the handle region of ATG3 is not visible, and the FR of ATG3 interacts with a distal groove in the "shoulder" of ATG7 NTD rather than packing against the E2 core domain of ATG3 (Fig. 15.17c). Based on the determined complex structures, ATG7 uses a conserved groove located at the shoulder of NTD to recruit ATG3 and ATG10 (Fig. 15.17c, d), mechanistically explaining why ATG3 and ATG10 are mutual exclusive in binding to ATG7. Furthermore, in vitro biochemical data and structural modeling reveal that ATG7 transfers ATG8 and ATG12 to their respective targets, ATG3 and ATG10, in a *trans* manner (Hong et al. 2011; Kaiser et al. 2012; Yamaguchi et al. 2012). In particular, the ATG7 NTD-mediated recruitment of ATG3 and ATG10, coupled with conformational flexibility, allows presentation of the ATG3 and ATG10 active sites to the remote thioester-linked ATG8 and ATG12, which are bound to CTD from the opposite ATG7 monomer in the homodimer (Fig. 15.17e). In this way, ATG8 and ATG12 are linked to ATG3 and ATG10 by a *trans* mechanism to form ATG3–ATG8 and ATG10–ATG12 thioester intermediates, respectively. However, owing to lack of ATG7/ATG3/ATG8 and ATG7/ATG10/ATG12 complex structures, the exact mechanism underlying how ATG8 and ATG12 are transferred from ATG7 to ATG3 and ATG10 is still unknown.

#### 15.3.3.4 The Ligations of ATG12 with ATG5 and ATG8 with PE

After formation of the ATG10–ATG12 and ATG3–ATG8 intermediates, ATG12 is directly ligated to ATG5 without an E3-like protein. In contrast, ATG8 is covalently linked to PE, catalyzed by the ATG12–ATG5 conjugate, which serves as an E3-like enzyme. Structural studies reveal that ATG5 forms a unique globular fold, consisting of an N-terminal α-helix, followed by a ubiquitin-fold domain (UFD1), a helix-rich domain (HR), and a C-terminal ubiquitin-fold domain (UFD2) (Fig. 15.18a) (Yamaguchi et al. 2012). Unfortunately, to date, there is no available structural information related to the ATG10–ATG12/ATG5 complex; therefore, the detailed basis for the transfer of ATG12 from ATG10 to ATG5 is still unknown. However, biochemical and NMR studies have suggested that ATG5 binds to the concave surface around the active site of ATG10, and the ATG12 segment of the ATG10–ATG12 intermediate may also participate in the recruitment of ATG5 (Yamaguchi et al. 2012).

The coiled-coil protein ATG16L1 (ATG16 in yeast) can directly interact with ATG5 and recruits ATG12–ATG5 to the autophagosome formation site by binding to WIPI2b (ATG21 in yeast), which can specifically recognize PI3P generated

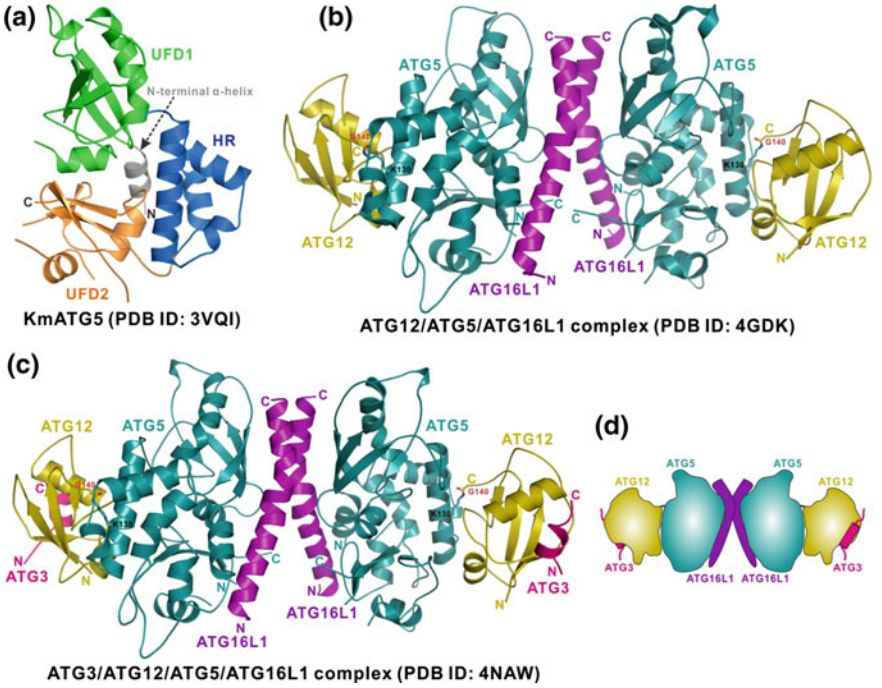


**Fig. 15.18** Mechanistic insights into the ligation of ATG8 with PE mediated by the ATG12/ATG5/ATG16L1 complex. **a** The overall structure of ATG5 from *Kluyveromyces marxianus* (Km) (PDB ID: 3VQI). The unique N-terminal helix, UFD1, HR, and UFD2 are highlighted and colored in gray, green, blue, and orange, respectively. **b** The overall structure of the dimeric ATG12/ATG5/ATG16L1 complex from human (PDB ID: 4GDK). The covalent linker between the side chain of ATG5 K130 and the carbonyl group of ATG12 G140 is also indicated. **c** The overall structure of ATG3/ATG12/ATG5/ATG16L1 complex (PDB ID: 4NAW). In this drawing, ATG16L1 is colored in purple, ATG5 in teal, ATG12 in olive, and ATG3 in pink. **d** A schematic diagram showing the overall architecture of the ATG3/ATG12/ATG5/ATG16L1 complex with the same color scheme as in panel (**c**)

by PI3KC3-C1 at the phagophore membrane (Dooley et al. 2014). The structure of the human ATG12–ATG5 conjugate in complex with the N-terminal region of ATG16L1 (ATG16N) reveals an integrated architecture through covalent and non-covalent contacts (Fig. 15.18b) (Otomo et al. 2013). In particular, ATG16N forms a parallel coiled-coil homodimer by two elongated α-helices, each of which has a slight kink in the middle and packs against the surface of ATG5 consisting of UFD1 and UFD2 (Fig. 15.18b). The ATG12–ATG5 conjugate exhibits E3-like activity and facilitates the transfer of ATG8 from the catalytic cysteine residue of ATG3 to PE. Structural and mutational analyses suggest that both ATG12 and ATG5 in the ATG12–ATG5 conjugate are directly involved in the E3 activity, but the interaction between ATG12–ATG5 with ATG3 is mainly mediated by ATG12 (Otomo et al. 2013). Recently, a structural study revealed that a stretch of peptides located in

the flexible FR region of ATG3 is responsible for ATG12 binding, forming a short α-helix and exclusively binding to a surface patch of ATG12 that is distinct from the ATG5-binding site (Fig. 15.18c, d) (Metlagel et al. 2013). Further structural investigations of the larger E2/E3 complex containing ATG12–ATG5 and ATG8–ATG3 conjugates would provide additional key information for the complete understanding of the molecular mechanisms underpinning the PE lipidation of ATG8.

## 15.4 Structural Studies of Proteins Involved in Autophagosome Transport

The spatial transport of autophagy vesicles is crucial for the maturation of autophagosomes. By far, only a small number of proteins involved in the intracellular transport of autophagy vesicles have been identified, such as FYCO1, the kinesin motor KIF5B and JIP1. At present, structural and mechanistic studies of these proteins have only just begun. In the following, we will only summarize the currently known structural data related to FYCO1.

### *15.4.1 FYCO1*

FYCO1 is a crucial autophagic adaptor protein and functions together with the kinesin motor KIF5B to mediate anterograde-directed transport of autophagosomes along the microtubule (Pankiv et al. 2010; Raiborg et al. 2015). FYCO1 contains an N-terminal RUN domain, followed by several coiled-coil domains and a FYVE domain, a C-terminal LIR motif that can specifically bind to ATG8 family proteins, and a GOLD domain with an unknown function (Fig. 15.19a). The interaction between FYCO1 and ATG8 family proteins is functionally different from that of currently known autophagy receptors, such as SQSTM1/P62, NBR1, Optineurin, and CALCOCO2/NDP52, as autophagy receptors specifically interact with ATG8 family proteins that are present on the inner membranes of autophagosomes, while FYCO1 only associates with ATG8 family members located on the outer surface of autophagosomes. Recently, a structural study systematically characterized the interactions between FYCO1 LIR and ATG8 family members and discovered that the unique FYCO1 LIR can selectively interact with the six mammalian ATG8 orthologues and preferentially and strongly binds to LC3A and LC3B (Cheng et al. 2016). The high-resolution crystal structure of FYCO1 LIR in complex with LC3A not only provides mechanistic insights into the selective interactions between FYCO1 and ATG8 family members (Fig. 15.19b, c) but also reveals a novel universal interaction mode of LIR motif binding to ATG8 family proteins; this observation highlights the notion that in addition to the canonical core sequence ΘxxΓ (where Θ is an aromatic

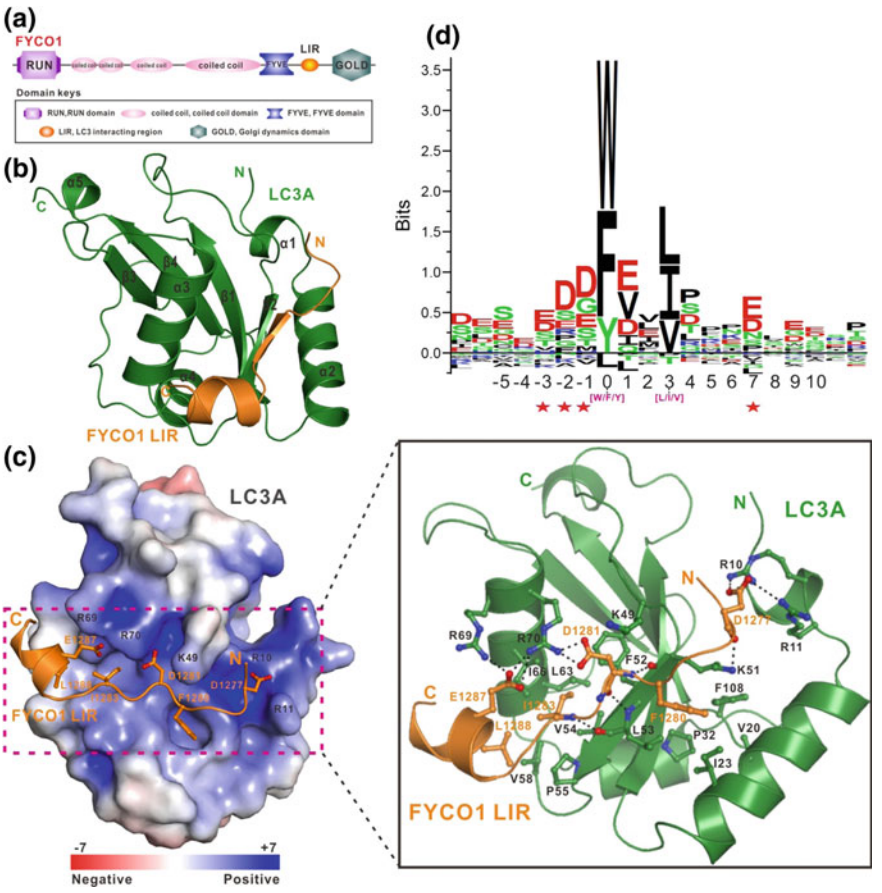


**Fig. 15.19** Mechanistic insights into the interaction of the FYCO1 LIR motif and LC3A. **a** The domain organization of FYCO1. **b** The overall structure of FYCO1 LIR/LC3A complex (PDB ID: 5CX3). **c** Detailed interactions between FYCO1 LIR and LC3A. The hydrogen bonds involved in the binding are shown as dotted lines. (D) A sequence logo of the multiple sequence alignment of the currently known 43 LIR motifs. The analysis of these 43 LIR motifs not only confirms the core consensus sequence [W/F/Y]xx[L/I/V] and the acidic residues preceding the hydrophobic LIR core sequence, it also reveals that the presence of a negatively charged residue at the seventh position C-terminal to the core aromatic residue (W/F/Y) is highly prevalent

residue (W/F/Y) and Γ is a bulk hydrophobic residue (L/I/V)), N-terminal negatively charged residues and extension sequences C-terminal to the LIR core motif are required for many LIR-containing proteins to specifically interact with ATG8 family proteins (Fig. 15.19d). Further structural characterizations are required to elucidate the detailed mechanism underlying the specific interactions of KIF5B with FYCO1 and JIP1.

## 15.5 Structural Studies of Proteins Involved in Autophagosome–Lysosome Fusion

Finally, autophagosomes fuse with lysosomes to form autolysosomes, and engulfed autophagic cargoes are degraded by lysosomal enzymes for recycling. The fusion between autophagosomes and lysosomes generally depends on specific SNARE proteins, tethering factors, and Rab GTPases. Recently, two autophagic SNARE complexes, the Synatxin17/VAMP8/SNAP29 complex and the YKT6/Synatxin7/SNAP29 complex, have been shown to mediate the fusion of autophagosomes and lysosomes in mammalian cells (Itakura et al. 2012; Matsui et al. 2018). In addition, several tethering factors, such as the tethering homotypic fusion and protein sorting (HOPS) complex, ATG14, PLEKHM1, TECPR1, BRUCE and EPG5, are also required for the autophagosome–lysosome fusion (Corona and Jackson 2018). In this section, we will summarize the currently known structural results related to these proteins.

### *15.5.1 Autophagic SNARE Complexes*

Membrane fusion is mediated by SNARE proteins, each of which contains a characteristic SNARE motif, an evolutionarily conserved stretch of 60–70 amino acids (Jahn and Scheller 2006). When appropriate sets of SNARE proteins are encountered, their SNARE motifs spontaneously assemble into a four-helical core complex with extraordinary stability. Structural studies of SNARE core complexes have revealed a remarkable degree of conservation, which is represented by four intertwined and parallel α-helices, with each helix being provided by a different SNARE motif (Sutton et al. 1998; Antonin et al. 2002). The centre of the helical bundle contains 16 stacked layers of interacting side chains. These layers are largely hydrophobic, except for a central "0" layer that contains three highly conserved Glu (Q) residues and one Arg (R) residue. Accordingly, SNARE proteins can be classified into Qa-, Qb-, Qc- and R-SNAREs (Jahn and Scheller 2006). SNARE proteins that are localized in "donor" membranes or "acceptor" membranes drive membrane fusion by using the free energy that is released during the formation of a four-helix bundle.

In 2012, Mizushima and colleagues discovered that Syntaxin17 (Qa-SNARE), in cooperation with SNAP29 (Qbc-SNARE) and VAMP8 (R-SNARE), mediates the fusion between autophagosomes and lysosomes in mammalian cells (Itakura et al. 2012). Specifically, Syntaxin17 is recruited to a completed autophagosome outer membrane from the cytosol and, together with cytosolic SNAP29, interacts with VAMP8 on a lysosome membrane to promote autophagosome–lysosome fusion. Syntaxin17 contains an N-terminal Habc domain, a Qa-SNARE motif, and a C-terminal hairpin-type structure formed by two unique tandem transmembrane domains, which is essential for its localization to a completed autophagosome (Fig. 15.20a). SNAP29 contains a short N-terminus, a Qb-SNARE motif, a linker

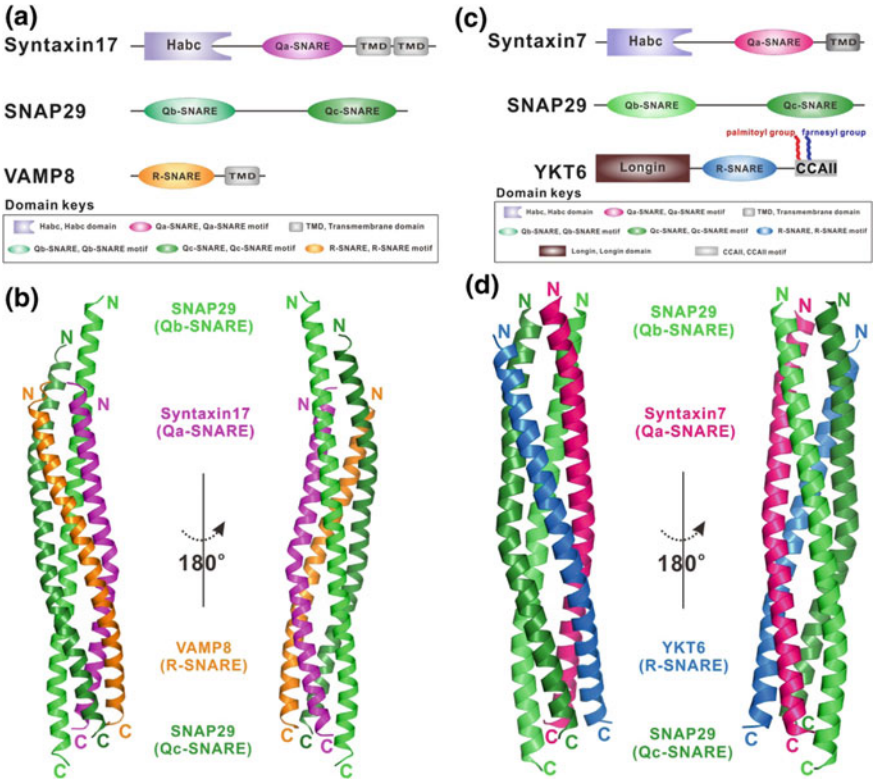


**Fig. 15.20** The components and structures of two autophagic SNARE complexes. **a** A schematic diagram showing the domain organization of the three components of the autophagic SNARE complex: Syntaxin17, SNAP29, and VAMP8. **b** Ribbon diagram representation showing the overall structure of the Syntaxin17/SNAP29/VAMP8 SNARE core complex. In this drawing, the Qa-SNARE motif of Syntaxin17 is shown in magenta, the R-SNARE motif of VAMP8 is in orange, the Qb-SNARE of SNAP29 is in green, and the Qc-SNARE of SNAP29 is in forest green. (PDB ID: 4WY4). **c** A schematic diagram showing the domain organization of the three components of the autophagic SNARE complex: Syntaxin7, SNAP29, and YKT6. **d** Ribbon diagram representation showing the overall structure of the Syntaxin7/SNAP29/YKT6 SNARE core complex

region, and a C-terminal Qc-SNARE motif (Fig. 15.20a). As there is no cysteine residue in the linker region of SNAP29, SNAP29 cannot attach to the membrane through the palmitoylation of a cysteine residue in its linker region. It is believed that SNAP29 is recruited to autophagosome membranes by binding to Syntaxin17. The lysosomal SNARE VAMP8 contains an R-SNARE motif, followed by a C-terminal transmembrane domain (Fig. 15.20a). A recent structural study revealed that the Synatxin17/VAMP8/SNAP29 core SNARE complex forms a four-helix bundle, with all four helices aligned in parallel, of which Syntaxin17 and VAMP8 each contribute one helix and SNAP29 contributes two helices to the bundle (Fig. 15.20b) (Diao et al. 2015). Despite rather limited sequence identities, the overall structure of the

Synatxin17/VAMP8/SNAP29 SNARE complex is highly similar to that of the known neuronal, early endosomal, late endosomal and yeast SNARE complexes (Diao et al. 2015).

Most recently, another SNARE complex consisting of Syntaxin7 (Qa-SNARE), SNAP29 (Qbc-SNARE), and YKT6 (R-SNARE) has been reported to be involved in mediating the fusion of autophagosomes and lysosomes in addition to the Syntaxin17/SNAP29/VAMP8 SNARE complex (Matsui et al. 2018). YKT6 contains an N-terminal longin domain, followed by an R-SNARE motif (Fig. 15.20c). Owing to the lack of a transmembrane domain, YKT6 uses a lipid anchor for transient association with the membrane. In particular, the C-terminal "CCAII" motif of YKT6 can be palmitoylated at the first cysteine and farnesylated at the second cysteine (Fig. 15.20c). Syntaxin7 contains an N-terminal Habc domain, a Qa-SNARE motif, and a C-terminal transmembrane region (Fig. 15.20c). When forming the YKT6/Synatxin7/SNAP29 SNARE complex, Syntaxin7 is localized on the lysosome, while YKT6 is localized on the completed autophagosome (Matsui et al. 2018). Interestingly, studies in *Drosophila* suggest that unlike in mammalian cells, YKT6 localizes on lysosomes through its C-terminal lipid anchors and can form a SNARE complex with SNAP29 and Syntaxin17, but it can be outcompeted from this complex by VAMP7, suggesting that YKT6 may serve as a non-conventional, regulatory SNARE protein in autophagosome–lysosome fusion (Takáts et al. 2018). In yeast, the R-SNARE YKT6 on the autophagosome associates with the Q-SNAREs Vam3, Vam7, and Vti1 on the vacuole to form the SNARE complex for promoting the fusion between autophagosome and vacuole (Bas et al. 2018; Gao et al. 2018). Recently, we have solved the crystal structure of the Synatxin7/YKT6/SNAP29 core SNARE complex, which forms a four-helix bundle and adopts a canonical SNARE core complex architecture (Fig. 15.20d). The overall structures of the two autophagic SNARE complexes are very similar. However, it remains unknown why the autophagic fusion process needs two SNARE complexes in mammals.

## *15.5.2 Tethering Proteins for Autophagosome–Lysosome Fusion*

### 15.5.2.1 HOPS Complex

The HOPS complex (the homotypic fusion and protein sorting complex), which is assembled by VPS33, VPS16, VPS11, VPS18, VPS39 and VPS41 (Fig. 15.21a), is an evolutionarily conserved membrane-tethering complex for vesicle membranes containing the small GTPase Rab7 (Balderhaar and Ungermann 2013). Previous studies have indicated that HOPS serves as a crucial tethering factor for autophagosome–lysosome fusion. In particular, the HOPS subunit VPS33A can directly interact with Syntaxin17, and links the HOPS complex with the Syntaxin17-positive autophagosomes upon starvation (Jiang et al. 2014). Importantly, the loss of a HOPS

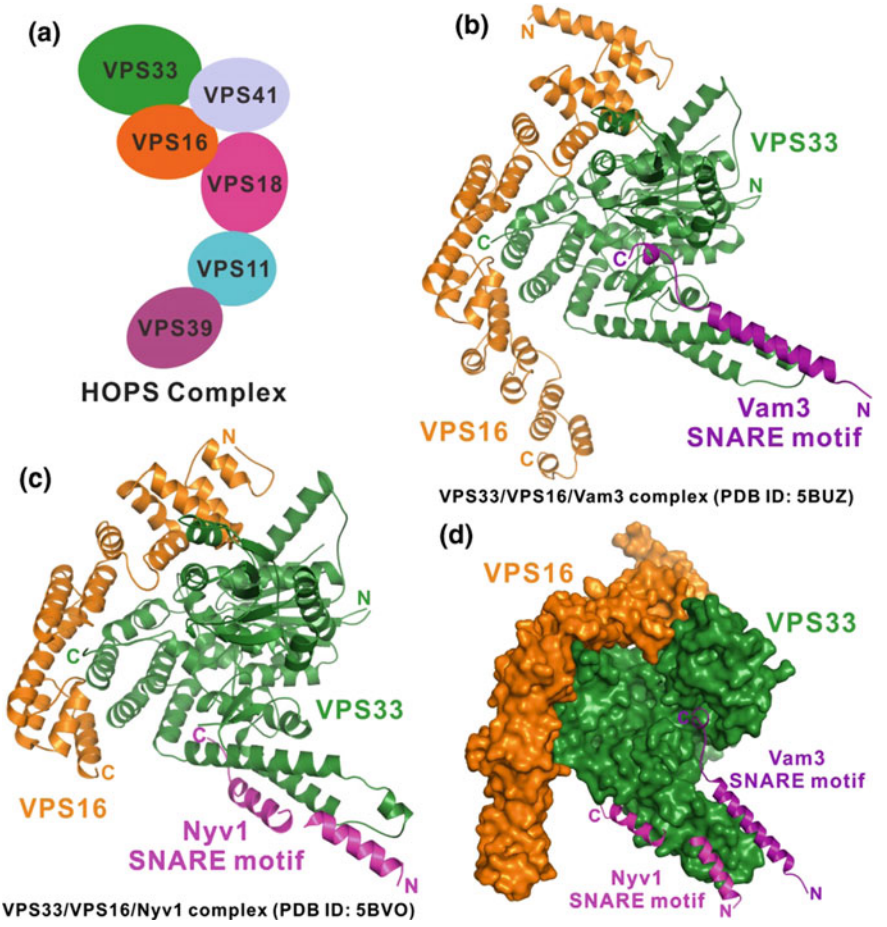


**Fig. 15.21** The components and determined structures related to the HOPS complex. **a** A schematic diagram showing the components and organization of the HOPS complex. **b** Ribbon diagram representation showing the overall structure of the yeast VPS33/VPS16 sub-complex in complex with the Vam3 SNARE motif (Qa-SNARE) (PDB ID: 5BUZ). In this drawing, VPS33 is shown in forest green, VPS16 in orange, and the bound Vam3 SNARE motif in purple. **c** Ribbon diagram representation showing the overall structure of the yeast VPS33/VPS16 sub-complex in complex with the Nyv1 SNARE motif (R-SNARE) (PDB ID: 5BVO). **d** A structural model showing the SNARE motifs of Vam3 and Nyv1 binding simultaneously to VPS33

subunit, such as VPS33A, VPS16, or VPS39 causes an accumulation of Syntaxin17- and LC3-positive autophagosomes and blocks the autophagic flux, indicating that HOPS promotes autophagosome–lysosome fusion with Syntaxin17 (Jiang et al. 2014). A recent structural study determined two crystal structures of the yeast VPS33/VPS16 sub-complex in complex with the SNARE motifs of Vam3 (Qa-SNARE) and Nyv1 (R-SNARE) (Fig. 15.21b, c) and demonstrated that VPS33 can promote SNARE assembly by precisely positioning and aligning different SNARE proteins (Fig. 15.21d) (Baker et al. 2015). However, how the HOPS complex associates with autophagic SNARE proteins in mammals remains elusive.

### *15.5.3 PLEKHM1*

PLEKHM1 (pleckstrin homology and RUN domain containing M1) is a ubiquitously expressed protein in mammalian cells and was initially identified as a homologue of rubicon (Tabata et al. 2010). Knockout of the *Plekhm1* gene inhibits in autophagy, leading to the accumulation of autophagic substrates and LC3B-II and a decrease in autolysosome formation in cells (McEwan et al. 2015). PLEKHM1 contains an N-terminal RUN domain that can specifically interact with VPS39, a subunit of HOPS, a canonical LIR motif sandwiched by two PH domains located in the middle region, and a C-terminal C1 domain (Fig. 15.22a). PLEKHM1 can bind to the GTP-bound Rab7 through its PH2 and C1 domains. A previous structural study showed that the LIR motif of PLEKHM1 adopts a canonical binding mode to interact with ATG8 family proteins located at the outer membrane of autophagosomes (Fig. 15.22b) (McEwan et al. 2015). Therefore, as a Rab7 effector protein, PLEKHM1 can directly bridge the autophagosome and lysosome via binding to ATG8 family members, Rab7 and HOPS, facilitating autophagosome and lysosome membrane fusion.

### *15.5.4 TECPR1*

TECPR1 (tectonin beta-propeller repeat containing 1) was identified as a component of the autophagy network through its interaction with ATG5, and importantly, TECPR1-depleted cells have impaired autophagic flux and accumulate autophagic substrates (Ogawa et al. 2011; Chen et al. 2012). Domain analysis reveals that TECPR1 mainly contains nine β-propeller repeats (TECPR), an internal AIR (ATG12–ATG5-interacting region), a PH domain, and two dysferlin domains (Fig. 15.22a). The PI3P binding of TECPR1 is mediated by its PH domain, which is regulated by the ATG12–ATG5 conjugate and is essential for the autophagic function of TECPR1 (Chen et al. 2012). In normal conditions, the AIR of TECPR1 packs with the PH domain and blocks its interaction with PI3P molecules. However, during autophagosome maturation, the ATG12–ATG5 conjugate binds to the AIR of TECPR1 that localizes at lysosomes and releases the PH domain of TECPR1. The

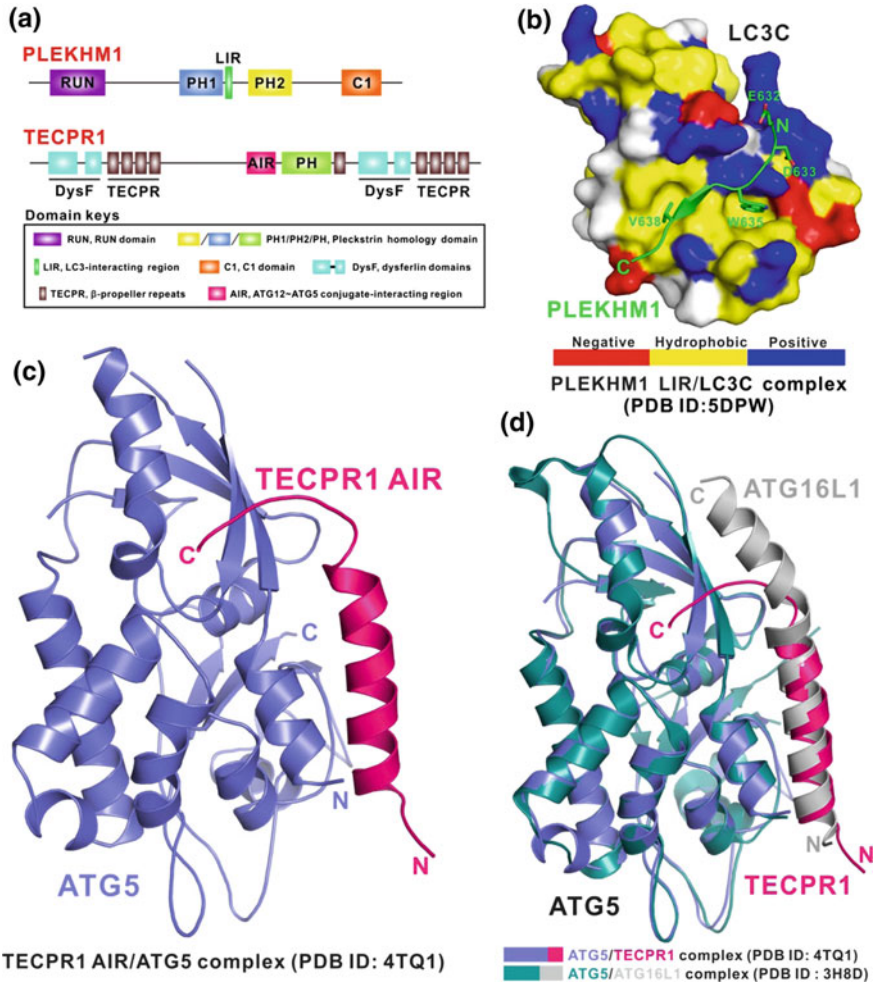


**Fig. 15.22** The domain organizations and related structures of the tethering factor PLEKHM1 and TECPR1. **a** The domain organization of PLEKHM1 and TECPR1. **b** The combined surface representation and the ribbon-stick model showing the structure of LC3C in complex with the PLEKHM1 LIR motif (PDB ID: 5DPW). In this presentation, the LC3C is shown in the surface model and the LIR motif of PLEKHM1 in the ribbon-stick model. In the surface model of LC3C, the hydrophobic amino acid residues are drawn in yellow, the positively charged residues are in blue, the negatively charged residues are in red, and the uncharged polar residues are in gray. **c** Ribbon diagram representation showing the overall structure of the TECPR1 AIR/ATG5 complex (PDB ID: 4TQ1). **d** A comparison of the overall structures of the ATG5/TECPR1 AIR complex (blue-pink, PDB ID: 4TQ1) and the ATG5/ATG16L1 sub-complex (blue-grey) in the ATG12/ATG5/ATG16L1 complex (PDB ID: 3H8D)

liberated PH domain of TECPR1 subsequently attaches to PI3P molecules located at the autophagosomal membrane, thereby tethering the autophagosome to a lysosome and facilitating the autophagosome–lysosome fusion (Chen et al. 2012). The crystal structure of the TECPR1 AIR/ATG5 complex revealed that the AIR of TECPR1 mainly forms an elongated α-helix (Fig. 15.22c) and occupies an overlapped binding site on ATG5 shared by ATG16L1 (Fig. 15.22d) (Kim et al. 2015b). Therefore, TECPR1 and ATG16L1 are mutually exclusive in binding to the ATG12–ATG5 conjugate. However, how the AIR of TECPR1 regulates the PI3P-binding ability of the PH domain remains unknown; therefore, additional structural information on the full-length or defined domains of TECPR1 is needed.

## 15.6 Conclusions and Future Prospects

Autophagy, especially in mammals, is an exciting and rapidly developing field, and previous genetic and functional studies have allowed us to identify and explore the functions of a large number of proteins that are critical for the autophagy process. Structural studies of these proteins have revealed many molecular details of autophagic proteins and complexes that regulate and control multiple aspects of the autophagy process. These determined structures have provided unprecedented mechanistic insights into the assemblies of the mTOR complex and AMPK for sensing cellular nutrient and energy levels, the class III PI3K complexes for generating a PI3P signal, the ATG8 and ATG12 ubiquitin-like conjugation systems for providing a membrane-linked platform for the biogenesis and maturation of autophagosomes, and the autophagic SNARE complexes for the fusion of autophagosomes and lysosomes. However, there are still many intriguing and critical unanswered questions regarding almost every step of the autophagy process. For example, how is the entire ULK1 complex assembled and regulated? How do the upstream mTOR1 complex and AMPK associate with and activate the ULK1 complex? How the information from the activated ULK1 complex is sequentially transferred to the downstream class III PI3K complexes, the PI3P-binding proteins, and the two autophagic ubiquitin-like cascades? How do tethering factors associate with autophagic SNARE complexes to promote the fusion between autophagosomes and lysosomes? These important mechanistic issues can surely be answered by further structural studies. In addition, recent studies have shown that there is substantial number of selective autophagy processes that are mediated by different autophagy receptor proteins. The molecular mechanisms and regulations of these selective autophagy processes are still largely unknown; thus, further mechanistic studies based on structural biology are highly recommended.

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# Part III
# Biological Function of Autophagy

# Chapter 16
# Autophagy and Energy Metabolism


**Jie Yang, Ruimin Zhou and Zhenyi Ma**

**Abstract** Autophagy is a lysosome-dependent catabolic process. Both extra- and intra-cellular components are engulfed in autophagic vacuoles and degraded to simple molecules, such as monosaccharides, fatty acids and amino acids. Then, these molecules can be further used to produce ATP through catabolic reactions and/or provide building blocks for the synthesis of essential proteins. Therefore, we consider autophagy a critical and fine-tuned process in maintaining energy homeostasis. The complicated relationships between autophagy and energy metabolism have raised broad interest and have been extensively studied. In this chapter, we summarize the relationships enabling autophagy to control or modulate energy metabolism and allowing metabolic pathways to regulate autophagy. Specifically, we review the correlations between autophagy and energy homeostasis in terms of oxidative phosphorylation, reactive oxygen species in mitochondria, glycolysis, metabolism of glycogen and protein, and so on. An understanding of the role of autophagy in energy homeostasis could help us better appreciate how autophagy determines cell fate under stressful conditions or pathological processes.

**Keywords** ATP · Amino acid · Autophagy · Carbohydrate · Energy · Glycogen

## Abbreviations

| | |
|---|---|
| 2-DG | 2-deoxy-D-glucose |
| 3-MA | 3-methyladenine |
| 4E-BP1 | Eukaryotic initiation factor 4E binding protein 1 |
| α-KG | α-ketoglutaric acid |
| ABCC1 | ATP-binding cassette C1 |
| ADI | Arginine deiminase |
| AICAR | 5-aminoimidazole-4-carboxamide ribonucleoside |

J. Yang · R. Zhou · Z. Ma (✉)
Department of Biochemistry and Molecular Biology, School of Basic Medical Sciences, Tianjin Medical University, 22 Qixiangtai Road, Heping District, Tianjin 300070, China
e-mail: zhyma@tmu.edu.cn



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| | |
|---|---|
| AMBRA | Activating molecule in Beclin1-regulated autophagy |
| AMPK | Adenosine 5′-monophosphate (AMP)-activated protein kinase |
| ASS | Argininosuccinate synthetase |
| ATF4 | Activating transcription factor 4 |
| ATG | Autophagy-related gene |
| ATP | Adenosine triphosphate |
| BNIP3 | Bcl2/adenovirus E1B 19-kDa protein-interacting protein 3 |
| CBM | Carbohydrate-binding module |
| CMA | Chaperone-mediated autophagy |
| DAPK-1 | Death-associated protein kinase 1 |
| eIF | Eukaryotic initiation factor |
| EGLN | Egl-9 family hypoxia-inducible factor |
| F2,6BP | Fructose-2,6-bisphosphate |
| FCCP | Carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone |
| FOXO | Forkhead box O |
| G6P | Glucose-6-phosphate |
| G6PC | Glucose-6-phosphatase α |
| G6PD | Glucose-6-phosphate dehydrogenase |
| GAA | Lysosomal acid α-glucosidase |
| GABARAPL1 | Γ-aminobutyric acid receptor-associated protein-like 1 |
| GAPDH | Glyceraldehyde-3-phosphate dehydrogenase |
| GCL | Γ-glutamate cysteine ligase |
| GILT | Interferon-γ-inducible lysosomal thiol reductase |
| GLS | Glutaminase |
| GLUD1 | Glutamate Dehydrogenase 1 |
| GSH | Glutathione, γ-L-glutamyl-L-cysteinyl-glycine (reduced) |
| GSK3 | Glycogen synthase kinase 3 |
| GSSH | Glutathione, γ-L-glutamyl-L-cysteinyl-glycine (oxidized) |
| HIF | Hypoxia-inducible factor |
| HK | Hexokinase |
| HSC70 | Heat shock cognate protein 70 |
| ICD | Immunogenic cell death |
| LAMP2A | Lysosome-associated membrane protein type 2A |
| LC3 | Microtubule-associated protein 1 light chain 3 (MAP1LC3) |
| LDHB | Lactate dehydrogenase B |
| MPC | Mitochondrial pyruvate carrier protein |
| mTOR | Mammalian target of rapamycin |
| $NADH/NAD^+$ | Nicotinamide adenine dinucleotide |
| NADPH | Nicotinamide adenine dinucleotide phosphate |
| NOX | NADPH oxidase |
| PARP1 | Poly(ADP-ribose) polymerase 1 |
| PFKFB4 | 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase 4 |
| PI3K | Phosphatidylinositol 3-kinase |
| PI3P | Phosphatidylinositol-3-phosphate |
| $PIP_3$ | Phosphatidylinositol 3,4,5-trisphosphate |

| | |
|---|---|
| PK | Pyruvate kinase |
| PKM2 | Pyruvate kinase M2 isoform |
| PKA | Protein kinase A |
| PKB | Protein kinase B, also known as Akt |
| PPP | Pentose phosphate pathway |
| PRODH/POX | Proline dehydrogenase/oxidase |
| Raptor | Regulatory-associated protein of mTOR |
| Rheb | Ras homolog enriched in brain |
| ROS | Reactive oxygen species |
| SIRT | Sirtuin |
| SLC1A5 | Solute carrier family 1 (neutral amino acid transporter) member 5 |
| SNAREs | Soluble N-ethylmaleimide-sensitive factor attachment protein receptors |
| SOD | Superoxide dismutase |
| SQSTM1/p62 | Sequestosome 1/p62 |
| Stbd1 | Starch-binding domain-containing protein 1 |
| TFEB | Transcription factor EB |
| TIGAR | TP53-induced glycolysis and apoptosis regulator |
| TRAF6 | TNF receptor-associated factor 6 |
| TSC1/2 | Tuberous sclerosis 1/2 protein |
| ULK1 | Unc-51-like autophagy activating kinase 1 |
| v-ATPase | Vacuolar H+-adenosine triphosphatase ATPase |
| Vps34 | Vacuole protein sorting 34 (class III phosphatidylinositol 3-kinase) |

Autophagy refers to a series of strictly regulated catabolic processes by which cytoplasmic components are transported to lysosomes for degradation. Then, the subsequent product is used to generate energy and replenish the intracellular metabolic pool for protein synthesis and renewal. The basic functions of autophagy include the regulation of cellular energy metabolism and maintenance of homeostasis. The regulation of energy metabolism in eukaryotes is achieved by balancing the synthesis and degradation of nutrients, including proteins, lipids, carbohydrates, and so on. The primary function of basic autophagy is to degrade intracellular long-lived proteins and remove damaged or aged organelles.

Autophagy is generally classified into the following three categories: macroautophagy, microautophagy and chaperone-mediated autophagy (CMA). Macroautophagy refers to the process by which double-membrane vesicles called autophagosomes sequester cytoplasmic proteins and organelles and then fuse with lysosomes to degrade the encapsulated components. Microautophagy refers to the invagination of lysosomes or endosomal membranes, resulting in the direct phagocytosis of the substrates, followed by degradation by lysosomal hydrolases. Molecular CMA differs from macroautophagy and microautophagy because the targeted proteins to be degraded are not restricted to vesicles. The target proteins carry a KFERQ motif that can be recognized by the chaperon molecule heat shock cognate protein 70 (HSC70).

HSC70 transports these target proteins to lysosomes via the lysosomal-associated membrane protein 2A (LAMP2A) receptor (see Chap. 20). These three types of autophagy processes play important but different roles depending on the biological contexts. However, each stage of the three types of autophagy requires the synthesis and utilization of ATP and plays a regulatory role in maintaining homeostasis in cellular energy metabolism. In this chapter, we mainly focus on macroautophagy (hereinafter referred to as autophagy).

During the process of autophagy, due to the production and extension of the membrane, proteins, organelles and other autophagic substrates are encapsulated into a bilayer membrane structure, forming autophagosomes, and then, autophagosomes fuse with lysosomes to form autolysosomes. Owing to the presence of various hydrolases, including lipases, proteases, glycosidases and nucleotidases, in the lysosome, the contents encapsulated by the autophagosomes are degraded by related hydrolases to produce lipids, amino acids, sugars and other degradation products. These products enter the cellular metabolic cycle and are reused. On the one hand, when the cells are under a low nutrient or low energy state, autophagy is induced to hydrolyze proteins and organelles through selective or nonselective autophagy. The intracellular nutrients are mobilized to produce amino acids, sugars and lipids. Then, these products are catabolized through glycolysis, the citric acid cycle, β-oxidation and oxidative phosphorylation to provide ATP. *Atg5* or *Atg7*-knockout newborn mice represent a typical example of mice that die without breastfeeding because the deletion of autophagy-related genes prevents the formation of autophagosomes. Therefore, these newborn mice cannot obtain the energy necessary for survival by the autophagic degradation of nutrients. The survival of *Atg*-knockout mice with insufficient autophagy can be prolonged by breastfeeding soon after birth.

On the other hand, the degradation products are involved in the biosynthesis of proteins, which is an energy-consuming process. The maintenance of physiologically essential proteins is necessary for survival. Both basic and induced autophagy are dynamic processes that control the balance of cellular energy metabolism. From an evolutionary perspective, autophagy is thought to be evolved from the ancient mechanism of "providing nutrition to the unicellular organism during energy crisis." In multicellular organisms, autophagy within a single cell type or tissue can break through local boundaries and affect the metabolic balance of surrounding tissues or organs. Thus, autophagy not only regulates the internal energy homeostasis of a single cell but also has a profound impact on the overall energy homeostasis of the body. Considering the example of a normal human body, autophagic tissue degradation is inevitably accompanied by the synthesis of new biomacromolecules to maintain homeostasis, and the synthesis process consumes a large amount of energy. Considering the example of protein synthesis, the formation of each peptide bond requires the consumption of four high-energy phosphate bonds, including two bonds for tRNA-loaded amino acids and two bonds for peptide bond formation in ribosomes. Under physiological conditions, assuming an average release of 50 kJ of energy per mole of ATP hydrolysis, 1.8 kJ of energy is consumed per 1 g of protein synthesized. Thus, in a normal human body, the turnover of 10% of protein consumes approximately 2000 kJ of energy, which is approximately 20% of the daily energy

intake. Thus, autophagy degradation is a major energy source for biomacromolecule synthesis. It has also been confirmed that a SQSTM1/p62 knockout in mice results in decreased energy expenditure and obesity (Kaur and Debnath 2015).

In summary, a complex relationship exists among energy metabolism, autophagy and cell fate determination to maintain energy homeostasis. This association is tightly tuned by various genetic and environmental factors. The function and underlying molecular mechanisms are of great interest in the field of autophagy research. This chapter reviews the interaction between energy metabolism and autophagy and its role in cell fate decision.

## 16.1 Autophagy and Carbohydrate Metabolism

Mitochondria are important sites for energy production that transform various forms of energy in cells into a common "energy currency," that is, adenosine triphosphate (ATP). The process of ATP production in mitochondria is called oxidative phosphorylation. Carbohydrates produce acetyl-CoA in mitochondria through glycolysis and glycogenolysis. ATP and reducing equivalents, such as NADH, are subsequently produced by the citric acid cycle. In the electron transport chain, NADH transfers electrons through a series of redox reactions to a protein complex with a larger reduction potential and finally to the oxygen molecule $O_2$. $H^+$ is refluxed to the mitochondrial matrix by ATP synthase, which drives ADP phosphorylation to form ATP. Carbohydrates are the most readily available direct source of energy supply. However, when exogenous sugar intake is insufficient, the synthesis of ATP cannot meet the needs of cell survival. Do cells provide additional substrates for degradation to produce ATP? This section focuses on the relationship between carbohydrate (sugar) metabolism and autophagy from the perspective of the mitochondrial respiratory chain, reactive oxygen species, glycolysis, glycogenolysis, aerobic oxidation and carbohydrate synthesis regulation.

### *16.1.1 Autophagy and the Respiratory Chain*

The half-life of long-lived proteins (a good marker for monitoring autophagy) in younger and healthier animal cells is generally shorter compared to that in older animals, indicating that the basal level of autophagy is generally higher in younger and healthier cells. This finding raises the following interesting question: what determines the level of basic autophagy activity in a cell? Clearly, mitochondrial oxidative phosphorylation is the most important source of ATP. The integrity of the inner mitochondrial membrane, particularly mitochondrial respiratory chain activity, is important for determining the autophagy activity. Mitochondrial respiratory defects significantly reduce autophagy levels in yeast and mammalian cells.

#### 16.1.1.1 Autophagy and Oxidative Phosphorylation

Mitochondrial inhibitors generally lead to a decrease in ATP levels, activation of AMPK (adenosine 5′-monophosphate (AMP)-activated protein kinase), inactivation of mTOR (mammalian target of rapamycin) and induction of autophagy. However, studies have found that low concentrations (approximately 5–10 nM) of antimycin A (a specific inhibitor of mitochondrial complex III) are effective in reducing both basal autophagy and rapamycin (an mTORC specific inhibitor)-induced autophagy. This autophagy inhibition is specific to mitochondrial complex III. First, inhibitors of other mitochondrial complexes do not produce the same effect; second, structural analogs of antimycin A that do not inhibit complex III have no inhibitory effect on autophagy; and third, compounds that differ in structure from antimycin A but inhibit complex III exhibit autophagy inhibition activity. The above three points indicate that mitochondrial complex III is an important "tuner" that can positively regulate basal and induced autophagy and is a key molecule linking basal autophagy and the cellular energy flux (Ma et al. 2011).

However, other studies have reported controversial results. For example, inhibitors of respiratory chain complex I, that is, rotenone and aumitin (a derivative of diaminopyrimidine), and inhibitors of complex IV, that is, oligomycin and the uncoupler FCCP (2,4-dinitrophenol), can inhibit autophagy in cells, suggesting that there is a tight correlation between oxidative phosphorylation in the mitochondrial respiratory chain and autophagy. This correlation is related to altered energy production, ROS production and oxidative stress. Although the above studies reached different conclusions regarding oxidative phosphorylation in autophagy, these studies all highlighted the important point that abnormal energy metabolism is naturally related to a change in the autophagy level (Robke et al. 2018). The mechanism of this connection still requires further investigation.

NAD is an essential substrate for oxidative phosphorylation and its oxidized form is $NAD^+$, while its reduced form is NADH. Nutritional deficiency leads to the continuous oxidation of NADH to produce $NAD^+$, which tends to accumulate and subsequently trigger the activation of the sirtuin family histone deacetylase, thereby promoting autophagy. In contrast, the activation of $NAD^+$-dependent enzymes, such as poly(ADP-ribose) polymerase 1 and PARP1, can reduce both intracellular $NAD^+$ and NADH levels. The enzymatic activity of sirtuins mainly depends on the $NAD^+$ levels. Sirtuins activation-mediated autophagy can be efficiently provoked by inhibiting PARP1 to maintain the endogenous NAD levels or artificially adding NAD precursors (e.g., nicotinamide and nicotinamide riboside). Therefore, both the relative abundance of NADH and $NAD^+$ and the total NAD content play an important role in autophagy regulation (Galluzzi et al. 2014).

#### 16.1.1.2 Autophagy and Reactive Oxygen Species

Endogenous ROS are produced by various mechanisms. The main producer of ROS is the NADPH oxidase (NOX) complex, which is located in the cell membrane, mitochondria, peroxisome and endoplasmic reticulum. The NOX complex has seven

isoforms. Mitochondria convert various forms of energy in cells into the common "energy currency" ATP. Under normal conditions, one molecule of $O_2$ is reduced to produce two molecules of $H_2O$. However, in vitro experiments using isolated mitochondrial components have shown that approximately 0.1–2% of electron transport causes a premature and incomplete reduction of $O_2$, thereby transforming into peroxide and superoxide, such as oxygen free radicals ($\bullet O_2^-$).

The high mitochondria-derived ROS activity can target and oxidize certain molecules, including proteins, lipids and nucleic acids. Normal cells require a certain amount of ROS for signal transduction pathways. However, environmental stresses, such as nutrient deficiencies and pathogen invasion, cause an excessive production of ROS, leading to the unexpected oxidation of a large amount of targeted molecules and mitochondrial damage. Consequently, autophagic and preapoptotic proteins are induced, and p53 is activated, leading to cell death. To benefit cell survival, induced autophagy degrades aggregates of damaged mitochondria and reduces ROS-induced damage, subsequently preventing cell death (Filomeni et al. 2015).

Notably, the dynamics of the intra- and extra-cellular environment affect the interaction between ROS and autophagy. For example, amino acid starvation and glucose deficiency can lead to autophagy activation. Amino acid starvation induces the production of both $H_2O_2$ and $O_2^-$, while glucose deprivation induces only the production of $O_2^-$. The cell type also has an impact on ROS-induced autophagy. Upon $H_2O_2$ treatment, tumor cells or transformed cells undergo autophagy-dependent cell death, while mouse primary astrocytes undergo apoptosis with no significant change in autophagy (Lee et al. 2012).

The molecular mechanism of ROS-triggered autophagy is still unclear. A possible interpretation of this missing link is the instability of rapidly changing ROS signals. For example, superoxide dismutase (SOD) converts $O_2^-$ to $H_2O_2$, and $H_2O_2$ is further reduced by catalase or peroxidase to form $O_2$ and $H_2O$. Then, $O_2$ can be converted back to $O_2^-$ by NOX (located on the mitochondrial inner membrane), thus resulting in a cycle between ROS molecules. Among these molecules, $H_2O_2$ may be a relatively stable signal regulating autophagy. $H_2O_2$ stabilizes lipidated LC3 (microtubule-associated protein 1 light chain 3/MAP1LC3) and gamma-aminobutyric acid receptor-associated protein-like 2 (GABARAPL2) by directly oxidizing ATG4. This observation further suggests that mitochondria may provide certain signals to promote autophagy. For instance, the membrane structure derived from mitochondria is involved in the biosynthesis of the autophagosome membrane. Other evidence suggests that the membrane structure derived from the endoplasmic reticulum is also involved in the formation of autophagosomes. Experiments using yeast have demonstrated that a part of the membrane structure derived from the Golgi apparatus can also participate in the formation of autophagosomes. Under certain conditions, the endomembrane system is also an ROS generating site, further complicating the correlation between ROS and autophagy. Accordingly, more detailed studies are required to further elucidate the connections and regulatory mechanisms.

### *16.1.2 Autophagy and Carbohydrate Catabolism*

Carbohydrate catabolism pathways, including the citric acid cycle, the pentose phosphate pathway and glycolysis, are important for energy metabolism at both the cellular and individual levels. Various mechanisms are involved in the regulation of energy metabolism, and autophagy is an important mechanism maintaining the homeostasis of cellular energy metabolism by regulating the central carbon cycle. On the one hand, autophagy participates in energy metabolism by degrading biomacromolecules, such as protein degradation (see Sect. 16.2 of this chapter) and glycogen hydrolysis (glycogen-specific autophagy termed "glycophagy"). The degradation of glycogen to produce glucose affects the cell's glucose metabolic pool, which, in turn, alters the metabolic flux of glucose and other carbohydrates. On the other hand, changes in the cellular energy status and changes in the glucose metabolism flux regulate autophagy in various ways. For example, the energy "sensor" AMPK monitors the change in the intracellular ATP levels and regulates autophagy through a series of signal transduction in an mTORC1-dependent or -independent manner. Several amino acids can be converted to α-ketoglutaric acid, which is an intermediate in the citric acid cycle, thereby entering the carbohydrate metabolic pathway to regulate autophagy (see Sect. 16.2 of this chapter). Furthermore, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and glucose-6-phosphate dehydrogenase (G6PD) participate in glycolysis and the pentose phosphate pathway as important catalysts that regulate autophagy but in a catalytic activity-independent manner. This section elaborates upon the interrelationship between autophagy and glucose metabolism, the molecular mechanism by which cells respond to glucose deficiency, and the autophagic degradation of glycogen.

#### 16.1.2.1 Autophagy and Glycolysis

Glycolysis involves ten reaction steps from glucose to pyruvate. This tandem of reactions not only produces ATP but also provides substrates for the subsequent aerobic oxidation or anaerobic fermentation. Therefore, glycolysis is fundamental for the cellular energy status. Glycolysis-related enzymes, such as hexokinase and pyruvate kinase, are also unsurprisingly associated with autophagy.

First, changes in enzymatic activities affect autophagy. Hexokinase (HK) is a key enzyme in glycolysis. HK catalyzes the conversion of glucose to glucose-6-phosphate. HK has at least four isoforms (HK1-4) in the human body, including HK1, which is constitutively expressed at a high level. In contrast, HK2 is induced to express under stressful conditions. The functions and mechanisms of HK2 in autophagy have been studied in detail. The glucose analog 2-deoxy-D-glucose (2-DG) is a competitive inhibitor of HK2. The addition of 2-DG reduces autophagy and increases myocardial cell death, highlighting the protective role of autophagy, whereas the constitutive HK1 has no impact on the autophagy process (Jiao et al. 2018). The structure of HK2 contains a TOS motif (MTOR signaling motif). Via

TOS, HK2 can directly bind the Raptor protein, which is an important component of mTORC1 that acts as a bait substrate to inhibit mTORC1 activity and consequently enhance autophagy. The binding of HK2 to mTORC1 is independent of its enzymatic activity. Additionally, researchers have compared the effects of 2-DG, 5-glucosamine (another glucose analogue and competitive inhibitor of HK2) and glucose on autophagy. HK2 can phosphorylate, but not bind, substrates; thus, there are no glycolysis reactions, but autophagy is triggered. When the substrates are not phosphorylated by HK2, neither glycolysis nor autophagy occurs. When the substrates are phosphorylated and bound by HK2, only glycolysis, but not autophagy, is accomplished (Roberts et al. 2014).

In tumor cells, HK2 is induced due to nutrient deficiency, and cells attempt to enhance glycolysis for the synthesis of ATP. However, as the HK2 protein is ubiquitinated by the E3 ligase TRAF6 (TNF receptor-associated factor 6) at the Lys41 residue, the ubiquitinated HK2 protein is recognized by the autophagy receptor SQSTM1/p62 for selective degradation. Therefore, HK2 expression induces autophagy, which, in turn, can negatively regulate glycolysis. This study showed that energy-demanding cells, such as tumor cells, depend more heavily on autophagic degradation than conventional glucose metabolism for energy.

PFKFB4 (6-phosphofructo-2-kinase/fructose-2, 6-bisphosphatase 4) is one of the four bifunctional kinases/phosphatases. PFKFB4 catalyzes the phosphorylation of fructose-6-phosphate, produces fructose-2,6-bisphosphate (F2,6BP) and dephosphorylates F2,6BP. F2,6BP is the most potent allosteric activator of glycolysis. PFKFB4 is highly expressed in cancer cells and can be induced by hypoxia. The induction of this enzyme promotes glycolysis and ATP synthesis, inhibits ROS production, and activates mTOR pathway, thereby inhibiting autophagy (Strohecker et al. 2015). Another isoform, that is, PFKFB3, promotes both basal and $H_2O_2$-induced autophagy by localizing to the nucleus and activating AMPK (Yan et al. 2017).

Interestingly, glycolysis and autophagy are also linked by TIGAR (TP53-induced glycolysis and apoptosis regulator). TIGAR has fructose 2,6-bisphosphatase activity, which can reduce the content of F2,6BP, thus inhibiting glycolysis. In lung tissue from patients with idiopathic pulmonary fibrosis, the expression level of TIGAR is increased, while the expression level of LC3 and p62 and the number of autophagosomes are decreased. A negative correlation between TIGAR and autophagy was observed in this scenario. In addition, TIGAR is activated in starved HeLa cells. TIGAR reduces the ROS level and inhibits autophagy. Taken together, the regulation of autophagy levels by TIGAR represents a potential therapeutic approach for metabolism-related diseases, such as cancers (Figs. 16.1, 16.2 and 16.3).

Pyruvate kinase (PK), which is another key enzyme in glycolysis, is also linked to autophagy. The acetylation at K305 of its alternative splicing isoform M2 (PKM2) increases the interaction between PKM2 and the molecular chaperone HSC70 and promotes CMA, which, in turn, promotes the lysosomal-dependent degradation of PKM2 and reduces PKM2 enzyme activity (Lv et al. 2011). However, the knockdown of PKM1 or PKM2 reduces the cellular ATP levels, thereby activating the LKB1-AMPK pathway to activate autophagy (Prakasam et al. 2017).

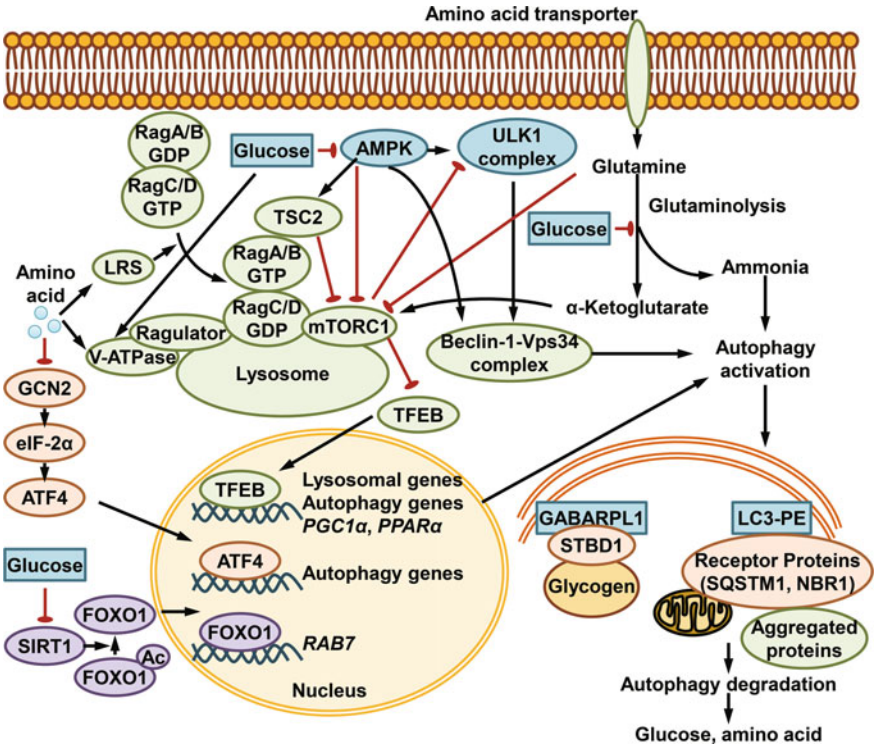


**Fig. 16.1** Regulation of autophagy by amino acids and glucose. In the presence of sufficient amino acids, the v-ATPase-Ragulator-Rag GTPase or LRS–Rag GTPase complexes activate mTORC1. Consequently, ULK1 and Beclin1–Vps34 complexes are inhibited, and autophagy is suppressed. Under conditions of amino acid deprivation, these pathways are inactivated, and autophagy is induced. ATF4 activated by GCN2–eIF-2α also contributes to this induction. TFEB induces genes associated with lysosomal biogenesis, autophagy and lipid catabolism. Glutamine produced by FOXO-induced glutamine synthetase can activate autophagy by inhibiting mTORC1 translocation to the lysosome; however, glutamine can also inhibit autophagy through the activation of mTORC1 via α-ketoglutarate production. Glucose starvation increases autophagic activity through the transcriptional regulation of autophagy-related genes or posttranslational modification. Ammonia promotes autophagy under glucose deprivation conditions. Glucose and amino acid produced by autophagy provide energy sources for ATP production or building blocks for protein synthesis. Abbreviations: Ac, acetylation; LRS, leucyl-tRNA synthetase; SIRT1, sirtuin-1; SQSTM1, sequestosome-1; TSC2, tuberin

GAPDH catalyzes a reversible reaction in glycolysis, namely, the conversion of glyceraldehyde 3-phosphate to 1,3-bisphosphoglycerate. In HEK293 cells, glucose deprivation results in an increased interaction between GAPDH and mTOR, which, in turn, promotes autophagy. A higher concentration of glucose enhances the interaction between GAPDH and its substrate glyceraldehyde 3-phosphate, leading to enhanced glycolysis. After staurosporine (an activator of apoptosis) treatment, HeLa cells transfected with GAPDH show a translocation of GAPDH to the nucleus and an

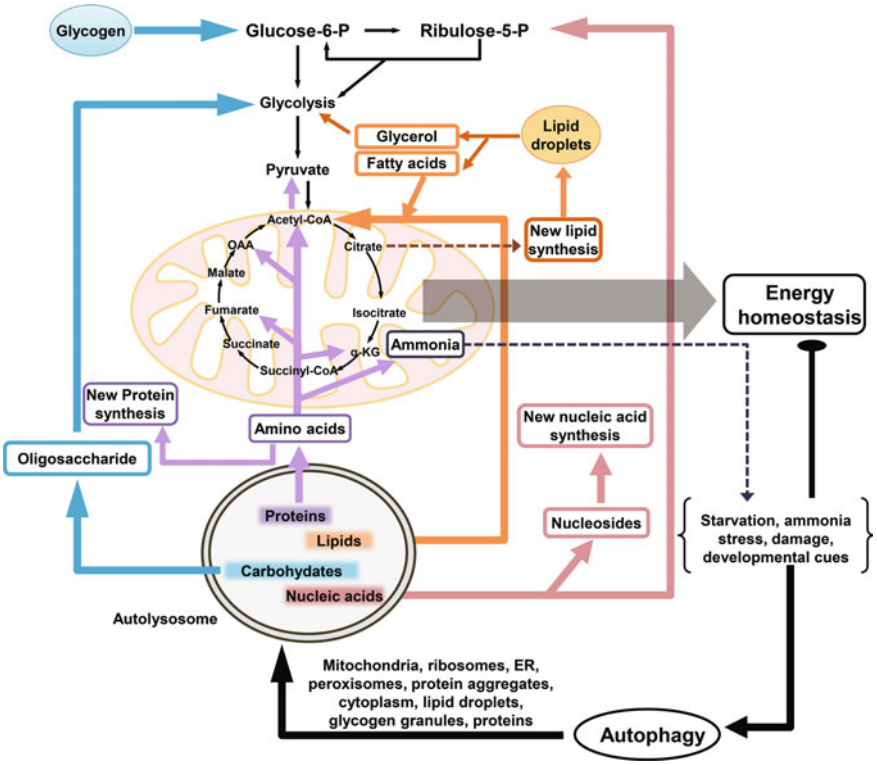


**Fig. 16.2** Use of the products of autophagy. Multiple forms of stress activate autophagy (bottom right). Degradation of proteins, lipids, carbohydrates and nucleic acids liberates amino acids, fatty acids, sugars and nucleosides, which are released into the cytoplasm for reutilization. Sugars, including glucose released from glycogen granules by glycogenolysis or autophagy, are catabolized by glycolysis and the PPP to generate ATP and pyruvate for subsequent TCA cycle metabolism. Nucleosides are used for new nucleic acid synthesis and catabolized by the combined action of the PPP and glycolysis. Amino acids are used as building blocks for new protein synthesis, ATP production by central carbon metabolism, and (in the liver) substrates for gluconeogenesis. Amino acids can also be combined to yield citrate, which drives lipid synthesis and membrane biogenesis. Catabolism of amino acids yields ammonia, which is an activator of autophagy. Fatty acids from lipolysis or autophagy of membranes or lipid droplets yield acetyl-CoA, which feeds the TCA cycle, supporting ATP production and citrate generation. OAA indicates oxaloacetate; α-KG, α-ketoglutarate; and ER, endoplasmic reticulum

upregulation of Atg12, which is a key player in autophagosome formation. GAPDH induces the upregulation of Atg12 to promote autophagy to protect cells against apoptosis. This protective effect of autophagy is associated with an increased metabolic flux of glycolysis upon the addition of the pan-caspase inhibitor zVAD (Dodson et al. 2013). In wild-type mouse embryonic fibroblasts (MEFs), the expression of H-Ras$^{V12}$ increases glucose uptake and the metabolic flux in glycolysis. However, the expression of H-Ras$^{V12}$ in Atg5$^{-/-}$ mice reduces glucose uptake and the metabolic

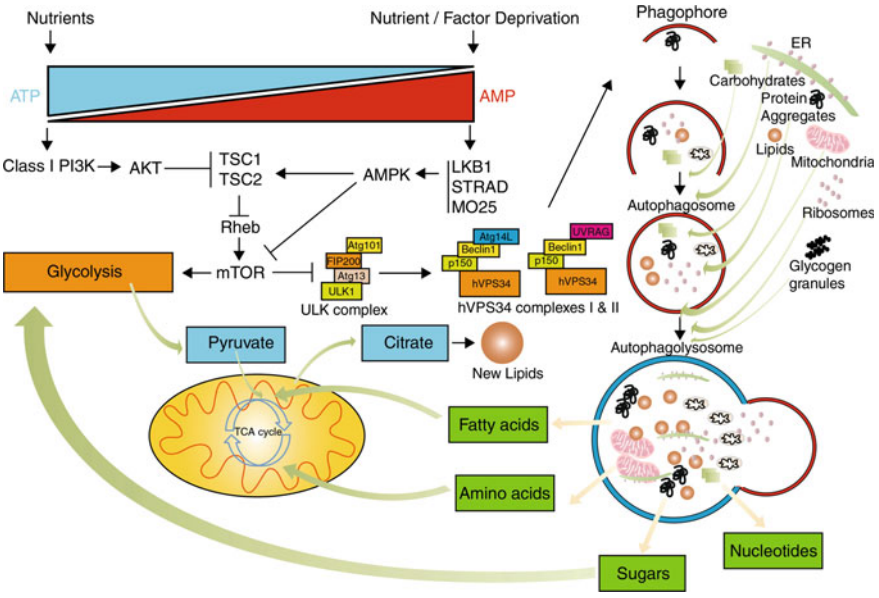


**Fig. 16.3** Regulation of autophagy under nutrient deprivation and its interaction with central carbon metabolism. The major extracellular nutrient sensing pathways, which are controlled by PI3K-I and AMPK, tightly regulate autophagy through mTOR signaling, although other mTOR-independent mechanisms exist. Under nutrient-replete conditions, autophagy is inhibited by mTOR and the inactivation of the ULK1 complex. Metabolic stress relieves this inhibition to activate autophagy and activates AMPK. AMPK inhibits mTOR by activating its negative regulator, that is, TSC2, and inhibiting its positive-regulatory subunit, that is, Raptor. The ULK1 complex activates the Vps34-containing and Beclin1-containing complexes to initiate phagophore formation. Phagophores nucleate and expand around the cargo encapsulating them and target it to lysosomes for degradation. The degradation products of autophagy substrates may re-enter glycolysis and the TCA cycle for anabolic and catabolic processes, leading to the generation of energy and biomass production

flux in glycolysis. Further studies have shown that H-Ras$^{V12}$Atg5$^{-/-}$ MEFs are more sensitive to glucose deprivation in culture medium than wild-type MEFs.

Pyruvate, which is the end product of glycolysis, is either oxidized to acetyl-CoA entering the citric acid cycle under aerobic conditions or reduced to lactic acid under hypoxic or anoxic states. Lactate dehydrogenase B (LDHB), which is an enzyme that catalyzes the reversible reaction between pyruvate and lactic acid, is also associated with autophagy. In tumor cells, a large amount of lactic acid accumulates due to prolonged hypoxia. LDHB converts lactic acid and $NAD^+$ to pyruvate, NADH and $H^+$. This reaction is accompanied by the pumping of $H^+$ into lysosomes via the lysosomal proton pump V-ATPase (vacuolar $H^+$-ATPase), which promotes lysosomal acidification and enhances autophagy in cancer cells (Brisson et al. 2016).

The pentose phosphate pathway (PPP), which is also known as the hexose monophosphate shunt, is a branch of glycolysis that maintains cellular redox homeostasis by providing NADPH. Glucose-6-phosphate dehydrogenase (G6PD) catalyzes the first reaction of PPP to produce NADPH, which is important for maintaining the reduced form of glutathione (γ-L-glutamyl-L-cysteinyl-glycine, GSH). Then, the reduced GSH controls the production of superoxide and hydrogen peroxide by NOX, which may further affect the signaling pathways in the cell. The equilibrium state of intracellular ROS and GSH has a remarkable impact on the regulation of autophagy. The proper function of PPP and the level of GSH are essential for maintaining basal autophagy. The abnormal expression of G6DP catalyzing the first and rate-limiting step of PPP causes the dysregulation of autophagy and consequently affects the whole body. For example, the lack of interferon-γ-inducible lysosomal thiol reductase (GILT) leads to a reduced level of GSH in fibroblasts but an increase in the GSSH level along with autophagy. In HeLa, HepG2 and H1299 tumor cells, starvation lowers the intracellular GSH level likely via the exocytosis of GSH through the membrane transporter protein ABCC1. Consequently, the decreased GSH level impairs the lipidation of LC3 and inhibits autophagy. However, whether the same mechanism can be applied to normal epithelial cells still requires further exploration. In addition, another enzyme, that is, γ-glutamyl cysteine-ligase (GCL), catalyzes the rate-limiting step in GSH synthesis. Upon starvation, a specific inhibitor of GCL, that is, buthionine sulfoximine, increases LC3B-II, while a GSH donor, that is, glutathione ethylester, reduces LC3B-II. TIGAR regulates both cell metabolism and autophagy, and its expression attenuates glycolysis and enhances PPP to increase the ratio of reduced GSH. These studies show that the GSH level is critical for regulating the induction of autophagy.

#### 16.1.2.2 Autophagy and Biological Oxidation

In eukaryotes, acetyl-CoA is mainly produced by pyruvate decarboxylation and fatty acid β-oxidation in mitochondria. Interconversions of acetyl-CoA occur in different metabolic pools, the mitochondria matrix and cytoplasm. Since mitochondrial acetyl-CoA mainly fuels the citric acid cycle and does not significantly change, the cytoplasmic CoA level is a good indicator of the cell energy state and a major regulator of autophagy. For example, the cytoplasmic acetyl-CoA level is significantly reduced after culturing mammalian cells for several hours under low nutrient conditions or after an overnight starvation in mice. The depletion of acetyl-CoA effectively stimulates autophagy likely because acetyl-CoA is the sole donor of the acetyl group of acetyltransferases, and posttranslational acetylation regulates the activity of several autophagy-related proteins. In turn, starvation-induced autophagy can be inhibited in both cultured cells and mice when the intracellular acetyl-CoA level is elevated due to artificial supplementations (Mariño et al. 2014).

Accumulating evidence suggests that acetylation and deacetylation modification are vital for regulating autophagy. Genes and proteins involved in autophagy regulation and the autophagy machinery can be epigenetically modified by acetyltransferase

(KAT/HAT) or deacetylase (KDAC/HDAC). In mammalian cells, under nutrient-rich conditions, the acetyltransferase p300 directly interacts with ATG7, acetylating the autophagy proteins ATG5, ATG7, ATG8 and ATG12. During starvation, p300 dissociates from ATG7, and these autophagy proteins are deacetylated and activated by the action of SIRT1, thereby inducing autophagy. Recent studies have shown that another acetyltransferase, that is, Esa1p (yeast), and its mammalian homolog, that is, TIP60, participate in the regulation of autophagy by modulating the LC3 lipidation and ULK1 complex activation, respectively. During starvation-induced autophagy, Esa1p is acetylated to induce the enzymatic activity of Atg3p, that is, lipid-binding interaction with Atg8p, which is a yeast homolog of mammalian LC3. In mammals, TIP60 has been shown to be a positive regulator of growth factor (or serum) deprivation-induced autophagy in which TIP60 is phosphorylated and activated by glycogen synthase kinase-3 (GSK3). Activated TIP60 interacts with acetylated and activated ULK1 to induce autophagy in response to growth factor (or serum) starvation. In general, acetylation/deacetylation modification is an important regulatory mechanism linking autophagy to the cellular metabolic status, energy levels and extracellular growth factor levels (Ha et al. 2015).

Acetyl-CoA is an important molecule in the citric acid cycle producing ATP and other high energy molecules, powering the electron transport chain and maintaining cellular energy homeostasis. The conversion of pyruvate to acetyl-CoA occurs only in the mitochondrial matrix. Therefore, to enter the matrix, pyruvate needs to be transported through the outer membrane, the intermembrane space and the inner membrane of mitochondria. Although pyruvate readily passes through the mitochondrial outer membrane (via porins or nonselective channels) and intermembrane space, its transport through the inner membrane is limited and requires specific transporters, that is, MPC1 (pyruvate carrier proteins 1) and MPC2. The deletion of MPC1 results in defects in mitochondrial pyruvate uptake and oxidation. MPC1 or MPC2 knockout results in a decrease in acetyl-CoA and citric acid cycle intermediates in certain cell types. An inhibitor of MPC, that is, MSDC-0160, acts on neurons and glioma cells in animal and cell models and promotes autophagy to alleviate neurodegenerative diseases, such as Parkinson's disease, by inhibiting the mTOR pathway (Quansah et al. 2018).

#### 16.1.2.3 Glycophagy

Glycogen is an effective means of energy storage and fast delivery. The cytoplasm and autophagy vacuoles represent two spatially distinct intracellular glycogen pools. Glycogen phosphorolysis is a process in which glycogen in the cytoplasm is degraded into glucose-1-phosphate by glycogen phosphorylase. Glycogen hydrolysis is a process in which glycogen is sequestered into autophagy vacuoles and degraded by lysosomal acid α-glucosidase (GAA) to release nonphosphorylated free α-glucose (termed "glycophagy"). Glycophagy is an important alternative to glycogen phosphorolysis in muscle fibers and cardiomyocytes. Glycophagy maintains glucose homeostasis at the whole-body level. Various different conditions, such as hypoxia, can

induce glycophagy. Changes in glycophagy activity have been associated with multiple diseases, such as glycogen storage type II (also known as Pompe's disease) and diabetic cardiomyopathy.

Under glucose starvation, in addition to conventional glycogenolysis by glycogen phosphatase and glycogen debranching enzymes, cells can undergo glycophagy. Starch-binding domain-containing protein 1 (Stbd1) is a receptor protein responsible for the autophagic degradation of glycogen in selective autophagy. Stbd1 is mainly distributed in the liver and muscles, which are the main organ and tissues that store glycogen. In vitro experiments have confirmed that this protein can directly bind glycogen; in vivo experiments in rat liver cell FL83B and rat fibroblast Rat-1 have indicated that this protein is localized in the perinuclear region. Overexpressed Stbd1 accumulates in the perinuclear region and colocalizes with glycogen molecules, endosomes, LAMP1 (lysosomal-associated membrane protein 1) and GABARAPL1 in African green monkey kidney COS M9 cells. Stbd1 overexpression in wild-type Rat1 cells results in the accumulation of a large amount of glycogen molecules in the perinuclear region. The accumulated glycogen molecules are degenerated upon glucose starvation. The above studies indicate that Stdb1 interacts with the ATG8 family member GABARAPL1 and mediates the transportation and anchorage of glycogen to the cell lysosomal membrane (Khaminets et al. 2016). In addition, lysosomal glycosidases are involved in glycophagy. Acute nutritional deficiency in newborns triggers autophagy in liver cells. Glucose can be obtained by the autophagic degradation of hepatic glycogen. Lysosomal mannose-6-phosphatase and glucose-6-phosphatase regulate the phosphorylation of glucose, assisting the transportation of glucose from lysosomes. Glycophagy is not limited to the liver. In fact, altered glycophagy is responsible for several muscle abnormalities called autophagic vacuolar myopathies. For instance, primary LAMP-2 deficiency causes X-linked vacuolar cardiomyopathy and myopathy (also known as Danon disease). Glycogen particles accumulate in the muscles of patients with Danon disease, leading to cardiomyopathy, proximal muscle weakness and mental retardation. The special feature of this disease is that the level of lysosomal acid maltase, which is an enzyme that breaks down glycogen, is at a basal level despite the accumulation of glycogen. Pompe disease is caused by a deficiency in lysosomal α-glucosidases. Moreover, the accumulation of dextran is associated with insufficient autophagy. Recent studies have shown that autophagy defects lead to the inability to transport the sequestered contents (including glycogen) in autophagosomes to properly fuse with lysosomes for degradation.

## *16.1.3 Autophagy and Carbohydrate Anabolism*

### 16.1.3.1 Autophagy and Glycogen Synthesis

Glycogen synthase kinase 3 (GSK3) phosphorylates and inactivates glycogen synthase to inhibit glycogen synthesis. GSK3 has also been reported to participate in

autophagy regulation. In pancreatic cancer cells, the inhibition of GSK3 enhances autophagy to promote cell survival and reduce apoptotic sensitivity. A rapid treatment with GSK3 inhibitors results in the dephosphorylation and nuclear localization of transcription factor EB (TFEB), which is a major regulator of autophagy and lysosomal biogenesis. GSK3 regulates mTORC1 by phosphorylating the mTOR-associated scaffold protein Raptor on Ser859. The inhibition of GSK3 reduces mTOR and Raptor interactions, resulting in the decreased phosphorylation of p70S6K1 and ULK-1 and an increased autophagic flux. The inhibition of GSK3β activity leads to an increase in the AMP/ATP ratio, triggering the activation of AMPK, which, in turn, phosphorylates TSC2 (tuberous sclerosis 2 protein), leading to mTORC1 inhibition and autophagy promotion (Marchand et al. 2015).

The autophagy pathway is regulated by AMPK, and the association between AMPK and glycogen synthesis is more complicated. On the one hand, glycogen can bind the AMPKβ1 subunit via a carbohydrate-binding module (CBM) and allosterically inhibit AMPK activity. This allosteric inhibition requires the presence of CBM. CBM also has isoamylase activity, which catalyzes the hydrolysis of α-1,6-glycosidic linkages at the branch sites of glycogen molecules. However, the CBM domain tightly binds the kinase domain of the AMPK alpha subunit in the absence of glycogen. The binding to glycogen weakens the interaction between the two subunits, thereby reducing kinase activity.

On the other hand, AMPK responds to metabolic stress and preserves cellular energy by inhibiting the anabolic pathway, which consumes ATP. AMPK inhibits the synthesis of fatty acids, cholesterol and proteins by directly phosphorylating and inactivating acetyl-CoA carboxylase, HMG-CoA reductase and mTORC1. In a cell-free system, AMPK phosphorylates the glycogen synthase Ser7 site and inhibits glycogen synthase activity. Studies using animal models have further confirmed that AICAR (5-aminoimidazole-4-carboxamide ribonucleoside), which is a widely used AMPK activator, inactivates glycogen synthase. This inhibitory effect can be eliminated by AMPKα2 knockout (not AMPKα1 knockout) in mice (Ha et al. 2015).

However, studies using several animal models have shown the opposite result following AICAR treatment. AMPK activation was found to be associated with glycogen accumulation but not depletion. One speculation regarding these apparently contradictory results is that other regulatory mechanisms are dominant under chronic AMPK activation, exceeding the inhibitory phosphorylation of glycogen synthase by AMPK. Alternatively, AMPK promotes glucose uptake, leading to an increase in the intracellular G-6-P level and the allosteric activation of glycogen synthase activity, resulting in a net increase in glycogen storage. In transgenic mice overexpressing a mutant glycogen synthase (Arg582Ala), the mutant glycogen synthase cannot be allosterically activated by G-6-P but retains its full catalytic activity. Hence, AICAR-induced glycogen synthesis is completely abolished in these mutant animals.

#### 16.1.3.2 Autophagy and Gluconeogenesis

The liver is the main site of gluconeogenesis. Upon a decrease in blood sugar, the liver produces free glucose through gluconeogenesis, providing energy sources for important tissues and organs, such as muscles and the brain. It is important to provide a sufficient amount of substrate to ensure liver gluconeogenesis for glucose production. During fasting or under stressful conditions, autolysosomes degrade their packed contents to recycle oligosaccharides, fatty acids and amino acids into the cytoplasm. In fasting animal models, liver cells degrade proteins by autophagy, providing glucogenic amino acid substrates for gluconeogenesis to maintain the blood glucose level. There are significant differences in both hepatic gluconeogenesis and autophagy regulation among hepatoma cell lines with different glycolysis patterns. For example, autophagy can be induced by HBSS treatment in both Hep3B and HepG2 cells, but only HepG2 cells exhibit enhanced gluconeogenesis activity, whereas Hep3B cells demonstrate higher glycolysis activity. Researchers have suggested that autophagy promotes the gluconeogenesis of HepG2, which, in turn, reduces glycolysis. However, the molecular mechanism is unclear. Another study showed that in glucose-6-phosphatase α (G6PC)-knockout cells, both the ATP level and TFEB nucleus localization were reduced. TFEB is a key transcription factor that regulates autophagy-related genes, and G6PC is a key enzyme that catalyzes the production of free glucose by gluconeogenesis. Thus, this pathway may be a key signaling pathway linking gluconeogenesis to autophagy (Farah et al. 2016).

Glucose metabolism is closely related to autophagy. Glucose deprivation decreases the intracellular ATP level and increases the ratio of AMP to ATP by activating AMPK. AMPK is an important signaling protein that regulates autophagy in cells (see Chap. 4). AMPK is inactivated under normal physiological conditions. AMPK regulates autophagy in an mTORC1-dependent or -independent manner. Activated AMPK phosphorylates the Raptor protein or inhibits Rheb by phosphorylating TSC2, thereby inhibiting mTORC1 activity and inducing autophagy. In addition, AMPK regulates autophagy in an mTORC1-independent manner. AMPK directly phosphorylates ULK1 and Beclin1 to positively regulate the Vps34 protein (vacuole protein sorting 34, also known as class III phosphatidylinositol 3-kinase, PI3K). The kinase activity of PI3K catalyzes the production of PI3P (phosphatidylinositol-3-phosphate), which regulates the initiation and maturation of autophagy.

In addition to AMPK, glucose can promote mTORC1 activity by the v-ATPase-Ragulator-Rag GTPase complex. Studies have shown that similar to amino acids, glucose can activate mTORC1 via a Rag-dependent pathway. When the glucose concentration is low, autophagy can be induced through two pathways. One pathway is similar to amino acid-regulated autophagy. For example, both the aforementioned mTORC1-dependent pathway and the ammonia produced from glutamine degradation can regulate autophagy. Specifically, ammonia produced by amino acid metabolism activates autophagy through pathways that are independent of ULK1 and mTORC1. The other pathway differs because amino acids regulate autophagy. For instance, sirtuin-1 mediates the deacetylation, activation, and localization of FoxO1 in cardiomyocytes. Then, activated FoxO1 induces the expression of the GTPase

Rab7, which mediates the fusion of autophagosomes with lysosomes. The overexpression of Rab7 activates autophagy, while Rab7-knockdown inhibits FoxO1-induced autophagy. This finding indicates that Rab7 is sufficient and necessary for FoxO1 to induce the autophagic flux. This pathway of autophagy regulation can be attributed to cell-type differences (e.g., primary cells derived from tissue versus immortalized cells) or experimental designs (e.g., times of different nutrient deficiencies).

## 16.2 Autophagy and Protein Metabolism

Protein degradation and synthesis are important for the turnover of proteins in the body. The following two major molecular mechanisms control the complete degradation of proteins in eukaryotes: ubiquitination and autophagy. The ubiquitination system degrades specific proteins via proteasomes, while autophagy is mediated by the lysosomal degradation of excessive or abnormal proteins, and most intracellular proteins can be degraded through autophagy. In particular, lysosomes provide an acidic environment for proteases to degrade proteins. The resulting substances, that is, amino acids, are then transported to the cytosol, supplementing the metabolic pool of amino acids.

Although both autophagy and ubiquitination pathways can degrade proteins into amino acids, only autophagy is regulated by the concentration of cytosolic amino acids. Several amino acids, such as glutamine, leucine, arginine and proline, regulate autophagy by acting as signal molecules. In addition, the metabolism of amino acids and the extracellular nutritional status play an important role in regulating autophagy. In general, the excessive intake of amino acids leads to the inhibition of autophagy in the body. For example, a glutamine concentration ten times greater than the normal concentration causes toxicity to lysosomes and inhibits autophagy.

Autophagy produces amino acids to fuel cells as an energy resource and provides building blocks for protein synthesis. Protein synthesis requires a large amount of energy, which is approximately 18 kJ (4.3 kcal)/kg body weight every day for adults, equaling approximately 20% of the total energy produced by basal metabolism. Therefore, the processes of the autophagic degradation of proteins to produce amino acids and the biosynthesis of new proteins are dynamically balanced, playing an important role in energy metabolism homeostasis and coping with environmental stresses. This section elaborates upon the molecular mechanisms involved in protein degradation, autophagy and amino acid metabolism. Moreover, the correlations between autophagy and protein and amino acid metabolism are discussed.

### 16.2.1 Autophagy and Protein Synthesis and Degradation

To date, most studies have focused on the functions and mechanisms of autophagy in protein degradation. In fact, for a long time, the activity of autophagy was monitored by the changes in the rate of long-lived protein degradation. In liver cells, autophagy degrades 1–1.5% of all proteins per hour under normal conditions and up to 5% per hour under starvation. More than 70% of the intracellular proteins in the liver are degraded by autophagy. Studies using mouse models have also confirmed this observation.

The autophagic degradation of proteins is significant in the following two ways: amino acids are used to provide energy for cells, and the intracellular amino acid pool is maintained to maintain the synthesis of essential proteins. Studies using both cellular and animal models have shown that protein synthesis is diminished by insufficient autophagy. The cross-talk between autophagy and protein synthesis is mainly mediated by mTORC1. mTORC1 is a major negative regulator of autophagy that stimulates protein synthesis (see Chap. 3). Nutritional deficiencies lead to mTORC1 inhibition, autophagy induction, nonessential proteins and senescent organelle degradation, providing the free amino acids required for the synthesis of critical proteins. Yeast cells maintain protein synthesis by autophagy during restricted supply of amino acids. ULK1 is a Ser/Thr kinase located at the initiation complex of autophagy and regulates other autophagy factors, such as AMBRA (activating molecule in Beclin1-regulated autophagy), ATG9 and BECN1 to stimulate autophagosome formation. mTORC1 inhibits ULK1 function by phosphorylating the S758 and S638 sites, whereas AMPK phosphorylates the Ser317 and Ser556 sites to activate ULK1. mTORC1 also phosphorylates and inactivates other autophagy proteins, including AMBRA and ATG13.

Some proteins associated with autophagy also engage in feedback regulation on mTORC1, thus indirectly affecting protein synthesis. For example, SQSTM1/p62 transports ubiquitinated substrates to autophagosomes, which interact with mTOR, raptor and RAG GTPases to sense changes in the amino acid content. Once the autophagosome is formed, it fuses with the lysosome to degrade its contents, releasing free amino acids, and mTORC1 activity is activated, stimulating protein synthesis and suppressing autophagy by self-regulation. Furthermore, methylpyruvate is a structural analog of pyruvate that is free to pass through the cell membrane and enter the citric acid cycle as a substrate. When ammonium induces autophagy, methylpyruvate can bind free ammonium and convert into alanine to inhibit autophagy.

Eukaryotic initiation factor (eIF) is an important component of protein biosynthesis. Changes in its expression and/or activity also affect autophagy. For example, elevated levels of eIF4G1 (eukaryotic initiation factor 4 gamma 1) are associated with the translational activation of mRNA involved in the regulation of cell survival and prevent autophagy and apoptosis. In addition, the association between mTOR and eIF2α phosphorylation affects autophagy. The inhibition of mTORC1 activates GCN2 (general control nonderepressible 2), leading to the phosphorylation of eIF2α. This inhibitory effect is phosphatase PP6C-dependent. The eIF2α/ATF4 (activating

transcription factor 4) pathway upregulates the transcription of many autophagy-related genes, including *p62, ATG16L, LC3B, ATG12, ATG3* and *BECN1*. Decreased PP6C attenuates mTORC1-inhibition-induced autophagy.

The posttranscriptional regulation of autophagy-associated mRNA is also associated with protein synthesis. The HuD protein binds the 3′-UTR of ATG5 mRNA, stabilizing the mRNA and increasing translation. In *Drosophila*, Orb (vertebrate CPEB homologous protein) and CCR4 (C-C chemokine receptor type 4) adenylase inhibit the translation of ATG12 mRNA, thereby inhibiting autophagy. Once nutrient restriction is restored, the decap reaction reduces the stability of autophagy protein-encoding mRNA and inhibits translation. However, some studies have shown that the capping of mRNA may inhibit autophagy (Lindqvist et al. 2018).

Thus, autophagy produces amino acids for protein synthesis, and protein synthesis and turnover continually consume amino acids that are degraded by autophagy. These two cellular processes work together to maintain cell homeostasis and energy balance through complicated molecular mechanisms.

### *16.2.2 Autophagy and Amino Acid Metabolism*

Amino acids not only regulate autophagy but are also the end products of this process. Amino acids produced by autolysosomal degradation can be used for ATP production, protein synthesis or gluconeogenesis. Thus, protein degradation by autophagy mainly regulates energy and nutrient balance and maintains the amino acid metabolic pool. In peripheral tissues, branched amino acids and nonessential amino acids can be obtained by the autophagic degradation of proteins. In peripheral blood vessels and tissues from autophagy-deficient neonates, the levels of these two types of amino acids are significantly lower. Under starvation, the amino acids produced by autophagy provide substrates for gluconeogenesis and ketogenesis in the liver. The molecular mechanisms regulating protein degradation vary during different periods of starvation. In cells cultured in vitro, the proteasome system maintains the amino acid pool within 1 h of starvation. Along with proteasomal degradation, amino acids are produced by autophagy though a process that typically peaks after 6–8 h of starvation. In fact, although the autophagosome still can be observed within 24 h of starvation, the peak form is achieved approximately 6 h later and then slowly declines. When the duration of starvation exceeds 8 h, the main protein degradation pathway switches from autophagy to CMA. After 10–12 h of starvation, CMA is maximally upregulated, and this rate can be maintained for 3 days. Switching from macroautophagy to CMA can protect cells from breaking down proteins and organelles essential for survival under starvation stress for a long time. Although the molecular mechanism regulating this process is not fully understood, the ketone bodies produced during starvation can induce the production of intracellular oxidized proteins. These oxidized proteins are used as substrates for CMA. The accumulation of oxidized proteins can further induce CMA. Therefore, the autophagic degradation of proteins is a self-regulating process. Forcibly terminating autophagy to degrade

proteins could adversely affect protein quality control and increase the protein toxicity caused by abnormal protein aggregates. In fact, insufficient autophagy is closely related to the formation of protein aggregates, which is the basis of the protein conformation abnormalities leading to Alzheimer's disease and Parkinson's disease (see Chap. 18).

#### 16.2.2.1 Autophagy and Glutaminolysis

Glutamine lysis regulates the process of autophagy. Glutamine is metabolized to glutamate in cells and further converted to alpha-ketoglutarate (α-KG) by the action of glutamate dehydrogenase. α-KG stimulates the binding of Rag to GTP, resulting in the activation of mTORC1 and inhibition of autophagy. However, the specific mechanism by which α-KG induces mTORC1 activation is not fully understood. Studies have shown that the presence of leucine can activate glutamate dehydrogenase. However, ammonium produced by glutaminolysis inhibits autophagy independent of the mTORC1 pathway. A possible mechanism is that the weak-alkaline ammonia neutralizes $H^+$ and weakens the functions of autolysosomes (Tan et al. 2017).

In 1977, scientists observed that amino acid deprivation induces the accumulation of autophagosomes in the perfused rat liver, providing the first evidence of the amino acid regulation of autophagy. Subsequently, the effect of amino acids on autophagy was found to be mediated by mTOR. mTORC1 recognizes the presence of glutamine and leucine by glutaminolysis. Then, α-KG produced by glutaminolysis increases the GTP-binding form of RAGB (a member of the RAG family), which, in turn, activates and translocates mTORC1 to the surface of lysosomes to inhibit autophagy.

The activity of EGLNs (egl-9 family hypoxia-inducible factor)/prolylhydroxylase is critical for the α-KG-dependent activation of mTORC1. EGLN is a cellular oxygen sensor that requires oxygen and α-KG to hydroxylate a protein of interest (e.g., hypoxia-inducible factor HIF). However, under normal conditions, when oxygen is not restricted, EGLN activity strictly depends on the intracellular α-KG levels. Thus, at a high rate of glutaminolysis, the α-KG level increases, which promotes mTORC1 activation and subsequent autophagy inhibition. Therefore, EGLN is a molecular mechanism linking α-KG production to mTORC1 activation. However, the interaction between glutamine and mTORC1/autophagy seems to be more complicated. It has been previously thought that since ammonium increases the pH of the lysosome, even at low concentrations, the presence of ammonium inhibits rather than promotes the autophagy flux. Recent studies have shown that α-KG activates mTORC1 and inhibits autophagy through a combined mechanism involving acetyl-CoA synthesis and protein acetylation. Furthermore, although glutaminase has an inhibitory effect on autophagy, the byproduct ammonium has a dual effect on autophagy, activating the process at low concentrations (2–4 mM) while inhibiting it at higher concentrations. However, the mechanism by which ammonium regulates autophagy remains largely unclear.

Another link between glutaminolysis and autophagy is related to the production of ROS. The association between ROS and glutaminase is as follows: the inhibition

of glutamate dehydrogenase 1 (GLUD1) or glutaminase 2 (GLS2) increases the cellular ROS levels. Since glutamate produced by GLS maintains the synthesis of GSH, the inhibition of GLS reduces the level of GSH and the ability of cells to resist ROS. A decrease in the level of GLUD1 increases the level of ROS likely due to a decrease in the production of NADPH and α-KG. Therefore, oxidative stress caused by glutaminolysis also regulates the level of autophagy.

#### 16.2.2.2 Autophagy and Other Amino Acid Metabolism

Essential amino acids, such as leucine and arginine, inhibit autophagy by upregulating the activity of mTORC1. Studies have shown that the lack of leucine or arginine blocks mTORC1 activation and leads to the dephosphorylation of the mTORC1 substrates S6K1 and 4E-BP1 (eukaryotic initiation factor 4E binding protein 1). Further studies have shown that the presence of leucine can activate mTORC1 only in the presence of L-glutamine. In human cervical cancer HeLa cells, the presence of leucine and L-glutamine synergistically activates mTORC1. The influx of leucine in cells and the efflux of L-glutamine are critical for the activation of mTORC1 and inhibition of autophagy activity. By using GPNA (L-γ-glutamyl-*p*-nitroanilide), which is an inhibitor of SLC1A5 (solute carrier family 1 (neutral amino acid transporter) member 5), and silencing the SLC1A5, SLC7A5 and SLC3A2 genes via mRNAi, researchers have confirmed that mTORC1-mediated autophagy is regulated by a complex bidirectional system. This system involves SLC1A5, which regulates L-glutamine uptake, and the bidirectional transporter SLC7A5/SLC3A2, which transports L-glutamine in and essential amino acids, such as leucine, out (see Chap. 3).

These molecules accumulate in the lysosomal lumen (rather than the cytoplasm), and mTORC1 is recruited to the lysosomal membrane via the amino acid receptor protein complex v-ATPase-Ragulator-Rag GTPase during sufficient availability of amino acids. v-ATPase contains V0 embedded in the lysosomal membrane and hydrophilic V1 subunits located in the cytoplasm. ATP is hydrolyzed by the V1 subunit via some mechanisms that are not fully understood. Hydrolysis can cause the v-ATPase axis to rotate such that the V0 subunit transports protons from the cytoplasm entering lysosomes via a rotation, leading to the acidification of the lysosomal matrix (Carroll et al. 2015).

In general, most upstream signals of mTORC1 are linked at the heterodimer TSC1/TSC2 (tuberous sclerosis 1 protein, TSC1; tuberous sclerosis 2 protein, TSC2). This dimer can negatively regulate Rheb (Ras homolog enriched in brain) and inhibit the activation of mTORC1 (see Chap. 3). However, amino acids regulate the mTORC1 pathway through Rag not Rheb. The Rag family includes RagA, RagB, RagC and RagD. The homology between RagA and RagB is over 98%, and the homology between RagC and RagD is approximately 87%. The human Rag protein exists as a heterodimer, that is, RagA or RagB forms a dimer with RagC or RagD. Under amino acid-rich conditions, Rag can recruit mTORC1 to the membrane of lysosomes and form a protein complex with mTORC1. Leucyl-tRNA synthetase (LRS) activates Rag, mediates leucine-induced mTORC1 activation and inhibits

autophagy. The formation of the mTORC1-Rag complex relies on a protein complex named Ragulator independent of growth factor stimulation, the activity of Rheb or mTORC1 itself. The Ragulator complex consists of p18, p14 and MP1 proteins (encoded by c11orf59, ROBLD3 and MAPKSP1, respectively). Ragulator anchors Rag to lysosomes. In vitro coimmunoprecipitation experiments have revealed a direct interaction between Ragulator and Rag. In vivo RNA interference experiments have shown that the Rag protein could not be localized to the lysosomal membrane in cells with p18 or p14 knockdown. The reintroduction of wild-type p18 could relocate Rag protein to the lysosome. The introduction of a mutant of p18 that targets mitochondria instead of lysosomes to a cell lacking p18 could also lead to Rag localization to mitochondria. This study shows that the p18 protein in the Ragulator complex is sufficient for the localization of Rag. More specifically, the N-terminal lipidation of the p18 protein, including myristoylation and palmitoylation, is required for its localization to lysosomes. In addition, the p18 protein is a platform for keeping the other two protein members, that is, p14 and MP1, in the Ragulator protein complex to localize on the lysosomal membrane. After mTORC1 is recruited to the lysosomal membrane, Rheb activates mTORC1, thereby inhibiting autophagy. In addition, p62 regulates amino acid-induced mTORC1 activity through an interaction with Rag. Taken together, the above studies indicate that amino acid-induced autophagy requires the coordinated regulation of amino acid transporters, mTORC1, v-ATPase, Rag, Ragulator and other protein/protein complexes.

Proline is the most abundant amino acid in the cellular microenvironment. More than 80% of the extracellular matrix is collagen, and more than 25% of the amino acid residues of collagen are proline or hydroxyproline. Proline dehydrogenase/oxidase (PRODH/POX) is the first enzyme in the proline metabolism pathway, which is localized to the mitochondrial inner membrane and catalyzes the dehydrogenation of proline to dihydropyrrole-5-carboxylic acid. One electron is generated in this reaction. This electron can form ROS directly or through the electron transport chain or generate ATP under nutrient-deficient conditions. Hypoxia can activate AMPK and induce the expression of POX, which, in turn, produces ROS and activates protective Beclin1-mediated autophagy in an mTORC1-independent manner.

L-arginine deficiency leads to slower T cell proliferation and functional damage. Arginine deprivation leads to endoplasmic reticulum stress and induces the binding of Beclin1 to Bcl-2 and the formation of autophagosomes. Silencing IRE1α attenuates autophagy induced by arginine deprivation, which, in turn, causes T cells to undergo apoptosis. Most melanoma cells do not express argininosuccinate synthetase (ASS); thus, arginine cannot be synthesized from citrulline. The arginine required for cell growth and proliferation depends on exogenous supply. Arginine deiminase (ADI) can degrade arginine, and ADI leads to cell growth inhibition and death. Studies have shown that melanoma cells lacking ASS exhibit enhanced autophagy, which is a protective mechanism required for cell survival. Upon *Beclin1* silencing via siRNA, the mortality of cells treated with ADI increased by 20–30%, and similar effects were also observed in prostate cancer cells.

## 16.3 Autophagy and Lipid Metabolism

Lipids represent an important part of living organisms as both energy storages (e.g., triglycerides) and important precursors in the regulation of metabolism and signal transductions (e.g., phospholipids and sphingolipids). Some lipid metabolites participate in pathophysiological processes, such as inflammation and the immune response.

Cross-talk exists between lipid metabolism and autophagy (Rabinowitz and White 2010). Lipids regulate autophagy mainly through the following four pathways. (i) Lipid-derived metabolites activate mTOR and downregulate the initiation of autophagy. For example, class I PI3K can catalyze the production of $PIP_3$ to activate AKT and trigger the activation of mTORC1 and its downstream signals to inhibit autophagy. (ii) Inner membrane lipid molecules mediate the morphological change in the double-membrane structure and the transportation of vesicles by binding effector proteins. For instance, PI3P binds its effector protein to regulate the formation and maturation of autophagosomes. (iii) Some lipid molecules facilitate protein modification to regulate autophagy, such as the lipidation of ATG8/LC3 family proteins (i.e., covalently binding to phosphatidylethanolamine), promoting the extension of the phagophores and the formation of autophagosomes. (iv) Lipid molecules regulate autophagy by controlling the distribution of lipid species in the double-membrane structure. For example, an increase in phosphatidic acid, diacylglycerol and ceramide helps the extension and fusion of these autophagic vesicles.

Autophagy of lipid molecules (termed lipophagy) regulates lipid metabolism. Intracellular lipids are mainly present in the form of lipid droplets. The cholesterol and triacylglycerols within the lipid droplets can be degraded by β-oxidation in mitochondria to provide ATP, maintaining a stable supply of cellular energy. The functions and mechanisms of lipophagy in the regulation of lipid metabolism are as follows. (i) Blocking autophagy increases the amount of lipids in lipid droplets. (ii) LC3 is localized on lipid droplets during lipophagy. The SNARE (soluble N-ethylmaleimide-sensitive factor attachment protein receptor) complex may mediate the fusion of lipid droplets and autophagosomes. (iii) ATG15 may act as an esterase. Hence, abnormalities in lipophagy lead to lipid metabolism disorders, such as obesity, arteriosclerosis and diabetes.

Lipophagy in hepatocytes is an important means of lipid degradation. Lysosomes contain many types of hydrolases, esterases, and so on. These enzymes are active in an acidic environment ($pH < 5.2$) and are capable of hydrolyzing the biological macromolecules and organelles transported to the lysosomes. The autophagosomes sequester the lipid droplets, fuse with lysosomes and finally degrade the lipid droplets. By using the autophagy inhibitor 3-MA or silencing the mRNA of the ATG5 and ATG7 genes, triglycerides can be accumulated in hepatocytes, which are accompanied by a decrease in β-oxidation. In summary, lipophagy has complicated regulatory functions for metabolism and intracellular signaling transductions (see Chap. 17).

## 16.4 Autophagy and ATP Synthesis and Utilization

The human body and cells need to constantly synthesize and utilize ATP. It is generally believed that cell survival requires energy, that is, ATP. AMPK is a sensor that monitors the energy state in cells. When the ATP/AMP ratio decreases and the energy supply is insufficient, AMPK subsequently activates and inhibits mTOR via the activation of the downstream molecules TSC1/TSC2, thereby inducing autophagy. During insufficient extracellular nutrition, mTOR is also inactivated by low amino acid concentrations or low growth factor signals. Autophagy inhibits anabolism, enhances catabolism, and provides nutrients and energy for cell survival. Once this dynamic balance between ATP synthesis and utilization is disrupted, autophagy cannot benefit cell survival. Consequently, cells undergo cell death in the form of apoptosis, senescence and necrosis (see Chap. 29).

Upon glucose deprivation, autophagy is induced to supplement the metabolic pool and provide ATP through various mechanisms. The key mechanisms are as follows (Galluzzi et al. 2014). (1) AMPK phosphorylates p53 at Ser53 (Ser18 in mice). At low glucose concentrations in medium (0.5 mM), p53 is phosphorylated, translocates to the nucleus and binds autophagy-related genes, such as the promoter regions of SESTRIN 2 and DAPK-1 (death-associated protein kinase 1), to activate autophagy. SESTRIN 2 activates AMPK and induces the phosphorylation of TSC2, thereby inhibiting mTORC1. APK-1 phosphorylates Beclin1 and blocks the formation of the Beclin1/Bcl-2 complex, thereby activating autophagy. p53 also regulates the AMPKB1 (B1 subunit of AMPK), DRAM and TSC2 genes to induce autophagy. Glucose deficiency induces the AMPK-mediated phosphorylation of p53, whereas p53 can activate AMPK through transcriptional regulation, highlighting the positive feedback between AMPK and p53 (see Chaps. 4 and 6). In summary, AMPK is an ATP sensor that directly regulates autophagy. The positive feedback loop between AMPK and p53 indicates that p53 is a key protein connecting energy metabolism to autophagy regulation. p53 plays a key regulatory role in the activation of autophagy under energy crisis.

(2) Glucose deficiency increases the LKB1/AMPK-mediated phosphorylation of cyclin-dependent kinase inhibitor $p27^{KIP1}$ at Ser83, Thr170 and Thr197 (mouse)/Thr198 (human). LKB1 is usually absent in HeLa cells. Even if cells are glucose-starved, the levels of total and phosphorylated $p27^{KIP1}$ are still low. When LKB1 is introduced to cells, glucose starvation induces an increase in total $p27^{KIP1}$ and an increase in Thr198 phosphorylation. Stable $p27^{KIP1}$ expression promotes autophagy and cell survival. Autophagy is insufficient, and cells undergo apoptosis upon $p27^{KIP1}$ knockdown. In addition, incubation with glucose-deprived medium results in an increase in the number of autophagic vesicles in $p27^{+/+}$ MEFs but not in $p27^{-/-}$ MEFs. Autophagy regulation by $p27^{KIP1}$ is mediated by RhoA. Cytoplasmic $p27^{KIP1}$ inhibits the activation of RhoA, which, in turn, inhibits the activation of calpains, which participate in the degradation of Beclin1. The above studies indicate that $p27^{KIP1}$ is another key protein that mediates the regulation of autophagy by AMPK.

In the absence of glucose, the phosphorylation of $p27^{KIP1}$ induces autophagy and promotes cell survival.

(3) Akt (also known as protein kinase B, PKB) is a serine/threonine protein kinase. Akt plays an important role in the PI3K/Akt/mTOR signal transduction pathway to regulate autophagy. Akt inhibits TSC2 by autophosphorylation, thereby activating mTORC1. The phosphorylation of Akt can also activate mTORC1 by regulating its substrate PRAS40 (proline-rich Akt substrate of 40 kDa). PRAS40 binds and suppresses mTOR via Raptor. As a substrate of Akt, PRAS40 detaches from and abolishes the inhibitory effect of mTORC1 when phosphorylated by Akt. In muscle cells, FoxO3 can be inactivated by Akt phosphorylation at Thr32, Ser253 and Ser315. The phosphorylation of the Thr32 and Ser253 sites induces the binding of Akt to the molecular chaperone 14-3-3, and the resulting complex enters the cytoplasm from the nucleus. In addition, the phosphorylation of Akt alters the binding of FoxO to target DNA sequences. Two autophagy-related genes, that is, BNIP3 (Bcl2/adenovirus E1B 19-kDa protein-interacting protein 3) and GABARAPL1, are regulated by FoxO3. In muscle tissues from Aktl and Akt2 knockout mice, the transcription of the BNIP3 and GABARAPL1 genes is significantly upregulated, highlighting the important role of Akt in the regulation of FoxO transcription and autophagy. In other cell types, FoxO1 and FoxO4 are also phosphorylated by Akt (see Chap. 9).

In contrast to the aforementioned protective autophagy, some researchers believe that autophagy activation promotes immunogenic cell death (ICD). In general, the immunogenicity of cancer cells is partially or completely eliminated, and thus, these cells can survive immune attacks. Some chemotherapeutic drugs, such as anthracycline and oxaliplatin, can reinitiate an immune response against tumor-associated antigens and lead to ICD of tumor cells, which is critical for successful cancer treatment. One of the three main features of ICD is the release of ATP from dying cells. Autophagy is thought to be required for ICD-associated ATP secretion, that is, exocytosis of ATP via a LAMP1-dependent lysosomal pathway. Calorie intake restriction or mimicking calorie restriction by pharmacological inhibitors can enhance tumor cell autophagy, thereby increasing extracellular ATP secretion and allowing cells to enter the ICD process (Bloy et al. 2017).

In summary, autophagy pathways and cell metabolism form a complex interacting network. In this multilevel regulatory network, autophagy plays an important role in sensing fluctuations in cellular energy metabolism. Through AMPK, mTORC1, protein kinase A (PKA) and other pathways, the energy demand of cells can be adjusted according to the autophagy flux to improve the utilization efficiency of cells. Stressed cells can struggle to survive if the changes in the autophagy flux can meet the needs of intracellular ATP; otherwise, the cells initiate programmed death. Different environmental factors force cells to undergo different death programs, such as apoptosis, necrosis or autophagy-dependent death (see Chap. 29). Studies investigating tumor cells suggest that autophagy regulation and energy metabolism are environment-dependent. More experimental evidence is required to provide detailed regulatory mechanisms for translational medicine.

## 16.5 Conclusion

ATP is a universal "energy currency" that maintains cell life and activities. ATP is produced primarily through a series of catabolic pathways and oxidative phosphorylation in aerobic cells. The oxidation of glucose, amino acids and lipids provides ATP in most types of cells; however, some types of cells, such as red blood cells, can only rely on the glycolysis pathway to generate energy since they lack mitochondria. Another example is the Warburg effect in tumor cells, that is, due to hypoxia, glycolysis functions more rapidly in tumor cells than normal cells and becomes a preferential pathway for ATP production. Recently, lactic acid has been recognized as a major energy resource in lung cancer cells.

Energy metabolism is a complex biological cycle, and autophagy plays a dual role in the regulation of this process. Autophagy consumes a large amount of intracellular ATP. Autophagy provides substrates for protein biosynthesis, and this process also requires a great amount of ATP. However, autophagy provides energy for cell survival and growth by degrading organelles, proteins, lipids, glycogens, and so on. Obviously, autophagy and its regulation play an important role in the maintenance of cellular energy homeostasis and cell fate decisions. The metabolism of amino acids, carbohydrates and lipids, which are the three major nutrients of the cell, is closely related to autophagy. Some key autophagy regulatory proteins and complexes, such as mTORC1 and AMPK, are also important regulators of energy metabolism. The complicated interaction between energy metabolism and autophagy maintains the dynamic balance of cell physiology. We can better understand the intrinsic molecular regulatory mechanisms only by linking the cellular metabolic processes with autophagy. Thus, we need to consider the autophagy process as an energy metabolism feedback loop that involves energy sensing, metabolite production, substrate consumption, and ATP synthesis and utilization. Therefore, autophagy controlling cell energy homeostasis can also be considered a steady-state feedback mechanism.

Under stress conditions (such as nutrient deprivation, hypoxia and amino acid deficiency), autophagy may progress through the following two stages: the signaling transduction stage, which does not involve transcriptional regulation, and the subsequent transcriptional regulation stage. Signaling pathways are responsive during the early stage of autophagy, which ranges from a few minutes to a few hours. Then, the subsequent transcriptional regulation of autophagy is initiated to better adapt to the stressful conditions. Numerous transcription factors, such as p53, NF-κB, STAT3, FOXO, TFEB, XBP1, SOX2, NRF2 and ZKSCAN3, are known to transcriptionally regulate autophagy. These transcription factors interact with their cofactors synergistically to identify the chromatin environment of the target genes and epigenetically regulate these target genes (see Chap. 11). For example, FOXOs are highly conserved transcription factors that play an important role in the regulation of autophagy and ubiquitin-proteasome-related genes. Autophagy and ubiquitin proteasome systems are two important ways to maintain cellular homeostasis. FOXO3 and/or FOXO4 can mediate the induction of GLS activity, and glutamine produced by this pathway inhibits mTORC1 activity and promotes autophagy. In the absence of amino acids,

TFEB (an important transcription factor that regulates lysosomal biogenesis) transfers from lysosomes to the nucleus, inducing the transcription of a series of autophagy genes and lipid metabolism genes, such as PPARα and PGC1α. The identification of the binding sites of these transcription factors by high-throughput screening may allow for a dynamic investigation of the transcriptional regulation of autophagy and its cellular effects. From this perspective, a complex relationship exists among energy metabolism, autophagy and cell fate determination. An improved mechanistic understanding of these autophagy machineries could offer therapeutic options via autophagy modulation in translational medicine.

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# Chapter 17
# Autophagy and Lipid Metabolism


**Muhammad Babar Khawar, Hui Gao and Wei Li**

**Abstract** Autophagy is a conserved catabolic process that delivers intracellular proteins and organelles to the lysosome for degradation and recycling. Evidences over the past decades have proved that autophagy participates in cell fate decision and also plays a key role in regulating cellular energy and nutrient stores. Lipid droplets (LDs) are the main lipid storage form in living organisms. The process of autophagic degradation of LDs is referred to lipophagy or macrolipophagy. Lipophagy is not only indispensable for the cellular lipid metabolism but also closely associated with several metabolic disorders such as obesity, hepatic steatosis, atherosclerosis, and so on. Here, we summarize recent progress in understanding the molecular mechanisms of lipophagy regulation and the emerging roles of lipophagy in various biological processes and metabolic disorders.

**Keywords** Autophagy · Lipid droplet · Lipid metabolism · Lipophagy · Metabolic disorder

## Abbreviations

| | |
|---|---|
| 3-MA | 3-methyladenine |
| ATGL | Adipose triglyceride lipase |
| CD36 | Cluster of differentiation 36 |
| CGI-58 | Comparative gene identification-58 |
| CHOP | C/EBP homologous protein |
| CYP2E1 | Cytochrome P450 2E1 |
| CMA | Chaperone-mediated autophagy |
| DHA | Docosahexaenoic acid |

M. B. Khawar · H. Gao · W. Li (✉)
State Key Laboratory of Stem Cell and Reproductive Biology, Institute of Zoology, Chinese Academy of Sciences, 1 Beichen West Road, Chaoyang District, Beijing 100101, China
e-mail: leways@ioz.ac.cn

M. B. Khawar · W. Li
University of Chinese Academy of Sciences, Beijing 100049, China



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| | |
|---|---|
| DMP | 3,5-dimethylpyrazole |
| DNM2 | Dynamin 2 |
| eIF4A | Eukaryotic initiation factor 4A |
| FXR | Farsenoid X receptor |
| FOXO1 | Transcription factor forkhead box O1 |
| GLP-1 | Glucagon-like peptide-1 |
| HBV | Hepatitis B virus |
| HCV | Hepatitis C virus |
| HOPS tethering complex | Homotypic fusion and vacuole protein sorting complex |
| HSL | Hormone-sensitive lipase |
| Hsp70 | Heat shock cognate protein of 70 kDa |
| LAMP2A | Lysosome-associated membrane protein 2A |
| LIPL-1 | Lysosomal acid lipases 1 |
| LPL-1 | Lipoprotein lipase-1 |
| MXL-3 | Basic helix–loop–helix transcription factor Max-like 3 |
| NAFLD | Nonalcoholic fatty liver disease |
| NASH | Nonalcoholic steatohepatitis |
| NDP52 | Nuclear dot protein 52 kDa |
| PDCD4 | Programmed Cell Death Protein 4 |
| PGC-1α | Peroxisome proliferator-activated receptor-γ coactivator-1 alpha |
| PI3KCIII | Phosphatidylinositol-3 kinase class III |
| PL | Phospholipid |
| PLINs | Perilipins |
| PLIN1 | Perilipin1 |
| PLIN2 | Perilipin2 |
| PLIN3 | Perilipin3 |
| PLIN4 | Perilipin4 |
| PLIN5 | Perilipin5 |
| ROS | Reactive oxygen species |
| Sidt2 | SID1 transmembrane family, member 2 |
| Sirt1 | Silent information regulator 1 |
| SNARE | Soluble N-ethylmaleimide-sensitive factor attachment protein receptors |
| SREBP2 | Sterol regulatory element-binding protein-2 |
| TBC1D15 | TBC1 domain family member 15 |
| TBC1D17 | TBC1 domain family member 17 |
| TG | Triglyceride |
| TFE3 | Transcription factor E3 |
| TFEB | Transcription factor EB |

## 17.1 Introduction

Lipids are not only the key integral components but also the main energy storage form in living organisms. The major types of lipids are triglyceride (TG), phospholipid (PL), cholesterol, cholesterol ester, and many others. Lipids usually exist in the form of lipid droplets (LDs) in the cytoplasm with a diameter of 0.1–100 μm. The core of LDs is mainly composed of neutral fats such as TG and cholesterol ester, and the outer side is composed of one monolayer phospholipids and a large number of lipoproteins (primarily the perilipins protein family, PLINs) (Fig. 17.1) (Wilfling et al. 2014). Cells tend to store excess energy in the form of LDs when there are abundant nutrients, while cells degrade these LDs via lipolysis or autophagy under starvation or other stimulations. The process of LDs degradation by autophagic pathway is referred to lipophagy or macrolipophagy. Lipophagy is not only indispensable for the cellular lipid metabolism but also closely associated with several metabolic disorders such as obesity, hepatic steatosis, atherosclerosis, and so on. Lipophagy research is an emerging field and has become a hot topic in the past few years. This chapter will review the major regulatory mechanisms and role of lipophagy in various cellular processes.

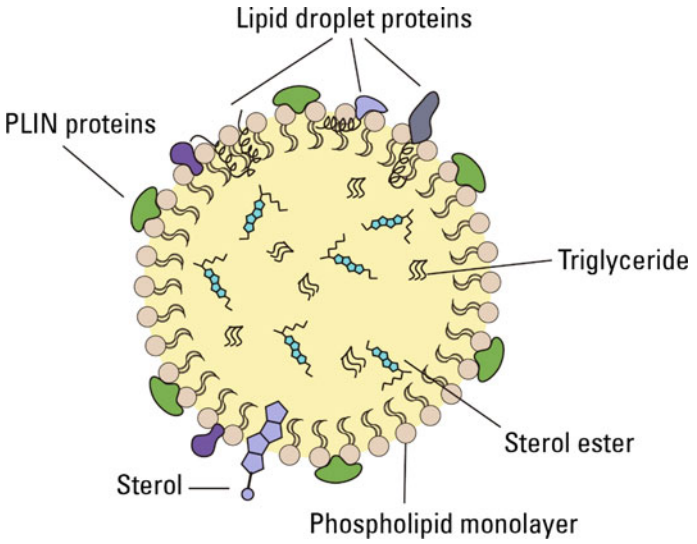


**Fig. 17.1** Structural organization of a lipid droplet. LDs are surrounded by a monolayer of phospholipids that encloses an organic core consisting of neutral lipids. The major proportion of core is filled by sterol esters and triglycerides. LDs are decorated by numerous types of proteins including structural proteins (PLINs) and trafficking proteins

## 17.2 Lipophagy

Singh et al. (2009) found a significant LDs accumulation accompanied by a considerable decrease in fatty acid oxidation in cultured hepatocytes either treated with 3-methyladenine (3-MA) or upon *Atg7* and *Atg5* knockdown via shRNA. In line with it, the accumulation of TG in $Atg5^{-/-}$ mouse embryonic fibroblasts also suggests that autophagy deficiency blocks the LDs degradation. LDs are encapsulated in autophagosomes, subsequently autophagosomes and lysosomes fuse to complete the degradation in autolysosomes. Thus, autophagy is found to regulate LDs degradation (Singh et al. 2009). Later on, the existence of lipophagy was confirmed in different species such as yeast, nematode, rice and in various cell types, that are, macrophages, lymphocytes, and hypothalamic neurons. Therefore, lipophagy is thought to be a conserved and widespread phenomenon.

## 17.3 Key Regulatory Proteins Involved in Lipophagy

### *17.3.1 Role of PLIN Protein Family and Lipolytic Enzymes in Lipophagy*

Hundreds of proteins are found on the LDs surface, which are widely involved in LDs metabolic regulation and lipid signal transduction. Many of these proteins also participate in lipophagy regulation. The most important ones are the PLINs family proteins that comprised of five members, PLIN 1–5. These PLIN members have different expression patterns and physiological functions in various tissues. PLIN1 and PLIN2 are located only on the surface of LDs and are degraded when not bound to LDs. PLIN3 and PLIN4 can bind or dissociate freely with LDs and remain stable even when released in to the cytoplasm. PLIN5 mainly expresses in some highly oxidized tissues of the body, such as the heart, skeletal muscle, and liver. These PLINs participate in lipophagy by regulating the binding of lipase to LDs. In adipocytes, PLIN1 can bind with the comparative similarity gene-58 (CGI-58). Once a lipolysis signal is detected, PLIN1 rapidly phosphorylates and releases CGI-58 that eventually activates adipose triglycerides lipase (ATGL) to initiate lipolysis. PLIN2 and PLIN3 proteins were found to contain a chaperone-mediated autophagy (CMA) recognition sequence (KFERQ) that binds with heat shock cognate protein of 70 kDa (HSP70) to guide the LDs toward lysosomes for CMA degradation. Inhibition of CMA led to LDs accumulation, indicating that PLIN2 and PLIN3 degradation acts as an upstream signal for lipophagy initiation (Fig. 17.2) (Kaushik and Cuervo 2016). Recent studies have found that PLIN2 and HSP70 interact with each other to activate the AMPK signaling pathway. The resulting AMPK signaling pathway can activate PLIN2 by phosphorylation, and phosphorylated PLIN2 dissociates from the LDs surface before the recruitment of ATGL or nearby autophagy-related proteins on LDs. Since no such

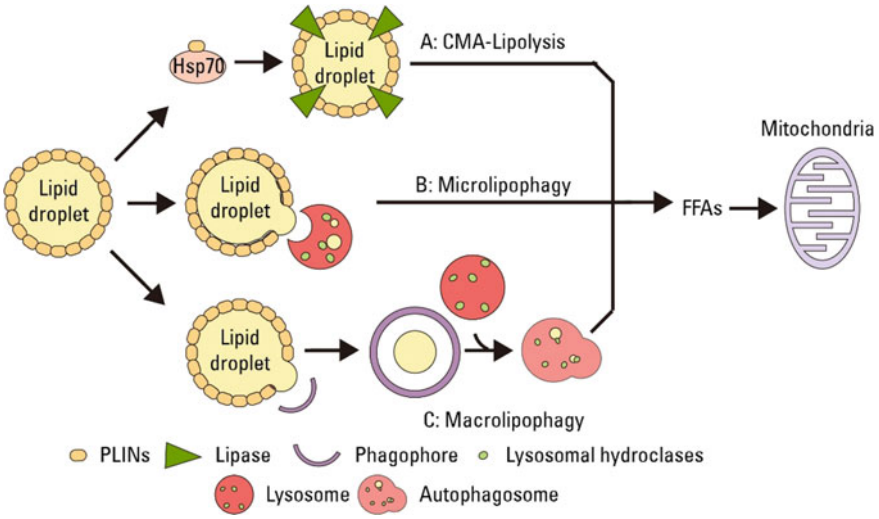


**Fig. 17.2** Major pathways of lipid droplet degradation. **A** HSP70 binds with LDs-associated proteins (PLINs) and activates CMA for PLINs protein degradation allowing access for lipases. Additionally, both micro- (**B**) and macro-lipophagy (**C**) facilitate LDs catabolism and generate free FAs that are channeled toward mitochondria for complete oxidation

phosphorylation was detected for PLIN3, it is speculated that PLIN3 may act differently from PLIN2 in the recognition and regulation mechanism, but its molecular mechanism remains unknown. During lipolysis, ATGL is recruited to LDs surface to initiate TG degradation upon detection of lipolysis signal. Subsequently, hormone-sensitive lipase (HSL) and monoacylglycerol lipase are also recruited to LDs for further degradation. A close relationship between ATGL, HSL and lipophagy has been found. ATGL has been found to colocalize with autophagy marker protein LC3 on LDs surface, and it can enhance LC3 binding with both lysosomes and LDs, thus endorses lipophagic activity. In addition, ATGL also enhances the expression of SIRT1 (NAD-dependent deacetylase), which in turn upregulates lipophagy (Sathyanarayan et al. 2017). The decreased lipophagic activity in *ATGL* knockout macrophages also suggests that ATGL is involved in regulation of LDs metabolism via lipophagy.

### *17.3.2 The Role of Rab GTPase in Lipophagy*

The Rab GTPase protein family is a key regulator of intracellular vesicular trafficking. The Rab family members mediate a series of molecular events by continuously cycling between an active state (bound to GTP) and inactive state (bound to GDP). The Rab protein family promotes the intracellular vesicular transport by interacting with cytoskeletal dynein and membrane fusion complexes. The Rab protein family

was recently found to play an important role in LDs biology. Nearly 30 Rab family members have been identified on the LDs surface. The most critical member is Rab7, which is widely involved in the regulation of autophagosome maturation and intracellular transport. Rab7 has been reported to mediate the fusion of autophagosome membranes and late endocytic membranes by coordinating the interaction of multiple SNARE proteins with the components of the HOPS tethering complex (Balderhaar and Ungermann 2013). Furthermore, Rab7 also promotes the autophagic phagocytosis of mitochondria by interacting with GTPase activator proteins TBC1D15 and TBC1D17, suggesting that it also participates in mitophagy (Yamano et al. 2014). Recently, starvation in hepatocytes was found to activate Rab7 on LDs surface, and promotes the transport of lysosomes to the LDs vicinity. siRNA mediated *Rab7* knockdown or overexpression of *Rab7* functional mutants led to the abnormal accumulation of LDs in cells. The expression of Rab7 was also significantly reduced in the liver of rats fed on alcohol-containing diet. These results indicate that *Rab7* is involved in the regulation of lipid metabolism via lipophagy. Rab10 is also a member of the Rab family localized on LDs surface. Rab10 has been found to play an important role in Golgi trafficking during epithelial polarization and insulin-stimulated GLUT4 vesicular trafficking. *Rab10* knockdown led to increased LDs accumulation in hepatocytes. It is noteworthy that activated Rab10 has been colocalized with autophagy protein LC3 and Atg16 on LDs surface in starved hepatocytes (Li et al. 2016). Other members of the Rab family are also involved in the regulation of lipid metabolism. For example, *Rab32* knockdown in hepatocytes can upregulate *ATGL* expression and hence, decrease LDs. An increased reactive oxygen species (ROS) production during hepatic stellate cells activation results in increased Rab25 expression. LDs degradation was inhibited upon *Rab25* knockdown by siRNA. Whether other Rab family members are also involved in lipophagy regulation remains to be further explored.

### *17.3.3 Role of Lipid Droplet Surface Receptors in Lipophagy*

The identification of recognition receptors in cargo-specific selective autophagy and their molecular mechanisms have been the key issues of autophagic research. A number of specific receptors have been found in most of the organelles. For example, nuclear dot protein 52 kDa (NDP52) and optic nerve protein optineurin are the key factors for the specific recognition of mitochondria during mitophagy. NDP52 is also involved in the regulation of xenophagy. However, what are the specific autophagic substrate receptors in LDs is unclear. Huntingtin is a known recognition receptor responsible for the degradation of several organelles. A mutation in *huntingtin* resulted in significant LDs accumulation and is suggestive of its important role in lipophagy regulation by acting as an LDs recognition receptor protein (Rui et al. 2015). ATGL also facilitates the degradation of LDs by binding to the autophagy marker protein LC3. However, it is not clear whether other proteins also mediate

lipophagy by binding to LC3. Therefore, studies on the initiation and regulation of lipophagy are still lacking.

In addition, many other proteins have also been found to regulate lipophagy. For example, SID1 transmembrane family member 2 (Sidt2) is a member of the lysosomal enzymes, and *Sidt2* knockout in mice resulted in LDs accumulation in hepatocytes. Moreover, significantly reduced fatty acid oxidation, and a decrease in plasma β-hydroxybutyrate levels were also reported in these mice. In contrast, p62 and LC3-II proteins were accumulated in hepatocytes, and autophagic flux was found to be decreased. Cluster of differentiation 36 (CD36) is a fatty acid transferase that widely expresses on the surface of various cells and promotes the absorption of long-chain fatty acids. *CD36* knockout in mouse hepatocytes was found to activate the AMPK signaling pathway. This activation not only phosphorylated the ULK1 but also increased the autophagy and fatty acid oxidation, along with a reduction in the level of steatosis (Li et al. 2019). Dynamin 2 (DNM2) is a large GTPase. Once the autolysosome completes the degradation of the internalized substance, the autolysosome membrane can be further recovered to produce a new prolysosome. DNM2 helps in the isolation of the nascent lysosomes from autolysosome. Furthermore, *DNM2* knockout led to an abnormal separation of new prolysosomes, eventually resulting in the accumulation of a large number of LDs in hepatocytes. It is believed that DNM2 plays a role as "lubricating oil" to help hepatocyte lipophagic machinery running smoothly (Schulze et al. 2013).

## 17.4 Mechanism of Lipophagy Regulation

### *17.4.1 Transcriptional Regulation*

Considerable progress has been made in exploring the transcriptional regulation of autophagy and lipophagy in the past few years. Transcription factor EB (TFEB) and transcription factor E3 (TFE3) are the most studied autophagy/lipophagy regulators in mammals. TFEB regulates the lipid metabolism by activating the expression of peroxisome proliferator-activated receptor-γ coactivator-1 alpha (PGC-1α) (Settembre et al. 2013). It has also been found to upregulate the expression of a series of lysosomal lipases, which in turn enhances the autophagy and lipolytic activity. HLH-30 is the TFEB homologous protein in *Caenorhabditis elegans*. During starvation, HLH-30 is activated to upregulate the expression of a series of lipase such as lipoprotein lipase 1 (LPL-1). *TFE3* knockdown in hepatocytes promotes steatosis, whereas *TFE3* overexpression reduces the extent of steatosis by inducing lipophagy. However, some studies have found that *TFE3* overexpression in adipocytes leads to obesity, suggesting that TFE3 may have diverse roles in the regulation of lipid metabolism in different types of cells. In addition, many other transcription factors have also been found to regulate lipophagy. For example, activation of transcription factor forkhead box protein O1 (FOXO1) in the starved adipose tissues upregulated

the expression of *lal* and *Atg14.* Similarly, *FOXO-1/-3/-4* knockout in mouse hepatocytes leads to liver steatosis accompanying hypertriglyceridemia, and a significant decrease in autophagic activity. In another study, activation of farnesoid X receptor (FXR) in hepatocytes was found to inhibit autophagic activity. During sterol depletion, SREBP2 in hepatocytes activates the expression of *Atg4b* and *Atg4d*, to promote autophagosome formation and LDs degradation (Seo et al. 2011).

### *17.4.2 Nutritional and Hormonal Regulation*

mTOR acts as a key node in the metabolic regulation of related signaling pathways. Various nutrients and hormones such as glucose, amino acids, and insulin can influence the metabolic pathways by modulating mTOR activity. The mTOR signaling pathway is believed to inhibit autophagy during abundant nutrient supply. Moreover, increased autophagy, lipid oxidation, and lipolytic activity were found in rapamycin (mTOR signaling pathway inhibitor) treated hepatocytes. Serum-starved hypothalamic neuronal cells also showed similar results. Furthermore, rapamycin treatment enhanced the lysosomal lipase activity in *C. elegans*. Therefore, mTOR is suggested to be involved in the regulation of lipophagy, but its mechanism still needs further exploration.

Interestingly, the hormones that activate metabolic pathways generally promote lipophagic activity. For example, β-adrenalin treatment enhanced autophagy by activating Rab7 in adipose tissue and hepatocytes. Similarly, thyroid hormone ($T_3$) also enhanced mitochondrial β-oxidation in hepatocytes and upregulated LDs degradation (Sinha et al. 2012). Lipids themselves were also found to regulate lipophagic activity. Oleic acid treatment was found to induce lipophagy in hepatocytes, and similar results were found in treated hypothalamic neurons. It is currently believed that autophagy activation helps in the elimination of sharp lipid influx. Chronic high-fat diet induced a high degree of liver steatosis accompanied by a decreased LC3 and LAMP-2A expression on LDs surface. These changes in LC3 and LAMP2A expression block the PLIN2 degradation and consequently inhibit lipophagy.

### *17.4.3 Regulation via Small Molecules*

A wide range of natural small molecules has also been reported to affect autophagy. For instance, epigallocatechin-3-gallate (EGCG) is a polyphenolic compound found in green tea. It has been found to enhance autophagy in hepatocytes (Kim et al. 2013) Caffeine was observed to enhance the fatty acid oxidation by activating autophagy in hepatocytes. Bergamot is a dietary polyphenol extracted from the bergamot peel. Bergamot diet was found to reduce the steatosis by promoting the LDs degradation. In line with it, resveratrol found in red wine also showed a similar effect. In contrast to the above-described molecules that promote autophagy/lipophagy, tetrandrine, a

bisbenzylisoquinoline alkaloid, led to lipid accumulation in the liver cell by impairing the normal autophagic process.

## 17.5 Lipophagy in Various Cellular Processes

### *17.5.1 Lipophagy and Cell Death*

Autophagy has been found closely associated with cell death, but the exact relationship between them is still not very clear. Lipophagy is generally thought to promote cell survival. Lipid accumulation in obesity, fatty liver or other metabolic diseases has been found to produce a large amount of ROS and free fatty acids may harm the cell by inducing cell death. Lipophagy can neutralize these excessive fatty acids and maintains cell energy homeostasis. For example, a higher autophagic activity, characterized by enhanced encapsulation of LDs and mitochondria in autophagosomes, was observed in ethanol-treated hepatocytes and liver of mice fed on alcohol-containing diet. However, the rate of autophagic degradation of some long-lived proteins did not change, suggesting the specific induction of lipophagy and mitophagy only. Furthermore, autophagy inhibition was found to induce massive cell death. In another study, rapamycin treatment in mice chronically fed on high-fat diet resulted in a significant decrease in lipid contents, whereas autophagy-deficient mice exhibited severe hepatic steatosis and necrosis upon chronic feeding. These findings clearly suggest that lipophagy has a protective effect against alcohol or lipid overload-induced liver injury. Furthermore, it can also be suggested that lipophagy may serve as a potential target for the treatment of hepatic steatosis and related diseases.

Ferroptosis is a newly discovered iron-dependent cell death different from apoptosis and necrosis. Excessive free iron or other small molecular compounds can induce lipid peroxidation by generating a large amount of ROS and eventually result in ferroptosis. The cytoplasmic membrane in ferroptosis remains intact while the mitochondrial volume shrinks. In addition, the outer membrane ruptures, and the number of internal mitochondrial cristae decreases. Lipophagy has been found to regulate ferroptosis in mice. The number of LDs in the mouse hepatocytes were found to be increased in the early stages of ferroptosis, but significantly decreased in the later stage. *Atg5* or *Rab7a* knockdown in cells can inhibit RSL3-induced lipid peroxidation and cellular ferroptosis (Bai et al. 2019), but its mechanism remains to be investigated.

### *17.5.2 Lipophagy and Atherosclerosis*

Atherosclerosis can cause several cerebral vascular and coronary artery diseases. It is characterized by the formation of lipid-rich atherosclerotic plaques in the intima

of the arteries that eventually cause stenosis of blood vessels restricting the blood supply to tissues and organs leading to ischemia and necrosis (Libby et al. 2011). Many factors can promote atherosclerosis, but it is generally believed that perturbed lipid metabolism in macrophages transforms them into lipid foam cells that are the main players in atherosclerotic plaques formation. The major type of lipids found in these foam cells are free cholesterol and cholesterol esters, which are stored in the form of LDs. Thus, an effective strategy to prevent the formation of foam cells and atherosclerosis is to promote the efflux of cholesterol from the macrophages by enhancing the LDs degradation. Programmed cell death protein 4 (*Pdcd4*) is a tumor suppressor gene that inhibits the translation of proteins by binding to eukaryotic translation initiation factor 4A (eIF4A). PDCD4 has been found to play an important role in macrophage foam cell formation and lipophagy. In *Pdcd4* knockout mice, the autophagic activity of macrophages was enhanced and the intracellular LDs were decreased. Moreover, the conversion of macrophage into foam cells was also inhibited, and the hardened plaques in the blood vessels of the knockout mice were correspondingly reduced. These results suggest that endogenous PDCD4 may promote macrophage foam cell formation and the development of atherosclerosis by inhibiting lipophagy. These features make PDCD4 an important future therapeutic target to treat atherosclerosis (Wang et al. 2016). Lysosomal inhibition has been found to promote the accumulation of cholesterol esters and slows down the rate of cholesterol efflux from macrophages. *Atg5* knockout in esterified macrophages inhibited the cholesterol efflux. Therefore, autophagy in macrophages may simultaneously regulate the degradation of LDs and enhance the efflux of degradation products. Although these studies have revealed the potential of lipophagy in atherosclerosis treatment; however, some problems still exist and need further investigations. For instance, the use of autophagy activator to promote LDs degradation can also induce inflammatory reactions at the same time. Moreover, a large amount of free cholesterol is produced following LDs degradation, which further promotes macrophage foam cell formation, endoplasmic reticulum stress and apoptosis further aggravating the situation. In addition, an enhanced autophagic activity was found during the esterification process, suggesting that increased autophagy also induces lipid accumulation but the molecular mechanism of this enhancement is still unclear. Therefore, a lot of problems remain to be solved prior to the formal intervention of lipophagy in clinical applications.

### *17.5.3 Lipophagy and Viral Replication*

Cells can clear the invaded pathogens via autophagy, but some pathogens were found not only to evade the autophagic machinery but also to promote their replication by enhancing autophagic activity (especially lipophagy). Hepatitis B virus (HBV) and hepatitis C virus (HCV) are the two most important pathogenic factors of chronic hepatitis. Both of them were found to enhance autophagic activity in host cells through unknown mechanisms and in turn promote their own replication (Dreux et al. 2009).

The nuclear protein of HCV promotes its own viral assembly by adhering to LDs probably utilizing β-oxidation. Dengue fever is a common hemorrhagic viral infection disease (Dreux et al. 2009). Dengue virus infection may trigger lipophagy in liver cancer cells. Moreover, it also enhances the β-oxidation and promotes the degradation of LDs in infected cells. In addition, the energy produced as a result of LDs degradation can be utilized for further viral replication. Furthermore, dengue virus infection has been found to activate the AMPK signaling pathway, further enhancing lipophagy by inhibiting mTORC1 activity. In contrast, autophagy inhibition resulted in blockade of viral replication. Therefore, autophagy induction may be a key event in the life cycle of these viruses. However, the exact molecular mechanism is yet unidentified and requires further research to be fully understood.

### *17.5.4 Lipophagy and Liver Diseases*

Lipophagy-mediated LDs degradation is an important way to maintain hepatic lipid homeostasis. Abnormal lipophagy in liver leads to excessive lipid accumulation and steatosis, consequently resulting in alcoholic and nonalcoholic fatty liver disease (NAFLD). Nonalcoholic steatohepatitis (NASH) has been associated with severe autophagy disorders in the liver. In a hepatitis animal model, autophagy inhibition caused deterioration of liver steatosis in mice supplemented with a methionine–choline-deficient diet (hepatic steatosis inducing diet) (Chen et al. 2016). Glucagon-like peptide-1 (GLP-1) receptor agonist, exendin-4, significantly reduced the accumulation of LDs in cultured human hepatocytes treated with a large amount of unsaturated fatty acids. Moreover, the expression of endoplasmic reticulum stress marker protein C/EBP homologous protein (CHOP) was significantly decreased and the expression of autophagy proteins BECN1 and LC3B were increased in these hepatocytes. Electron microscopic observations also showed that the number of autophagosomes containing LDs was significantly increased, and hepatocyte survival was considerably improved. At the same time, supplementation of liraglutide in diet, another GLP-1 receptor agonist, to chronic high-fat diet fed mice showed similar results. Therefore, GLP-1 receptor agonists may enhance the lipophagy and reduce the lipid contents in the liver, eventually slowing down the steatosis process (Sharma et al. 2011). There are only a few studies describing the role and molecular mechanism of lipophagy in the development of fatty liver-related diseases. ROS intermediates produced as a result of ethanol metabolism may be the primary factor involved in lipophagy induction in acute ethanol-induced in vitro and in vivo models of liver steatosis and injury. In line with it, the expression of a pro-oxidase enzyme, namely CYP2E1, was continuously upregulated while glutathione (main antioxidants in the liver cells) was significantly reduced in hepatocytes of mice chronically fed on an alcoholic diet, whereas autophagy inhibition led to a further decrease in glutathione levels. These results suggest that induced autophagy may be a protective mechanism for hepatocytes against oxidative stress and liver damage. Lipophagy degrades LDs and helps the cells to avoid excessive lipid accumulation to prevent

the oxidative stress. The resultant fatty acids (byproducts of LDs degradation) are oxidized to produce ATP to facilitate cell energy homeostasis.

Lipophagy can affect the development of fatty liver disease by regulating the metabolism of TG and other lipids. Ceramide sphingolipids were found to be increased significantly in the liver of *Atg7* knockout mice. This increase in sphingolipids resulted in autophagy activation in hepatocytes. Therefore, it can be suggested that cells need to degrade LDs to prevent excessive accumulation of sphingolipids. In addition, an increase in ceramide levels was also detected in some metabolic diseases such as diabetes and obesity. These findings suggest that sphingolipids may be an attractive therapeutic target and expands the spectrum of autophagy interventions in fatty liver disease.

Although a large number of studies have described the relationship between lipophagy and hepatic lipid homeostasis, the degree of steatosis has not been reduced or even altered in some autophagy-deficient animal models. This inconsistency is perhaps based on experimental design and the choice of animal models. For example, there have been reports that lipophagy promotes fatty acid turnover in LDs rather than degrading them. However, these results were obtained by long-term physiological saline treatment instead of the serum-free medium in fibroblasts with fewer lipid contents; thus, its physiological significance remains to be discussed. For the time being, studies on steatosis and hepatocyte damage in NAFLD-like diseases still require extensive in vivo experiments to further clarify the role of lipophagy and related molecular mechanisms.

### *17.5.5 Role of Lipophagy in Hepatic Stellate Cell Activation and Fibrosis*

Long-term liver damage can cause liver fibrosis and cirrhosis, which can also lead to organ failure, as a result of excessive accumulation of extracellular matrix. Activation of hepatic stellate cells is the leading cause of liver fibrosis. Hepatic stellate cells store a large number of lipids in the form of vitamin A in the resting state. Once these cells are activated, they tend to proliferate rapidly; the intracellular LDs reduce dramatically; and a larger amount of extracellular matrix components are produced. Consequently, these cells are transformed into myofibroblasts, and finally, the liver parenchyma is gradually replaced by scar tissue. Recently, lipophagy has been found to participate in the process of hepatic stellate cell activation. Intraperitoneal injection of tetrachloromethane induced the hepatic fibrosis in mice, and the autophagy activity was significantly increased in hepatic stellate cells. In vitro treatment of human and mouse hepatic stellate cells with the autophagy inhibitor bafilomycin A1 significantly inhibited the expression of stellate cell activation genes such as *Acta2, Procol1a1*, and *Pdgfr-β*, and inhibited their proliferation (Thoen et al. 2012). Increased Rab25 expression is often associated with stellate cells activation. Rab25 has been found to guide the recognition of autophagy-associated organelles and

LDs by binding with phosphatidylinositol 3-kinase type III complex (PI3KCIII). Docosahexaenoic acid (DHA) is a polyunsaturated fatty acid that is indispensable for the development of the brain. The researchers have found that the treatment of DHA can block the degradation of LDs and alleviates the progression of fibrosis in tetrachloromethane-induced hepatic stellate cells. Further studies found that DHA treatment can inhibit the expression of RAB25, and impairs the recognition of intracellular LDs by lipophagy-related mechanisms, thereby inhibiting the activation of hepatic stellate cells. In addition, ROS has also been shown to play a role in the binding of RAB25 with PI3KCIII. Treatment of hepatic stellate cells with antioxidants such as glutathione and N-acetylcysteine, or PLIN5-overexpression can inhibit the production of intracellular ROS, and thus prevents the binding of RAB25 with PI3KCIII, but it is still unclear how this binding is prevented. *Atg7* knockout in stellate cells was found to reduce the stellate cell activation. Furthermore, a significant reduction in the accumulation of extracellular matrix and the extent of fibrosis was found as a result of *Atg7* knockout. Free fatty acid-oleate supplementation to autophagy-deficient cells can initiate the activation of stellate cells again. These results indicate that autophagy may promote the transdifferentiation of cells by providing the energy required for activation. The degree of fibrosis was found to be reduced in autophagy-deficient stellate cells. However, it is unclear whether the fibrosis of organs other than the liver is also related to lipophagy. Moreover, stellate cell activation suggests that lipophagy may play a key role in the initiation and development of various fibrotic diseases.

### *17.5.6 Lipophagy and Metabolic Disorders*

Autophagy and lipophagy have been associated with a number of metabolic disorders. The size of the fat depots in different degrees and different types of obese people were found directly related to the level of autophagy. For example, autophagy in omental adipose tissue was found to be significantly increased in obese patients. The autophagic activity in many organs was also increased in obese patients with insulin resistance (Kovsan et al. 2011). These results indicate that autophagy participates in the regulation of lipid stores in adult individuals in addition to adipose tissue differentiation during development. Autophagy activity often began to increase abnormally before obesity-associated morbidity, indicating that enhanced autophagy may be a defense strategy to cope with lipid accumulation. However, it should be noted that the symptoms of metabolic disorders and the effects of autophagy may vary in different metabolic states. For example, mTOR attenuation was observed in the adipocytes of type 2 diabetes patients with insulin resistance, partly explaining why autophagy in fat cells is enhanced. However, lipids in these patients continue to accumulate, and its molecular mechanism is still unclear. An enhanced lipophagy has been found to improve insulin resistance in patients with type 2 diabetes. Metformin, a biguanide compound, commonly used in the treatment of type 2 diabetes can upregulate the FOXO1 expression in adipocytes of mice promoting the expression of lysosomal-associated lipase and enhances autophagic activity. The free fatty

acids, produced as a result of lipophagic degradation of LDs, are further oxidized in the mitochondria. This oxidation improves the energy homeostasis in adipocytes and reduces the pressure of ATP production by glycolysis and eventually reduces the insulin resistance. Therefore, it is necessary to comprehensively consider the situation to establish a reasonable therapeutic lipophagy intervention strategy for clinical applications.

### *17.5.7 Lipophagy and Aging*

The autophagic activity continues to decline with aging (Cuervo 2008). A decrease in autophagy especially lipophagic activity leads to the accumulation of LDs, which can further reduce the autophagy activity via negative feedback. Consequently, this decrease in autophagy results in a number of aging-related metabolic disorders, such as hypercholesterolemia, insulin resistance and increased lipid accumulation in organs. Intraperitoneal injection of 3,5-dimethylpyrazole (DMP) resulted in the reduction of plasma levels of free fatty acids, glucose, and insulin. In addition, DMP treatment also significantly upregulated the autophagy and proteolytic activity in hepatocytes. Furthermore, DMP supplemented food not only effectively improved the phenotype of aging-related hypercholesterolemia but also prolonged the lifespan of aging mice. Collectively, the above results suggest that DMP likely increases the lipophagic activity in hepatocytes and thus, in turn, affects the lifespan of mice.

Recently, lipophagy has also been found to regulate the lifespan of the *C. elegans*. A number of lysosomal acid lipases LIPL 1-5 have been found in the *C. elegans*. Among these lipases, LIPL-1, LIPL-2, and LIPL-3 are mainly located in the lysosomes of small intestinal cells. Starvation results in a significant increase in the expression of these lipases. LIPL-1 and LIPL-3 have been found to promote the degradation of lipids by enhancing lipophagy in *C. elegans*. In well-fed *C. elegans*, the basic helix–loop–helix transcription factor Max-like 3 (MXL-3) inhibits the expression of lipases, such as LIPL-1, LIPL-2, LIPL-3, and LIPL-5. However, under acute nutrient depletion, the expression of MXL-3 was decreased, which in turn activated HLH-30, causing an increase in the expression of LIPL-1 and LIPL-3 in the cells. The lifespan of *mxl-3* mutant *C. elegans* was prolonged compared with the control group. Moreover, the lipolytic activity in these mutants was found to be significantly enhanced, but the autophagic activity did not change significantly. However, the lifespan of *hlh-30* mutant *C. elegans* was shortened, while overexpression of HLH-30 significantly prolonged its lifespan (O'Rourke and Ruvkun 2013). These results indicate that HLH-30 participates in the regulation of *C. elegans* lifespan by increasing autophagy and lipophagy, suggesting a relationship between autophagy, lipid metabolism, and longevity. The specific target of lipases and its mechanism in autophagy regulation is still unclear, but it still provides new horizons for the cure of aging-associated diseases.

## 17.6 Conclusion and Perspective

Research in the field of lipophagy has not only extended our understanding of autophagy and lipid homeostasis and related mechanisms in the last decade but also provided new avenues to target and treat a variety of metabolic disorders, such as obesity, fatty liver disease, and atherosclerosis that have done great harm to humans. However, a lot of questions in this field still remain to be solved: (1) The effects of different *Atgs* knockout on lipid metabolism were highly variable in different animal and cell models. We have recently found that autophagy is not only involved in the degradation of lipids but also maintains the lipid homeostasis by regulating the cholesterol uptake (Gao et al. 2018). The effect of autophagy deficiency was more stable in lipid absorption-oriented cells or lipid consumption-oriented cells than others. Therefore, these results revealed the difficulty of the lipophagy application as a molecular therapeutic target for disease and also provide new options for its precise regulation. (2) Different lipophagy functions exist in different types of cells, what are their specific molecular mechanisms? (3) How does lipophagy sense and ensure the recognition of LDs? How does the nutritional status of cells affect this recognition? With the continuous development of biomedical technologies, continuous marvels in basic scientific research, and clinical medical transformation, it is foreseeable that in the future the research on lipophagy will provide a deep insight into molecular mechanisms and will also open new and better therapeutic avenues to benefit humans.

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# Chapter 18
# Autophagy and the Metabolism of Misfolding Protein


**Chao Cheng and Zhen-Guo Liu**

**Abstract** Autophagy is a major intracellular degradative process that delivers cytoplasmic materials to the lysosome for degradation. An increasing number of studies on the physiological and pathological roles of autophagy in a variety of autophagy knockout models and human diseases were carried out. Among them, the clearance of misfolded proteins is the important function of autophagy. Impairment at different steps of the autophagy system, such as the ubiquitin-proteasome and the autophagy-lysosome pathways, may result in the accumulation of misfolded proteins in insoluble aggregates. Abnormal accumulation of misfolded proteins in cells can lead to a variety of human diseases. Here, we review the major advances in autophagy and the metabolism of misfolding protein in human diseases. Current studies about the promising therapeutic strategy in autophagy-modulating are also summarized.

**Keywords** Autophagy · Misfolding protein · Metabolism · Aggregation

## 18.1 Introduction

Intracellular proteins are in a dynamic balance of constant synthesis and degradation, which facilitates the implementation of cell-specific functions and maintenance of homeostasis. However, human cells are often subjected to various stressors (such as oxygen-free radicals, ultraviolet radiation, etc.), and these challenges can lead to various types of protein damage that can damage normal cell function and disrupt cell homeostasis. When a specific protein is abnormal in the intracellular structure and aggregates in a toxic structure, it will collect and inactivate the normal functional protein, cause cell damage and eventually cause cell death, and cause degenerative diseases. For example, some neurodegenerative diseases are characterized by abnormal protein conformation in pathology. The change of protein conformation may be caused by insufficient folding during protein synthesis. Some sites of protein are not folded; Abnormal cell division; Gene insertion, deletion or abnormal modification

C. Cheng (✉) · Z.-G. Liu
Department of Thoracic Surgery, The First Affiliated Hospital of Sun Yat-Sen University, 58 Zhongshan 2nd Road, Guangzhou 510080, People's Republic of China
e-mail: drchengchao@163.com



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of amino acid sequence, etc. In the cell, from protein synthesis to release, the intracellular system constantly checks the quality of synthetic proteins in order to timely repair and remove abnormal proteins. Drugs aimed at improving the quality control system of intracellular proteins are being developed, which may bring new hope for the clinical treatment of this disease.

Protein misfolding is a common form of abnormal protein conformation, this chapter mainly introduces the occurrence of protein misfolding and the corresponding degradation pathways, including the ubiquitin proteasome system (ubiquitin-proteasome system, UPS), autophagy-lysosomal pathway (autophagy-lysosome system), also introduces the degradation pathway caused by abnormal nerve vascular lesion and its mechanism of the system, may potentially effective treatment strategies, etc.

## 18.2 Protein Misfolding and Degradation Pathways

### *18.2.1 Protein Misfolding and Protein Polymer Formation*

#### 18.2.1.1 Basic Principles of Protein Folding and Molecular Chaperones

Protein folding refers to the physical process by which proteins form their intrinsic three-dimensional structure from polypeptide chains. According to the law of genetic center, the transmission of biological genetic information is generated by DNA transcription into RNA, which is then translated into polypeptide chains. Polypeptide chains with the complete primary structure are processed and modified, and finally folded to form proteins with specific spatial conformation. Protein usually has a quaternary structure, among which, the primary structure of a protein refers to the sequence of amino acids in the peptide chain, which is the basic structure of protein molecules and the basis of protein spatial structure and function. Protein spatial structure also includes secondary, tertiary and quaternary structure, which is a three-dimensional conformation formed by folding and coiling on the basis of the primary structure. Newly synthesized polypeptide chains in cells can only perform specific biological functions if they are folded correctly to form specific proteins with three-dimensional structures. So why do newborn peptides spontaneously fold to form proteins with a specific spatial conformation? What is the regulatory mechanism for this folding process? At present, it is believed that protein folding is a spontaneous process, which is determined by hydrophobic bond interaction, hydrogen bond formation, van der Waals force, and other factors in the polypeptide chain. The folding process of the new peptide chain follows the "thermodynamic hypothesis" and is controlled by kinetics.

In the 1960s, Anfinsen et al. found in the denaturation and renaturation experiments of bovine pancreas RNA hydrolases that the denaturated RNA hydrolases could renature only by removing the denaturants and reducing agents without the

help of any other substances, and the bioactive RNA hydrolases could be formed again. Based on this, Anfinsen proposed the classical "thermodynamic hypothesis" of protein folding, and believed that natural protein polypeptide chain is the most thermodynamically stable form under certain environmental conditions. Since the conformation of natural protein is the lowest or the most stable thermodynamic free energy under certain environmental conditions (such as solution component, PH, temperature, ionic strength, etc.), denatured protein can spontaneously fold to form its natural conformation with biological activity under appropriate environmental conditions. The "thermodynamic hypothesis" of protein folding has been confirmed by some experiments and widely accepted.

With the development of research, it has been found that many protein polypeptides have low renaturation efficiency in vitro and form some non-natural conformations or nonspecific polymers. And the renaturation rate is much lower than the body level. In the 1990s, Joseph et al. proposed the protein folding energy theory and introduced the principle of minimum frustration. Bakei et al. thought that the natural conformation of some proteins might not be the lowest energy or the most stable energy state, and proposed that a protein polypeptide chain might have two low-energy states: one is a kind of natural conformation, the other is a kind of non-natural conformation, and the two mutual transformations in a lower energy state of a polypeptide chain need to overcome a high energy barrier, thus both mutual transformation is often difficult to complete, so there are two ways in the process of protein folding competing with each other, one way is correct folding form stable natural conformation, another kind is abnormal folding form stable than native conformation. For example, human insulin growth factor type I has two stable conformations, one natural and one non-natural conformation with mismatched disulfide bonds, both of which are in a similarly low-energy state.

At present, the theoretical model of protein folding assumes that the local conformation of a protein depends on the local amino acid sequence. Finally, the secondary structure frames were spliced with each other, and the peptide chain was gradually tightened to form the framework model of the tertiary structure of the protein. Hydrophobic collapse model in which hydrophobic forces play a decisive role in protein folding; A diffusion–collision–adhesion model of complex structures was established by the diffusion, collision and mutual adhesion of unstable secondary structural units generated by several sites of the extensional peptide chain in a nonspecific Brownian motion model. A nucleation–condensation–growth model in which a region of the peptide chain forms a "folded crystal nucleus" and continues to fold to form a natural conformation. Polypeptide chains can be folded along several different pathways, and in the process of folding along each pathway are more and more natural structures, eventually forming a natural conformational puzzle model.

Studies in the 1990s found that intracellular peptide folding was generally helpful, and some cofactors have been isolated that can promote the correct folding of polypeptide chains on the kinetics. For example, molecular chaperones (a particular protein) and folding enzymes (catalyze the covalent bond changes necessary to form a functional conformation directly related to protein folding). These facts strongly

show that the correct folding of the polypeptide chain is controlled by dynamics as well as thermodynamics.

In 1978, Laskey et al. called the particular protein that binds to histone and mediates the ordered assembly of nucleosomes as molecular chaperone. In 1993, Ellis extended the concept to "a class of proteins that are sequentially unrelated but have common function: they help other structures containing polypeptides to complete the correct assembly, and separate from these structures after assembly, not forming the functional parts of these proteins" (Finn et al. 2005). Molecular chaperones are required to fold about 10–20% of the new peptide chains into proteins with specific spatial structures. Researchers found, significantly different from the condition of in vitro environment where protein polypeptide chain to fold, the environment inside the cell is very crowded, full of high concentrations of proteins, nucleic acids and other molecules, so the interaction between the molecules is very frequent, and the environmental conditions in the cell aggregation is easy to cause the nascent peptide chain or interaction between different protein peptides that hinder their correct folding of the natural conformation. In order to minimize the risk of protein misfolding, molecular chaperones are involved in a complex regulatory system. On the one hand, they can help ribosome-synthesized polypeptide chains fold as quickly as possible to form natural proteins; on the other hand, they can also make the hydrophobic surface of the aggregation protein recede and promote correct folding. In the cell fluids of prokaryotic and eukaryotic cells, molecular chaperones of different structural types enable the transition of substrate polypeptide chains from the primary synthesis state of ribosomes to the final folding state. Therefore, molecular chaperones play an important role in the interpretation of genetic information and the formation of biologically functional proteins. If the function of the molecular chaperone is inhibited, the misfolded protein will accumulate and deposit in the cell and directly cause toxicity to the cell.

Molecular chaperones are found in a wide variety of organisms, including many types of proteins, such as Chaperonin family (Cpn), heat shock protein 100 (HSP100), heat shock protein 90 (HSP90), heat shock protein 70 (HSP70), heat shock protein 60 (HSP60), small heat shock protein (smHSP), nucleoplasmin and chaperonin containing t-complex polypeptide 1 (CCT) (Finn et al. 2005). Molecular chaperone HSP90 is the most abundant cytoplasm, which consists of two highly similar to 90 kDa subunit HSP90 alpha and HSP90 beta form dimers, HSP90 by combination with ATP hydrolysis and conformational change and plays its role, its activity is a steroid hormone receptor and protein kinase must be mature, HSP90 is able to combine with hundreds of the substrate at the same time, participate in DNA damage repair, immune response process. Mammalian cells contain six HSP70 family members, and HSP70 and its homologous HSC70 are the main members, which are very similar and have similar activity. They can recognize misrecognized proteins and hydrophobic surfaces of some misfolded proteins, and regulate their binding and release to substrate proteins through ATP binding and hydrolytic activity. Increased intracellular HSP70 expression will reduce apoptosis. CCT is a chaperone located in eukaryotic cytoplasm. The substrate protein is folded and trapped in the central lumen of CCT protein, and CCT wraps the substrate protein through its helix protrusions.

Co-chaperone regulates its activity by interacting with molecular chaperones. These include HSP40/DnaJ family protein, bcl2-related athanogene (BAG) family protein, HSP70/HSP90 tissue protein (Hop), HSP110, HSP70 binding protein carboxyl terminal (CHIP), and GimC. HSP40 is located in the endoplasmic reticulum, mitochondria, and nucleus, and is involved in protein transport. DnaJ was first discovered in *E. coli*, which is called HSP40 in eukaryotes. HSP40/DnaJ family proteins contain J binding region and the ATPase binding region of HSP70 to bind and promote the ATPase activity. These proteins can also bind to the substrate protein and regulate the activity of HSP70. The protein family is divided into three categories: DnaJA, DnaJB, and DnaJC, which play various roles in protein folding, assembly, translocation, and degradation, respectively. All the BAG family proteins have a conservative BAG binding region and bind to the ATPase binding region of HSP70. They affect the exchange of HSP70 nucleotide and its binding/release with the substrate protein. HSP110 is also a nucleotide exchange factor of HSP70 and regulates the activity of HSP70. Both Hop and CHIP are common molecular chaperones that can interact with two different molecular chaperones. Hop is HSC70/HSP90 tissue protein, also known as stress-inducible protein 1 (STI1). Hop binds to HSP90 and HSC70/HSP90 through the tetratricopeptide repeat (TRP) binding region and regulates the interaction between molecular chaperones, so that it is easier to produce accurately folded proteins and form functional protein complexes. These effects include the transport of substrate proteins between them. CHIP, or C terminal HSP70 binding protein, can interact with HSP70 and HSP90 and play an important role in the quality control of ubiquitination of misfolded proteins. For example, Bag2 overexpression will inhibit CHIP activity and stimulate the maturation of other molecular chaperone functions. GimC helps to correctly fold CCT-dependent actin and microtubule proteins by transferring folded protein intermediates to CCT. GimC has six tentacle-like structures like jellyfish that trap unfolded proteins inside.

Under certain environmental conditions, newborn polypeptide chains are folded correctly to form natural proteins with biological activities. In general, the formation of the protein's natural conformation is determined by the linear amino acid sequence of its primary structure. During the folding process, the protein polypeptide chain follows the "thermodynamic hypothesis" to change from high energy state to low-energy state and is controlled by the dynamics. For some proteins, the correct folding of cellular proteins requires the assistance of molecular chaperones.

#### 18.2.1.2 Production and Recognition of Misfolded Proteins

The natural conformation of the protein is mainly composed of α-helix and irregular crimp structure, while the conformation of the misfolded protein is mainly composed of β-folding structure which is rich in hydrophobic. For example, neurodegenerative diseases caused by prions are caused by the accumulation in brain tissue of prion proteins (PrP), the pathogenic proteins formed by abnormal folding of normal proteins. There are two forms of PrP: wild-type $PrP^{c}$ and mutant $PrP^{sc}$. Among them,

the sequences of wild-type $PrP^c$ were dominated by α-helix and only 11.9% by β-folding, when the α-helix structure in wild-type $PrP^c$ is converted to β-folding, it becomes a mutant $PrP^{sc}$. At this time, β-folding accounts for 43% of the protein structure and aggregates are formed outside the cell. The α-helix/β-folding structural transformation results in the exposure of hydrophobic groups and the embedding of hydrophilic groups in the protein, resulting in the formation of cross-over β-folding structures between protein molecules. β-folding structures are linked together by side chains and hydrogen bonds in the main chain to form a polymer dominated by β-folding, leading to disease.

Polypeptide chains with complete primary structure can only play their biological functions when they are folded correctly to form specific spatial conformation. Once the folding is abnormal, the wrong spatial structure will be formed, leading to the loss of biological functions and the occurrence of a series of diseases. Misfolding of proteins in vivo is caused by mutation induction, increased protein concentration, oxidative stress, aging, and other related reasons. In the cell, there is a protein folding quality control system, which can monitor the folding of proteins in the cytoplasm and timely remove misfolded proteins. This system mainly includes molecular chaperones and protease systems. Their action process is divided into two steps: One is to identify errors; Second, correct mistakes. First, the molecular chaperone recognizes misfolded protein monomers and attaches them to the hydrophobic terminal surface to prevent their polymerization and promote protein refolding and assembly. If the misfolded protein cannot be repaired, it will be delivered to the ubiquitin-proteasome system and the chaperon-mediated autophagy system for degradation under the molecular chaperone mediation. If the misfolded protein monomers accumulate, the proteasome system will lose its function, and the protein aggregates will be degraded and cleared by the macroautophagy pathway. In addition, the microtubule-dependent transport system can transport soluble oligomers/aggregates to inclusion bodies for degradation. The intracellular quality control system is regulated by stress-induced transcription factors, co-chaperone, and other cofactors. If the quality control system obstacle, protein misfolding hydrophobic surface cannot be exposed by molecular partner or protease recognition, or the formation of aggregates is faster than molecular chaperone and protease recognition speed, those who are not protected by molecular chaperone, or who has not been protease degradation of abnormally folded proteins may occur polymerization, leading to the accumulation of abnormal folding proteins inside cells and causing cell damage and even death (Kubota 2009).

So how do these toxic misfolded proteins cause cell death? One of the most important mechanisms is that misfolded proteins induce endoplasmic reticulum stress (ER stress), increasing of protein synthesis, the expression of misfolded proteins, calcium ion imbalance, virus infection and nutrition deprivation, glycosylation changes and cholesterol overload, and then these conditions affect the endoplasmic reticulum folding ability, leading to unfolded protein accumulation in endoplasmic reticulum. In order to maintain the balance of demand and endoplasmic reticulum protein folding ability, endoplasmic reticulum evolved highly specific intracellular signaling pathways—the unfolded protein response (UPR), and the induction of UPR also suggests the activation of the compensatory mechanism, which affects the normal

physiological function (Senft and Ronai 2015). In addition, because the degradation of misfolded proteins occurs in the endoplasmic reticulum, it is also known as ER-associated degradation (ERAD). Misfolded proteins inhibit the proteasome function and inhibit the protective effect of ERAD. Some proteins, such as 1-antitrypsin Z mutants, activate ER related caspase 4 and caspase 12, although they do not induce the UPR reaction.

In order to stabilize their environment, cells degrade abnormally folded proteins through a variety of pathways (as described below), and conformational changes in proteins are key factors for degradation. At present, most proteins that can be degraded and cleared by autophagy are known to be mutated proteins with unstable conformation and easy to form oligomers. There are many reasons for protein conformation instability, not only gene mutation. In mouse models lacking the autophagy regulatory gene atg5 or atg7, it was found that the nerve and liver tissues of the mice had accumulated a large amount of ubiquitinated proteins, and these proteins were not mutated. The accumulation of these proteins suggests that they cannot be cleared by the proteasome pathway, at least this pathway is not very efficient. However, the exact mechanism that causes these proteins conformational instability is unclear. It has been found that disrupting the environment or function of ER can lead to the accumulation of misfolded proteins in cells. In the process of cell development, some special metabolic changes will cause stress of ER, which will lead to the accumulation of misfolded proteins. In addition, oxidative stress, hunger, and other stress factors can also induce the production of misfolded proteins, which can also be ubiquitinated and formed into polymers, similar to polymers formed by mutant conformation changes. Aggresome-like induced structure (ALIS) is the structure of these protein polymers (Szeto et al. 2006). But, how did these proteins turn into misfolded proteins under stress is unclear. Dendritic cell ALIS, a structure called dendritic cell ALIS, is produced when the ribosome products of dendritic cells are insufficient in the process of maturation. Thus, it is possible that ALIS proteins are modified or destroyed under stress, leading to protein misfolding and ubiquitination.

### *18.2.2 Degradation of Misfolded Proteins*

Cell proteins are in a dynamic balance of continuous synthesis and degradation, which is related to the specific biological functions of cells and the maintenance of cell homeostasis. However, cells are often affected by a variety of environmental factors, such as oxidative stress and ultraviolet radiation. Excessive environmental stress will cause cell protein damage, affect the cell's normal function and cell homeostasis, and eventually lead to cell death. Therefore, timely clearance of damaged and harmful proteins in cells is crucial, especially for those non-proliferating cells such as neurons.

Normally, misfolded proteins in a cell are first identified by a molecular chaperone, and then refolded and assembled with the help of a molecular chaperone. In some cases, however, misfolded proteins mutate so much that the intracellular conditions are insufficient for selective refolding, then molecular chaperones will transport the

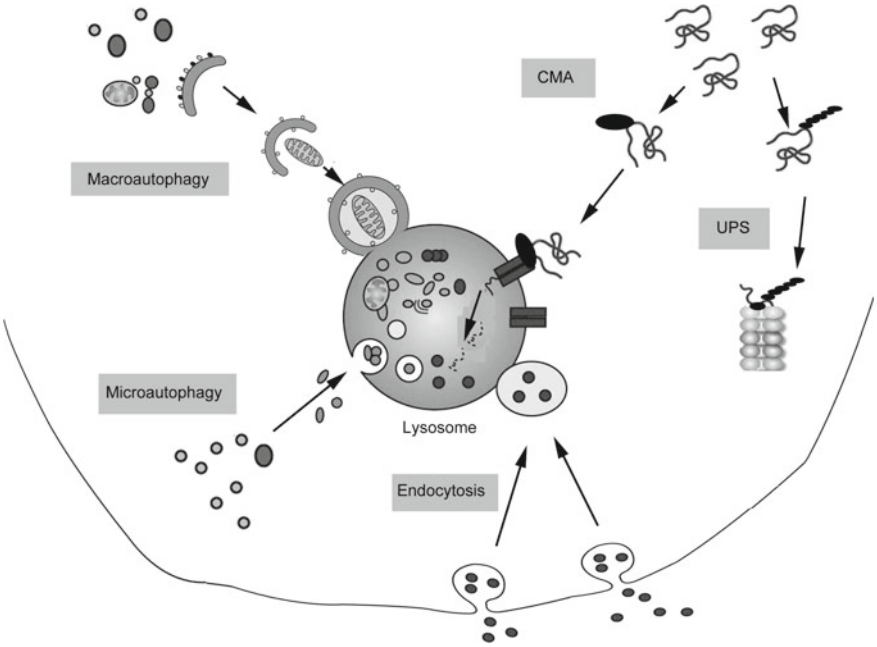


**Fig. 18.1** Proteolytic systems in mammalian cells (Martinez-Vicente and Cuervo 2007)

misfolded protein products to the proteolytic system for degradation. The intracellular proteolytic system consists of two major pathways: the UPS pathway and the autophagy pathway (Martinez-Vicente and Cuervo 2007) (as shown in Fig. 18.1).

Lysosome protein substrates have two sources: from extracellular (swallowed foreign body) and from cells (autophagy). In mammalian cells, there are three types of autophagy, namely macroautophagy, microautophagy and chaperone-mediated autophagy (CMA). In the macroautophagy pathway, intracellular substances can be separated by some double membrane structure and autophagy vesicle formation, and then merge with lysosomes. In microautophagy pathway, lysosome membrane directly by swallowing function within the substrate protein intake. CMA pathway is different from the former two ways of "batch" degradation, CMA-degraded substrate proteins are selective and bind to lysosomal receptors (lamp-2a) before translocation into lysosomes. Ubiquitin-proteasome pathway (Daw et al. 2015) is another important pathway for intracellular protein degradation. The UPS pathway substrate protein is ubiquitinated and then degraded by protease recognition.

#### 18.2.2.1 UPS Pathway

The UPS pathway, also known as the ubiquitin-proteasome pathway, is an important pathway for protein selective degradation in cells (Martinez-Vicente and Cuervo

2007). Proteasome exists in both the nucleus and the cytoplasm and it is a proteolytic system other than lysosome. The 26S proteasome, the most common form of proteasome, has a molecular weight of about 2.5MDa and contains one 20S core particle and two 19S regulatory particles. The core particle (CP) is a double-sided symmetric hollow cylindrical structure, which surrounds the active site of the spliced protein in the "hole". By opening the ends of the core particles, the target protein can enter the "hole". Each end of the core particles is connected with a 19S regulating particles (RP), the RP consists of a central part and a cap-like part, the central part is formed by 6 ATPases, which can hook CP through the C terminal and regulate the switch of degradation channel. The cap-like structure of RP is composed of 9 non-ATPase subunits, and the regulatory particles can recognize the ubiquitinated proteins and deliver them to the core particle degradation chamber. The phosphorylation of CP and RP subunits is related to the activity and stability of proteasome. Proteasomes hydrolyze a variety of ubiquitinated protein substrates from the carboxyl side of basic, acidic, and neutral amino acids. Protein degradation by proteasome is isolated from the intracellular environment. The UPS pathway degrades two types of proteins: proteins that are misfolded and proteins that require quantitative regulation.

The ubiquitin-proteasome system is an important part of the intracellular quality control system, it includes ubiquitin and its promoter system and proteasome system. The ubiquitin promoter enzyme system is responsible for activating ubiquitin and binding it to proteins to be degraded to form target protein polyubiquitin chain, namely ubiquitination. Proteasome systems can recognize and degrade ubiquitinated proteins. Proteasome degradation regulation is an important way to control the proteasome substrate pathway. Ubiquitinated proteins are directly recognized by proteasomes through three ubiquitin receptors: Rpn1, Rpn10, and Rpn13. More and more evidence shows that cells regulate proteasome-mediated protein degradation to meet their own needs by coordinating the expression of proteasome subunits and molecular chaperones, and the core of proteasome-mediated assembly regulation is TOR complex 1 (TORC1), which is a major regulator of cell growth and stress. The ubiquitin-proteasome pathway is involved in a variety of intracellular processes, including apoptosis, MHC I antigen presentation, cell cycle, intracellular signal transduction, etc., and is closely related to some physiological functions and pathological states of cells. For example, some misfolded proteins still maintain their solubility, and these proteins can be selectively degraded by the UPS pathway. The high selectivity of the UPS pathway ensures that the abnormally folded proteins in the cell can be degraded and cleared without affecting the normal cellular components. However, the specific pathway by which an abnormal protein is degraded is not clear, which may be related to the effectiveness of the proteolytic system at a specific time or the characteristics of the substrate protein. For example, certain proteolytic enzymes of the lysosome may be more effective at degrading certain proteins; Only unfolded monomer proteins can be degraded by both the UPS pathway and the autophagy pathway, while proteins that have formed oligomers, fibrils, or fibrous structures can only be processed by batch degradation pathway, namely the autophagy pathway (Martinez-Vicente and Cuervo 2007).

#### 18.2.2.2 Macroautophagy-Lysosome System (MALS)

Lysosomes, an organelle used for degradation and recycling of intracellular and extracellular substances, were first described and identified more than 60 years ago. The lysosomal system plays an important role in regulating cell surface molecules and plasma membrane receptors as well as resisting the invasion of extracellular substances. The degradation of exogenous substances by lysosomes is called phagocytosis. In contrast, autophagy refers to the degradation of intracellular components by lysosomes (Fig. 18.1). In the past 10 years, some molecular characteristics of autophagy have been discovered by using relatively simple experimental models of gene manipulation such as yeast, worms, and flies and so on, which is of great help for us to better understand and understand autophagy.

In summary, autophagy is a "batch" protein degradation process involving lysosomes. At present, studies have confirmed that almost all neurodegenerative diseases have the accumulation of autophagic vesicles and the aggregation of proteins in the cytoplasm. Autophagy in mammals can be divided into three types according to the route of intracellular material transport to lysosomal degradation: macrophage, microautophagy and molecular chaperon-mediated autophagy (Fig. 18.1) (Martinez-Vicente and Cuervo 2007). All three autophagy pathways have the ability to transport cytoplasmic substrates to lysosomes for degradation. The three types of autophagy have a common endpoint, that is, they all form autophagosomes, but their substrates, regulatory modes, and activation conditions are different.

Macroautophagy Pathway

The macroautophagy pathway can degrade a whole region of the cytoplasm in the cell in batches. This region is first surrounded and isolated by a plasma membrane, forming a closed chamber with a bilayer membrane, namely the autophagosome. Autophagosome membrane is formed by coupling microtubule-associated protein 1 light chain 3 with lipid, Atg5, Atg16, Atg12, and other autophagocyte-related proteins. These bilayer membrane vesicles are formed by intracellular lipid phosphorylation of organelles such as the endoplasmic reticulum, mitochondria, and Golgi bodies, triggered by a kinase complex regulated by beclin-1. There are no enzymes in autophagosome, and lysosomes contain all the enzymes needed for content degradation, so the content can only be degraded after the fusion of autophagosome and lysosomes. Autophagy-related protein (Atg) family is involved in the whole process of autophagy degradation, which involves a series of interactions (including protein–protein, protein–lipid, etc.) and several major kinase families in the cell. Autophagy is most easily activated under stress conditions, and its activation has two main functions: one is that autophagy can be used as a source of macromolecules and energy in cells in the state of nutritional deficiency, and the other is to remove abnormal intracellular components. Many tissues have persistent autophagy activity, which is essential to maintain cell stability. Changes in the autophagy pathway have

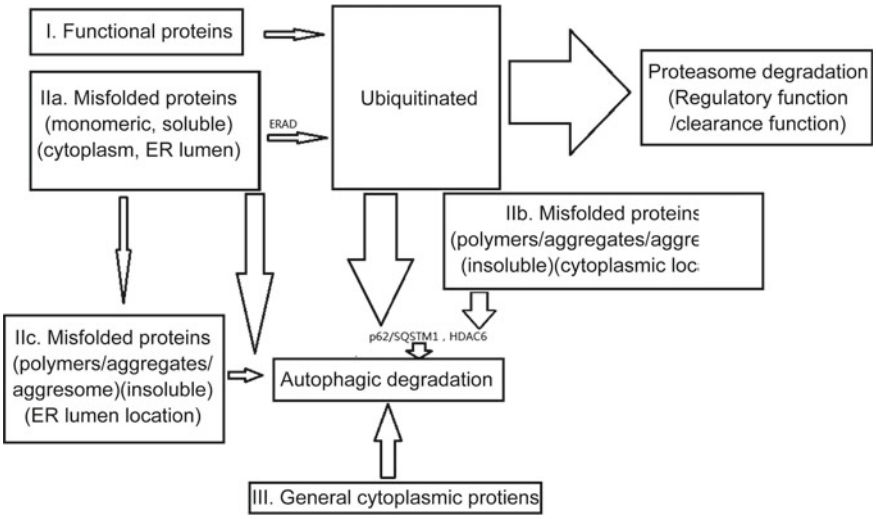


**Fig. 18.2** Protein classification based on different degradation pathways

been shown to be strongly associated with tumorigenesis, bacterial and viral infections, severe myopathy, metabolic diseases such as diabetes and neurodegenerative diseases.

Macroautophagy is the main pathway of autophagy, and recent studies on autophagy mainly focus on this pathway (Menzies et al. 2011). Macroautophagy is an important pathway of protein degradation in vivo. According to different protein degradation pathway, the protein can be divided into three types, namely type I, type II, type III, and classification through the ubiquitin-proteasome pathway or Macroautophagy pathway for degradation (Fig. 18.2). These two degradation pathways are related to each other in mechanism, but their ability to degrade different kinds of proteins seems to be different. Macroautophagy degrades almost all forms of misfolded proteins, whereas the ubiquitin-proteasome pathway degrades only those proteins that are soluble. In the state of starvation, the protein in the cell will be degraded in batch without selectivity. Although it is generally believed that the autophagy degradation pathway is nonselective to the substrate protein, the conformational changes of the protein and subsequent ubiquitination and further autophagy degradation suggest that the autophagy pathway has a certain degree of specificity in the degradation of misfolded proteins. At present, it is believed that this specificity is closely related to the recognition of ubiquitinated proteins P62/SQSTM1 and HDAC6 and the promotion of the degradation of these substrate proteins by autophagy pathways. These two molecules can recognize the ubiquitin sites of misfolded proteins and LC3/Atg8 of autophagosomes. The C terminal of P62 contains a ubiquitin-associated ubiquitin-binding region, and the cytoplasm of P62 overexpression can form a large number of p62 positive corpuscles, which are located in the UBA region and are also ubiquitin-positive. This suggests that P62 attracts ubiquitinated

proteins to form large complexes. Currently, P62 has been found in the inclusion bodies of various protein aggregation diseases, including Lewy body that is Parkinson's disease, tau protein that is Alzheimer's disease, and Huntington's aggregates. These protein aggregates are composed of misfolded mutant proteins and ubiquitin is often positively altered. Mutant protein aggregates (II b and II c protein) is the target of autophagic degradation, thus P62 also played an important role in it. P62 can also bind to LC3/Atg8 directly through a special sequence near the UBA region (aa.321–342), LC3 and P62 protein co-localizes and is degraded by autophagic lysosome in Huntington. Interestingly, even large p62-positive structures (2 μm) can be degraded by autophagy, suggesting that autophagy can degrade not only aggregates but also some large inclusions and aggregates. In addition, P62 was also involved in autophagy clearance of ALIS. The inhibition of autophagy P62 was significantly up-regulated, suggesting that even at the basic level, P62 was continuously degraded by the autophagy pathway. HDAC6 is another protein that binds to ubiquitinated proteins through the BUZ region, and has been found to co-localize with Lewy bodies and ubiquitin (Pandey et al. 2007). Inhibition of HDAC6 significantly slows down the formation of autophagosome aggregates, indicating that HDAC6 is important for the formation of aggregates. HDAC6 is a microtubule-associated deacetylase that binds to dynein, which has been found that the protein is closely related to the reverse transport of the aggregation protein to the center of the microtubule to form a cluster. HDAC6 can directly act on dynein to lead to the formation of aggregates, and the autophagy degradation of mutant huntington's protein aggregates also requires the participation of HDAC6. In addition, the activation of autophagy after proteasome inhibition also depends on HDAC6. These findings suggest that HDAC6 is important for the degradation of typeIIb misfolding proteins and type IIa soluble misfolded proteins. Because both of these proteins can be produced after proteasome inhibition, but the degradation of HDAC6 whether involved in IIc aggregated proteins is unclear, and how HDAC6 is involved in autophagic degradation of misfolded proteins remains also unclear. Some evidence suggests that this may be related to HDAC6-mediated microtubule activity, which allows some autophagic components, such as lysosomes, to be reversely transported to the microtubule center for autophagy degradation. P62 and HDAC6 have similar and synergistic effects, and both interact with misfolded proteins and autophagic vesicles, which also show some specificity in autophagy degradation.

In autophagy studies, another autophagy carrier receptor NBR1 (neighbor of BRCA1 gene 1) was also found. NBR1 receptor may be related to the degradation of the ubiquitin-labeled target in autophagosomes. Studies show that when autophagy occurs, NBR1 began to accumulate, it is linked to autophagy-related ubiquitin through its own UBA domain (Ub-associated domain) at the C-terminal, and the proteins to be degraded by ubiquitin are also linked to and aggregated with NBR1 through ubiquitin, and then NBR1 interacts with LC3. The autophagy carrier receptor is formed and the ubiquitin marker is transported to the autophagy for degradation.

In addition, NBR1 has been found to have a variety of biological functions. In the M line of the skeletal muscle segment, it can be observed that NBR1 is directly

connected to the giant sarcomeric protein kinase titin and P62. If the mutation of the giant sarcomeric protein kinase breaks the connection with NBR1, it will cause genetic muscle diseases, and NBR1 and P62 will form the "P62 corpuscle" mentioned above. However, it is still uncertain whether autophagosomes play a role in this disease. Knock out of NBR1 in mouse inhibited T cells activation and Th2 cell differentiation, while T cells play an important role in allergic airway inflammation. Therefore, it can be inferred that NBR1 may play a role in allergic airway inflammation by regulating the function of T cells. It can be seen from the above studies that compared with P62, NBR1 undoubtedly has more complex biological functions.

It can be seen from the above that both P62 and NBR1 can serve as the carrier receptors for autophagosomes to degrade the ubiquitinated target, so how do these ubiquitinated degradation targets enter the autophagosome? In general, there are three pathways: P62 body pathway, HDAC6-p62 pathway, and ATGs degradation pathway.

Abnormal folding proteins are generally degraded by the P62 corpuscles, pathway. Studies have shown that P62, ALFY (autophagy-linked FYVE protein), and NBR1 are assembled into P62 corpuscles, which act as autophagy carrier receptors and mediate misfolded proteins into autophagy vesicles. ALFY is a protein molecule with large structure, which plays the role of the skeleton in P62 corpuscles. As cells age, more and more misfolded proteins accumulate, and the stress effect of these misfolded proteins can induce the high expression of P62. In addition, the formation of P62 bodies also requires the assistance of specific molecular chaperones. Recent studies have shown that Hsp70 (Heat Shock Protein70) and its molecular chaperone Bag3 and HspB8 can induce the formation of P62 bodies. Endoplasmic reticulum stress caused by protein misfolding will induce Bag1 into Bag3, and Bag3, Hsp70, HspB8 and E3 ubiquitin kinase CHIP (carboxyl terminus of Hsc70-interacting protein) constitute a complex. The complex can recognize folded abnormal proteins, then the composite experience gathered P62, ALFY and NBR1 form P62 small body, P62 bodies bind to the folded abnormal protein labeled by ubiquitinated protein 1–4 to form autophagic vesicles of different sizes. Finally, P62, as the carrier receptor of the autophagosome, enables autophagic vesicles to enter autophagosome by endocytosis.

The HDAC6-P62 pathway is mainly used for aggresome degradation. Abnormal protein aggresome refers to the structure formed when proteins are labeled by ubiquitin and transported to microtubule organizing center (MTOC) through microtubule and then encased by intermediate filaments so that they can be effectively degraded by autophagy. HDAC6 is critical for the formation of abnormal protein aggresome by binding to ubiquitinated and dynein proteins, allowing it to transport soluble ubiquitin markers into the abnormal protein aggresome. Another of its roles is to induce the construction of dynein networks to promote the fusion of autophagosomes with abnormal aggresome and lysosomes. When proteins accumulate in large quantities, ubiquitinated proteins are labeled and bound to HDAC6, which is transported to the nucleus of autophagosomes via microtubules to form abnormal protein aggresome, and mediate the fusion of autophagosomes containing abnormal protein aggresome with lysosomes. The degradation process after binding with lysosome is related to P62 and its related factors. After the formation of a complex between ALFY and

ATG5, it binds with P62 as a carrier that mediates the fusion of autophagosomes and lysosomes, and after autophagy-lysosome formation, the abnormal protein aggresome degraded in autophagy.

The selective autophagy pathway independent of ubiquitin-protein has been most thoroughly studied in yeast, which can be divided into the following three pathways: Cvt pathway, mitochondrial autophagy pathway, and peroxisomal pathway. Cvt pathway is the most mature selective autophagy pathway. Its main function is to transport cytoplasmic and autophagosome related substrates, such as aminopeptidase (Ape1), to phagophore assembly site (PAS) for its hydrolytic enzyme activity. Ape1 is present in the cytoplasm as an inactive precursor of PrApe1. When PrApe1 forms Ape1 complex with Atg11 and Atg8, Atg11 leads the complex into PAS, and Atg8 leads the complex to bind Atg19 as carrier receptor, which mediates the complex into an autophagosome. The mitochondrial autophagy pathway is easily activated during nutrient deficiency or when cells enter the quiescent phase. This pathway is mainly regulated by a protein on the outer membrane of mitochondria: Atg32. After the damaged mitochondria were combined with Atg11 and Atg8 to form a complex, Atg32 was used as the carrier receptor to mediate the damaged mitochondria into PAS. The peroxisomal pathway refers to the combination of intracellular peroxides with Atg11 to form a complex, and Atg30 as the carrier receptor mediates the peroxisomal entry into PAS.

Activation of autophagy pathway is related to the stress of endoplasmic reticulum (ER). ER controls the degradation of abnormally folded proteins through the proteasome system and macroautophagy-lysosome system (MALS). Both pathways are activated under ER stress induced by misfolded proteins. When the UPS pathway is inhibited, the MALS pathway will be compensatorily activated. However, when the MALS pathway is inhibited, whether the UPS pathway can be compensatorily activated is uncertain, suggesting that the ubiquitin-proteasome pathway may be more limited in the degradation of misfolded proteins. ER stress activates the proteasome pathway through ER-associated degradation (ERAD). On the other hand, ER stress activates the autophagy system through ER-activated autophagy (ERAA). In addition, the release of calcium ions in ER to activate multiple downstream regions including CaMMK/AMPK, calcium protease, and DAPK is also an important mechanism of ERAA. In addition, molecular regulation of G protein signals (e.g., RGS16) may also be involved in ERAA activation. In rapidly growing tumor cells, the rapid metabolism of unfolded or abnormally folded proteins makes endoplasmic reticulum more prone to excessive load. Therefore, compared with normal cells, tumor cells are more prone to endoplasmic reticulum stress. Cells respond to endoplasmic reticulum stress with unfolded protein response (UPR) and enhanced autophagy. The unfolded protein reaction is also an important pathway of ERAD and plays an important role in ERAA. UPR is the main protective and compensatory mechanism in ER stress, it has three main feelings of endoplasmic reticulum stress sensors, endoplasmic reticulum protein kinase sample kinase (pancreatic ER kinase-like ER kinase, PERK), activation of transcription factor 6 (ATF6), and the endoplasmic reticulum inositol-requiring enzyme 1 (IRE1). The accumulation of unfolded or abnormally

folded proteins in the endoplasmic reticulum induces the dissociation and activation of these receptors and their molecular chaperones in the endoplasmic reticulum, glucose-regulated protein78 (GRP78). Activated PERK has been found to inhibit the synthesis of unfolded or misfolded proteins by phosphorylating the subunit of eukaryotic initiation factor2 (eIF2). In addition, the activated PERK and its molecular targets eIF2α in the process of autophagy LC3-I to LC3-II played an important role in transformation. The Huntington mutant protein transcriptionally up-regulates CHOP and Atg12 in an eIF2α phosphorylation-dependent manner, whereas Atg12 is a ubiquitin-like protein that modifies Atg5 by covalently binding. These processes are important for autophagosome formation. Activated ATF6 can regulate the expression of endoplasmic reticulum molecular chaperones (e.g., GRP78 and GRP94), X box-binding protein 1 (XBP1) and protein disulfide isomerase (PDI) to reduce the folding, secretion, and degradation of unfolded and abnormal folded proteins in the endoplasmic reticulum. In addition, autophagy can be induced by some drugs, such as A23187, tunicamycin, and toxic carotene, that directly interfere with the environment and function of ER, and this process seems to be dependent on the IRE1 signaling pathway. IRE1 also plays an important role in autophagy induced by proteasome inhibitors. In mammals, autophagy induction requires IRE1 kinase activity. IRE1 can activate the transcription factor XBP-1 to form sXBP1 (spliced XBP1) through the endoribonuclease activity, and sXBP1 can activate the transcription-translation of related proteins in the ER-associated protein degradation pathway (ERAD), promoting the degradation of unfolded or abnormally folded proteins. In addition, IRE1α can also activate ASK-1 and stress kinase JNK by binding to TRAF2. Overexpression of ASK-1 (Apoptosis signal, regulating kinase I) can mediate several cell death, suggesting that ASK-1 is significantly correlated with cell apoptosis induced by ER stress. It has been found that JNK is not only involved in autophagy induced by protease inhibitors but also plays an important role in autophagy induced by ER stress inducers. CHOP (C/EBP homologous protein) and JNK are the main factors in this process. Intense ER stress causes CHOP up-regulation of GADD34 (growth arrest and DNA damage-inducible gene 34) expression. GADD34 proteins can interact with protein phosphatase 1 (PP1) to dephosphorylate eIF2α, resulting in the uninhibited synthesis of folded or abnormally folded proteins. After the phosphorylation of Bcl-2 (B-cell lymphoma 2) by JNK, Beclin1, an autophagy gene, was released and activated from its inhibitory factors to induce autophagy, leading to apoptosis.

Recent studies have found that many factors in the UPR pathway play a role in tumor formation and development. For example, GRP78 has been found to play a role in tumor progression, including the proliferation of tumor cells, escape of apoptosis, tumor angiogenesis, tumor metastasis, and resistance of tumor cells to chemical agents. In addition, GRP78 protects quiescent tumor cells from chemical damage by inhibiting apoptosis. In liver cancer tissues, the GRP78 expression level was higher in low-differentiated liver cancer cells than in high-differentiated liver cancer cells.

The IRE1/XBP1 axis in UPR is another mediator that has been found to play a wide role in tumors. It was found that the growth of fibroblasts in transgenic mice with XBP1 dysfunction was significantly inhibited. On the contrary, the growth of multiple

myeloma tumor cells in transgenic mice with sXBP1 overexpression was exuberant after human multiple myeloma cell transplantation. In breast cancer patients, the accumulation of estrogen caused by breast cancer can promote the overexpression of XBP1, and the overexpression of XBP1 can induce the chemical resistance of tumor cells.

The autophagosome pathway is another pathway for the endoplasmic reticulum to degrade unfolded or abnormally folded proteins, which includes the following processes: autophagy guidance, nuclear formation of autophagosome, autophagy vesicle growth, and vesicle formation. ULK1/2 (Unc-51-like kinase 1/2) activates autophagy induction. Beclin1-VPS34 (phosphatidylinositol-3-kinase, PI3KIII)-p150 nuclear complex mediates autophagosome nuclear formation. Ubiquitinated protein is a key factor in the growth stage of autophagic vesicles. It can be mediated into autophagosome by Atg3, Atg5, Atg7, Atg12, LC3 (microtubule-associated protein 1 light chain 3) to promote the growth of autophagosome and eventually form autophagosome. Endoplasmic reticulum stress induced by unfolded or misfolded proteins up-regulated the expression of Atg12 and promoted the transformation of LC3-I into LC3-II. In addition, the endoplasmic reticulum stress can lead to caspase (cysteinyl aspartate specific proteinase) activation of autophagy function can make the Bcl-2 phosphorylation, and activated caspase and phosphorylated Bcl-2 can separate Beclin1 from its inhibitor to form a state of shear activation, and activated Beclin1 is then transported to mitochondria to promote the release of cytochrome C, which itself becomes a free form of Beclin1, free Beclin1 can induce autophagy again and form a positive feedback pathway.

Endoplasmic reticulum stress caused by unfolded or abnormally folded proteins can lead to the occurrence of autophagy. The endocytic degradation of autophagosomes to intracellular abnormal proteins or senescent organelles is an important source of cellular amino acids under endoplasmic reticulum stress. Therefore, in more metabolically active tumor cells, autophagy can increase the resistance of tumor cells to stress such as chemotherapy drugs and radiotherapy; similar studies have shown that inhibition of autophagy can increase the efficiency of chemotherapy drugs. For example, in tumor cells with Ras gene mutations, autophagy is still at a high level even under nutrient-rich conditions. The effect of chemotherapy drugs is not obvious, but if the autophagy pathway is blocked, the inhibition of tumor cell growth is significantly improved. In addition, malignant melanoma has a higher level of expression of LC3, and LC3 levels in advanced malignant melanoma cells are higher than the early stage. The expression of the autophagy gene Beclin-1 can be used as a prognosis for patients with nasopharyngeal carcinoma. The higher the expression level of Beclin-1, the worse the prognosis of patients.

So, what is the significance of ER-activated autophagy in clearing misfolded proteins? Clearly, the separation and removal of misfolded proteins reduce the toxicity of these proteins. The accumulation of misfolded proteins that have been cleared will eventually lead to cell death. The formation of aggregates avoids the distribution of misfolded proteins throughout the cell and is believed to have a protective effect on the cells. Inhibition of HDAC6 interferes with the formation of aggregates and increases cell death. Autophagy separates the misfolded protein and also protects the

cells. In some over-expressed misfolded proteins, such as mutant Huntington protein cells, autophagy can be promoted to reduce cell death, while autophagy inhibition can increase cell death (Arrasate et al. 2004). Autophagy has the ability to ameliorate the pathological changes caused by misfolded proteins, which has been confirmed in the animal models of Huntington's and Kennedy's diseases. So how does autophagy inhibit cell death? This is related to the upstream signaling pathway of autophagy inhibiting cell death. ER stress-induced apoptosis is largely mediated by the mitochondrial pathway and ultimately depends on the Bcl-2 family proteins, Bax and Bak. Current research suggests that Bax and Bak are not equivalent in response to a death signal stimulus. Bax appears to be more sensitive. When autophagy decreased, the death signal stimulation also acted more on sensitive Bax and less on Bak.

In summary, autophagy and UPS pathways work together to provide another compensatory mechanism for the degradation of misfolded proteins, which attenuates ER stress and reduces cell death. Among them, ER plays an important regulatory center in various degradation pathways. At present, the mechanism of autophagy induction has been continuously discovered. Of course, it is necessary to clarify how various abnormal folding proteins activate autophagy and the recognition mechanism of these proteins.

Proteins can be divided into three types based on the pathway of protein degradation: type I, type II, and type III. Type I proteins are completely degraded by the ubiquitin-proteasome pathway (Daw et al. 2015) and are used to regulate cellular homeostasis; type III proteins are completely degraded by large autophagy pathways for nutrient cycling; type II proteins are nonfunctional, abnormally folded proteins that are capable of being cleared by the UPS pathway, and the macroautophagy pathway. According to different protein solubilities, protein structures, and subcellular localizations, type II proteins can be divided into three subtypes: type IIa, type IIb, and type IIc. Type IIa is a soluble type II protein that is degraded by the UPS pathway; where protein degradation from the endoplasmic reticulum (ER) requires the involvement of the endoplasmic reticulum-associated degradation pathway (ERAD). Macroautophagy can degrade all abnormally folded proteins, but is more important for highly folded proteins (types IIb and IIc). Most misfolded proteins can also be ubiquitinated, and ubiquitinated proteins can be degraded by the protease pathway (pathway 1) and autophagy pathways (pathways 2 and 3). Among them, P62/SQSTM1 and HDAC6 promote the process of path 3. Some type IIa proteins, such as IKK complexes; IIc type proteins, such as a1-antitrypsin mutants, are not clear whether they are ubiquitinated. If they are ubiquitinated, they are degraded via pathways 2 and 3; if there is no ubiquitination, they are degraded via other degradation pathways (pathways 4 and 5).

## Microautophagy Pathway

More than 50 years ago, Duve and Wattiaux first proposed the concept of microautophagy, in which a small portion of mammalian cytoplasm can directly form vesicles and be engulfed by lysosomes. The microautophagy pathway was first identified in

the liver. Microautophagy has a variety of molecular mechanisms. In early studies, it was noted that the cytoplasm was partially extended and encapsulated by the lysosomal membrane. Degradation of vacuolar membrane structure was observed in yeast. Therefore, the definition of microautophagy was redefined in terms of membrane kinetics. Microautophagy was divided into three types: microautophagy with lysosomal membrane protrusion, microautophagy with lysosomal membrane entrapment, and microautophagy with nuclear endosomal membrane entrapment. In yeast, microautophagy involves degradation of a variety of substrates including peroxidase, nuclear fragments, mitochondrial fragments, lipid droplets, and so on. In plants, microautophagy mediates the degradation of anthocyanins. With the endosome membrane invagination, microautophagy depends on the endosome transport complexes (ESCRT) system, resulting in a major or selective cytoplasm protein degradation. Some small molecular mechanisms across different types of autophagy, the formation of vesicle membrane structure is a mammalian cell with lysosome membrane outstanding small autophagy and yeast cells with lysosome membrane invagination of the common features of autophagy, and ESCRT system in yeast cells with small lysosome membrane invagination autophagy and tracing the endosome membrane invagination little autophagy plays a key role. Autophagy-related proteins, such as Hsc70, interact in different types of autophagy pathways, so microautophagy is associated with other types of autophagy. In microautophagy, Hsc70 is recruited to mature endosome through the electrostatic interaction between protein and phosphatidylserine, so that it shows membrane deformation. In the microautophagy pathway, lysosomes deliver substances from different regions of the cell fluid into the lysosomal cavity for rapid degradation through endocytosis or tubulogenesis. Microautophagy is involved in the recycling of intracellular components under normal cell conditions. In addition, autophagy is also responsible for selectively removing excess organelles within the cell. For example, clinical drugs induce the production of peroxisomal enzymes, which maintain a normal number of intracellular organelles through the degradation of the microautophagy pathway. The microautophagy pathway also plays a role in maintaining the stability of the intracellular environment. However, so far, there is still a lack of effective methods to understand the relationship between microautophagy and human diseases.

## Chaperon-Mediated Autophagy Pathway

Molecular chaperone-mediated autophagy pathway is abbreviated as CMA. The most important characteristic of CMA is its selective substrate. Some proteins in the cell have lysosomal target sequences that molecular chaperones can recognize and bind to and transport to the lysosomal surface. Once the substrate protein reaches the lysosomal membrane, the substrate protein will interact with the receptor protein on the lysosomal membrane, and with the assistance of the molecular chaperone in the lysosomal body, the substrate protein passes through the plasma membrane into the lysosomal body for degradation. Like the macroautophagy pathway, CMA activity exists in most tissue cells, but the maximum activity of CMA occurs in the stress

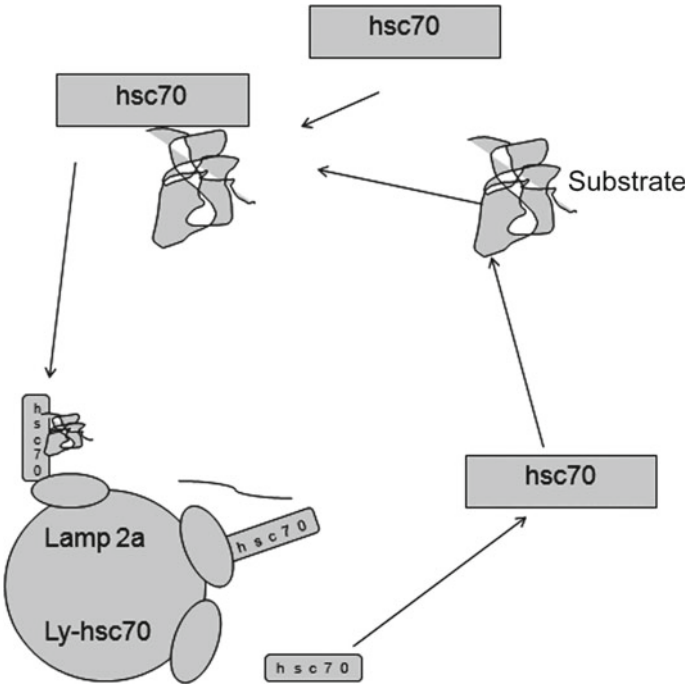


**Fig. 18.3** CMA pathway

state. As mentioned earlier, the initial response to nutrient deficiency is the activation of the autophagy pathway to provide an amino acid source for tissues to maintain normal physiological functions of the body. However, if nutrient deficiency persists, it is difficult to maintain cell metabolism merely by the degradation of intracellular components, and then CMA becomes a new source of amino acids needed for protein synthesis. When certain conditions (oxidative stress, exposure to toxins, etc.) cause protein damage, the selectivity of the CMA pathway can specifically remove the damaged protein without affecting the surrounding normal components.

The CMA action process is sophisticated and complex, which requires the coordination of multiple components. The specific functions and related mechanisms are described as follows (Fig. 18.3):

(1) HSC70 and common molecular chaperones (including hip, hop, hsp40, hsp90, bag-1, etc.) identify the region containing KFERQ related peptide in the substrate protein and form a complex. (2) the complex binds to the subunits of LAMP-2A on the lysosomal membrane. (3) the substrate protein was folded before translocation through the lysosomal membrane, and then entered the lysosome via the transmembrane segment (transposon) of LAMP-2A. (4) lysosomal HSC70 (ly-HSC70) is necessary for the transmembrane transport of substrate proteins. (5) once the substrate protein enters the lysosomal cavity, it is rapidly degraded by enzymes. (6) HSC70

molecular chaperone complex is released from the lysosome. (7) HSC70 molecular chaperone complex binds to other CMA pathway substrate proteins.

(1) The KFERQ-like motif of substrate protein (KFERQ is a lysosomal targeted signal peptide). Most known CMA substrate proteins contain the KFERQ peptide sequence, and only protein substrates with this sequence can bind and interact with molecular chaperone specificity. Accurate information on this sequence has previously been reported. Specifically, the sequence is centered on glutamine (Tabone et al. 1994), and four amino acid compounds form four flanks, including an alkaline end (K, R), an acidic end (D, E), a large hydrophobic end (F, I, L, V), and a repeating basic or large hydrophobic end (K, R, F, I, L, V). Such sequences are present in 30% of cytoplasmic proteins, some of which may produce kFERQ-like sequences through posttranslational modifications such as deamidation of posttranslational proteins.

(2) Association of molecular chaperone complexes in the cytoplasm with lysosomal membranes. There are a variety of molecular chaperones in the cytoplasm, and HSP70 is taken as an example to illustrate the process of the molecular chaperone. Continuously expressed heat shock protein 70 (HSP70) is a molecular chaperone that plays an important role in substrate proteins entering the lysosome through the plasma membrane. Cytoplasmic HSP70 can recognize the KFERQ sequence of the substrate protein. Co-chaperones interact with HSP70 to form a molecular chaperone complex that binds to the substrate protein recognized by HSP70 and transports it to the lysosomal surface. In addition, multiple mechanisms are involved in the regulation of this process. Heat shock protein 40 (HSP40) can stimulate the ATPase activity of HSP70 and increase the binding and release of HSP70 to a substrate protein. Hip (Huntington-interacting protein) promotes protein and HSP70, HSP40 substrate assembly; Heat shock protein 90 (HSP90) can prevent the aggregation and/or refolding of unfolded proteins. Bcl-2-associated immortal gene-1 (bag-1) is composed of several subtypes that positively or negatively regulate HSP70 activity.

The molecular chaperone complex is also closely related to the substrate protein located in the cytoplasmic surface of the lysosomal membrane and the LAMP-2A on the lysosomal membrane. Substrate proteins need to be folded and unfolded before they can be translocated into the lysosome, a process that requires the assistance of most molecular chaperones. Therefore, HSP70, HSP40, hip, and hop antibodies can prevent substrate proteins from entering the lysosome to varying degrees.

(3) HSP70 in the lysosomal cavity. The HSP70 in the lysosomes is called Ly-HSP70. Ly-HSP70 is not associated with other molecular chaperones, and most of them are acidic HSP70 subtypes. It is possible that all of the HSP70 subtypes and the substrate protein and the molecular chaperone complex can enter the lysosomes through a variety of routes, such as by autophagy, but only the acidic HSP70 subtype can be stably present in the lysosomes. The most reasonable explanation is that all HSP70 subtypes can enter lysosomes, but more basic HSP70 subtypes are transformed into acidic HSP70 subtypes by lysosome modification. An increase in Ly-HSP70 was found in the liver of chronically hungry rats when the CMA pathway was activated, and one of the characteristics of activated lysosomes was the presence of a large amount of Ly-HSP70 in the liver. Just as proteins enter ER and mitochondria

require the involvement of Ly-HSP70s, Ly-HSP70 is necessary for CMA-mediated substrate proteins to enter lysosomes.

(4) LAMP-2A. LAMP-2A is a receptor on the lysosome membrane and plays an important role in substrate binding and translocation. In the CMA pathway, the substrate protein binding to the molecular chaperone complex binds to the LAMP-2A on the lysosome membrane, and the binding is saturated. The binding of one substrate protein to LAMP-2A is inhibited by the competitive inhibition of other substrate proteins, but there is no competitive binding site between one substrate protein and other non-substrate proteins. These characteristics of LAMP-2A directly affect the protein degradation efficiency of the CMA pathway. Binding to LAMP-2A is the rate-limiting step of CMA, so LAMP-2A can up-regulate or down-regulate the level of CMA on the lysosome membrane. During oxidative stress or T cell activation, the synthesis of LAMP-2A increased, but the degradation of LAMP-2A decreased under the condition of starvation. LAMP-2 A receptor has three different subtypes, namely, LAMP-2A, LAMP-2B, LAMP-2C. But only LAMP-2A can be used as the receptor of CMA, and the function of the other two subtypes is not clear.

The number of LAMP-2A on the lysosomal membrane was regulated by several different pathways. A complete LAMP-2A can form complex with liposomes in the lysosomal cavity. When the CMA pathway is activated, these molecules can be transposed and reinserted into the lysosomal membrane. By regulating the degradation rate of LAMP-2A, the cells could better facilitate the transport of substrate protein to lysozyme for degradation. The LAMP-2A subregion on the lysosomal membrane is responsible for its dynamic regulation. In a resting state, the LAMP-2A is periodically separated into lipid regions for degradation which is released by the enzyme A on the membrane and rapidly degraded in the lumen. The transport of LAMP-2A may be a regulatory mechanism of CMA. In addition, two different proteases are responsible for the degradation of LAMP-2A, one of which is lysosome cathepsin A. The cleavage of cathepsin A determines the stability of monomer LAMP-2A, and HSP 90 located on the membrane of cytoplasm and lysosome can help stabilize the structure of LAMP2A.

The CMA substrate binds to LAMP-2A with 12 short amino acid chains. Using specific antibodies to block this region, the amino acid residues were exchanged with the corresponding region in LAMP-2B or LAMP-2C to reduce the CMA level. Therefore, the short chain of amino acids in this region exists only in the tail of LAMP-2A. When the substrate binds LAMP-2A, it triggers its multistep conversion from monomer to polymer to form a 700 kDa CMA transposition complex. The transposition complex decomposes immediately after the substrate is transferred to the lysosome.

At present, through the research on a transgenic mouse model, the important role of CMA-related molecules has been established, the kinetic characteristics of CMA substrate degradation have been clarified, and the physiological role of CMA has been verified in vivo. It was found that the regulation of LAMP-2A on the lysosomal membrane made the CMA mechanism better understood. However, the number of known signaling pathways in CMA regulation is still incomplete. In species without LAMP-2A, whether microautophagy is an alternative to CMA or whether there are

other CMA equivalent autophagy pathways in these species remains controversial. More and more studies have proved that there is a common autophagy-related protein between CMA and the macroautophagy pathway, and these problems provide new research directions for the autophagy mechanism.

## Autophagy and the Quality Control System

Autophagy system is an important part of the intracellular quality control system, which is of great significance for maintaining the stability of the intracellular environment (Kubota 2009). The intracellular quality control system also includes another important proteolytic system, ubiquitin-proteasome system, and molecular chaperone.

Changes in intracellular proteins are attributed to intracellular and extracellular influences (e.g., oxidative stress, ultraviolet radiation, exposure to toxic substances, etc.). In addition, the protein translated by the mutant gene is prone to aggregation due to incorrect folding. These abnormal proteins tend to form complex structures such as oligomers, aggregates, and fibrous structures. These form complex structures that can be identified by molecular chaperones. Molecular chaperones can prevent the aggregation of abnormal proteins by facilitating their refolding into proteins with normal structures. In some cases, the intracellular abnormally folded proteins exceed the repair capacity of the molecular chaperone, which will choose to transport these abnormal proteins directly to the proteolytic system (ubiquitin-proteasome system and autophagy system) for degradation and clearance. The imbalance of various clearance systems in the quantity and quality control system of intracellular abnormal proteins will lead to the accumulation and aggregation of intracellular abnormal products. Although the exact mechanism by which abnormal proteins cause cytotoxicity remains controversial, it has been demonstrated that the greatest pattern of cytotoxicity is in complex tissue structures, such as oligomers or fibrous structures. The quality control system is a strong defense against the cytotoxicity of misfolded protein aggregates. If the system fails to function properly, cells are more likely to form protein aggregates rather than oligomers or fibrous structures. Protein aggregates interfere with the normal transport of cell contents and occupy space within the cell and can form an absorption pool. Proteins with normal functions often fall into the absorption pool and affect their normal function.

UPS and autophagy are two major evolutionarily conserved degradation and circulatory systems in eukaryotes. Early studies have shown that their work is not interdependent, but recent studies have shown that there are connections and overlaps between the two systems. Mitochondrial autophagy is an example of two interconnected systems. Functional studies have shown that damage to one of the UPS or autophagy degradation systems leads to compensatory up-regulation in the other system. In order to maintain homeostasis, cellular material accumulated after one degradation system is inhibited needs to be cleared by another system, thus forming a compensatory mechanism. However, this compensation is not always effective and largely depends on the cell type, the intracellular environment, and the load of the

target protein. Inhibition of the UPS pathway or gene regulation by different compounds will lead to up-regulation of autophagy. For example, proteasome inhibitors and chemotherapy drugs can lead to increased expression of autophagy-related genes ATG5 and ATG7 and induce autophagy. The up-regulation of autophagy-related gene expression was caused by the activation of ER stress-related pathways and AMPK activation (Kouroku et al. 2007). Similarly, decreased autophagy levels were associated with UPS activation. In colon cancer tumor cells, chemical inhibition of autophagy and ATG knockout will lead to increased levels of proteasome subunits, which in turn activate UPS. Since proteasome is the target of autophagy degradation, the enhancement of proteasome activity after autophagy inhibition may be related to the continuous accumulation of proteasome. In some cases, however, autophagy inhibition is associated with the accumulation of ubiquitinated proteins. For example, accumulation of ubiquitination conjugates was observed in the brains and livers of ATG5 or ATG7 knockout mice. Ubiquitination is considered to be a link between the substrate and the appropriate degradation system or even the UPS system and the autophagy system.

So how do proteasome and autophagy systems cooperate in protein degradation? Firstly, most soluble abnormally folded proteins are preferentially degraded by the proteasome, and only when the proteasome system is overloaded can autophagy pathways be initiated to compensate. For example, after inhibiting the activity of protease, the autophagy pathway is activated and the intracellular ubiquitinated abnormally folded protein is degraded and cleared by the autophagy pathway. Second, whether the autophagy pathway is activated may depend on the stress level of the ER. When the stress level of ER reaches a certain level, the autophagy degradation pathway is activated. Third, the composition of misfolded proteins themselves is also critical. For example, misfolded proteins in the form of soluble monomers can be degraded by proteases, while insoluble misfolded proteins in the form of polymers cannot be degraded by proteases. In addition, misfolded proteins in the form of polymers are harmful to proteases. At present, it has been found that the proteasome function is significantly weakened in the presence of a large number of aggregates. Some misfolded proteins exist in both soluble and insoluble forms, such as α1-antitrypsin Z mutant, whose soluble form can be degraded by proteasome and autophagy system, while the insoluble form can only be degraded by autophagy.

In cells, proteolytic systems are usually rapidly adapted to changes in the concentration of abnormal constituents. However, the accumulation of protein aggregates occurs when a large amount of protein damage occurs in a short time and exceeds the degradation capacity of the proteolytic system. In most cases, cells will eventually overcome this problem. Accumulation of toxic protein products or protein aggregates can have different consequences depending on the extent to which the cell is affected. In cells that are dividing rapidly such as skin fibroblasts, aggregates are distributed to mother and daughter cells to dilute the aggregates. However, in some anaphase tissue cells (those that do not divide) such as brain neurons, the persistence of cytotoxic protein products results in cell death. We all know that neurons don't regenerate, so the constant loss of neurons eventually leads to disease. This explains why the central nervous system is often affected by a high concentration of

proteins with harmful conformational abnormalities in cells. Proteasome system and autophagy system exist at all levels of protein degradation. In addition to ubiquitination, some proteins and signaling pathways are involved in the communication and mutual regulation of these two systems. Discussing the interconnection between the two systems is helpful to understand the biological significance and clinical value of protein quality control from the perspective of basic medicine.

## 18.3 Autophagy, Proteins Misfolding, and Diseases

### *18.3.1 Factors Affecting the Function of Autophagy and Related Diseases Caused by Its Abnormalities*

#### 18.3.1.1 Dynein Abnormality-Associated Diseases

Signal transmission within and between cells depends on efficient internal transport systems. Neurons accurately convert external stimulus into various reactions between cells through the movement of internal substances. In numbers of motor neurons, long-distance transportation of substances depends on microtubules, dynein, and kinesin. The dynein pushes the substance centripetally to the negative end of the microtubule, and transports the substance to the central part of the nucleus. While the kinesin moves centrifugally to the positive end of the microtubule, transporting the substance into the cytoplasm.

Nocodazole is a kind of antitumor drug and also an antiparasitic. It can depolymerized microtubules and inhibit the fusion of autophagosome and lysosome. Thus, the flow of autophagosome is also associated with microtubules. The disintegration of microtubules results in decreasing ability of autophagy in clearing the substrate. Under these circumstances, the maturity of autophagosome and the fusion of autophagolysosome are affected, so the autophagosomes cannot pass through the microtubules to the microtubule organizing center (MTOC). Similar results are obtained by attenuating the effects of dynein, suggesting that dynein is a key protein that transports intracellular substances along the microtubules to lysosomes. In addition, HDAC6, a histone deacetylase-like protein is associated with microtubules and can regulate autophagy. However, it is not clear that how dynein and HDAC6 participate in the transportation of autophagosomes on microtubules.

At present, it was found that mutations of certain genes affecting microtubule transportation can cause motor neuron disease (MND) in the mouse model and humans (Cipolat Mis et al. 2016). MND refers to a group of sporadic, familial diseases characterized by motor neuron degeneration. In transgenic mice,

material transportation through the axon is affected by overexpression or deletion of dynein, leading to progressive degeneration of motor neuron. Pathological changes and symptom progression observed in MND mouse model were similar to those of MND patients. In MND model, protein aggregates accumulate in neurons, suggesting that autophagic dysfunction is one of the pathogenesis of these diseases. Dynamin-dependent microtubule transportation dysfunction is one of the causes of this kind of diseases. The partial reason for protein aggregates accumulating in motor neurons is the disruption of the links such as microtubule-dependent autophagosome dyskinesia, autophagosome maturation, and autophagosome-lysosomal fusion dysfunction. The importance of autophagy decline in the development and progression of MND remains to be elucidated.

#### 18.3.1.2 The Role of ESCRT and Related Diseases (Endosomal Sorting Complexes Required for Transport, ESCRT)

Most of the recycling of intramembrane proteins of cell membranes is done by various functions of endocytic pathways, including some receptor proteins, such as the epidermal growth factor receptor (EGFR) protein. Some proteins, called endosomes recycles proteins, perform a simple classification of these membrane proteins and return them to the membrane. More complex degradation systems rely on the multivesicular body (MVB), which is a luminal vesicle formed by the inclusion of body membrane and invaginated to produce a specific porous structure. After MVB is fused with lysosomes, vesicles are released into the acidic lysosomal cavity, where the hydrolytic enzymes degrade the vesicles and the substances they carry with. The classification of membrane proteins and their entry into MVB depend on the ubiquitination of proteins. This specific process can interact specific proteins with lumen like vesicles of MVB. Four kinds of ESCRTs ensure the high fidelity of protein classification to the endosome and fusion with the lysosome. The specific interaction of the ESCRT complexes is necessary for the formation of MVB and the normal fusion of endosome with lysosomal. Each complex performs its specific function through multiple interactions with proteins, cell membranes, endosomes, and other complexes. ESCRT plays an important role in the formation of autophagosomes and the fusion with lysosomes, which may be related to the fact that ESCRT can polymerize SNAREs and Rab7. Soluble N-ethylmaleimide-sensitive fusion attachment protein receptors(SNAREs) in autophagy body play an important role in the process of formation and fusion. In mammalian cells, vesicle-associated membrane protein7 (VAMP7), syntaxin-7 (syntaxin-7), syntaxin-8 (syntaxin-8), VTI1B (vesicle transport through interaction with t-snares 1B) promote the mutual fusion of Atg16L protein on vesicle precursor membrane. And they form a tubular network, and eventually, these networks fuse to form an autophagosome. Vamp3-mediated fusion of endosomes in mature autophagosomes to form Amphisomes has also been observed in some special cells. Inhibition of these SNAREs can lead to a reduction of the volume of autophagy precursors and delayed maturation of autophagosome. In yeast, the functions of Sec9p and Sso2p in SNAREs are similar to those of VAMP7

in mammals, which can promote the fusion of Atg9 proteins in autophagy precursor membrane to form tubular reticular structure and then fuse with each other to form autophagosome. In Yeast, the fusion of autophagosomes and lysosomes requires the Vam3 (vacuolar syntaxin homolog), Vam7, GTP binding protein transport 7 in Rab family, and in mammalian cells, it needs the mediation of VAMP7, syntxin-7, and VTI1B. Studies have shown that when a large amount of cholesterol accumulates in the body, it leads to the immobilization of VAMP7 by abnormal organelles hinders the fusion of autophagic lysosomes, and prevents the entry of substances to the lysosomes by endocytosis. As mentioned above, the acidification of autophagosomes and the activation of hydrolases in lysosomes are important preconditions for autophagosomes to perform their functions and also signs of their maturation. Recent studies have found that syntaxin-5 in SNAREs is involved in this process.

Rab7 is an important GTPase in the Ras family, together with SNAREs playing a role in the process of endosome maturation and lysosomal fusion in autophagosomes. In the process of maturation, an endosome is transported from the cell edge to lysosome by microtubule system, which is called forward transportation. This direction is determined by dynein-dynactin, which is constantly consumed during transportation. ORP1L (oxysterol-binding protein-related protein 1L) and Rab7 together form the complex that positioning on the membrane of the endosome, when it moves forward in the microtube it will activate ORP-Rab7 complexes to connect with RILP (Rab7-interacting lysosomal protein). Then ORP interacts with the cholesterol contributed at the endosome membrane to constitute a relatively stable conformation that can make the Rab7-RILP complex concentration dynein, complementing the consumption. When endosome gets near to the late endocytic compartments forming near the edge of the cell membrane, ORP1L will induce the Rab7-RILP complex to become β-III contractile protein, which is necessary for the extracellular transportation of the endosome through microtubules. In the process of autophagosome-lysosomal fusion, Rab7 first binds to GTP under the induction of HOPS (homotypic fusion and protein sorting complex) and becomes into an activated form. Cis-SNAREs complex which is located at the membrane of autophagosome and lysosome that is about to be fuse with each other dissociated into monomer. Subsequently, under the mediation of rab7-GTP protein complex, monomers of SNAREs on the fusing membrane get close to each other to form a compact trans-SNAREs complex and start to fuse with each other. After Rab7 aggregation was inhibited by toxic carotene, it was found that autophagosome and lysosome could not fuse with each other, which proved the important role of Rab7 in this process.

The classification of the early substances transportation is mainly finished by the ESCRT-0, -I, -II interacting with ubiquitin substances. ESCRT complex decomposition and the involvement of DE-ubiquitinating enzymes (DUBs) make the ubiquitin dissociate from the substrate before the substrate degradation. The process requires ESCRT-III. Although specific ESCRT mutations are associated with neurodegenerative diseases, the mechanisms of the disease are still not well understood. Now it has been found that the ESCRT-III who has the changed gene-splicing sites, its CHMP2B subunits point mutations is associated with frontotemporal dementia linked to chromosome 3(FTD3) at its familial autosomal dominant type.

The expression of deletion mutations and splicing mutations of CHMP2B aggravates the reduction of neurons and dendrites through non-apoptotic pathways, so some current studies aim at observing the effect of CHMP2B deletion mutations on autophagy. In cells and fly model, the expression of the deletion mutation increased raising of LC3-II level caused by autophagy accumulation, and reduce the formation of MVB. The experiment compared CHMP2B wild-type and mutant SKD1, the latter prevents the ESCRT-III complexes separate from the endosome. The experiment shows that proper dissociation of ESCRT-III complex is the key to autophagy body maturation and autophagy-lysosome. The deficiency of msnf7-2 which is the key component of ESCRT-III, or the mutation of CHMP2B protein may lead to the functional deficiency of ESCRT-III which will cause accumulation of ubiquitinating marker protein, and finally leads to FTD3 and amyotrophic lateral sclerosis (ALS). Studies on mature cortical neurons have found that the functional deficiency of ESCRT-III will lead to the accumulation of autophagosomes and block the fusion of autophagosomes and lysosomes leading to the clearance of aging proteins and organelles.

It was found that the autophagy function decreased to different degrees through the construction of the gene mutation expression of ESCRT complex or the reduction of its expression. Data from a variety of cell types showed that the functional deletion of various ESCRT genes would result in immature autophagosomes or failure in fusing with lysosomes. The accumulation of autophagosomes without the ability to degrade the substances leads to neurodegeneration. In addition, polyubiquitinated endosomes increased in these tissues. It is not clear whether the decline in autophagy function is caused by the destruction of autophagosome by the ESCRT complex or by the indirect destruction of the endosome and lysosome. In conclusion, current studies suggest that neural degeneration caused by decreased autophagy function with decreased ESCRT function is one of the important pathogenesis of FTD3 and ALS.

#### 18.3.1.3 Lysosomal Storage Disorder (LSD)

Lysosomes are the ubiquitous organelles in cells surrounded by monolayer membrane, which contain a variety of acid hydrolytic enzymes which have the functions of dissolution or digestion. Normal functional lysosomes are involved in the circulation of cellular components, cholesterol homeostasis, down-regulation of surface receptors, inactivation of pathogenic microorganisms, repair of the cytoplasmic membrane, and cell bone remodeling. Lysosomes can be divided into two types: primary lysosomes are vesicular structures formed by bulking at the edges of the Golgi capsule, actually they are secretory vesicles containing a variety of hydrolytic enzymes. These enzymes are synthesized on the ribosome of the rough endoplasmic reticulum and transferred to the Golgi capsule. The enzymes in the primary lysozyme do not digest. Secondary lysosomes are the fusion products of phagocytic vesicles and primary lysosomes. In the secondary lysosomes, the remaining substances in phagocytic vesicles are excreted after digestion.

The fusion of autophagosomes and lysosomes is necessary for the degradation of substances in autophagy, so it plays an important role in the process of autophagy. Without this fusion, autophagy accumulates and carries a large amount of undegraded material, which is toxic to cells. Currently, LSDs is considered to be a group of nearly 60 different genetic diseases, each with a single-gene defect that causes the lysosomal system malfunction and fails to degrade specific substances within the cell. As a result, many tissue and organ systems are affected, including the brain, internal organs, bones, and cartilage, with early-onset central nervous system (CNS) dysfunction predominating. Although the clinical characteristics of these diseases vary from each other, most of the survival keep within 20 years from onset. Progressive phenotypic development is one of the hallmarks of LSDs.

Generally speaking, LSD is a single-gene disease caused by complete or partial dysfunction of lysosomal proteins (mainly lysosomal hydrolases) due to gene mutation. It is currently estimated that lysosomes contain 50–60 hydrolases that are active in acid environments in lysosomes. Although LSD varies greatly in pathology, all of these diseases result in the accumulation of undegraded substances in and out of the lysosomal cavity at the molecular level. This accumulation affects the normal functioning of a large number of intracellular signaling pathways. Recent biochemical and cell biological studies on LSDs have shown that LSDs have abnormalities in a variety of cell functions. These defects include signal pathway defects, calcium homeostasis imbalances, lipid biosynthesis and degradation defects, and intracellular transportation disorders. Because lysosomes and autophagosomes fuse and digest their contents, they play a fundamental role in the autophagy pathway, which is called the high integrity of lysosomes and autophagosomes. The researchers believe that the lysosomal accumulation in LSDs will have an impact on autophagy.

Mucopolysaccharidoses (MPSs) is a kind of disorder for glycosaminoglycans (GAGs) degradation caused by the inactivation of hydrolytic enzyme mutation in a set of lysosomes. As long as one of the mucopolysaccharides hydrolytic enzyme is inactivated, mucopolysaccharides will gradually accumulate leading to the occurrence of tissue and organ dysfunction. Deficiency of 11 enzymes is known to be the cause of 7 different MPSs.

Multiple sulfatase deficiency (MSD) is a typical disease of LSD, and the patient has a complex multisystem phenotype due to the impairment of all sulfate esterase activities. It is caused by a gene mutation which code the FGE (formylglycine-generating enzyme) of SUMF1 (sulfatase modifying factor 1). The posttranslational modification of sulfate esterase requires the participation of FGE. Without this modification, the sulfatase activity is reduced and the lysosomal substrate cannot be degraded. Autophagy injury is believed to play an important role in the pathogenesis of this disease.

MSD mouse chondrocytes have severe lysosomal storage defect, which presents as abnormal autophagy activity, and eventually leads to energetic metabolism imbalance and cell death. The number of positive cells in the co-localized staining of LAMP1 and LC3 of the autophagosome marker in MSD mice decreased, suggesting that the fusion of lysosomal and autophagosome may be impaired. In MSD mouse

body, we also found that not only the substrate of lysosome and degradable lysosome increased, autophagosome also appears in a large number of accumulation. It can be proved by western blot and immunofluorescence technology which found LC3-II level increasing obviously. The MSD mouse SUMF1 mutation inhibited the degradation of lysosomal substrates and the fusion of lysosomal and autophagosome. Accumulation of ubiquitination aggregates was also found in brain tissues. A large amount of lysosomes in the cell will lead to the inhibition of autophagy, thus affecting two important pathways: (1) protein recycling is inhibited, and toxic protein polymers accumulate in the cell in large quantities, inducing inflammatory reactions, damaging mitochondria, and forming a vicious cycle. (2) the regeneration and circulation function of mitochondria decreases and damaged mitochondria accumulate continuously, which will induce apoptosis.

Two kinds of LSD mouse models which are related to severe nerve degeneration are often used in the study: MSD and mucopolysaccharidosis type IIIA (MPS IIIA) model. The generality of these two models includes: (1) autophagy body increase in number, (2) the clearance of autophagy substrate of endogenous and exogenous decreases, (3) organelles circulatory disturbance.

The mechanism of weakened autophagosome-lysosomal fusion in LSD remains to be elucidated. The possible mechanisms are (1) microtubule-based transportation system dysfunction. (2) lipid composition of the lysosomal membrane was changed. Some lipid components (mainly cholesterol and glycosphingolipids) have been found to accumulate in various LSD diseases. The lipid components are an important part of the cell membrane where is rich in lipid, called lipid rafts. Lipid rafts played an important role in the physiological function of the cell membrane by determining the cell membrane elasticity. The accumulation of lipid raft in LSDs may affect the dynamics of the lysosomal membrane and its fusion with autophagosomes. It has been suggested that the abnormal distribution of cholesterol in the lysosomal membrane and the rupture of lipid rafts in the plasma membrane may be the cause of dysfunction of lysosomal and autophagosome in MSD.

Sphingolipidosis is a heterogeneous inherited sphingolipid metabolic disorders. Children are the main population of s sphingolipidosis. The accumulation of sphingomyelin, glycolipids, glycosides, gangliosides, unesterified cholesterol, sulfides, and other compounds may be caused by abnormal hydrolytic enzyme function or secondary to the accumulation of other lipids. The increase in sphingomyelin will change the level of autophagy. Studies have shown that sphingomyelin addition to cell culture medium can induce autophagy and reduce the clearance efficiency of autophagosomes. It can be observed in multiple models. Therefore, the accumulation of sphingomyelin can change the function of the autophagy pathway, leading to the occurrence of such diseases.

Niemann-pick type C disease (NPC) is an autosomal recessive inherited fatal neurovisceral lipid deposition disease, which is mainly manifested as liver damage, progressive neurological dysfunction, and mental symptoms. Most of them are caused by the mutation of the NPC1 gene on chromosome 18, while a few are caused by the mutation of the NPC2 gene on chromosome 14. The protein products of NPC1 and NPC2 genes are associated with the outflow of cholesterol in the late

lysozyme. Researchers found that the fibroblasts of patients with NPC and the brain of NPC mouse can find a large accumulation of autophagosomes. It was regarded that the combination of class III phosphatidylinositol kinase and BECN1 induce the occurrence of autophagy and maturity of the autophagosome. They put forward the deficiency of NPC1 caused the autophagy induction and autophagy pathway is unusual, autophagy substrate degradation dysfunction, neurons, and glial cells eventually develop a large amount of lipid, cause-related symptoms.

Gaucher disease (GD) is a chromosomal recessive inherited disease of glucose and lipid metabolic abnormality. The deficiency of glucocerebrosidase causes the accumulation of glucocerebrosidase in mononuclear macrophages of the liver, spleen, bone and central nervous system, causing related symptoms. Like NPCs, autophagy induction, autophagosome formation, and autophagosome substrate accumulation can be seen in neurons and glial cells in GD model, while lysosomal degradation ability in gaucher's cells is insufficient.

Neuronal ceroid lipofuscinoses (NCLs) is the most common neurodegenerative disease in children due to the deficiency of fatty acid peroxidase. Severe pigmentation of the retina, waxy and lipofuscin deposits in the skin, internal organs, and nerve cells, mainly manifesting as blindness, epilepsy, progressive cognitive impairment, and loss of motor function. NCLs are genetically and phenotypically heterogeneous. Autophagosomes were significantly increased in both NCLs animal models and patient cells. Mutations in the CLN3 gene cause juvenile-onset NCL (JNCL). Mitochondrial dysfunction, down-regulation of the MTOR pathway and formation of autophagosome can be seen in JNCL mouse model neurons.

Autophagy-related molecules play different roles in the pathogenesis of different types of LSDs. In most cases, the autophagy pathway is blocked, leading to the accumulation of autophagy substrate and the dysfunction of mitochondria, resulting in an increase of autophagosomes. At the same time, synthesis of related molecules inducing autophagy increases to compensate for the blocked autophagy pathway. Therefore, LSDs is mainly manifested as autophagy disorder.

### *18.3.2 Neurodegenerative Diseases Caused by Autophagy and Misfolded Proteins*

In the research of special neurodegenerative diseases, it has been found that autophagy dysfunction is closely related to neurodegenerative changes (Choi et al. 2013). Although the pathogenesis of each neurodegenerative disease is complex, they all share some common characteristics, which are related to the passway that neurons process abnormal intracellular proteins. Recent studies have shown some common pathogenesis of this disease. Abnormal proteins that still maintain their own solubility can undergo normal, targeted degradation via the ubiquitin-proteasome system (Daw et al. 2015) or the CMA pathway. These degradation pathways are relatively selective in that they remove abnormal proteins without affecting the surrounding

intracellular components. However, as mentioned before, the specific pathway by which an abnormal protein is degraded is not clear, which may be related to the effectiveness of the proteolytic system at a specific time or the characteristics of the substrate protein. For example, certain proteolytic enzymes of the lysosome may be more effective at degrading certain proteins; Only unfolded monomer proteins can be degraded through both the UPS pathway and the autophagy pathway, while proteins that have formed oligomers, fibrils or fibrous structures can only be processed by batch degradation pathways, such as microautophagy or macroautophagy. Now it is thought that macroautophagy can remove the aggregation protein. Although the mechanism that determines macroautophagy activity under these conditions is unclear, it seems that abnormal proteins that cannot be degraded by other proteolytic systems can only be degraded by macroautophagy. In cultured cells, oligomers and specific protein fibers block the activity of the ubiquitin-proteasome system and CMA, and the blocking of these two pathways increases the activity of the macroautophagy pathway (Martinez-Vicente and Cuervo 2007).

In normal individuals or in the early stages of the disease, most soluble abnormal proteins are typically degraded in a targeted manner by either the ubiquitin-proteasome system or the CMA. However, some abnormal proteins sometimes have toxic effects on these two proteolytic pathways and partially inhibit their activity (red arrow). In addition, once these toxic proteins form complexes (oligomers or cellulose), they cannot be degraded through the UPS or CMA pathway. At the compensatory phase, the macroautophagy pathway is activated (green arrow) to compensatorily scavenge toxic proteins. In the late stage, the UPS and CMA pathways are further blocked, the macrophage activity decreases and the toxic proteins accumulate in the cells and the aggregates leak out from the autophagy vesicles. Then the cells begin to show dysfunction or even death (the red circle represents the non-degradable autophagy vesicles). Although the exact cause of proteolytic system failure is still not clear, however, factors such as oxidative stress and senescence promote cells to enter the late stages (Martinez-Vicente and Cuervo 2007).

Studies have shown that macroautophagy consistently clears intracellular abnormal proteins. Thus, the absence of macroautophagy in neurons will lead to accumulation of agrin and neurodegeneration (Metcalf et al. 2012). At the compensatory phase, the appropriate activity of the macroautophagy pathway is essential to maintain cell survival. In fact, if the activity of the macroautophagy pathway is enhanced by drugs at this stage, the accumulation of intracellular aggregates and the occurrence of symptoms will be significantly delayed. Unfortunately, the normal course of these diseases inevitably leads to depletion after the compensatory phase. The ubiquitin-proteasome system and CMA activity are further reduced and the macroautophagy pathway begins to lose activity. With the inactivation of these systems, abnormal proteins and normal intracellular components begin to accumulate, leading to progressive cell damage and death.

For these reasons, studies of clinical treatment have focused on how to prevent primary blocking of the UPS and CMA pathways or remove abnormal proteins in cells by enhancing autophagy activity, thereby prolonging the asymptomatic compensatory period. Following we outline several major neurodegenerative diseases

(Parkinson's disease, Alzheimer's disease, polyglutamine disease, etc.) to help people understand the role of autophagy in neurodegenerative changes and the characteristics of these diseases (Rubinsztein et al. 2005).

#### 18.3.2.1 Parkinson Disease

Parkinson's disease is characterized by progressive diffused loss of the substantia nigra and striatum dopaminergic neurons. A pathological hallmark of the disease is the inclusion bodies and Lewy bodies in the cells, mainly containing aggregated α-synuclein proteins. Oxidative stress (caused by mitochondrial dysfunction) and damage to the proteolytic system may be responsible for the accumulation of these abnormal proteins and other aggregation-prone proteins, but the reason of the loss of selectivity of dopaminergic neurons and the accumulation of α-synuclein protein still not clear (Sheehan and Yue 2019). There is also evidence that neurons with more inclusion bodies can survive. In contrast, large pyramidal neurons and Purkinje cells appear to be vulnerable to be attacked by abnormal proteins and die before large inclusion bodies formed. Therefore, the formation of inclusion bodies in neurons is harmful or beneficial to cells still has much controversy.

The α-synuclein protein is known to be a major component of Lewy bodies, but studies have found that less than 2% of patients (familial Parkinson's disease) develop mutations in this protein. The increase in the concentration of intracellular non-mutated α-synuclein protein (such as the triploid of α-synuclein) also causes Parkinson's disease, which confirms that α-synuclein protein plays a key role in the pathogenesis of Parkinson's disease. Posttranslational modification of the non-mutated α-synuclein protein also promotes the formation of oligomers and fiber intermediates, which usually develop into insoluble α-synuclein fibers, which are the major constituents of Lewy bodies. In the neuron, dopamine reacts with α-synuclein to induce posttranslational modification of α-synuclein, which inhibits fibrosis of α-synuclein protein, resulting in accumulation of α-synuclein protein in cells in a soluble toxic form, thus ultimately causes damage and death of neurons. This may explain why dopaminergic neurons are sensitive to neurodegenerative changes in Parkinson's disease.

Some symptoms of Parkinson's disease may result from loss of function due to protein aggregation, and other symptoms may be the result of a direct action of toxic proteins. Although the physiological function of α-synuclein is still unclear, it is generally localized at the presynaptic end of the neuron and has a certain correlation with the vesicle structure in the cell. This is consistent with the previously proposed theory that α-synuclein plays a certain role in the dopamine synaptic sac recycling. The mechanism of action of fibrin structure to produce cytotoxicity is still unclear, but they can bind to and promote the permeabilization of secretory vesicles (increased permeability), which may play an important role in the transmission of neurotransmitters and the maintenance of cell homeostasis.

Whether the α-synuclein protein is degraded by the ubiquitin-proteasome system or the autophagy pathway depends on its conformation and the conditions which

the cells are located. Only soluble forms of the protein can be degraded by the proteasome pathway; proteins in the fibrotic form are usually trapped inside certain proteasomes and block the activity of the enzyme. These soluble proteins can also be degraded via the CMA pathway in the lysosome. Pathogenic α-synuclein variants bind tightly to lysosomal membranes, but they are difficult to degrade via the CMA pathway due to their inability to translocate into lysosomes. More importantly, due to its high affinity with the CMA receptor, α-synuclein hinders the uptake and degradation of other substrate proteins by lysosomes, causing blockade of the CMA pathway. Inappropriate posttranslational modifications also affect the degradation of the α-synuclein protein through the CMA pathway. On the one hand, α-synuclein, which cannot be degraded, causes an increase of the concentration of the protein in the cytoplasm, which aggravates the oligomerization or aggregation reaction of the protein; on the other hand, the normal α-synuclein protein accumulates in the cell due to the inability to degrade through this pathway. The ubiquitin-proteasome system and CMA pathway inhibitors promote up-regulation of autophagy, thereby maintaining the function of cell-degrading and removing cytotoxicity, thereby controlling α-synuclein at normal levels. Of course, this process is costly for cells from highly selective protein degradation pathways (the ubiquitin-proteasome pathway and the CMA pathway) to the initiation of bulk nonselective protein degradation pathways. This makes the cells more sensitive to stressors and less resistant to stress states such as oxidative stress. We can imagine that as the disease progresses, autophagy will eventually fail to maintain the stability of the intracellular environment, and neurotoxicity becomes more apparent. Accumulation of abnormal proteins and oxidative stress (typically present in damaged neurons) can directly impair large autophagy, and as the substrate proteins from other degradation pathways increase, the large autophagy pathway may be overloaded and eventually being exhausted. In addition, as the age increases, the activity of the human protein hydrolyzing system will progressively decline, which inevitably leads to cell decline.

In general, Parkinson's disease mainly manifests as Parkinson's syndrome or tremor paralysis. All conditions are characterized by the absence of dopaminergic neurons; some of them also have characteristic Lewy bodies. In addition, some abnormal proteins other than α-synuclein were found in patients with Parkinson's disease, which is helpful for people to further understand the pathogenesis of the disease. Recent studies on Parkinson's disease (PD) have focused on two genes involved in the occult formation of autophagosomes, PINK1 (PTEN-induced putative kinase 1) and Parkin (Jiang and Mizushima 2014). Among them, PIK1 is associated with mitochondrial clearance, and Parkin is associated with autophagy. Parkin can re-aggregate and clear damaged mitochondria by autophagy, which requires the stable expression of PINK1 in mitochondria. Blockade of the above association can be seen in familial Parkinson's disease (FPD). Recent studies have also reported that the direct association of PINK1 and Beclin-1 can promote the formation of autophagosomes. Other studies have shown that impaired mitochondrial function is associated with mutations in the PARK7(Parkinson autosomal recessive kinase 7). The loss of expression of this gene increases the oxidative stress of neurons and the sensitivity of cell death, while the expression of PINK1 and Parkin can avoid this phenomenon.

The most common genetic risk factor for PD is the glucocerebrosidase (GBA) mutation. Deletion of GBA results in the accumulation of its substrate glycosylceramide in the lysosome, resulting in lysosomal dysfunction which may lead to autophagic damage. In PD patients without GBA mutations, enzyme levels and activities were reduced in brain regions where a-synuclein protein levels were elevated at an early stage of the disease.

Early-onset Parkinson's disease (EOPD) is a Parkinson's disease with an onset age of less than 50 years due to a loss of P-type ATPase ATP13A2, a defective function of PARK9 mutation. ATP13A2 mutant cells have impaired lysosomal degradation ability, causing a-synuclein accumulation, which may lead to toxicity of ATP13A2 mutation. Mutation of ATP13A2 resulted in a decrease in the expression level of another PD-related gene, synaptotagmin 11 (SYT11), which further impaired lysosomal function and interrupt autophagosome degradation. LRRK2 is the most common mutein in late-type familial PD. Mutations in LRRK2 result in hyperactivation of proteases and are involved in the regulation of autophagy activity.

Synaptic vesicles (SVs) are sites where presynaptic membrane nerve endings store and release transmitters. In addition to the autophagy-lysosomal pathway, PD gene studies suggest that synaptic vesicle dysfunction is another neuro membrane transport pathway, and that two pathways may share some regulatory proteins, i.e., synaptic vesicle dysfunction is PD. Potential pathogenesis. Early studies suggest that autophagy and SV transport are two independent pathways, but recent studies suggest that if the SV cycle is abnormal, synapses may lose function over time, causing abnormal neuronal signaling, ultimately leading to neurodegenerative. Many PD-related proteins, including LRRK2, EndophilinA (EndoA), synaptojanin1 (synj1), dynamin, and auxilin, have a clear role in SV endocytosis, and these proteins also play a role in autophagy pathways, some PD-related genes. It also plays a role in regulating SV transport and autophagy, thus suggesting a wide relationship between SV transport and synaptic autophagy, and the mechanism of synaptic autophagy in PD patients may be impaired. Due to the complexity of autophagy regulation at the central nervous system. Further research is needed to address the specific mechanisms of SV transport and autophagy regulation, and to understand the interaction mechanism between SV transport and autophagy, which may help to reveal new therapeutic targets for PD.

#### 18.3.2.2 Alzheimer's Disease

The gradual loss of neurons in Alzheimer's patients and progressive dementia are closely related to the tangles of fibers in neurons and the appearance of extra-neuronal senescence. The tangles are mainly formed by the aggregation of highly phosphorylated taurine (a microtubule-associated protein); the amyloid beta-peptide (Aβ) is a transmembrane protein hydrolysate that is a major component of senile plaques (Choi et al. 2013). Over 80% of Alzheimer's disease is sporadic, and the rare genetic defects that have been found are mainly caused by mutations in some enzymes that produce Aβ. Among them, soluble amyloid has a high toxic effect on neurons, and

the content of insoluble senile plaques is not significantly related to the severity of clinical symptoms.

The association between abnormalities in the ubiquitin-proteasome system and the onset of Alzheimer's disease is currently uncertain. Although in vitro experimental evidence indicates that amyloid protein has a negative effect on the activity of the proteasome, the intracellular proteasome activity of this type of patient has not changed. In contrast, changes in lysosomal systems and their association with disease pathogenesis have been confirmed and widely accepted. Up-regulation of intracellular lysosomal system activity is an early cellular change in Alzheimer's disease, which is already obvious before amyloid precipitation. The up-regulation of autophagy in the early stage of the disease is related to the proliferation of lysosomes and the growth of lysosomal enzymes. Up-regulation of autophagy can increase the successful clearance of aggregates and toxic products. As the disease progresses, the removal efficiency of the lysosomal system decreases progressively, resulting in insufficient clearance of harmful components in the cell. Among them, most of the components to be removed are still isolated in autophagic vesicles, and their structures are not significantly affected. Many autophagic vesicles cannot fuse with lysosomes, so they are unable to obtain the enzymes required for protein degradation; although some vesicles can fuse with lysosomes, their contents cannot be degraded by enzymes. The detailed reason is not clear. The result is that a large number of autophagic vesicles accumulate in the neurons. The presence of these autophagic vesicles may interfere with the normal intracellular transportation of the cells, affecting the normal function of the neurons, leading to damage or even death of the neurons. In addition, autophagic vesicles may begin to leak after a period of time in the cells, and the free proteases and undegraded toxic substances are toxic to cells. A new perspective has recently been proposed: in Alzheimer's disease, persistent autophagic vesicles eventually transform into a source of Aβ because they contain transmembrane proteins and the enzymes which are needed to produce Aβ leading to amyloid accumulation. At the same time, the association between autophagy and apoptosis has been confirmed by more and more studies.

Aβ balance in the brain plays a key role in the pathogenesis of AD. Abnormal brain tissue Aβ deposition can lead to neuronal axoplasmic transportation disorders and trigger neuronal cell death. Different clearance mechanisms of Aβ play different roles in the progression of AD. Aβ is produced by two sequential cleavages of amyloid precursor protein (APP). Aβ and APP are degraded by autophagy, and up-regulation of autophagy levels will reduce Aβ production.

Studies have shown that mitochondrial function is impaired in brain tissue of AD patients, which promotes the production and deposition of Aβ. Inhibition of impaired mitochondrial clearance, while an increase in oxidative stress levels, leads to dysfunction of AD neurons. In order to remove damaged mitochondria, autophagosomes containing mitochondria must fuse with lysosomes to form autolysosomes, which in turn degrade mitochondria. Experiments have found that normal cell mitochondria damage has a phenomenon similar to PD neurons. Autophagy is one of the most characteristic downstream pathways regulated by mTOR and plays an important role in AD neurodegeneration. The accumulation of protein aggregation may be due to

excessive activation of the PI3K/Akt/mTOR axis. Induction of autophagy can reduce Aβ accumulation and reduce cognitive decline in transgenic AD mice. As one of the autophagy markers, Beclin1 is an important marker of autophagy and the initiation of autophagosome formation. Decreased expression levels of Beclin1 can lead to Aβ deposition and neurodegeneration in the AD mouse model. In addition, the expression of Beclin1 in the olfactory cortex and hippocampus is also significantly reduced, which can accelerate the progression of AD. Recent studies have found that the expression of Beclin1 is significantly decreased in neurons of AD patients, and the decrease in Beclin1 expression impairs microglial phagocytosis and increases Aβ deposition and neurodegeneration. Inhibitors of mTOR pathway induce neuronal autophagy. For example, rapamycin, a selective inhibitor of TORC1, can reduce Aβ deposition and inhibit Tau protein phosphorylation in an AD mouse model.

On the other hand, in vitro experiments showed that the production of Aβ was reduced after the addition of autophagy inhibitors, Aβ transport and plaque formation after autophagy-related gene ATG5 knockdown were significantly reduced, and Aβ secretion returned to normal after induction of autophagy. It is suggested that Aβ is produced by the autophagy process.

Therefore, the role of autophagy in Aβ deposition is still controversial. Autophagy inducers may provide a new effective therapeutic strategy by degrading AD early Aβ deposition. However, activation of autophagy may increase the deposition of Aβ in AD and aggravate the condition.

#### 18.3.2.3 Polyglutamine Diseases

Polyglutamine diseases are a general term for a group of diseases characterized by intracellular toxicity of agglomerated abnormal proteins containing abnormally extended glutamine ends. Huntington's disease is the most characteristic of polyglutamine disease. It also contains a group of diseases such as spinal cord medullary muscle atrophy, spinocerebellar ataxia, and dentate globus pallidus atrophy (Martin et al. 2015).

Huntington's disease is an autosomal dominant genetic disease caused by the abnormal amplification of the IT-15 gene in the 4p16.3 region of chromosome 4, the CAG trinucleotide repeat in the huntingtin gene. The CAG repeat in the Huntington gene is translated into the Huntington's protein (HTT) N-terminal polyglutamine (polyQ) sequence extension. Amplified mutations in polyQ are generally considered to be functional manifestations of HD cytotoxicity.

The main features of the disease are neuronal loss and progressive damage, abnormal motor function, and abnormal Huntington's inclusion body structure in neurons, which are manifested as dance-like symptoms, cognitive and mental disorders. At present, people have insufficient understanding of the physiological functions of Huntington's protein, so it is not possible to correctly distinguish whether the disease symptoms are caused by the toxic effects of the mutant protein or the malfunction of Huntingtin itself. Huntingtin interacts with other intracellular proteins and participates in different physiological processes of the cell such as gene transcription,

signal transduction, intracellular transport. The interaction of most proteins with Huntingtin occurs in the glutamine prolongation region of the n-terminal region; interacting proteins are often dragged into inclusion bodies, which exacerbates the loss of physiological function of the protein.

In Huntington's disease, the amount of glutamine repeated is directly related to its toxicity. It was initially thought that aggregates would directly lead to cell death; however, there have been recent observations that protein aggregates may have protective functions on neurons. In fact, in a Huntington's mouse model, Huntington's inclusions are widely distributed in neurons, and the model does not exhibit any characteristic neurodegenerative changes, while in other mouse models, there is no Huntington inclusion. The neurons also die.

By detecting different proteasomal subunits in Huntington's aggregates, it has been found that there is a correlation between Huntington's disease and the abnormal ubiquitin-proteasome system. In cultured cells, filaments formed by the mutated polyQ fragment (formed before the appearance of inclusion bodies) inhibit the ubiquitin-proteasome system. Abnormalities in macroautophagy are also associated with Huntington's disease. Autophagy is currently considered to play a dual role in toxicity and protection in HD. On the one hand, this pathway can degrade Huntington's protein aggregates. The expression of autophagy-related genes was altered in HD patients, with increased expression of LAMP2, ULK2, and LC3A, and decreased expression of PINK1, FKBP1A, and EEF1A2. In HD, the recognition function of autophagosomes and the transport efficiency of substrates are decreased due to the failure of autophagy substrate-associated protein p62 to interact with mutant HTT. Mutant HTT can also interact with autophagy regulatory gene Rhes. And inactivate it, so that autophagy activity is inhibited. Mutant HTT clearance is impaired, causing HTT accumulation and neurotoxicity. In fact, blocking large autophagy by drugs or the like in an animal model of Huntington's disease exacerbates the pathological changes of the disease. Some researchers believe that the activation of large autophagy may be the result of proteasome inhibitors, and some scholars have suggested that aggregates may isolate endogenous autophagy inhibitors (such as mTOR) to activate autophagy pathways, activation of large autophagy. Conducive to the clearance of intracellular toxic Huntington's protein, thereby improving the symptoms of the Huntington's mouse model. Because of this, it is envisaged that macroautophagy activators may be used for the treatment of these diseases in the future. Other scholars believe that mTOR function is inhibited in polyQ, causing autophagy to activate, leading to cell death and accelerating neurodegeneration.

As with other neurodegenerative diseases, the specific cause of the disease's autophagy failure is unclear. It has now been found that isolation of autophagy-associated proteins in aggregates may result in a decrease in autophagy activity.

#### 18.3.2.4 Amyotrophic Lateral Sclerosis (ALS)

Amyotrophic lateral sclerosis, also known as atrophic lateral sclerosis, is a progressive and fatal neurodegenerative disease. ALS is a kind of chronic progressive degenerative disease involving upper motor neurons (brain, brain stem, spinal cord neurons), which affects lower motor neuron (cranial nucleus, spinal anterior horn cells) and their dominating trunk, limbs with head muscles. Clinically, the combination of upper and lower motor neurons combined with impaired paralysis. Venkatachalam et al. found that abnormal accumulation of macromolecular substances in motor neurons is a direct result of autophagic dysfunction, which leads to the degradation of motor neurons. They found that autophagic dysfunction in motor neurons of ALS rats was accompanied by accumulation of large autophagic vesicles, which often caused significant impairment of motor function (Evans and Holzbaur 2019; Ramesh and Pandey 2017). Superoxide dismutase 1 (SOD1) is a key enzyme that inhibits intracellular toxic superoxide radicals. More than 100 different mutations in SOD1 are associated with the onset of ALS. Alterations in autophagy activity in the mouse ALS model of mutant SOD1 G93A can affect the degradation of motor neurons. When the experimenter gave lithium (lithiium) or rapamycin, some autophagic vacuoles were observed in the cells, and all autophagy markers were up-regulated, indicating that autophagy was enhanced, while damaged cells were observed significantly recovery occurred in pathological morphology. Excessive macroautophagy vesicles in diseased neurons were removed by enhanced autophagy, and large autophagic vesicles were replaced by newly formed small autophagic vesicles. In addition, in the G93A mice (control group) administered with saline, a large amount of damaged mitochondria appeared in the motor neurons; after the ALS mice were treated with the lithium salt, the damaged mitochondria in the motor neurons were cleared. In contrast, when autophagy blocker 3-MA was used to treat G93A mice and wild-type mouse primary motor neurons (which were derived from primary cultured cells of the embryonic abdominal spinal cord), we found that after the autophagy pathway was blocked, only primary motor neurons of G93A mice died. But under basal conditions, there was no significant difference in spontaneous cell death rates between cultured cells of G93A mice and wild-type mice. From the above studies, it can be inferred that autophagic damage leads to a significant increase in motor neuron death of the SOD1 G93A mutation. SOD1 is an antioxidant enzyme that protects neurons from oxygen-free radicals. After the mutation, the accumulated abnormal proteins cause apoptosis due to the continuous attack of oxygen-free radicals. The mutated protein transcribed from the mutated SOD1 is misfolded and aggregated, and the binding of the autophagy receptor p62 to the mutated SOD1 protein enhances the action of the SOD1 protein and LC3. The false aggregation of SOD1 protein will affect multiple early autophagy processes, such as inhibiting mTOR function and inducing autophagy. During the process of binding with lysosomes after autophagosome formation, SOD1 protein impairs the retrograde transport of axons and causes the failure in fusion of autophagosomes and lysosomes, which plays a toxic role.

In the ALS-G93A mutant, the beneficial effects of autophagy were further confirmed by Kabuta et al. They found that autophagy cleared the cells' toxic substances,

including the removal of the mutated SOD1. Similar results were obtained in in vivo experiments by Fornai et al.: Mutant SOD1 aggregates in spinal anterior horn neurons were cleared by induced autophagy. Recent experiments have found that although most familial ALS and sporadic ALS do not have SOD1 mutations, abnormal SOD1 occurs in cells, and administration of SOD1 antibodies can inhibit disease progression. Activated autophagy can accelerate the clearance of intracellular SOD1, so enhancing autophagy function by autophagy inducer can be used to treat familial ALS and sporadic ALS. α-synuclein mutations can accumulate in the spinal cord of ALS and cause motor neuron death, while lithium salts promote the clearance of α-synuclein. Similarly, in the SOD1 mouse model and other forms of ALS, autophagy also clears ubiquitinated aggregates. All of these proteins (SOD1, α-synuclein, ubiquitin, etc.) are characteristically present in the neurons of most familial ALS and sporadic ALS patients, and they are substrates for the autophagy pathway. Therefore, these proteins accumulate when autophagy is depleted, which is very common in familial ALS and sporadic ALS. Only riluzole is currently available for the treatment of this disease, but it only extends the average life expectancy by about 2–3 months. Recent studies have found that due to the more complex relationship between SOD1 and autophagy, SOD1-related autophagy seems to be different at the different stage of ALS progression. In a recent study, inhibition of autophagy activity resulted in the early onset of ALS symptoms in SOD1G93A mice after knockout of the autophagy gene ATG7, suggesting that autophagy has neuroprotective effects in the early stages of the disease. However, subsequent inhibition of autophagy prolonged the lifespan of mice, delayed the progression of the disease and showed that autophagy played a detrimental role in the late stages of the disease. This phase-dependent effect of autophagy was also confirmed in another study. Starvation-induced autophagy significantly reduced the accumulation of neurotoxic mSOD1 in the early pathological stage, but mSOD1 was significantly increased in SOD1G93A mice in the late stage of ALS.

TDP-43 is a regulatory factor of cellular RNA. Recent studies have found that TDP-43 gene mutation in ALS patients impairs its binding and stabilizes the function of autophagy-associated protein ATG7 mRNA, suggesting that the accumulation of TDP-43 in the cytoplasm will lead to autophagy damage, and then leads to the ubiquitinated protein and p62 accumulation. TDP-43 regulates autophagy by affecting the localization of TFEB and regulates DCTN1 levels to help autophagosome-autolysosomal fusion. In mice and drosophila, the deposition of TDP-43 is neurotoxic, and when autophagy is chemically inhibited, TDP-43 deposition is significantly reduced and neuronal survival is improved.

The exact role of autophagy regulation in the pathogenesis of ALS needs further study, but there is a unique common clue between ALS genes. Therefore, it is particularly important to study the interaction between ALS mutant genes or proteins and autophagy. These interactions will be able to bring greater benefits to patients.

### *18.3.3 Autophagy and Heart Disease Caused by Abnormal Folding Protein*

Heart failure (HF), is often an end-stage change in a variety of heart diseases, including coronary heart disease, hypertension, and idiopathic cardiomyopathy. Mutations in the genes of cardiac muscle fibrin, cytoskeletal proteins, and other related proteins cause a variety of myocardial diseases. These mutations promote or cause abnormal folding of proteins, which in turn affect the normal function, interaction, localization, stability of other proteins. Among them, some gene mutations in cardiomyopathy also involve some molecular chaperone. For example, mutations in the desmin or its chaperone (a small heat shock-like protein αβ-crystalline) can cause desmin-related myopathy (DRM). DRMS is characterized by the accumulation of intracellular insoluble molecules and other interacting proteins, and cause muscle weakness and dilated cardiomyopathy. Protein misfolding can lead to dysplasia of the myocardium and amyloidosis.

The accumulation of incorrect conformational proteins in the intracellular and extracellular space is a common feature of neurodegenerative amyloidosis diseases, including Alzheimer's disease, Huntington's disease, and Parkinson's disease. Recent studies suggest that these insoluble protein aggregates may not be the direct cause of disease, but the toxicity of pre-amyloid oligomer (PAO), an intermediate product of fibril formation, maybe the main cause of disease. Regardless of the gene sequence, these soluble PAOs share a common conformation-dependent protein structure and this structure can be detected by conformation-specific antibodies. Using staining PAOs in DRM and HF model mouse, it was found that there was PAO accumulation in cardiomyocytes of various cardiomyopathy, but not in normal cardiomyocytes. The presence of intracellular PAO in patients with HF suggests that there may be a common pathogenic mechanism between neurodegenerative amyloidosis and some cardiomyopathy that can cause advanced HF. Regrettably, the relationship between intracellular PAO production, protein accumulation, as well as cardiac pathological changes is still unclear.

So, can the high expression of PAO substances in cardiomyocytes cause heart failure? In order to confirm the relationship between the expression and the accumulation of PAO in cardiomyocytes and the development of cardiomyopathy, people use PQ83 (an ectopic peptide containing 83 glutamine repeating structure, similar to PAO) transgenic mice to study this relationship, taking PQ19 (a polypeptide containing 19 repeating inactivated starch polypeptide-like structure) transgenic mice as control to eliminate the effect of ectopic protein expression. If the expression and accumulation of PAO in cells produce toxic effects, the expression of PQ83 will lead to pathological changes in cardiomyocytes, potential cell loss, and successive HF. The results showed that low levels of expression of the peptides PQ83 can cause the deposition of PAOs and PQ83 aggregates, resulting in loss of cardiac cells and heart dilation in mice, and the mice eventually died of HF within 5–7 months. Further study of cardiomyocytes of dead mice revealed increased autophagosome activity

and increased lysosomes number. In addition, cell necrosis was evident, but the initiation of apoptosis and the markers of cytoplasmic endoplasmic reticulum stress were not found. Cellular ultrastructure study showed an increase in the number of labeled lysosomes and autophagic vesicles filled with polyglutamate aggregates. However, in the development of PQ83-related cardiomyopathy, the good or bad effect of the increase of intracellular autophagosomes and lysosomal contents on the survival of cardiomyocytes are still unclear. Further research will explore the relationship between autophagy and PAO-induced cardiomyocyte cytotoxicity and death, as well as the specific stage of the disease.

What role does autophagy play in heart failure? Whether the up-regulation of autophagy is a protection or damage to the heart is a controversial topic. Autophagy has been observed in HF myocardium caused by dilated cardiomyopathy, heart valvular disease, and ischemic heart disease. In animal models, autophagy was also observed in death and sudden death of cardiomyocytes. However, it does not mean that autophagy is a sign of failure of myocardial cell repair or a way to remove damaged cardiomyocytes. We already know that autophagy is a key pathway to eliminate abnormal folding proteins and protein aggregates in cells. In some cases, autophagy is a form of cell death but it plays a vital role in cell survival in starvation. Autophagy is very important for the maintenance of the basic functions of cardiomyocytes, and abnormalities in the regulation of autophagy can cause lesions in many types of cells including cardiomyocytes. It has also been shown that the loss of autophagy or lysosomal function is detrimental to the heart in some cases. The production of β-adrenergic antibodies that cause cardiomyocyte death is associated with a decrease in autophagy, and the rate of death of cardiomyocytes due to toxic effects is decreased after autophagy is up-regulated. Activation of autophagy during acute stress, such as ischemia and myocardial perfusion, can play a role in acute cardio-protection. Cardiac hypertrophy is a precursor to HF and is also a hallmark of myocardial remodeling in most cases. Angiotensin II is an important mediator of cardiac remodeling and is involved in hypertrophic responses to stress and acute injury, and is involved in the regulation of cardiomyocytes through the interaction of angiotensin II type 1 (AT1) and type 2 (AT2) receptors. Of course, a significant up-regulation of autophagy or lysosomal function is also detrimental. In cardiomyocytes, direct evidence of autophagy overactivation associated with autologous cell death is unclear. Thus, for the maintenance of the normal function of cardiomyocytes, autophagy may require precise regulation to maintain cell homeostasis.

Some evidence suggests that blocking the formation of amyloidogenic proteins can eliminate their cytotoxic effects. These problems have not been fully confirmed in human diseases. Whether the aggregation of PAO observed in human HF specimens is due to abnormal folding of proteins, loss of function of degradation system, or damage to the transport pathway of protein degradation pathways, has not yet been clearly answered. Abnormal folding of proteins and intracellular accumulation of PAO are the causes of HF progression? In many tissues, cellular stress and aging can lead to aggregation of insoluble proteins and massive formation of abnormally folded proteins. Hundreds of proteins can undergo abnormal folding, amyloidosis, and then produce cytotoxicity. Cardiomyocytes are in a postmitotic state, which reduces the

ability of cardiomyocytes to clear these aggregates. This feature is reflected in many cell types in neurodegenerative diseases. The effect of autophagy/lysosomal regulation on cardiomyocyte survival, the relationship between autophagy/lysosomal interaction and myocardial necrosis, the pathway by which PAO aggregation induces cell death, as well as the generation of PAO Commonality, etc., all of these issues need to be further clarified in future research.

At present, through the study of the proteasome pathway, it has been found that the proteasome plays an important role in the pathophysiological processes of various heart diseases. Cardiomyocyte proteases have at least 34 different subunits, and variations in these subunits alter the specificity and selectivity of proteases, so they play an important role in the regulation of degradation pathways in cardiac proteins. Currently, most studies focus on the effects of decreased proteasome function on the myocardial disease, and some studies have shown increased proteasome activity in certain heart diseases. Evidence for a decrease in proteasome function is mainly due to an increase in the concentration of ubiquitinated proteins leading to intracellular accumulation, and the other is the activity of the proteasome in vitro. Proteasome dysfunction exists to varying degrees in myocardial ischemia, heart failure, atherosclerosis, and other heart diseases.

Based on the ischemia-reperfusion injury rat heart model, researchers found the deletion of activity of the proteasome 20S and/or 26S in accordance with the increase of oxidation of the protein ubiquitin-proteasome after myocardial ischemia and reperfusion injury in myocardial ischemia. The mechanism of ischemia-induced proteasome inhibition is still unclear. Studies have found that some proteasome subunits are significantly inhibited or inactivated after oxidative stress. We know that the UPS pathway can degrade a large number of proteins including pre-apoptotic proteins and regulate multiple signaling pathways, so the decline in proteasome function in myocardial ischemia has an important effect on cardiac function. Similarly, a large amount of ubiquitinated protein was found in cardiomyocytes of heart failure, suggesting a decrease in UPS activity in heart failure cells. In the mouse model of pressure-overloaded heart, the level of ubiquitinated protein was increased and the activity of protease was decreased, indicating that the ability of proteasome to clear abnormal proteins in cardiomyocytes of HF was insufficient. In human dilated cardiomyopathy, the expression of the apoptosis regulator P53 was also found to be associated with dysregulation of the UPS system. Abnormal proteasome function in cardiac hypertrophic cells may cause accumulation of pre-apoptotic proteins leading to heart failure. In addition, it has recently been found that the UPS system also plays an important role in atherosclerosis. Atherosclerosis is thought to be induced by oxidative stress. In the high cholesterol-fed pig model, the proteasome inhibitor MLN-273 caused an increase in coronary oxidative stress and led to early atherosclerosis. UPS can also regulate insulin signaling pathways by altering the internalization of insulin receptors, controlling insulin receptor substrate levels, and insulin degradation. Therefore, changes in the UPS system can lead to insulin resistance and diabetic complications. In cardiac pathology, autophagy is a potentially promising therapeutic target. However, since there is no noninvasive imaging

method for directly monitoring autophagy, there are difficulties in the experimental intervention targeting autophagy in the treatment of heart disease.

## 18.4 Aging and Autophagy

We know that some abnormal proteins exist since birth; However, for some patients with neurodegenerative disorders, pathological changes only occur in the later stages of life. For example, many symptoms of neurodegeneration appear only after 60 years old. Progressive deterioration of intracellular quality control systems with age is considered to be the main cause of these neurodegenerative pathological changes. The homeostasis function associated with the autophagy and the longevity protein metabolism, the removal of damaged organelles and cell debris is considered an anti-aging process.

The ubiquitination system and the decline in autophagy activity are common features of all older animals. The activity decline of the proteasome-degrading protein, the dysfunction of the assembling and disassembling proteasome, and the changes of enzyme activity that catalyzes the proteasome will lead to the whole system activity decline as the age increase. For the autophagy pathway, both large autophagy and CMA activity also decreased with age. For example, an important abnormality in the signaling mechanism of insulin that controls autophagy leads to diminished activity of the entire pathway during the stress. In addition, as aging progresses, the body's ability to clear autophagosome also decreases. The accumulation of undigested products in the lysosome results in a decrease in the ability of the substance to degrade in the autophagy. With the aging of the body, whether the function change of important autophagy-related proteins, resulting in autophagy failure, is a focus of current research.

An important reason for the decrease in the activity of the CMA pathway with age is that the levels of CMA receptors in lysosomes are significantly reduced in older animal cells (Choi et al. 2013). The substrate proteins that are recognized by molecular chaperones in the cytosol cannot be combined with the lysosomal membrane in the absence of receptors, accumulating in the cytoplasm for a long time, eventually causing irreversible damage to the cells.

## 18.5 Treatment Strategy for Misfolded Protein Disease

Many of the neurodegenerative diseases mentioned above are associated with abnormal folding or aggregation of intracellular proteins. At present, there is no effective treatment for slowing or preventing neurodegenerative and muscular atrophy diseases. Due to the narrow scope of action of the proteasome system and the high specificity of the degradation process, most proteins with aggregation tendency (such

as Huntingtin) are more dependent on the autophagy pathway for degradation. Certain chemical regulators significantly reduced the aggregation-prone proteins (e.g., mutant huntingtin or α-synuclein protein) removal efficiency after autophagy inhibition, which results in a large accumulation of the protein in the cell.

The induction of autophagy by rapamycin significantly enhances the clearance of proteins with aggregation tendency and also reduces the formation of protein aggregates. In addition, in the Drosophila HD model and the HD transgenic mouse model, it has been found that the symptoms of neurodegenerative diseases are improved after the induction of enhanced autophagy.

The same situation occurs in other abnormally folded protein disease models. For example, rapamycin increases the clearance of aggregation-prone proteins to reduce cytotoxicity, such as tau protein. Autophagy can eliminate aggregation-prone proteins suggesting that the development of drugs that act on autophagy pathways may improve the symptoms of such diseases and prevent disease. Importantly, rapamycin also plays a better role in the fly model of these diseases, which are autophagy-dependent, and rapamycin has little effect.

Unfortunately, long-term use of rapamycin causes complications such as poor wound healing and decreased immune function. mTOR can regulate a series of pathways (such as translation of some proteins, cell division, etc.), and the main side effects of this drug are caused by non-autophagy-dependent pathways. In addition, it is still unclear how mTOR regulates autophagy in mammals. An in-depth study of this problem will help provide a safer, more specific, long-lasting drug target for the clinic.

At present, there are few effective treatments for neurodegenerative proteinopathy. Disaccharide trehalose can induce autophagy with mTOR-dependent manner thereby to reduce the accumulation of intracellular abnormal proteins, but the specific mechanism of action remains unclear. Recently, it was discovered that another non-mTOR-dependent pathway induces autophagy: inhibition of inositol monophosphatase (IMPase) reducing the free inositol and myoinositol-1,4,5-triphosphate, (IP3) levels, and up-regulated autophagy activity. Other drugs are to treat a range of neuropsychiatric disorders by this route induce autophagy, such as lithium, valproate. Like Rapamycin, these drugs in insects HD model increase the clearance of the aggregation-prone proteins (such as mutant huntingtin) and protect the cells.

Recently, it has also been found that lithium also has a good performance in the treatment of ALS diseases. Compared to a single standard drugs riluzole, the combination of Lithium carbonate and riluzole can significantly delay the onset of disability and death in patients with ALS. Lithium carbonate treatment of G93A mutant ALS mice can significantly delay the progression of disease and death in mice. The autophagy activity of these mice is increased, and the accumulation of ubiquitin and α-synuclein is generally reduced.

Thus, induction of autophagy can protect a series of neurodegenerative lesions caused by aggregation-prone proteins (Scrivo et al. 2018). In addition, the combination of rapamycin with another non-mTOR-dependent drug may exert a more effective effect on neurodegenerative diseases through stronger autophagy induction and reduce some of the side effects of rapamycin used alone.

## 18.6 Summary and the Prospects

The defect of the cell quality control system is based on the pathogenesis of a series of diseases represented by common neurodegenerative diseases, and this view is strongly confirmed. Among them, some defects are caused by the direct toxic effects of abnormal proteins on the clearance system. In addition, factors such as aging and oxidative stress can aggravate damage to the quality control system and accelerate system failure. Future research should focus on the study of the mechanisms of action of abnormal protein damage clearance systems in order to develop therapeutic drugs that block their toxic effects. Activation of large autophagy is beneficial for the clearance of abnormal proteins in the cell, and this view has been confirmed by recent research, increasing the possibility of treating diseases caused by abnormal proteins such as neurodegenerative diseases, heart diseases, liver diseases, etc. It can also be said that the activation of autophagy can be regarded as a compensatory mechanism induced by the failure of other proteolytic systems in the cell. The biggest challenge now is how to find new ways to activate the autophagy pathway. In summary, a better understanding of the autophagy mechanism may help to discover the new targets for the diagnosis and treatment. Drug screening of autophagy agonists or antagonists, including upstream regulators of autophagy and downstream targets, may provide a more useful treatment for human disease.

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# Chapter 19
# Autophagy in Mitochondrial Quality Control


**Rui Wang and Guanghui Wang**

**Abstract** Autophagy plays an important role in the renewal of cellular components, which function in energy production, metabolism, and clearance of damaged organelles. Both macroautophagy and microautophagy are involved in these processes. Although it was thought that nonselective macroautophagy is responsible for the clearance of damaged or old organelles, recent studies show that the clearance of cellular organelles depends on selective processes. Mitophagy is a process for selective degradation of mitochondria, which is well documented. The selective autophagy for other organelles includes endoplasmic reticulum autophagy (reticulophagy) and peroxisome autophagy (pexophagy). Autophagy is a routine pathway for cells to degrade unused proteins and damaged organelles in cells. Autophagy selectively removes dysfunctional cellular components but not damages the normally functioning organelles, to maintain the homeostasis of cells. In addition to the maintenance of the homeostasis of cells, autophagy clears the damaged organelles in disease or injury conditions to achieve cellular quality control. In some differentiated cells, such as red blood cells, some organelles are removed during the maturation, including mitochondria. The autophagy system can selectively clear the mitochondria and other organelles, which lead to the maturation of red blood cells. Dysfunction of autophagy impairs the clearance of damaged organelles, which results in injury of cells. In the maturation of red blood cells, failure to clear the cellular organelles by autophagy will disturb the normal differentiation of red blood cells, leading to a series of diseases such as anemia.

**Keywords** Autophagy · Mitochondria · Mitophagy · Fission · Fusion · PINK1 · Parkin

R. Wang · G. Wang (✉)
Laboratory of Molecular Neuropathology, Jiangsu Key Laboratory of Neuropsychiatric Diseases & Department of Pharmacology, College of Pharmaceutical Sciences, Soochow University, Suzhou 215123, Jiangsu, China
e-mail: wanggh@suda.edu.cn



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## Abbreviations

| | |
|---|---|
| AIM | Atg8-family interacting motif |
| Atg | Autophagy-related protein |
| CCCP | Carbonyl cyanide m-chlorophenyl hydrazone |
| Drp1 | Dynamin-related protein 1 |
| Fis1 | Mitochondrial fission 1 protein |
| LC3 | Microtubule-associated protein light chain 3 |
| LIR | LC3-interacting region |
| MFN1/2 | Mitofusin1/2 |
| mtDNA | Mitochondrial DNA |
| OPA1 | Optic atrophy 1 |
| PE | Phosphatidylethanolamine |

## 19.1 Mitochondria

In the selective autophagic clearance for cellular organelles, mitophagy is the most studied. Because of the linkage of mitochondrial damage to various neurological diseases, especially in Parkinson's disease in which the disease-related proteins Parkin and PINK1 are associated with selective autophagy, it blooms the field of mitophagy research.

### *19.1.1 Mitochondria*

The word mitochondrion comes from a combination of the Greek words that "mitos" means "thread" and the "khondrion", "granule". Mitochondria are organelles existing in most eukaryotic cells, including plants, animals, fungi, and others. The protozoan trypanosome has only one large mitochondrion, but most cells have hundreds of mitochondria in cells. The specific number of mitochondria in cells is related to the metabolic level of the cells. The cells that have higher metabolic activity have more mitochondria, even occupying 25% of the cytoplasmic volume. The mitochondria are double-membrane-bound organelles. A mitochondrion contains outer and inner membranes composed of phospholipid bilayers and proteins, with a cavity between the two membranes that is called intermembrane space of mitochondria (ISM). The mitochondria contain all the enzymes required for the tricarboxylic acid cycle. On the inner membrane, there are numerous cristae, which expand the surface area of the inner membrane. There are respiratory chain system and an ATPase complex on the inner membrane, which produces ATP. Mitochondria are placed to provide energy for cell activities. Mitochondria have a variety of shapes. The diameter is generally

between 0.15 and 0.5 μm, and varies greatly in length from 1.5 to 3 μm, even as long as 10 μm.

In addition to providing energy to cells, mitochondria are also involved in the process of apoptosis. The Bcl-2 family is one of the most important proteins in the apoptotic system, which is divided into proapoptotic and antiapoptotic families. Once apoptosis is induced, the proapoptotic proteins damage the mitochondria, leading to a release of proteins from mitochondria, such as cytochrome c and Bid, which cause apoptosis. The anti-apoptotic Bcl-2 family functions in maintaining the stability and integrity of the mitochondrial membrane and inhibiting mitochondrial damage and protein release. Therefore, the functional mitochondria with balanced dynamics are extremely important for cellular physiology. The biogenesis of new mitochondria and the degradation of aging mitochondria are dynamically balanced. Autophagy is an important system for the removal of damaged mitochondria (El-Hattab et al. 2018; Tilokani et al. 2018).

Mitochondria are dynamic networks of double-membrane organelles with energy-production sites in the inner membrane of mitochondria (IMM). The mitochondrial electron transport chain transfers electrons to $O_2$ through four protein complexes and two membrane electron carriers. During electron transfer, the mitochondrial respiratory chain complexes I, III, and IV pump protons from the mitochondrial matrix across the IMM to the ISM, which form an electrochemical gradient of $H^+$, the mitochondrial membrane potential ($\Delta\Psi m$). The $\Delta\Psi m$ of –160 mV (a high proton concentration in the ISM) drives the protons across the F0 subunit of the ATP synthase to produce ATP via the F1 subunit of the ATP synthase. The aerobic respiration coupled to the ATP synthase via an electron transport chain refers to oxidative phosphorylation (OXPHOS). In addition to energy production, mitochondria are the metabolic site for amino acids, fatty acids, and carbon, thus providing sources for protein, lipid, and carbohydrate synthesis. Dynamic changes by mitochondrial fusion and fission form a mitochondrial network for cellular metabolic demands. Mitochondrial fusion is dependent on normal OXPHOS and $\Delta\Psi m$. Mitochondrial fusion increases the mitochondrial volume and space, further enhancing the function of the electron transport chain. Meanwhile, the fusion of mitochondria protects mitochondria from degradation by mitophagy. In contrast, mitochondrial injury causes depolarization of mitochondria, which increases mitochondrial fission and degradation (El-Hattab et al. 2018; Tilokani et al. 2018).

### *19.1.2 Mitophagy*

Although it is thought that mitochondria can be eliminated by macroautophagy, it is currently realized that mitochondria undergo a selective degradation by the autophagic system, which is called mitophagy. The earliest report on mitochondrial clearance by autophagy came in 1962. Using electron microscopy, the intact mitochondria and mitochondria in various stages of degradation are engulfed in lysosomes, which are the basis for the conception of autophagy/autophagosome that was

proposed in 1963. Although there are other organelles in those autophagosomes, almost every lysosome contains a mitochondrion. At that time, the process for mitochondrial degradation and the fusion process of lysosomes were not understood, but it demonstrated that mitochondria can be degraded by lysosomes. In 2001, Elmore et al. proposed mitochondrial selective autophagy, a new conception of mitophagy. After that, more and more evidence suggest that the clearance of mitochondria in cells is specific and selective. The molecular mechanism for selective degradation of mitochondria has been revealed in both yeast and mammalian cells.

In mitophagy, mitochondrial fission is an important step for their degradation. Fission of mitochondria produces new mitochondria with a smaller size, which facilitates the engulfment of mitochondria by autophagosomes and fusion of autophagosomes to lysosomes. Fission of mitochondria also removes damaged parts from a mitochondrion, which allows normal parts to form a new healthy mitochondrion. It is therefore that the fission of mitochondria plays an important role in the clearance process for mitochondria. In mitophagy, the recognition of damaged mitochondria is critical for their degradation by lysosomes.

## 19.2 Mitochondrial Dynamics, Homeostasis, and Mitochondrial Autophagy

Mitochondria are dynamic organelles that undergo motion, fusion, fission, and degradation. The fusion and fission of mitochondria controls mitochondrial shape, size, and number. Mitochondria form a dynamic interconnecting network through fission and fusion, which accommodates the metabolic demands of the cells. Mitochondrial fission produces new mitochondria, allowing them to be transported and redistributed in cells. Mitochondrial fission promotes mitochondria to remove damaged parts that undergo mitophagy. Mitochondrial fusion promotes the exchange of mitochondrial components and enhances mitochondrial function. Mitochondria are the energy-production organelles in cells, which are distributed according to the energy demand in different regions in the cells. Intracellular movement of mitochondria is very important, which allows mitochondria to be transported intracellularly to areas of high metabolic demand. When mitochondria are damaged, they can be selectively engulfed by autophagosomes and fused to lysosomes for degradation, which maintains the healthy mitochondrial population and homeostasis of mitochondria. Mitochondrial dynamic processes, including movement, fusion, division, and autophagy, are regulated by a number of proteins and signals that maintain mitochondrial morphology, distribution, and function.

### 19.2.1 Mitochondrial Fission

A major regulator of mitochondrial fission is the dynein-related protein, Dnm1p in yeast, and Drp1 (Dynamin-related protein 1) in mammals. Mitochondrial fission is regulated by mitochondrial fission protein FIS1 and Drp1. Drp1 is a GTPase with a GTPase effector domain. When mitochondrial fission is initiated, the cytosolic distributed Drp1 is recruited to mitochondria, forming a polymer and wrapping around the scission site of the mitochondria. When GTP is bound and hydrolyzed, oligomerized Drp1 changes the conformation to cut mitochondria. FIS1, Mff, and MiD49/51 are anchored to the OMM and bound to Drp1 to recruit Drp1 to the OMM. Mitochondrial fission also requires actin and myosin IIA that provide a mechanical force for the cleavage of mitochondria.

### 19.2.2 Mitochondrial Fusion

Mitochondrial fusion is mainly performed by dynamin-related GTPases, the mitochondrial fusion protein MFN1/2 (Mitofusin 1/2), and OPA1 (Optic atrophy 1 protein). The fusion of mitochondria undergoes two processes, fusion of the OMM and fusion of the IMM, which occur simultaneously.

The fusion of the OMM is mediated by MFN1 and MFN2 that are anchored to the OMM via the C-terminal transmembrane domain and that have a conserved catalytic GTP-binding domain at the N-terminus. MFN1 and MFN2 form homo- or heterodimers that mediate fusion of the OMM dependent on GTP hydrolysis. Interactions of MFN1 and MFN2 on the OMM from two mitochondria pull them across each other, promoting fusion of the OMM.

The fusion of the IMM is mediated by OPA1, a dynamin-like GTPase that is anchored to the IMM via the N-terminal transmembrane domain, and that exposes the GTP-binding and GTPase effector domains to the ISM. OPA1 has different forms due to alternative splicing and proteolysis. Alternative splicing of OPA1 produces a long-form OPA1 (L-OPA1) that is cleaved into a short form (S-OPA1) via a proteolytic enzyme on the IMM. The proteolysis of L-OPA1 forms S-OPA1 that promotes the fusion of IMM.

Another protein that affects mitochondrial fusion is the F-box and leucine-rich repeat 4 (FBXL4), a mitochondrial protein localized on the IMM. It forms a quaternary protein complex through its leucine-rich repeat domain that is responsible for protein interactions. FBXL4 acts as a fusion protein or interacts with other mitochondrial fusion proteins to regulate mitochondrial fusion.

### 19.2.3 Mitochondrial Homeostasis

The fusion of mitochondria produces tubular or elongated mitochondria that are interconnected to form a dynamic network. Mitochondrial fusion allows material exchange between mitochondria and promotes molecules to diffuse throughout different parts of the mitochondria. Exchange of mitochondrial DNA, proteins, lipids, and metabolites among mitochondria is essential to maintain genetic and biochemical homogeneity within the mitochondrial population. It improves mitochondrial function and avoids accumulation of mitochondrial DNA (mtDNA) mutations during aging. In addition, mitochondrial fusion prevents mitochondria from mitophagy.

Mitochondrial fusion is critical for mtDNA maintenance. The cells require a sufficient amount of mtDNA to transactivate complex subunits that are mtDNA-encoded and function in a tricarboxylic acid cycle for energy production. Defects in mitochondrial fusion lead to an impairment of mtDNA synthesis, which causes mtDNA depletion or mtDNA mutations.

When mitochondria are damaged, mitochondrial fission increases. The damaged part is separated from the mitochondria, which forms a new healthy mitochondrion and a damaged mitochondrion. The damaged mitochondria are cleared by mitophagy, thus maintaining the normal function of mitochondria. Defects in mitochondrial fission or mitophagy lead to an accumulation of damaged mitochondria, which is tightly associated with diseases.

### 19.2.4 The Effect of Mitochondrial Fission and Fusion on Autophagy

In mammalian cells, mitochondria have a dynamic property. Fission of mitochondria makes mitochondria smaller, but fusion increases mitochondrial volume and makes mitochondria to form a connecting network. For mitophagy, the smaller mitochondria after fission are easily engulfed by autophagosomes. Decrease of $\Delta\Psi$m caused by mitochondrial damage is important for mitophagy induction and also affects the process of mitochondrial dynamics. The healthy mitochondria with normal $\Delta\Psi$m can be fused, but mitochondria with low $\Delta\Psi$m cannot be fused and are localized to microtubule-associated protein light chain 3 (LC3)-positive autophagosomes, suggesting that damaged mitochondria undergo mitophagy. Since the loss of $\Delta\Psi$m occurs before mitophagy, a depolarized state of mitochondria prevents mitochondrial fusion and promotes mitophagy.

Mitophagy is extremely important for mitochondrial homeostasis and the clearance of damaged mitochondria. Upon mitochondrial damage, the mitochondrial fission process “cuts off” the damaged parts of the mitochondrion to form two mitochondria, a damaged one and a healthy one. The healthy mitochondrion is fused to other healthy mitochondria by the action of MFN1/2 and OPA1. The damaged mitochondrion after mitochondrial fission has lower respiratory capacity and $\Delta\Psi$m. Since

mitochondrial fusion depends on normal $\Delta\Psi m$, the damaged mitochondria with low $\Delta\Psi m$ are unable to fuse to other mitochondria. Meanwhile, loss of $\Delta\Psi m$ is an important signal for mitophagy. Decreased $\Delta\Psi m$ induces OPA1 to be hydrolyzed by proteases and MFN1/2 to be degraded by the proteasome. Degradation of these two mitochondrial fusion proteins reduces mitochondrial fusion capability, which in turn promotes mitochondrial fission and mitophagy. A loss of the function of Drp1 in mammalian cells causes a decrease of mitophagy and induces the formation of tubular mitochondria. However, in *Drp1* knockout cells, although LC3-II is increased, the colocalization of mitochondria and LC3 labeled autophagosomes is rare. Overexpression of Drp1 promotes mitochondrial fission and induces mitophagy. Therefore, mitochondrial fusion and fission are directly associated with mitophagy.

## 19.3 Key Signals and Proteins in Mitophagy

Autophagy-related proteins (Atg) are critical for autophagy, and many Atgs are involved in mitophagy although they may not directly act on mitophagy. Atg is extremely important for the basal autophagy and selective mitophagy in the cells. The functional defects of Atg that affect the autophagy will also influence mitophagy. Recently, mitophagy has been extensively studied. Many proteins and signaling pathways are involved in mitophagy regulation, and more diseases have been found to be associated with mitophagy.

### *19.3.1 Mitophagy and Mitochondrial Receptors in Yeast*

In 2009, two groups, Ohsumi and Klionsky, independently identified that Atg32 specifically participates in mitochondrial selective autophagy. In addition, Atg33 that was identified by Klionsky group is also a factor that selectively affects mitophagy. Thus, Atg32 is the earliest identified mitophagy receptor on the mitochondrial membrane.

During growth, yeast cells are converted from anaerobic to aerobic respiration when they are cultured in media containing lactose, ethanol or glycerol. Mitochondrial oxidative stress and damage occur during aerobic respiration. The aerobic respiration in yeast induces the expression of Atg32 that is recruited to the OMM. Atg32 is an Atg protein unique in yeast, for which no homolog has been identified in animals. Atg32 is required for mitophagy. Atg32 is a 59 kDa transmembrane protein on the OMM. The N-terminus of Atg32 faces to the cytosol and the C-terminus is located in the ISM. Inhibition of Atg32 expression reduces mitophagy, while overexpression enhances mitophagy. The 43 kDa N-terminus of Atg32 that faces to the cytosol carries two conserved motifs that bind Atg8 and Atg11, thus greatly influencing mitophagy. The binding motif of Atg32 bound to Atg8 is W/YXXI/L/V that is called AIM (Atg8-family interacting motif), which can bind to free form of Atg8 (a homolog of mammal

LC3) or Atg8 coupled to PE (phosphatidylethanolamine) (Fig. 19.1). Interestingly, the AIM of Atg32 is also presented in Atg19 and the mammalian p62 protein, in which this functional motif binds to Atg8 and LC3. Mutation in AIM significantly decreases the binding of Atg32 to Atg8, which decreases mitophagy. In addition to the effects of Atg8 on Atg32-mediated mitophagy, interactions between Atg11 and Atg32 also greatly influence Atg32-mediated mitophagy. Atg32 contains two serines (114 and 119), and mutation of the serine site greatly reduces mitophagy. In yeast, the damaged mitochondria are accumulated in *Atg11* and *Atg32* deletion mutants after exposure to ROS. Thus, Atg32 is an important autophagy receptor identified in yeast mitochondria, which binds to Atg8 coupled PE on autophagophores to transfer mitochondria to autophagosomes. It is now well accepted that Atg32 is a mitochondrial receptor for mitophagy (Fig. 19.1).

Similar to Atg32, Atg33, approximately 20 kD, is a protein resident on the OMM. In yeast, the *Atg33* deletion mutant strain has lower mitophagy than wild-type stain after starvation. Mitophagy cannot be induced after logarithmic growth phase in *Atg33* mutants, suggesting a role of Atg33 in mediating aging mitochondria for mitophagy, rather than mediating mitophagy in all phases by Atg32.

Among the genes in the regulation of mitophagy in yeast, Atg32 has been recognized as a selective autophagy receptor for mitophagy. *Atg* genes are necessary for autophagy as well as mitophagy. No autophagy or mitophagy can be induced in yeast strains even with single-gene deletion of *ΔAtg1/ΔAtg6/ΔAtg8/ΔAtg12*. The mitochondrial protein levels are significantly increased in *Atg* deletion mutants of yeast strains, suggesting that the core ATG functions in autophagy as well in the selective mitophagy. In addition, autophagy inactivation caused by *Atg* deficiency limits mitochondria-dependent cell growth, resulting in a significant increase in the

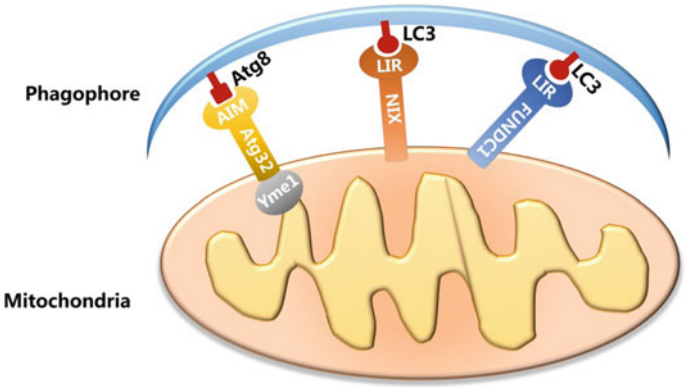


**Fig. 19.1** In yeast, the mitochondrial outer membrane protein Atg32 binds to Atg8 through its AIM (Atg8-family interacting motif) that can bind to free form of Atg8 or Atg8 coupled to PE (phosphatidylethanolamine), which anchors mitochondria to phagophore. In mammals, NIX or FUNDC1 has an LC3-interacting region (LIR) that binds to LC3 that is coupled to PE that is bound to the phagophore a membrane. Binding of LIR on NIX to LC3 drives mitochondria to be engulfed by autophagosomes, thus inducing mitophagy

number of cells at G1 phase. The mutants show decreases in $\Delta\Psi m$ and mitochondrial electron transport chain activity and increases in ROS levels and mtDNA mutations. Thus, mitophagy plays an important role in the maintenance of mitochondrial normal function.

### *19.3.2 Membrane Receptors on the OMM in Mammals*

Atg32 is an autophagy receptor on the OMM, which mediates mitophagy through its binding to Atg8 in yeast. In mammalian cells, LC3 is a homolog of Atg8 and has a similar function as yeast Atg8. On the OMM of mammalian cells, there are two proteins that are functionally similar to yeast Atg32 and are considered to be receptors for mitophagy (Kanki et al. 2009; Okamoto et al. 2009). One is the OMM protein NIX identified by Dikic group (Novak et al. 2010), and another is FUNDC1 that is identified by Chinese scientist Chen's Group (Liu et al. 2012).

NIX, also known as BNIP3L, is homologous to BNIP and was first identified as a protein that binds to Bcl-2 and that is involved in the regulation of apoptosis. NIX is a protein localized to the OMM, partially distributed in the endoplasmic reticulum. The involvement of NIX in mitophagy was first noticed in the regulation of maturation of reticulocytes. It was found that NIX knockout animals show residual mitochondria in reticulocytes, leading to failure of maturation for red blood cells, suggesting that NIX is involved in mitophagy. NIX has a LC3-interacting region (LIR) that binds to LC3. Binding of LIR on NIX to LC3 drives mitochondria to be engulfed by autophagosomes, thus inducing mitophagy, which is similar to AIM of yeast Atg32, binding to Atg8 in yeast for mitophagy induction (Fig. 19.1). NIX is therefore considered to be a mammalian mitophagy receptor. However, in NIX knockout animals, some reticulocytes can undergo maturation and develop to red blood cells, suggesting that some other factors may be also involved in reticulocyte maturation.

FUNDC1 is an OMM protein with its N-terminus facing to the cytosol and carrying a typical LIR motif, the YXXL amino acid sequence, in which tyrosine (Y) 18 and leucine (L) 21 are required for FUNDC1 bound to LC3. Mutations of Y18 and L21 or deletion of LIR in FUNDC1 lead to FUNDC1 failure to binding to LC3, thus failing to induce mitophagy. Interestingly, FUNDC1 seems to be involved in the induction of mitochondrial fission before mitophagy. Overexpression of FUNDC1 induces massive mitochondrial fission prior to autophagy, whereas knockdown of FUNDC1 promotes mitochondrial fusion. In addition, FUNDC1, in the absence of LIR, is still able to induce mitochondrial fission although it is completely unable to induce mitophagy. Thus, FUNDC1 is a membrane receptor that not only mediates mitophagy but also plays an important role in mitochondrial fission.

## 19.4 Ubiquitin-Dependent Mitophagy

Mitochondrial receptor-mediated mitophagy occurs through a direct binding of LC3 to the mitochondrial receptor on the OMM, thereby mediating mitophagy. However, in most cases, mitophagy is initiated by a series of signals, followed by ubiquitination of mitochondria on which the OMM proteins are polyubiquitinated. The autophagy receptors such as p62, NBR1, and OPTN recognize the polyubiquitin chains coupled to mitochondria. Meanwhile, the autophagy receptors also bind to LC3, thus driving polyubiquitinated mitochondria to be engulfed by the autophagosomes. In mammalian cells, loss of $\Delta\Psi m$ is required for mitophagy induction. In addition, mitochondrial dynamics, mitochondrial fission and fusion, is extremely important for mitophagy. Recently, the functions of Parkinson's disease-related proteins PINK1 and Parkin in mitochondrial ubiquitination and mitophagy have been well studied.

### *19.4.1 Parkin and PINK1*

Parkin is a cytosolic E3 ligase that was first identified to be linked to familial Parkinson's disease by Shimizu group in 1998. Point or deletion mutations in *PARK2* that encode Parkin cause autosomal recessive familial Parkinson's disease. Since Parkin is a cytosolic E3 ligase, early studies focus on the identification of Parkin's cytosolic ubiquitination substrate. Subsequently, using the Drosophila model, Pallank group found that Parkin plays an important role in the maintenance of mitochondrial morphology and function (Greene et al. 2003). Parkin-deficient Drosophila exhibits mitochondrial morphological abnormalities and degeneration of dopaminergic neurons and muscle tissue.

PINK1 was first identified as a PTEN-inducible kinase that is widely expressed in various tissues and organs, which was considered to be a tumor-associated factor at the beginning of its discovery. Soon, PINK1 was identified as a protein associated with Parkinson's disease. Mutations of PINK1 encoding gene *PARK6* cause autosomal recessive Parkinson's disease. PINK1 is a protein of 581 amino acids with a mitochondrial targeting signal, a transmembrane helix and a serine/threonine kinase domain at the N-terminus. In *Drosophila*, PINK1 deletion mutants show phenotypes similar to Parkin mutants. Overexpression of Parkin in PINK1-deficient *Drosophila* can partially rescue phenotypes, while overexpression of PINK1 in Parkin-deficient *Drosophila* cannot rescue phenotypes, suggesting that Parkin acts downstream of PINK1 (Clark et al. 2006; Park et al. 2006).

Under normal conditions, PINK1 is transported into mitochondria through the recognition of PINK1 N-terminal mitochondrial targeting sequence by the mitochondrial complexes, the translocase of the outer membrane (TOM), and the translocase of the inner membrane (TIM). The N-terminal mitochondrial targeting signal of PINK1 is cleaved by mitochondrial processing peptidase in the matrix and rhomboid family protease presenilin-associated rhomboid-like protein (PARL) on the IMM, resulting

in a cleavage at N-terminal Ala104. The N-terminal cleaved PINK1 returns to the cytosol and is degraded by the proteasome. In 2008, Youle group first showed that Parkin is accumulated on mitochondria after mitochondrial uncoupler CCCP (carbonyl cyanide m-chlorophenyl hydrazone) treatment (Narendra et al. 2008). Due to the uncoupling of mitochondria by CCCP, an increase of proton permeability eliminates $\Delta\Psi m$ of the IMM. Loss of $\Delta\Psi m$ induced by CCCP allows PINK1 to cross only the OMM but not the IMM, so that its N-terminal mitochondrial targeting signal cannot be processed by MPP in the matrix or the PARL on the IMM, resulting in an enrichment of the full-length PINK1 on the OMM. Enrichment of PINK1 on the OMM promotes Parkin translocation from cytosol to mitochondria and induces mitophagy (Narendra et al. 2009, 2010). Since Parkin is an E3 ligase that ubiquitinates mitochondrial proteins after its recruitment onto mitochondria, PINK1-induced Parkin recruitment onto mitochondria produces the polyubiquitin chains that are recognized by autophagy receptors (also see the section "Protein modification and autophagy activation"), thus the mitochondrial ubiquitination by PINK1/Parkin pathway is an important process for mitophagy (Fig. 19.2).

PINK1 is autophosphorylated on the OMM, and its autophosphorylation is important for PINK1 activation and enrichment on the OMM (Aerts et al. 2015). Enrichment of PINK1 on the OMM recruits Parkin to mitochondria and phosphorylates Parkin at serine (S) 65 site on Parkin ubiquitin-like domain (Ubl) (Ordureau et al. 2015). Phosphorylation of Parkin at S65 alters Parkin conformation, which removes Parkin's self-inhibition and increases its E3 ligase activity, allowing Parkin to catalyze its substrate ubiquitination. PINK1 also phosphorylates the S65 site on ubiquitin. Phosphorylation of ubiquitin on S65 activates ubiquitin and leads to resistance to deubiquitinases, thus promoting mitochondrial ubiquitination (Wauer et al. 2015). In addition, activated ubiquitin binds to Parkin to further activate Parkin, promoting

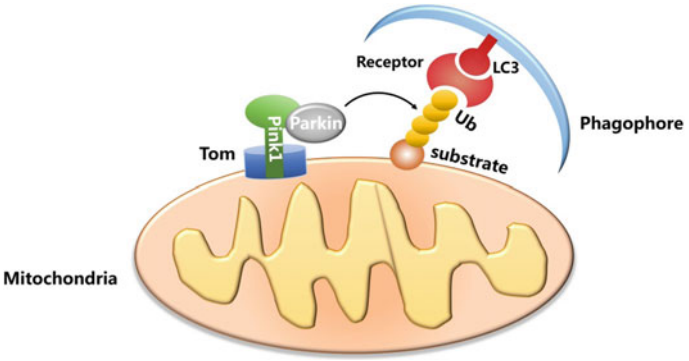


**Fig. 19.2** In most cases, mitophagy is mediated by autophagy receptors such as p62, NBR1, and OPTN that can interact with polyubiquitin chains as well as LC3. The accumulation of PINK1 on the outer membrane of mitochondria (OMM) recruits Parkin to mitochondria, leading to ubiquitination of substrates by Parkin. Thus, the autophagy receptors bind to ubiquitinated mitochondria (the ubiquitinated proteins on the OMM) and LC3, driving ubiquitinated mitochondria to be engulfed by the autophagosomes

Parkin to ubiquitinate substrates. Therefore, phosphorylation of Parkin and ubiquitin by PINK1 plays an important role in PINK1/Parkin-mediated mitophagy.

### *19.4.2 Autophagy Receptor and Mitophagy*

One of the important features of the autophagy receptor proteins is that they have ubiquitin interacting motif, which allows them to bind to ubiquitinated substrates. Meanwhile, they also have a LIR binding to LC3, so that the bound ubiquitinated substrates can be delivered to autophagosomes. Youle group found that NDP52 and OPTN are repaired for mitophagy using rescue approaches in the cells where all five autophagy receptors, p62, NBR1, NDP52, OPTN, and TAX1BP, are knocked out (Vargas et al. 2019). PINK1 kinase activity is required for autophagy receptor enrichment on the OMM. Enrichment of PINK1 and Parkin on the OMM and interaction between autophagic receptors and ubiquitin activate TBK1. TBK1 activation increases mitochondrial recruitment of phosphorylated OPTN, NDP52, and p62. In addition, phosphorylation of OPTN by TBK1 at serines 473 and 513 increases the affinity of OPTN to ubiquitin chains as well as phosphorylated ubiquitin chains. Furthermore, phosphorylation of OPTN by TBK1 induces retention of OPTN on mitochondria, which further recruits ubiquitin and autophagy receptors onto mitochondria.

In mitophagy, phosphorylated ubiquitin is functionally similar to an autophagy receptor that induces Parkin and autophagy receptors onto the OMM. On mitochondria, Parkin is activated by PINK1 and phosphorylated ubiquitin, which promotes TBK1 to activate OPTN and NDP52. The positive feedback pathway further functions in mitophagy through phosphorylation of ubiquitin and activation of PINK1/Parkin.

## 19.5 Physiological Functions of Mitophagy

Mitophagy plays an important role in the formation and maturation of lens, red blood cells, and sperm as mitochondria are degraded for the maturation and development of these organs and tissues. These organs serve as ideal models for exploring the mechanism of mitophagy as the clearance of mitochondria is required for the development. In *ATG5*$^{-/-}$ mice, no mitochondrial clearance defects occur in the lens and red blood cells. In *ATG7*$^{-/-}$ mice, the clearance of mitochondria in reticulocytes occurs normally. Thus, the induction of mitophagy seems unrelated to ATG5 or ATG7. However, more reticulocytes with mitochondria appear in *ULK1*$^{-/-}$ mice. The clearance of mitochondria in reticulocytes is delayed in *ULK1*$^{-/-}$ mice. Upon CCCP treatment, the efficiency of the clearance of mitochondria in reticulocytes from *ULK1*$^{-/-}$ cells is similar to wild-type cells in vitro although the efficiency is lower in *ULK1*$^{-/-}$ cells than wild-type cells without CCCP. It seems that NIX is

more related to mitophagy than ULK1 or ATG5 and ATG7. *NIX*$^{-/-}$ reticulocytes are immature and susceptible to apoptotic stimulation, leading to anemia, suggesting that NIX-mediated mitophagy is closely related to the pathology in blood cells.

## 19.6 Mitochondrial Autophagy and Cell Quality Control

Mitochondrial damage is closely related to apoptosis. During a mitochondrial injury, many mitochondrial proteins are released from damaged mitochondria to cytosol, leading to apoptosis. As mitophagy is the only way to clear the damaged mitochondria, mitophagy has an important role in protecting cells from mitochondrial-associated apoptosis. Under normal conditions, mitochondria pump protons across the IMM into the ISM, thus forming $\Delta\Psi$m, which can block release of mitochondrial components to cytosol. Inhibition of the respiratory chain increases mitochondrial oxidative metabolites and causes a decrease in $\Delta\Psi$m, resulting in an increase of mitochondrial membrane permeability. The superoxides produced by mitochondrial respiratory chain further damage the mitochondria that allow cytochrome c and other apoptotic factors releasing to the cytosol. Cytochrome c activates caspase-9 that further activates caspase-3, leading to apoptosis.

Mitophagy is an important cell quality control system for the clearance of damaged mitochondria. Injury of mitochondria induces mitochondrial fission so that the damaged mitochondria will be recognized and undergo mitophagy. Mitochondrial damage can be induced by increased ROS production during cellular stress, disease, and aging. Mitophagy effectively removes damaged mitochondria to avoid the release of mitochondrial proteins, which protect cells from apoptosis.

The selective autophagy in mitochondria interests scientists much in recent years, because mitochondria play crucial roles in the maintenance of physiological functions in cells, as well as in the pathogenesis of many diseases. Studies on selective mitophagy can expand our knowledge to understanding the functions of mitochondria as well as their roles in diseases.

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# Chapter 20
# Chaperone-Mediated Autophagy


**Qian Yang, Ronglin Wang and Lin Zhu**

**Abstract** Protein homeostasis is essential for maintaining cell survival. Protein synthesis and degradation coordinately regulate protein homeostasis. Chaperone-mediated autophagy (CMA) was the first lysosomal process to be discovered by which intracellular components are selectively degraded. This process involves the recognition of the substrate, the unfolding and translocation of the substrate, and the degradation of the substrate. By degrading specific target proteins in a timely manner, CMA is involved in a variety of cellular activities. In the past few years, we have acquired a better understanding of how CMA is regulated. It has been reported that peroxide accumulation, aging and/or other pathological signals interfere with CMA function, which in turn induces neurodegenerative diseases, cancer, and other diseases. Combining results from the current research, we summarize the basic processes, regulatory mechanisms, and physiological functions of CMA and discuss its critical role in the development of diseases.

**Keywords** Chaperone-mediated autophagy (CMA) · Basic process · Regulation · Physiological function · Microautophagy

Protein degradation machinery is composed of proteases, ubiquitin-proteasome systems, and lysosome-dependent autophagic processes. There are three kinds of autophagic processes: macroautophagy, microautophagy, and CMA. Among the three autophagic processes, CMA is unique in that it does not require the formation of autophagosome-vacuoles and it removes proteins with selectivity. By studying the transgenic mouse model, it was found that by the selective degradation of the substrate, CMA had the ability to regulate a variety of cellular activities.

Q. Yang (✉) · R. Wang · L. Zhu
Department of Neurosurgery, Tangdu Hospital, The Fourth Military Medical University, Xi'an, China
e-mail: qianyang@fmmu.edu.cn



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## 20.1 The Basic Process of CMA

Initially, the CMA process was found only in mammalian cells. Subsequently, key components of this process were also found in birds. Recently, CMA-like processes have also been found in fish, fruit flies, and lower species such as *C. elegans*. These results help us to understand the basic process of CMA, which involves the following three processes: (1) The recognition of substrates. Hsc70 recognizes the KFERQ motif in the substrate protein and forms a chaperone–substrate complex together with heat shock protein of 90 KD (Hsp90), heat shock protein 40 (Hsp40) and other co-chaperones. (2) Unfolding and translocation of the substrate. The chaperone–substrate complex binds to lysosome-associated membrane protein type 2A (LAMP2A), and the substrate protein undergoes unfolding with the assistance of chaperones. At the same time, multimerization of LAMP2A forms a "temporary pathway" through which the unfolded substrates are translocated to the lysosome. (3) Degradation of the substrate. The lysosomal hydrolase decomposes the substrate into amino acids, which are reused by the cells. LAMP2A dissociates from the chaperone–substrate complex and returns to a monomeric state (Cuervo and Wong 2014).

### *20.1.1 Substrate Selectivity*

Since the discovery of lysosomes in 1955, it has been widely believed that the degradation of substrates in lysosomes is a nonspecific process. In 1982, American Biochemist Fred Dice and his team found that the half-life of exogenous ribonuclease A (RNase A) in cells was approximately 90 h. However, the degradation rate of RNase A was increased by 1.6 fold during nutrient deprivation. Moreover, the degradation rate was closely related to a specific amino acid sequence of RNase A. These results suggest that lysosomes may function to specifically degrade target proteins. In 1986, Fred Dice and his team further studied the specific sequence and simplified it into the pentapeptide sequence KFERQ motif (Dice 1990).

Both upstream and downstream, the KFERQ motif is always flanked by glutamine (Q). In addition, the element usually contains one or two positively charged amino acids such as lysine (K) and arginine (R), one or two hydrophobic amino acids such as leucine (L), isoleucine acid (I), valine (V) or phenylalanine (F) and a negatively charged amino acid such as glutamic acid (E) and aspartic acid (D). These amino acids form a classic KFERQ-like motif.

However, it has been suggested that posttranslational modifications sometimes alter the properties of amino acids, which in turn contribute to the formation of bona fide CMA substrates. For example, the phosphorylation of serine (S), threonine (T) or tyrosine (Y) confers a similar function to negatively charged amino acids. Acetylated lysine (K) has a property similar to that of glutamine (Q) in that it helps convert proteins into CMA substrates. Posttranslational modifications not only change the amino acid properties but can also induce conformational changes in the protein to

mask or expose the KFERQ motif. It has been reported that ubiquitination changes the conformation of hypoxia-inducible factor 1α (HIF1α) and exposes its KFERQ motif, which in turn promotes its degradation by CMA.

The properties of the KFERQ motifs, rather than the amino acid sequence, determine whether a protein is a potential substrate for CMA. Therefore, the better way to determine whether a protein is a CMA substrate is to reproduce its binding and uptake with isolated lysosomes rather than to identify the CMA targeting motif in the amino acid sequence.

### *20.1.2 Recognition of Substrates*

In 1989, Chiang et al. found that a 73 kDa protein in the cytoplasm specifically recognizes and binds to KFERQ motifs and promotes degradation of the target protein through the CMA pathway under nutrient deprivation. Subsequent studies found that this protein belongs to the heat shock protein (Hsp70) family and was eventually identified as heat shock cognate 70 (Hsc70) (Agarraberes et al. 1997). It was the first component determined to be involved in the CMA process.

In addition to Hsc70, other chaperones are also reportedly involved in CMA substrate recognition, binding, and translocation processes. These chaperones include heat shock protein 90 (Hsp90), heat shock protein 40 (Hsp40), Hsp70–Hsp90 organizing protein (Hop), Hsp70-interacting protein (Hip), and bcl2-associated athanogene 1 protein (BAG-1). It was found that Hsp90, Hip, and Hop stabilize the chaperone—substrate complex. Hsp40 is reported to enhance Hsc70 ATPase activity and promote the release and recombination of the substrate from the chaperone–substrate complex. These chaperones and Hsc70 form a chaperone complex that assists Hsc70 in recognizing and binding the KFERQ motifs of the CMA substrates, but the specific mechanism of this action is currently unclear (Kaushik and Cuervo 2018).

### *20.1.3 Unfolding of the Substrate*

In 2000, Natalia discovered that dihydrofolate reductase (DHFR) is one of the CMA substrates. Lysosomes fully bind and uptake DHFR in vitro. However, when DHFR was maintained in a folded state with methotrexate treatment, the uptake of DHFR by lysosomes was reduced by 80%. When the DHFR was reestablished in an unfolded state by washing off the methotrexate or adding a natural substrate of DHFR, the lysosomal uptake of DHFR was significantly increased. Moreover, the folding state of the substrate had little effect on the binding of the substrate to the lysosomal membrane receptor; that is, it only affected the substrate translocation process. Thus, it was inferred that the CMA substrate must be in an unfolded state when it passes through the lysosomal membrane (Salvador et al. 2000).

### *20.1.4 Substrate Translocation*

After the formation of chaperone–substrate complexes in the cytoplasm, little is known about how the substrate is translocated into the lysosome. In 1996, Ana found a specific receptor, LAMP2A, on the surface of the lysosomal membrane that acts as a transport channel in CMA substrate translocation (Cuervo and Dice 1996).

There are three kinds of LAMP2 gene products, and LAMP2A is one of them. Mostly extending into the lysosomal matrix, LAMP2A has a single transmembrane region and a C-terminal tail located in the cytoplasm. The C-terminal tail comprises 12 amino acids and plays an important role in binding the chaperone–substrate complex. LAMP2A is present on the lysosomal membrane in the form of a monomeric protein, but during the process of recognition by the chaperone–substrate complex, LAMP2A forms a homotrimeric complex. Transmembrane helices in the homologous complexes are intertwined to form a parallel coiled-coil conformation that acts as a transmembrane channel. The GXXG sequence of the LAMP2A transmembrane region is significant for its multimerization. Any mutation in this region makes it is difficult for LAMP2A to multimerize. Both Hsc70 and the CMA substrates in the chaperone–substrate complex bind to the C-terminal tail of LAMP2A in the homologous complex, and the substrate in the unfolded state is ultimately translocated into the lysosomal lumen. It has been reported that the CMA substrate fails to enter the lysosomal lumen if the cytoplasmic tail of LAMP2A is blocked by a specific antibody or synthetic peptide.

### *20.1.5 Degradation of Substrate*

After the substrate is translocated into the lysosomal lumen, the relevant hydrolase breaks down the substrate into amino acids, providing material for the cell to use in synthesizing necessary proteins. LAMP2A dissociates from the homologous complex and is relocated to the lysosomal membrane. The basic process of CMA is summarized in Fig. 20.1.

## 20.2 Regulation of CMA

CMA activity is dynamic, and it responds to extracellular and intracellular signals in a timely manner. Similar to other cellular processes, CMA is tightly regulated by a variety of factors and signaling pathways. These regulatory pathways adapt CMA to address the need for cellular activities.

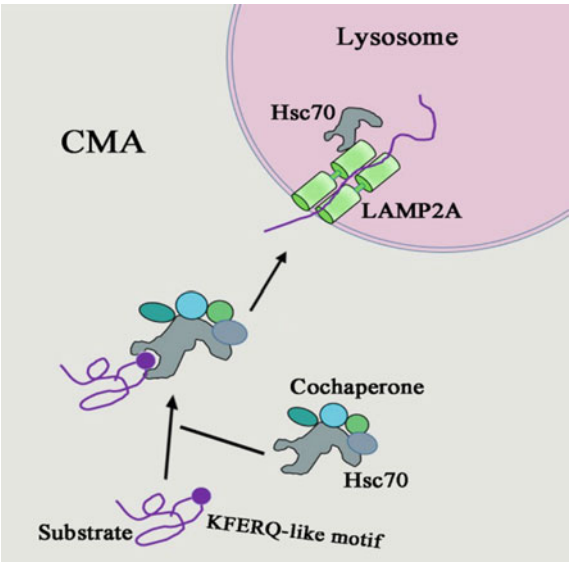


**Fig. 20.1** The basic process of CMA

### *20.2.1 Regulation of LAMP2A*

First, LAMP2A is precisely regulated at the transcriptional level. In T cells, the calcineurin–NFAT pathway is the first signaling pathway found to regulate LAMP2A transcription and CMA activation. There are several potential NFAT1 binding sites in the promoter region proximal to the LAMP2 gene. After T cell activation, reactive oxygen species (ROS) promote nuclear translocation of the transcription factor NFAT1 and bind to the LAMP2 promoter region, inducing transcriptional expression of LAMP2A. A recent report confirmed that NRF2 is also a transcription factor for the LAMP2A gene. Overexpression or knockout of NRF2 causes an increase or decrease in LAMP2A levels, which ultimately affects CMA activity.

Second, signaling pathways regulate the stability and function of LAMP2A. Previous studies have found that endoplasmic reticulum stress can activate macroautophagy, but whether and how endoplasmic reticulum (ER) stress can induce CMA remains to be explored. Recently, we demonstrated that ER stress can regulate the stability and function of LAMP2A by activating p38 kinase. When exposed to endoplasmic reticulum stress, MKK4 can accumulate and activate p38MAPK in lysosomes. Activated p38MAPK directly phosphorylates LAMP2A at 211 and 213 threonine. This dual phosphorylation modification increases LAMP2A levels and activity. We refer to the coupling between endoplasmic reticulum stress and CMA as ERICA (ER stress-induced chaperone-mediated autophagy). In the neurotoxin-induced Parkinson's disease model, we found that uncoupling induces neuronal death, suggesting that ERICA has a crucial role in protecting cells from oxidative stress (Li et al. 2019).

Abnormal transport of LAMP2A in the cell often affects the level and function of LAMP2A. Precise pathways also regulate the intracellular transport of LAMP2A.

Cystine disease is a lysosomal storage disorder characterized by a lack of the cystine transporter cystinosin. Researchers have found that the Golgi complex–lysosomal transport pathway of LAMP2A is defective and that CMA activity is significantly reduced in this disease. However, overexpression of the cystine protein RILP can repair the erroneous transport of LAMP2A, thereby maintaining the normal activity of CMA in vivo. Vps35 plays an important role in the endosome–Golgi transport of membrane proteins. For Parkinson's disease, researchers found that the lack of Vps35 hinders the endosome–Golgi transport of LAMP2A, which in turn promotes degradation of LAMP2A. As a result, the activity of CMA in dopamine neurons is impaired, ultimately promoting the development of Parkinson's disease.

As a transmembrane protein, LAMP2A is highly glycosylated, which protects it from lysosomal proteases. However, damaged LAMP2A is ultimately degraded by cathepsin A and an unidentified metalloproteinase in the lysosomal lumen.

### *20.2.2 Regulation of Chaperones*

CMA chaperones are composed of Hsc70, Hsp90, Hsp40, and other important proteins. They play a key role in modulating the formation of chaperone–substrate complexes, substrate recognition, substrate unfolding, and translocation. The level and function of these chaperones directly affect the activity of CMA.

Hsc70 is the only chaperone known to bind directly to CMA substrates (Chiang et al. 1989). Studying human B cells, researchers found that Hsc70 functions can be regulated by the level of LAMP2C. Overexpression of LAMP2C can block the binding between cytoplasmic Hsc70 and CMA substrates, thereby regulating the level of the target substrate and ultimately modulating presentation of the antigen in B cells. As Hsc70 is abundantly expressed and stable in the cytoplasm, to date, few reports have focused on the regulation of Hsc70 in the cytoplasm.

Hsc70 not only exists in the cytoplasm but is also present in the lysosomal lumen (Ly-Hsc70). Ly-Hsc70 was reported to promote the translocation process of the CMA substrate. Although cytoplasmic and lysosomal Hsc70 are transcribed from the same gene, Ly-Hsc70 has a higher acidic isoelectric point, ensuring that Ly-Hsc70 remains stable in an acidic environment with proteases (Agarraberes et al. 1997).

The chaperone Hsp90 helps maintain the stability of the chaperone–substrate complex. It has been reported that the mitochondrial-related peptide Humanin (HN) can promote the binding between the chaperone protein and CMA substrate by affecting Hsp90, thereby regulating CMA function of primary cardiomyocytes and finally increasing cell viability. These results demonstrate the existence of complex regulatory mechanisms in the body to regulate chaperones and thus affect overall CMA activity.

## *20.2.3 Signaling Pathways Regulating CMA*

### 20.2.3.1 NFAT and Calcium Signaling

As described above, the calcineurin–NFAT pathway regulates CMA activity in T cells. Exposed to oxidative stress, the transcription factor NFAT binds to the LAMP2A promoter region and increases the transcriptional expression of LAMP2A. When calcineurin activity is inhibited by cyclosporin A and ROS, the activity of CMA in the cells is also inhibited. CMA selectively degrades T cell antigen receptor (TCR) signaling inhibitors and maintains T cell activation under such conditions.

### 20.2.3.2 RARα Signal

It has been reported that the nuclear receptor family member RARα can inhibit CMA activity. Inhibition of RARα signaling by genetic or chemical means not only increases LAMP2A protein levels but also promotes the transport of LAMP2A to lysosomes. Effective RARα inhibitors have been developed based on chemical structural design techniques. Importantly, they increase only the CMA activity without affecting RARα transcriptional activity. Previous studies have shown that chemical activation of CMA protects cells from oxidative stress, providing a new application for RARα inhibitors.

### 20.2.3.3 TORC2–AKT1–PHLPP1 Axis

mTOR is a serine/threonine kinase that acts as a cellular nutrition sensor. mTOR has two different complexes, mTORC1 and mTORC2. It has been reported that activation of lysosomal mTORC1 can inhibit macroautophagy and that activation of mTORC2 can inhibit CMA. Lysosomal TORC2 is always present on the membrane of CMA-active lysosomes. mTORC2 phosphorylates AKT1, which subsequently phosphorylates and inactivates GFAP. As a positive regulator of CMA, phosphorylated GFAP is unable to accelerate the cycle of LAMP2A assembly and disassembly. Another phosphatase, PH domain leucine-rich repeat-containing protein phosphatase 1 (PHLPP1), suppresses the effect of mTORC2 by dephosphorylating AKT1. mTORC2 and PHLPP1 cooperate to modulate cellular CMA efficiency.

### 20.2.3.4 Regulation of CMA by Endoplasmic Reticulum (ER) Stress

As described above, endoplasmic reticulum stress can regulate the phosphorylation of LAMP2A by activating p38 kinase, thereby enhancing the stability and function of LAMP2A and finally regulating the activity of CMA and maintaining cell survival.

## 20.3 Physiological Roles of CMA

Early studies mainly focused on the protein quality control function of CMA and considered damaged or abnormally synthesized KFERQ-containing proteins as the main substrates of CMA. With the advancements in research, it has been found that damage is not required for a protein to serve as a CMA substrate. Normal, properly folded proteins can also become CMA substrates in specific cellular processes. By selectively degrading these proteins, CMA is involved in the regulation of cellular signaling pathways in which the proteins participate. The diversity of CMA substrate proteins and the potential substrates generated by protein modification suggest that CMA is involved in a variety of cellular physiological processes.

For most organ and cell types, nutrient deprivation effectively activates CMA. After nutrient deprivation, macroautophagy is first activated and can last for 8–10 h. Then, the nonessential protein will be degraded by CMA to generate free amino acids useful for synthesizing essential proteins. In addition, alanine and glutamine can be produced to provide energy for cells through gluconeogenesis. Liver and kidney are the main organs of gluconeogenesis, and they were found to have high CMA activity. Studies have demonstrated that inhibition of CMA activity can significantly reduce ATP levels in liver and kidney cells under conditions of nutrient deprivation in vivo and in vitro. Ketone bodies are the main energy source during starvation and can be generated by the α-ketoacids produced by the hydrolysis of amino acids. It was found that ketone bodies can effectively activate CMA, thus establishing a positive feedback loop for CMA and energy generation (Finn and Dice 2005).

CMA is involved in the regulation of glucose and lipid metabolism by selecting and degrading the key enzymes of glucose and lipid metabolism. A study on glucose metabolism in fasting rats found that CMA can effectively degrade key glycolysis enzymes, such as glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Timely degradation of the key enzymes of glycolysis can reduce the glycolysis rate in the liver and maintain a normal energy supply. Studies have confirmed that CMA is involved in the regulation of lipid metabolism by selectively degrading lipogenesis enzymes, lipid carriers, and proteins that coat lipid droplets. CMA initiates lipolysis and lipophagy by degrading and removing lipid droplet coat proteins such as perilipins 2 and 3 (PLIN2 and PLIN3) to expose the internal lipid droplets. By reducing lipogenesis and increasing lipolysis, CMA contributes to the maintenance of cellular lipid levels.

Protein quality control is another important function of CMA. CMA can maintain the normal physiological function of cells by selectively degrading damaged or abnormal proteins. For example, myocyte-specific enhancer factor 2D (MEF2D) is an important transcription factor that maintains the survival and homeostasis of neurons. After inactivation, it is transported to the cytoplasm and combined with Hsc70 for CMA degradation to maintain its normal transcription function. Paired paired-box protein 2 (PAX2) is an important cell proliferation and differentiation factor. In renal epithelial cells, CMA can degrade PAX2 in a timely manner to maintain the normal size of the kidney and prevent excessive hyperplasia and hypertrophy.

Nucleic acid deglycase (DJ-1) is an important mitochondrial quality control factor that helps maintain the normal morphology and function of mitochondria. CMA can degrade oxidized DJ-1 in timely fashion to maintain the morphology and function of mitochondria and protect the normal energy metabolism of cells (Wang et al. 2016).

In the immune system, extracellular antigens are internalized and processed in the endosomes and lysosomes of antigen-presenting cells (APCs) and then presented to T cells in combination with MHC class II molecules. It is now known that when LAMP2A is decreased, the cytoplasmic antigenic epitopes of the APCs decrease significantly. Overexpressing both LAMP2A and Hsc70 can effectively reverse the above process in APCs to increase cytoplasmic autoantigen presentation. The study revealed the role of CMA in promoting immune recognition and antigen presentation. The activation of $CD4^+$ T requires the activation of the TCR, which can be negatively regulated by the ubiquitin ligase ITCH and the calcineurin inhibitor RCAN1. Studies have shown that CMA can activate T cells by degrading ITCH and RCAN1 and promote the proliferation and cytokine secretion of $CD4^+$ T cells. In addition, studies have shown that CMA can contribute to the regulation of innate immune function by degrading the stimulator of interferon gene protein (STING). The trigger for STING degradation is desumoylation, which leads to unmasking of its KFERQ-like motif during the late phase of viral infection and the termination of the innate immune response.

The normal cell cycle is regulated by several protein factors. Studies have found that cell cycle checkpoint kinase (Chk1) can be activated after DNA damage and delay cell cycle progression. During this time, the cells repair the damaged DNA, and when the repair is complete, the cell cycle resumes. The mechanism of Chk1 activation and degradation is of great significance for maintaining the balance of the cell cycle. CMA has been shown to selectively degrade activated Chk1 after DNA repair completion to maintain a balanced cell cycle. In addition, hypoxia-inducible factor 1α (HIF1α) can be activated under hypoxia stimulation to affect cell cycle progression. CMA can degrade ubiquitinated HIF1 to regulate the cell cycle under hypoxia.

## 20.4 CMA Dysfunction and Disease

Peroxide accumulation, aging, and other pathological signals often cause CMA dysfunction. Because CMA is closely related to cellular physiological functions, it is believed that CMA dysfunction is involved in the pathogenesis of many diseases.

### *20.4.1 CMA and Neurodegenerative Diseases*

#### 20.4.1.1 Parkinson's Disease (PD)

One of the pathological markers of PD is the gradual, selective loss of midbrain dopaminergic neurons, but the underlying pathological mechanism is not well understood. Increasing evidence has shown that CMA dysfunction is involved in the pathogenesis of PD. By analyzing animal models and brain samples from PD patients, researchers found that LAMP2A levels were reduced in PD, suggesting impaired CMA activity in PD. Recently, CMA was reported to degrade wild-type α-synuclein under physiological conditions. However, mutant α-synuclein (A53T or A30P) can occupy the LAMP2A receptor, thereby inhibiting the degradation of wild-type α-synuclein and other substrates such as MEF2D by CMA and ultimately inducing neuronal death (Yang et al. 2009). Similarly, wild-type leucine repeat kinase 2 (LRRK2) can be degraded by CMA, but the LRRK2 G2019S mutant interferes with the formation of the CMA chaperone–substrate complex, ultimately leading to defects in CMA activity and the induction of neuronal death (Alvarez-Erviti et al. 2010).

#### 20.4.1.2 Alzheimer's Disease (AD)

β-Amyloid plaque formation and abnormal tau aggregation are considered to be the main pathological processes of AD. Studies have shown that CMA can degrade wild-type tau, but the A152T mutant tau will compete with wild-type tau for LAMP2A receptors, blocking CMA from degrading the wild-type tau, eventually causing abnormal accumulation of tau and inducing AD.

#### 20.4.1.3 Huntington's Disease (HD)

The pathological hallmark of HD is the abnormal aggregation of huntingtin in the nucleus. CMA can degrade wild-type huntingtin, but mutant huntingtin interferes with the uptake of CMA substrates by lysosomes, which affects the degradation of wild-type huntingtin. In addition, posttranslational modifications such as phosphorylation and acetylation alter the property of the KFERQ motif in the mutant huntingtin and ultimately interfere with CMA degradation of this protein (Lv et al. 2011). CMA activity is elevated in the early stages of HD but is decreased in the late stages of HD. Therefore, we need to measure the dynamics of CMA activity in HD to discover the underlying mechanism of HD pathogenesis.

### *20.4.2 Cancer*

Although CMA activity is generally reduced in neurodegenerative diseases, CMA activity is highly activated in most cancer cell lines. LAMP2A is the rate-limiting enzyme of CMA, and its level is significantly elevated in most tumor samples, suggesting an increase of CMA activity in most tumors (Kon et al. 2011). Genetic or chemical means used to inhibit CMA activity suppress cancer cell proliferation and metastasis.

In some cancers, CMA can upregulate glycolysis at both the transcriptional and translational levels, thereby promoting tumor cell proliferation and metastasis. In addition, there is evidence that CMA can promote tumor cell proliferation and metastasis by degrading oxidative and damaged proteins.

### *20.4.3 Other Diseases*

CMA is involved in the development of immune diseases by regulating antigen processing, presentation, and other immune processes. For example, knocking down cellular LAMP2 impedes the MHC II presentation of endogenous antigens, whereas overexpression of LAMP2A reverses this process. In diabetic-induced nephropathy, a damaged CMA process is unable to eliminate abnormal proteins with sufficient speed to prevent damage to normal kidney cells.

## 20.5 Microautophagy

Microautophagy, one of the three main autophagy pathways, plays an important role in the cellular activities of the body. Due to the lack of research tools and research methods, our understanding of microautophagy is still very limited. Some crosstalk exists between microautophagy and CMA; therefore, in this section, we briefly introduce the basic process of microautophagy and research advancements in the study of microautophagy.

In 1996, de Duve and Wattiaux first proposed the concept of microautophagy. Over the subsequent two decades, microautophagy was referred to as a phenomenon in which lysosomal-like organelles in mammalian cells contain numerous vesicles. Today, many studies suggest that microautophagy refers to the process in which the lysosomal membrane is either invaginated or projects arm-like protrusions to sequester cytoplasmic constituents into intralysosomal vesicles. There are many different classifications of microautophagy. For example, one of the classifications is based on lysosomal or vacuole membrane protrusion or invagination, and another is classified according to species. However, more researchers tend to classify microautophagy according to selectivity. In this way, microautophagy can be divided

into nonselective microautophagy (NSM), selective microautophagy and endosomal microautophagy (eMI). Among them, selective microautophagy can be divided into micromitophagy, micronucleophagy (or piecemeal microautophagy of the nucleus, PMN), and micropexophagy (Li et al. 2012).

### *20.5.1 Nonselective Microautophagy*

Demarcated by morphological changes, biokinetic analysis, and specific gene identification, microautophagy is characterized by four sequential stages: (1) Microautophagic invagination and autophagic tubes; (2) Vesicle formation and expansion; (3) Vesicle scission; and (4) Vesicle degradation and recycling (Mijaljica et al. 2011).

#### 20.5.1.1 Microautophagic Invagination and Autophagic Tubes

At the early stage of microautophagy, in the membranes of the lysosomes or vacuoles, the lipids and lipid-modifying proteins are segregated, and large transmembrane proteins are excluded to maintain areas of low protein density. The segregation of the lipids and lipid-modifying proteins drives the lysosome or vacuole membrane to form a depression into the interior. The dynamin-related GTPase Vps1p was confirmed to be involved in the invagination process of the membrane. The invaginated membrane will eventually extend into the autophagic tube, a characteristic tubular structure. At the tip of the autophagic tube, the protein density of the membrane is drastically reduced, while the lipid content is significantly increased.

Previous studies have suggested that many pathways and complexes are involved in this process. Two ATG7-dependent ubiquitin-like conjugation systems (Ublc systems) have been shown to be involved in this process. In the first system, ATG8, with the aid of ATG7, ATG3, and ATG4, participates in the process by combining with lipid phosphatidylethanolamine (PE) on the membrane. In the second system, ATG7 and ATG10 promote the binding of ATG5 and ATG12. The ATG5–ATG12 complex binds to ATG16, which subsequently promotes the binding of ATG8 to PE. In addition to these two systems, the vacuolar transporter chaperone (VTC) complex plays an important role in the formation of the autophagic tubes in yeast. The VTC complex regulates the distribution of proteins on the autophagic tubes and initiates calmodulin-mediated membrane invagination.

#### 20.5.1.2 Vesicle Formation and Expansion

Due to the high level of lipids and the obvious decrease in protein at the tip of the autophagic tube, vesicles gradually form at the tip of the autophagic tube. It has been reported that low temperature can inhibit the formation of vesicles in microautophagy, suggesting that lipids are involved in the autophagic lipid enrichment and protein

elimination process, which in turn affects the formation and expansion of the vesicles. In another study, inhibition of ATPase, GTPase, and other enzyme activities repressed the expansion of autophagic vesicles. This finding suggests that these enzymes are involved in the formation and expansion of vesicles. In addition, the EGO (exit from rapamycin-induced growth arrest) complex has also been reported to be involved in vesicle formation and scission (Dubouloz et al. 2005).

#### 20.5.1.3 Vesicle Scission

The vesicles at the tip of the autophagic tube are always in a dynamic state. The vesicles tend to fall off the autophagic tube and enter the lysosome or vacuole lumen. Usually, only one or two vesicles will fall into the lumen. It was found that rapamycin treatment and knocking down ATG7 and ATG1 blocked the scission process of vesicles in lysosomes or vacuoles, suggesting that ATG7 and the signaling pathway between TOR and ATG1 are involved in the regulation of vesicle scission. In addition, V-ATPase can pump hydrogen ions into the lysosome or vacuole lumen to maintain the electrochemical gradient. The proton kinetics are important for the normal scission of vesicles.

#### 20.5.1.4 Vesicle Degradation and Recycling

After the vesicles fall off the autophagic tube, they move freely in the lysosomes or vacuole lumen at high speed. ATG15p and hydrolase mediate the degradation of these vesicles while ATG22p is responsible for the recycling of nutrients and energy after degradation.

### *20.5.2 Selective Microautophagy*

In yeast, in some kinds of microautophagic processes, only specific substrates are phagocytosed and degraded. These selective microautophagy types include micromitophagy, micronucleophagy or piecemeal microautophagy of the nucleus (PMN) and micropexophagy, as summarized in Fig. 20.2.

#### 20.5.2.1 Micromitophagy

In yeast, micromitophagy can be divided into two types. The first mode is Uth1-dependent. Uth1 is a protein localized on the outer membrane of the mitochondria and mediates the binding of mitochondria and vacuoles. In this mode of micromitophagy, the mitochondria are in direct contact with the vacuole, and the vesicles generally contain little cytoplasm. The second mode is Uth1-independent. In Uth1 wild-type

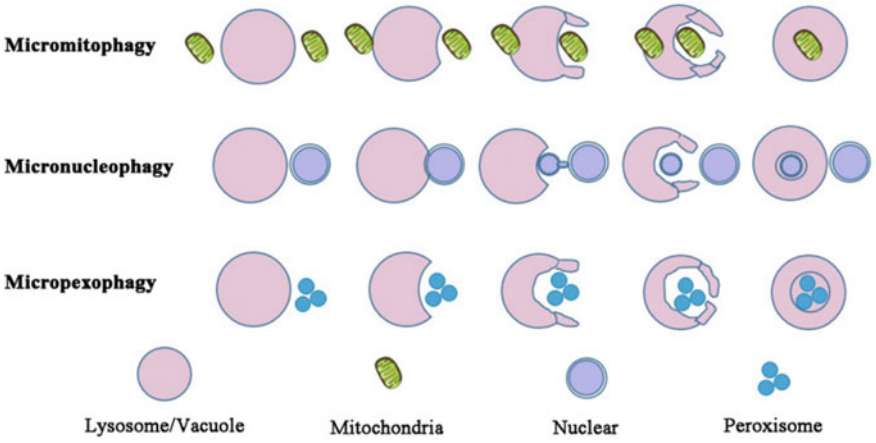


**Fig. 20.2** The process of selective microautophagy

and knockout cells, vesicles were observed to encapsulate the mitochondrion to be degraded and become part of the cytoplasm. Due to the genetic background of the strain and the lack of detection methods, there is room to question the role of Uth1 in micromitophagy, and therefore, the role of Uth1 in micromitophagy needs to be verified with more experiments. At present, no one has staged the process of micromitophagy.

Decreased intracellular glutathione pool, nitrogen starvation, and mitochondrial damage all induce micromitophagy. These factors result in mitochondrial permeability transition (MPT), which ultimately causes mitochondrial depolarization and swelling. One study showed that the mitochondrial inner membrane protein Mdm38 regulates the mitochondrial $H^+/K^+$ exchange system while participating in micromitophagy. Both Vac8 and ATG7-dependent Ublc systems are thought to modulate micromitophagy.

#### 20.5.2.2 Micronucleophagy or Piecemeal Microautophagy of the Nucleus (PMN)

PMN mainly degrades part of the nucleolus and nucleoplasm. Morphologically, PMN can be divided into the following steps: first, the vacuole receives the signal from the nucleus and moves close to the nucleus; then, with the help of Vac8 and the membrane protein Nvj1, a nucleus–vacuole junction (NV junction) is formed between the vacuole and the nucleus; next, with the increase in Nvj1 protein, part of the nucleus is sequestered at the vacuole membrane; and finally, some of the nuclei are detached from the vacuole, and vesicles enter the vacuole lumen and are degraded by hydrolase.

Rapamycin treatment, as well as nitrogen or carbon starvation, can induce PMN. Core ATG machinery proteins such as ATG1–ATG10, ATG12–16, ATG18, and ATG22 are essential for PMN. In addition, Osh1 and Tsc13, which are reported to be involved in lipid metabolism, participate in the formation of vesicles during PMN, suggesting an important role of lipids in the process of PMN. V-ATPase contributes to the formation of a diffusion barrier at the vacuole side of the NV junction and modulates PMN.

#### 20.5.2.3 Micropexophagy

Micropexophagy primarily degrades damaged or excess peroxisomes. Upon receipt of the signal from the peroxisomes, the vacuole gradually approaches the peroxisome to be degraded. Subsequently, the vacuole membrane invaginates and extends to form the two arm-liked vacuolar sequestering membranes (VSMs). The VSMs slowly entrap the peroxisome. With the help of the micropexophagic membrane apparatus (MIPA), the VSMs fuse with each other and eventually encapsulate and degrade the peroxisome.

Studies have shown that knocking out Pfk1, Vps15, Vps34, and ATG18 prevents vacuoles from being in proximity of the peroxisomes, but the specific mechanism remains to be elucidated. Gcn3 can initiate membrane transfer from peri-vacuolar dot-like structures (PVSs) to VSMs, and Gcn2 can regulate this process. The ATG core protein is essential for micropexophagy. ATG8 and ATG26 are involved in the formation of MIPA while ATG24 is involved in the fusion of MIPA with vacuoles to regulate VSM action.

### *20.5.3 Endosomal Microautophagy (eMI)*

eMI mainly occurs in late endosomes (LEs) and multivesicular bodies (MVBs), which mainly degrade the cytoplasmic components encapsulated by the vesicles in the LE/MVB (Sahu et al. 2011). One kind of eMI can encapsulate cytoplasmic components into vesicles with the help of proteins such as ESCRT, Alix, and Vps4, similar to nonselective microautophagy in yeast. Ultimately, cytoplasmic components can be directly degraded in the LE/MVB or degraded by fusing with lysosomes. However, there is another form of eMI that can specifically degrade the substrate by recognizing the KFERQ element (Mukherjee et al. 2016). The selective eMI process can be divided into the following steps: first, Hsc70 recognizes and binds a specific substrate by its KFERQ motif and also binds to phosphatidylserine (PE) on the endosomal membrane through its C-terminal LID domain. With the help of the ESCRT I and III complexes, the LE/MVB next forms vesicles, and the substrate is internalized into the vesicles. Next, the vesicles containing the substrate in the LE/MVB are eventually degraded by hydrolases. It should be noted that Hsc70 is degraded with vesicles in eMI, whereas Hsc70 is returned to the cytoplasm after the substrate is delivered to

the lysosome in CMA. Although Hsc70 is a common chaperone used by both eMI and CMA, its fate in these two autophagic processes is quite different.

In mammals, eMI is often highly activated 24 h after starvation. In *Drosophila*, ATG1 and ATG13 are essential for eMI activation. In fission yeast, NBR1 has been shown to be one of the receptors involved in eMI that mediates the entry of hydrolases into an LE/MVB and induces eMI. Hsc70 is abundant and stable in cells, making it an unlikely key regulator in selective eMI. However, the content and assembly dynamics of ESCRT directly affect the formation of vesicles, making ESCRT a likely key modulator of eMI (Saksena et al. 2007).

### *20.5.4 Physiological Function of Microautophagy*

In the process of membrane invagination, it is necessary to enrich lipids and exclude membrane proteins, suggesting that microautophagy plays an important regulatory role in lipid metabolism. Studying synapses, researchers found that Hsc70-4 regulates neurotransmitter secretion by promoting the eMI process. If eMI is inhibited, then the neurotransmitter secretion is attenuated. In addition, recovery of eMI can increase neurotransmitter activity. Other studies have suggested that RAB7-dependent microautophagy can mediate lysosomal signal transmission and play an important role in early mammalian embryonic development. However, in general, the study of microautophagy is still limited compared with the attention paid to the two other autophagy types, and more research is urgently needed to reveal the important functions of microautophagy in cellular activities.

## 20.6 Concluding Remarks

Approximately 30 years ago, Prof. Fred Dice discovered the selective autophagic process of CMA based on the KFERQ motif. Later, Ana et al. found that the chaperone–substrate complex and LAMP2A play key roles in the CMA process. Protein quality control is the most basic function of CMA, and CMA is involved in a variety of cellular activities by modulating the quality and level of the target protein. After CMA is disturbed, organs and tissues can develop relevant diseases. In summary, we have made tremendous progress in the study of CMA. However, compared to the knowledge of the other two forms of autophagy, the understanding of CMA is limited. There is still a long way to go to fully understand CMA, and there are still many questions to be answered.

One of the questions in this area is whether LAMP2A is the only receptor involved in CMA. It has been reported that, in some animal models, defects in LAMP-1 and -2 have little effect on CMA activity. Therefore, other receptors may coordinate with LAMP2A to regulate CMA activity.

Another unresolved area involves the relationship between CMA and other autophagy types. Nutrient deprivation activates both macroautophagy and CMA; therefore, a potential relationship between these two forms of autophagy may remain to be undiscovered. In endosomal microautophagy, Hsc70 is used to bind substrates containing KFERQ motifs. It can be seen that endosomal microautophagy and CMA share some regulatory components (Tekirdag and Cuervo 2018).

Of course, many challenges remain to be overcome in this field, such as the identification and development of CMA inhibitors and a systematic method of identifying CMA substrates. The means to answer the remaining questions and address the challenges will help in leveraging CMA for disease-targeted therapy that will ultimately benefit humans.

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# Chapter 21
# Autophagy in Reproduction


**Hui Gao, Muhammad Babar Khawar and Wei Li**

**Abstract** Autophagy, a major degradation/recycling pathway, plays an essential role in cellular homeostasis maintenance, cell fate decision, and reproductive development. During reproduction, sperms and eggs, the specialized haploid gametes produced by the meiotic process of the germ cells in male and female respectively, are fused to form a new zygote that develops into fetus through embryogenesis and maternal–fetal crosstalk. Researches carried out in the past few years have proved that autophagy plays a key role in the regulation of reproduction process, and blockage of autophagy process likely contributes to reproductive abnormalities and even infertility. Here we summerize the recent progress in exploring the functional roles of autophagy in reproductive processes, such as spermatogenesis, folliculogenesis, fertilization, embryogenesis, and maternal–fetal crosstalk, in both animals and plants.

**Keywords** Autophagy · Embryogenesis · Fertilization · Gametogenesis · Reproduction

## Abbreviations

| | |
|---|---|
| 3-MA | 3-methyladenine |
| Ambra1 | Activating molecule in beclin1-regulated autophagy |
| CTB | Cytotrophoblast |
| Dcp1 | Drosophila caspase-1 |
| enEVT | Endovascular extravillous trophoblast |
| epg-2 | Ectopic PGL granules 2 |
| epg-3 | Ectopic PGL granules 3 |
| epg-4 | Ectopic PGL granules 4 |

H. Gao · M. B. Khawar · W. Li (✉)
State Key Laboratory of Stem Cell and Reproductive Biology, Institute of Zoology, Chinese Academy of Sciences, 1 Beichen West Road, Chaoyang District, Beijing 100101, China
e-mail: leways@ioz.ac.cn

M. B. Khawar · W. Li
University of Chinese Academy of Sciences, Beijing 100049, China



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| | |
|---|---|
| epg-5 | Ectopic PGL granules 5 |
| epg-8 | Ectopic PGL granules 8 |
| ES | Ectoplasmic specialization |
| EVT | Extravillous trophoblast |
| FIP200 | 200-kDa FAK-family interacting protein |
| FOXO/DAF-16 | Forkhead box O transcription factor |
| GATA1 | GATA binding protein-1 |
| GOPC | Golgi-associated PDZ-and coiled-coil motif-containing protein |
| HIF1α | Hypoxia-inducible factor 1α |
| iEVT | Interstitial extravillous trophoblast |
| IL-1β | Interleukin-1β |
| IUGR | Intrauterine growth retardation |
| LAMP1 | Lysosome-associated membrane protein 1 |
| LOX | Lectin-like oxidized low-density lipoprotein |
| MOs | Membranous organelles |
| mtDNA | Mitochondria DNA |
| MYBL2/B-MYB | MYB-related protein B |
| NHERF2 | $Na^+/H^+$ exchanger regulatory factor 2 |
| oxLDL | Oxidized low-density lipoprotein |
| PCD | Programmed cell death |
| PDLIM1 | PDZ and LIM domain 1 |
| PGL-1 | P granule components 1 |
| PGL-3 | P granule components 3 |
| PtdIns(3)P | Phosphatidylinositol-3-phosphate |
| PTEN/DAF-18 | Phosphatase and tensin homolog |
| ROS | Reactive oxygen species |
| SEPA-1 | Suppressor of ectopic P granules in autophagy mutants-1 |
| Sirt1 | Silent information regulator 1 |
| SR-BI | Scavenger receptor class B type I |
| STB | Syncytiotrophoblast |
| VDAC2 | Voltage-dependent anion channel 2 |

## 21.1 Introduction

Reproduction is the process by which the new generation (offspring) is produced from the previous generation (parents). Reproduction can generally be divided into two basic types, asexual reproduction and sexual reproduction. The sexual reproduction is far more complex, in which the specialized haploid gametes (sperms and eggs in male and female, respectively) produced by the meiotic process are fused (fertilized) to form a zygote. A new generation (offspring), with genetic information coming from both the parents, is then formed as a result of embryonic development (embryogenesis and maternal–fetal crosstalk). Autophagy plays an extremely

important role in the process of reproduction. Selective and bulk autophagy both participate in various physiological processes, by degrading and recycling the cellular components, to ensure successful reproduction. Several autophagy-deficient organisms, such as yeast, nematode, fruit fly, and mice, exhibit a variety of reproductive abnormalities and sometimes infertility. In this chapter, we will focus on the role of autophagy in the reproduction of different species with a special emphasis on *Caenorhabditis elegans* and mammals. Furthermore, we will also focus on various roles of autophagy in the regulation of gametogenesis, embryonic development, and maternal–fetal crosstalk.

## 21.2 Role of Autophagy in Yeast Sporulation

The gametogenesis of yeast is called sporulation. Under nutrient deprivation, yeast exits the mitotic cycle and initiates meiosis to eventually produce four haploid spores (Tsukada and Ohsumi 1993). Autophagy has been recently reported to play an important role in the process of yeast sporulation. Nitrogen starvation endorsed *Saccharomyces cerevisiae* to enter in the meiotic process, whereas autophagy-deficient cells were unable to initiate meiosis. Autophagic activity was found to be enhanced in the early stages of meiosis in budding yeast, and autophagy deficiency resulted in DNA replication failure (Wen et al. 2016). In fission yeast, *atg1*, *atg7,* or *atg14*-deficiency resulted in overextension of spindle and abnormal chromosomal segregation during metaphase that eventually leads to the formation of additional nuclei. Further studies have shown that aurora kinase (a key regulator of spindle length and chromosomal segregation) expression was abnormally increased in autophagy-deficient cells, suggesting that aurora kinase is degraded via the autophagy–lysosome pathway. Knockdown of aurora kinase effectively restored the meiotic process. These results indicate that autophagy regulates sporulation by regulating the meiotic processes in yeast (Matsuhara and Yamamoto 2016).

## 21.3 Role of Autophagy in the Reproduction of Nematodes

Autophagy was found to play an important role in the reproduction of *C. elegans*. P granules are special protein–RNA complex derived from the maternal genome. After fertilization, P granules are randomly partitioned into the cytoplasm of the newly formed embryos. Later, P granules are found in the cytoplasm of blastomeres P1, P2, P3, and P4 and finally after several asymmetric divisions only in germ precursor cells Z2 and Z3 which are derived from P4. Afterward, the P granule components PGL-1 and PGL-3, restricted to somatic cells as a result of asymmetric cell divisions, are degraded via autophagy. Degradation of P granules not only supplies nutrition for the development of *C. elegans* but also prevents them from the toxic effects of P granule aggregation. Autophagy-deficient *C. elegans* embryos failed to remove

these PGL-1 and PGL-3 components and they are aggregated in somatic cells. Further studies have shown that autophagic degradation of PGL-1 and PGL-3 depends on the interaction of autophagy receptor proteins and scaffold proteins. It is generally accepted that SEPA-1 directly bind to PGL-3, GABARAP, and LGG-1/Atg8 to form a receptor required for the degradation of PGL granules, and then recruits EPG-2 to directly binds to SEPA-1 for the initiation of PGL granules degradation (Fig. 21.1). EPG-2, as a scaffold protein, can recruit and bind a variety of Atg proteins to PGL granules. Furthermore, SEPA-1 in early embryos was also found to form a complex independent of PGL-1 and PGL-3 and is cleared by autophagy, resulting in decreased expression of SEPA-1 in late embryos (Zhang and Baehrecke 2015). P granules have also been observed to bind with SQST-1 (*C. elegans* p62/SQSTM1 homolog) for the recruitment of LGG-1 and other Atg proteins present in the vicinity to initiates autophagic degradation. In autophagy-deficient embryos, SQST-1 was significantly increased and aggregated into a large number of complexes different from those of PGL granules. Other SEPA-1 family proteins and W07G4.5 were also eliminated by autophagy in the process of embryogenesis. Current research suggests that the purpose of autophagic degradation of these protein aggregates is mainly to provide

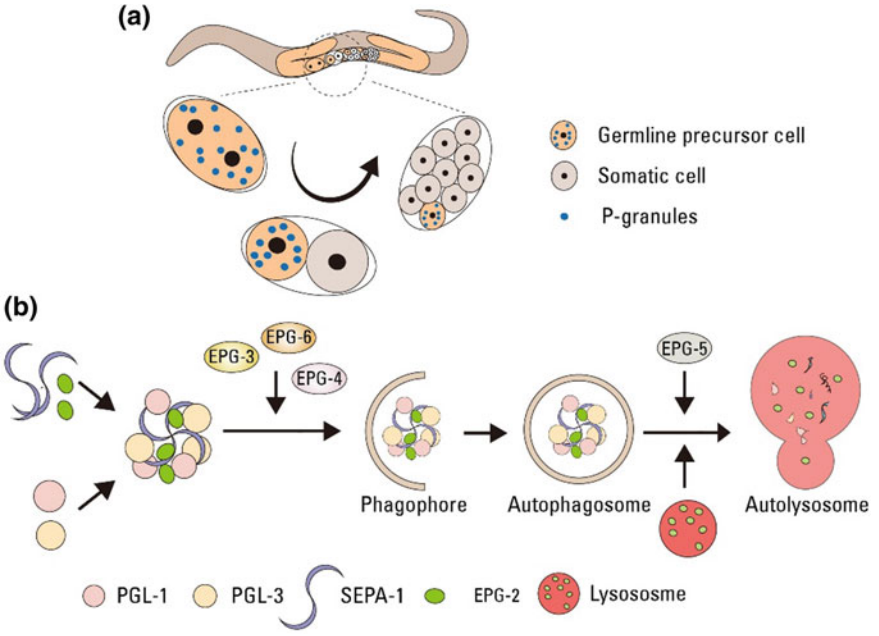


**Fig. 21.1** **a** P granules are distributed exclusively in the germline precursor cells via asymmetric cell divisions and are then degraded via autophagy. **b** P granules proteins PGL-1 and PGL-3 bind with receptor protein SEPA-1, and recruit Atg proteins to trigger the formation of phagophore via binding with scaffold protein EPG-2. Moreover, EPG-3, -4, and -6 are involved in the progression of phagophore formation. Afterwards, autophagosomes are fused with lysosome to complete the degradation of PGL-1 and PGL-3

nutrition for embryonic development in *C. elegans*. Some protein factors among them only play a role at specific stages of embryonic development, and that time-dependent degradation may prevent them from having an unforeseen effect on future embryonic development (Zhang et al. 2018).

In animals, the mitochondrial DNA (mtDNA) inherited by the offspring is only originated from the maternal side. The mitochondria carried by the sperm are randomly separated into blastomeres at 2-cell to 4-cell stage and are completely cleared in the 64-cell stage via autophagy in *C. elegans*. In autophagy-deficient embryos, the parental mitochondria and mtDNA retained and may persist until late embryonic or even larval stages. It has been found that paternal mitochondria are cleared perhaps via LGG-1 labeled autophagosomes in early *C. elegans* embryos (Sato and Sato 2011). However, it was found that mitochondria did not undergo ubiquitination, so how they are identified and degraded via autophagy still needs further exploration. Moreover, in addition to parental mitochondria, *C. elegans* spermatozoa also contain membranous organelles (MOs), whose degradation has also been associated with LGG-1 labeled autophagosomes. Mutations in a series of autophagy-related genes, such as *atg-7, atg-13, bec-1, lgg-2, lgg-3,* and *rab-7*, can impair MOs degradation (Al Rawi et al. 2011). However, MOs degradation via autophagy is different from the parental mitochondrial clearance. The ubiquitination of MOs can be detected in newly fertilized embryos, which may partly explain the mechanism by which MOs are cleared via autophagic recognition.

Autophagy is also involved in the clearance of apoptotic cell remnants during the embryonic development of *C. elegans*. It has been found that 113 somatic cells in nematode undergo apoptosis during the embryogenesis. As a result of apoptosis, a phosphatidylserine signal on the apoptotic cell surface is recognized by the surrounding cells, which then engulf, and internalize the dying cells eventually forming a phagosome. Autophagy genes (*epg-1, epg-5*, *epg-8,* and *atg-9*) knockout at different stages showed an increase in the number of apoptotic cell remnants in *C. elegans* embryos, which were found to persist there for longer times. The frequency of apoptosis, the phosphatidylserine signal on the surface of apoptotic cells, and the internalization process of cell remnants in these autophagy-deficient embryos did not change significantly. However, a decrease of PtdIns(3)P observed in phagosomes reduced the binding of RAB-5 and RAB-7 to the phagosome, indicating that the phagosome maturation and the autophagic activity were significantly inhibited (Cheng et al. 2013). It is now believed that autophagy is likely to affect the phagocytic activity of cells by regulating the formation of different VPS-34 complexes. On the other hand, some autophagy structures directly participate in the clearance of apoptotic cells by direct fusion with phagosomes.

Autophagy also plays an important role in germ cell proliferation in *C. elegans*. Germ cell proliferation was found to be inhibited in *bec-1*, *atg-16.2*, and *atg-18* mutants. Moreover, cell cycle analysis found a reduction in M-phase and S-phase duration while most of the cells were found arrested in the G-2 phase. It is speculated that *bec-1* and *atg-16.2* may activate DAF-18/PTEN while *atg-18* activates DAF-16/FOXO transcription factor to regulate the expression of cyclin proteins in germ

cells of *C. elegans* (Ames et al. 2017). However, the specific molecular mechanism still remains to be elucidated.

## 21.4 Role of Autophagy in *Drosophila* Reproduction

Autophagy was found to regulate the gametogenesis and early embryonic development of *Drosophila*. During oogenesis in *Drosophila*, certain stages of egg chamber development such as germarium (prior to follicle cell layer formation), the previtellogenic, and near maturation stages are characterized by massive programmed cell death (PCD). PCD was merely observed during the early and mid-stage of oogenesis under sufficient nutrient supply. However, during nutrient depletion or in case of abnormal development, a high frequency of PCD was observed. Egg maturation required the PCD of nurse cells during the late stage of oogenesis, and the resulting nutrients are pumped to the developing egg to support further development.

Autophagy was reported to be involved in this PCD regulation. Dcp-1 was found to clear the defective egg chamber in early and mid-oogenesis by simultaneously activating apoptosis and autophagy pathways. *Dcp-1* mutation led to a decrease in autophagic activity and decondensation of the nuclear structure. Germ cells specific autophagy deficiency retarded the DNA fragmentation of nurse cells during starvation induced-cell death suggesting autophagy plays an important role in the later stage of oogenesis (Hou et al. 2008). These results strongly suggest the role of autophagy in maintaining the interaction between germ cells and follicular cells by providing the nutrients for egg maturation via apoptosis of nurse cells. However, the specific molecular mechanism remains unknown.

## 21.5 Role of Autophagy in Mammalian Reproduction

### *21.5.1 Spermatogenesis*

Spermatogenesis refers to the production of haploid gametes from pluripotent diploid spermatogonial stem cells via sequential mitosis, meiosis, and spermiogenesis. Spermatogenesis is strictly dependent on germ cell-somatic cell interactions in the testes. There are mainly two types of somatic cells in the testes, Leydig and Sertoli cells. Interstitial cells (Leydig cells) are mainly located in the interstitial region of the testicular seminiferous tubules. They are mainly involved in the synthesis of androgens such as testosterone, androstenedione, and dehydroepiandrosterone. These cells also maintain the secondary sexual characteristics and sexual behavior of male animals. Sertoli cells are mainly located in the seminiferous tubules, which protect and support

the development of germ cells and construct the microenvironment required for spermatogenesis. Recent investigations have found that autophagy widely participates in the regulation of spermatogenesis.

#### 21.5.1.1 Role of Autophagy in Testosterone Production

Testosterone is an indispensable steroid hormone involved in male reproduction and plays an important role in sexual function and also maintains secondary sexual characteristics. About 90% of testosterone in the body is synthesized by Leydig cells. Leydig cells showed a very active type of autophagy in previous studies carried out in the past few decades. With the rapid progress in understanding the field of autophagy, especially lipophagy, researchers have found that autophagy participates in testosterone biosynthesis likely by regulating the lipid metabolism. Leydig cell-specific autophagy deficiency in mice led to a significant decrease in testosterone levels and adversely affected the sexual behavior of mice as both mounting and mating frequency and duration were pronouncedly decreased compared to the control. Further investigations showed that autophagy deficiency caused the $Na^+/H^+$ exchanger regulatory factor 2 (NHERF2) accumulation that led to a significant decrease in lipoprotein receptor scavenger receptor type B type I (SR-BI) protein expression. SR-BI down-regulation blocked the cholesterol uptake in Leydig cells and eventually resulted in reduced testosterone biosynthesis (Gao et al. 2018). This study also revealed the association between autophagy deficiency and late-onset hypogonadism.

#### 21.5.1.2 Role of Autophagy in Ectoplasmic Specialization (ES) Assembly

The ectoplasmic specialization (ES) is a unique intercellular junction found in the testis. ES is divided into two different types, the basal ES and the apical ES. The basal ES is a connecting structure formed by actin and endoplasmic reticulum between the adjacent Sertoli cells. The apical ES is localized at the contact surface between Sertoli cells and the spermatids and is closely connected to the sperm head via the acrosome. Apical ES participates in the sperm head shaping, ensures the right movement of spermatogenic cells in the seminiferous epithelium, and regulates the spermiation process. Autophagy-deficient Sertoli cells were found to produce a large number of deformed sperm heads. Further in-depth studies showed that PDLIM1 (a negative regulator of cytoskeleton) was found to be accumulated in these Sertoli cells. PDLIM1 accumulation led to cytoskeletal disassembly resulting in abnormal ES formation and malformation of the sperm head. Therefore, autophagy was suggested to participate in the assembly of extracellular specialized structures in the Sertoli cells by regulating cytoskeletal assembly (Liu et al. 2016).

#### 21.5.1.3 Role of Autophagy in Acrosome Biogenesis

The acrosome is a specialized membranous organelle that covers the mammalian sperm head. It is located between the nucleus and the plasma membrane and contains a large amount of hyaluronidase, acid hydrolase, proteolytic enzymes, etc. Its main function is to promote sperm-egg fusion via acrosome reaction. The acrosome biogenesis has four different phases. During the Golgi phase, proacrosomal granules are transported to and accumulated in the concave region of the nuclear surface, followed by fusion with each other to form a single large acrosomic granule that associates with the nuclear membrane. The acrosomal vesicle flattened and extends from the center to the sides along with the nucleus, and covers the nucleus in the capping phase. In the maturation phase, the acrosome stops expanding and begins to change its shape, eventually becoming an arc-shaped and covers the sperm nuclei. We have found malformed acrosome in germ cell-specific *Atg7* knockout mice. The sperms of these infertile mice were found similar to clinical globozoospermia phenotype. Further study found that Golgi-derived acrosome vesicles failed to be normally transported and fuse to form a complete acrosome in these *Atg7* knockout mice. At the same time, germ cell-specific *Atg7* knockout also impairs the localization of another Golgi protein GOPC and further affects the formation of the acrosome. These results demonstrate that autophagy is involved in the regulation of acrosome biogenesis process. Since both acrosomes and lysosomes share low internal acidic pH, and they contain many lysosomal related enzymes, we proposed acrosomal lysosomal origin hypothesis that acrosome is thought to be a form of modified lysosome or a lysosome-related organelle (Wang et al. 2014). *Sirt1* is a homolog of the yeast *Sir2* and is an important member of the mammalian sirtuin gene family. *Sirt1* is widely involved in the regulation of physiological processes such as gene transcription, metabolic regulation, cellular stress, apoptosis, and DNA damage repair as a deacetylase. *Sirt1* knockout in mouse germ cells led to the accumulation of acetylated LC3 and Atg7 and resulted in the failure of LC3 to be recruited to Golgi-derived vesicles ultimately resulting in acrosome vesicle recruitment failure, affecting acrosome biogenesis and eventually caused infertility (Liu et al. 2017). These results also favor the acrosomal lysosomal origin hypothesis.

#### 21.5.1.4 Role of Autophagy in Spermiogenesis

Spermatogonial stem cells undergo the process of meiosis to produce haploid round spermatids. These round spermatids are subjected to a series of dramatic morphological and functional changes to form elongating spermatids and mature spermatozoa in a process named as spermiogenesis. This process is characterized by spermatogenic cell chromatin condensation, change in nuclear morphology, acrosome biogenesis, sperm tail assembly, mitochondrial sheath formation, and cytoplasm removal along with many other events. Any problem or change in these sequential events

directly lead to abnormal sperm differentiation and may result in various sperm-associated disorders such as teratozoospermia, asthenozoospermia (or asthenospermia) and azoospermia (no sperm in the ejaculate). Germ cell-specific *Atg7* deficiency resulted in the accumulation of PDLIM1, thus rendering manchette deformation, abnormal assembly of the tail axis, and cytoplasmic removal failure. Consequently, there were found a large number of abnormal and malformed sperms with compromised movement (Shang et al. 2016). Further comprehensive studies found that autophagy is closely associated with spermatogenesis, and the abnormal autophagy in spermatogenesis can induce male infertility.

### *21.5.2 Oogenesis*

Follicles are the basic unit of gametogenesis in females. During oogenesis, follicles undergo a series of dramatic changes in their morphology and function prior to their maturity. Follicles can simply be divided into primordial follicles, primary follicles, secondary follicles, and mature follicles according to the morphologic phenotype in different developmental stages. About 99% of follicles are arrested during development, and only 1% of follicles can develop to ovulate during the entire reproductive lifespan. Therefore, follicle formation, development, maturation, and ovulation have always been hot topics in reproductive biology for a long time. Autophagy is involved in the establishment of primordial follicle pools in mammalian ovaries. During the perinatal phase, pro-granulosa cells invade the germ cell cyst and form the primordial follicle by wrapping the oocyte. More than half of the primordial follicles were lost in *Becn1*$^{+/-}$ mice ovary just 1 day after birth (Gawriluk et al. 2011). Similar results have also been observed in germ cell-specific *Atg7* knockout mice (Song et al. 2015). After birth, the neonatal mouse shifts the nutrition absorption from the placenta to lactation. Therefore, before the establishment of lactation relationship, fetuses need to rely on autophagy to degrade the substances stored in the cells for their survival.

During folliculogenesis, a large number of follicles undergo massive degradation at different developmental stages in a process termed as follicular atresia. This atresia was suspected to be a result of apoptosis of granulosa cells. A large number of studies have shown that autophagy is involved in the regulation of granulosa cell apoptosis in different species such as rats and human. The arrested oocytes found in immature rat ovary can simultaneously activate the apoptotic signaling protein caspase-3 and the autophagy signaling protein LAMP1. Follicle stimulating hormone can activate the PI3K/AKT signaling pathway, enhancing the activity of the downstream mTOR pathway that inhibits autophagy and reduces granulosa cell apoptosis. Moreover, the expression of lectin-like oxidized low-density lipoprotein (LOX) can be detected in degenerating antral follicles in the human ovary, while treatment of oxidized low-density lipoprotein (oxLDL) can induce autophagy activation in granulosa cells. Furthermore, granulosa cell apoptosis and autophagy were associated with binding of BCL-2 family proteins to BECN1. In the developing ovary, the transcription factors GATA1 and MYBL2 regulate the expression of VDAC2 by binding to the promoter

region of *Vdac2*. Thus, VDAC2 continuously expresses in oocytes and granulocytes after developmental initiation. A decrease in the level of LC3B, ATG16L1, ATG12, and ATG5 protein has been observed in a VDAC2-overexpressed transgenic pig model. *Vdac2*-knockout resulted in autophagy activation. Further studies have shown that VDAC2 can inhibit autophagy by enhancing the binding of BECN1 to BCL2L1 in granulosa cells (Yuan et al. 2015). However, it is noteworthy that some of the above results revealed the relationship between the autophagic pathway and the apoptotic pathway in folliculogenesis but the exact mechanism of autophagy and apoptotic regulation during follicular atresia is not known. Therefore, it is necessary to carry out more research for its full exploration.

The corpus luteum is a special, transient structure that plays a significant physiological role in female reproductive function. The postovulatory granulosa cells remnants proliferate slightly and the ruptured follicular cavity is closed. Moreover, the capillaries along with follicular cells invade, and the granulosa cells rapidly transform into luteal cells. Luteal cells are the main site of progesterone synthesis. The mother needs to maintain a certain level of progesterone to promote and maintain the development of embryos in vivo during the whole gestation period. *Becn1*-knockout in ovarian granulosa cells of mouse resulted in a significant decrease in lipid droplets in the luteal cells after ovulation. Moreover, the expression of various cholesterol synthesis-related genes such as *Hmgcr, Insig1, Acat1, Lhgc,* and *Star* was also decreased in luteal cells. Consequently, insufficient progesterone secretion resulted in preterm birth. However, the mechanism of the down-regulation of cholesterol synthesis-related genes expression is not known (Gawriluk et al. 2014). Moreover, it has been shown that autophagy is also involved in the regulation of the process of luteal regression. At the end of pregnancy, the uterus releases prostaglandin-2α to initiate corpus luteum regression, consequently, a large number of luteal cells degenerate and die. In rat ovaries, LC3B signaling is increased in degenerating luteal cells and caspase-3 level also upregulated while 3-methyladenine (3-MA) treatment resulted in increased luteal cell apoptosis and luteal regression. Therefore, it is suggested that both the autophagic and apoptotic pathway were involved in the corpus luteum regression. However, its specific regulatory mechanism remains to be studied.

### *21.5.3 Role of Autophagy in Early Embryogenesis*

During mammalian development, the autophagic events firstly take place in the oocytes after fertilization. Soon after fertilization, the oocyte is transformed from the previously highly differentiated gamete state to the undifferentiated embryo state. The transcription of the zygote genome in the fertilized egg can be detected in the late 1-cell stage, while a large amount of maternal protein and mRNAs stored in the oocyte are degraded in 2-cell stage, and eventually, the zygotic genome initiates new protein synthesis. Once the 4- to 8-cell stage is reached, the proteins inside the cells have all been renewed from maternal to newborn. Studies have found that the autophagic activity was relatively low in unfertilized oocytes, and a rapid increase

in autophagy activity was detected within 4 h after fertilization (Tsukamoto et al. 2008). This increase in autophagy activity was not due to starvation of oocytes after ovulation because no rapid changes in autophagy activity were detected in unfertilized oocytes. Further studies have found similar kind of autophagy activation in the parthenogenetic oocyte, so it is believed that this increase in autophagy activity is probably related to calcium oscillations in oocytes. It was found that autophagy activity in zygotic cells was transiently inhibited from the late 1-cell stage to the mid-phase of 2-cell, and was reactivated after entering the late phase of 2-cell stage. It has been suggested that this transient autophagy inhibition is probably to avoid the excessive autophagic degradation of some important maternal protein during cell division. The development of autophagy-deficient embryos formed by the fusion of *Atg5*$^{-/-}$ sperm and *Atg5*$^{-/-}$ oocytes was arrested in the 4-cell to 8-cell stage and finally led to embryo death (Tsukamoto et al. 2008). These results suggest that autophagy plays an essential role in early embryonic development. At the same time, the protein synthesis rate was decreased by 30% compared with the control group in these autophagy-deficient mouse embryos, suggesting that autophagy is likely activated to degrade maternal proteins to provide nutrition to the newly formed embryo. It was further found that during embryo implantation, some environmental factors such as hormonal disorders, and nutrient deficiencies may lead to delayed embryo implantation, causing the embryo to enter in a resting state. For instance, ovariectomy can delay the implantation of the mouse embryo. In these ovariectomized mice, decreased estrogen levels can increase the expression of ATG7 and LC3 proteins, and upregulate autophagy activity in blastocysts suggesting that autophagy may maintain the survival of these resting state embryos by degrading embryonic materials (Lee et al. 2011).

It is noteworthy that although *Atg5*$^{-/-}$ deficient embryos die due to developmental arrest prior to implantation, most of the autophagy-deficient embryos such as *Atg3*$^{-/-}$, *Atg7*$^{-/-}$, *Atg9*$^{-/-}$, and *Atg16L1*$^{-/-}$ successfully passed through the embryonic stage and successfully born by the small amount of maternal-derived proteins carried in the oocyte. However, some other autophagy-related genes deficient embryos, such as *becn1, Ambra1,* and *FIP200,* could not survive and died. *Becn1*$^{-/-}$ embryos showed severe developmental delay, and their volume was abnormally reduced at embryonic day (E)7.5. Moreover, a large number of cells were died and the anterior amniotic tube closure defect was observed in the embryo. These *Becn1*$^{-/-}$ embryos were found to accumulate a large amount of reactive oxygen species (ROS) and inflammatory factors during development, and the loss of autophagy made it impossible to effectively eliminate them, hence, their accumulation resulted in apoptosis (Yue et al. 2003). However, *Becn1*$^{-/-}$ embryonic stem cells can survive, indicating that *Becn1* is an alternative gene in vitro, but it is an indispensable key factor for in vivo development. In addition, autophagy is also involved in organ differentiation during embryonic development. *Becn1* regulates activating molecule in beclin1-regulated autophagy (Ambra1) to activate autophagy by binding to BECN1. The neural tube development of *Ambra1*$^{-/-}$ embryos showed over-proliferation and resulted in E10-14 embryonic lethality. The 200-kDa FAK family interaction protein (FIP200) can form a complex with ATG13, ULK1, and

ATG101, and participates in the regulation of the autophagy initiation. *FIP200*$^{-/-}$ embryos showed embryonic heart and liver tissue development defects, showing E13.5-16.5 embryonic lethality. It is still unclear why different autophagy-related gene knockout models lead to so diverse phenotypes. Some studies suggested that these phenotypes are perhaps because of different Atg genes that participate in the different steps of the autophagy process. *Becn1* and *FIP200* play a main role at the early autophagy nucleation stage, while *Atg3, Atg5, Atg7,* and *Atg16L1* all play a role in the relatively advanced stage of autophagosome elongation. Therefore, the upstream effects of *Becn1* and *FIP200* may be more preferential, and their deletion led to severe developmentally defected phenotypes. However, these results are contrary to the phenotype of *Atg9*$^{-/-}$. Atg9 plays a role in the early stage of autophagy, but its defective phenotype is not serious, which may indicate that there may be some functional redundancy between these Atg genes, or different compensatory mechanisms exist in different autophagy-related genes-knockout mice models. Therefore, it is necessary to further explore in future, whether autophagy-related genes have any non-autophagic functions and whether these functions contribute to embryonic development.

### *21.5.4 Role of Autophagy in the Placenta*

The placenta is a unique, temporary, and complex organ that begins to form after blastocyst implantation into the uterus. It mediates maternal–fetal interactions, plays an important role in fetal development and maintains pregnancy. The trophoblast cells including cytotrophoblast (CTB) cells, syncytiotrophoblast (STB) cells and extravillous trophoblast (EVT) cells are the major cell types in the placenta. EVT cells can be further differentiated into intravascular cytotrophoblast (iEVT) cells and endovascular trophoblast (enEVT) cells. In the early stages of pregnancy, enEVT can enter in mother's blood vessels and accumulate inside its lumen, forming a structure of a "trophoblastic plug" that maintains embryonic and placental development in a hypoxic state. enEVT can also invade the uterine spiral arteries and replace the arterial vascular endothelial cells to promote blood perfusion in the placenta, thereby promoting fetal development. iEVT can invade the maternal myometrial tissue, anchoring the placenta to the uterine wall. The autophagy activity in the cells was found to be increased during the differentiation of EVT in early pregnancy. EVT autophagic activity, invasion, and vascular remodeling activity were found to be decreased significantly in ATG4B-mutant EVT cells. At the same time, the expression of hypoxia-inducible factor-1α (HIF1α) in autophagy-deficient enEVT cells did not change significantly compared with the control cells, suggesting that autophagy is involved in the regulation of invasion activity of enEVT cells independent of HIF1α. The expression of ATG9L2 and the autophagy activity was upregulated in the trophoblast cells to increase its resistance to external pathogenic factors during the formation of STB. In addition, when the mother is in a state of starvation, autophagy activity is induced

in the placenta to maintain energy supply to the fetus, especially the fetal brain, by degrading the intracellular substances of the placenta.

Autophagy is also involved in the regulation of labor. The autophagic activity of cells in the central region is found lower than that in the marginal region of the placenta of caesarean section women. Moreover, autophagic activity in the placenta of caesarean section women was higher than that in the normal labor group. Previous studies have found that in *atg16l1*-knockout mice, the secretion of interleukin-1β (IL-1β) was much higher after lipopolysaccharide stimulation of macrophages. Further experiments demonstrated that autophagy can inhibit the synthesis of IL-1β and interleukin-18 (IL-18) by inhibiting the activation of inflammatory bodies. According to the above results, labor pain may promote the release of IL-1β by inhibiting autophagy activity in placental cells during normal labor. Consequently, IL-1β causes cervical ripening and further aggravates labor pain, thereby promoting the process of childbirth. In inflammation-induced preterm labor, the expression of *Atg4c* and *Atg7* in the uterus and placenta decreased significantly, which in turn caused a decrease in intracellular autophagy activity, accompanied by activation of nuclear transcription factor-κB (NF-κB) signaling pathway and increased secretion of various inflammatory factors (Agrawal et al. 2015). Considering the above-described results, it can be suggested that autophagy may affect the delivery process of the fetus in many different ways, but its exact molecular mechanism remains to be studied.

Autophagy is also involved in the regulation of some pregnancy-associated diseases. Preeclampsia is a serious pregnancy-related syndrome that is prone to cause preterm birth, intrauterine growth restriction (IUGR) and neonatal death. Researchers found that in the placenta of patients with preeclampsia, the LC3B protein signal in the trophoblast cells was increased, and at the same time, similar observations were also made in the placenta of some IUGR patients. It is speculated that autophagy activity in the placenta of these patients was enhanced. However, it must be noted that in other studies, P62/SQSTM1 protein was detected in the EVT cells of the preeclampsia patients, suggesting that autophagy was inhibited in EVT cells (Nakashima et al. 2013). Therefore, many problems still remain to be solved in the application of autophagy in the detection and treatment of clinical pregnancy complications.

## 21.6 Role of Autophagy in the Reproduction of Other Animals

There are only a few studies on the role of autophagy in the reproduction of other species at present. However, some studies have reported that autophagy (lipophagy) is involved in the regulation of lipid droplet metabolism in Sertoli cells of Chinese soft-shelled turtle. *Ol-Epg5*-knockout resulted in autophagy defects in the medaka fish, and as a result the removal of mitochondria and germplasm was blocked in germ cells. Moreover, autophagy deficiency resulted in abnormal spermatogenesis in *Ol-epg5*-knockout medaka. Autophagy also participates in the clearance of follicles

undergoing atresia in certain oviparous teleost. These studies have greatly expanded our understanding of autophagic functions in the reproductive system of different species, but still, there are many more unsolved mysteries.

In plants, reproduction is accompanied by the orderly sequence of a large number of PCD events, and once the PCD is affected, it often leads to plant infertility. For example, when microspores are released, the tapetum cells undergo PCD to provide various nutrients for the development of pollen and sporopollenin. In rice, *Osatg7-1* deficiency causes the reduction of autophagosomes in the mononuclear tapetum cells, which led to the reduction of anther dehiscence. The pollen of the *Osatg7-1* mutant was premature due to the defects in the anther during pollen maturation. The *Osatg9* mutation also produces a similar phenotype. These results indicate that autophagy mainly takes place in tapetum cells after meiosis, and its function is perhaps related to the metabolism of intracellular substances such as pigment bodies and lipid droplets during pollen maturation. Furthermore, *Osatg7-1* mutant exhibited a special dense layer of tapetum tissue, indicating that autophagy is involved in the regulation of PCD in tapetum cells. ROS was suspected to be an important regulator of the PCD. In the *mads3* mutant rice, the level of ROS abnormally fluctuated, leading to premature PCD of tapetum (Hu et al. 2011). In some autophagy mutants of *Arabidopsis* such as *ATG5*$^{-/-}$, ROS accumulation in the leaves indicates that autophagy may regulate the PCD events in tapetum cells by affecting the ROS signaling pathway. In addition, in the embryogenesis of Norway spruce, *Picea abies,* the embryo-suspensor cells need to undergo vacuolar death, which is essential for embryonic development. Autophagy was also involved in the regulation of this process. The autophagic activity in the suspensor cells was continuously enhanced during the growth of lytic vacuole. While inhibition of ATG5 and ATG6 causes improper autolysis of suspensor cells resulting in cell necrosis, impaired cell elongation and morphologically abnormal suspensor leading to the embryonic arrest.

## 21.7 Conclusion and Perspectives

The reproductive process is a special part of the individual's life activities that includes various unique biological events such as gametogenesis, fertilization, and embryonic development. Reproduction involves a series of biological activities, such as cell proliferation, differentiation, and apoptosis (Fig. 21.2). Autophagy is also an integral part of these basic life laws. Autophagy promotes cell survival, intercellular interactions, and guides cell function by degrading excess proteins and organelles during the reproductive process. This degradation and renovation play an extremely important role in the entire reproductive process.

Although there have been some studies on molecular mechanism and role of autophagy in the reproductive process, still a number of questions remain to be solved. For example, gametogenesis is accompanied by a large number of changes in the microenvironment of the reproductive organs, and the relationship between these changes and autophagy is largely a "black box". Early embryogenesis is accompanied

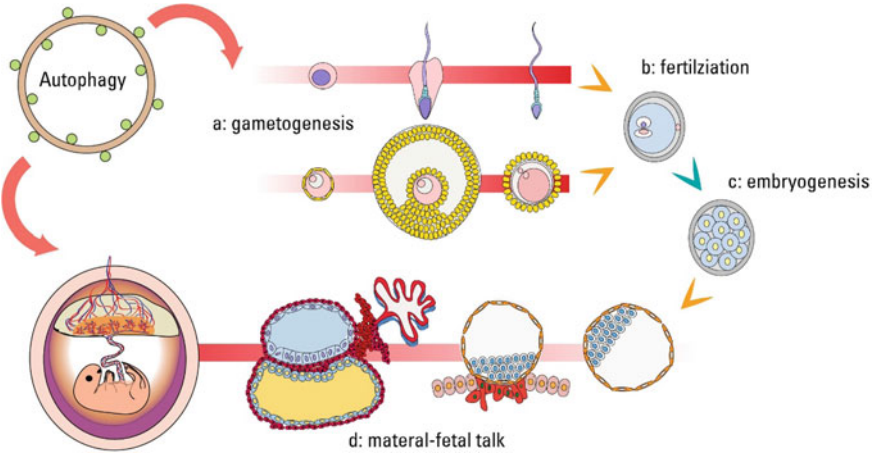


**Fig. 21.2** Autophagy widely participates in mammalian reproduction processes. Autophagy regulates a number of mammalian reproduction events, such as gametogenesis, fertilization, embryogenesis and maternal–fetal talk

by the dramatic changes in autophagy activity, where did these changes come from? Autophagy activity also found to be changed in a number of reproductive disorders. What is the relationship between autophagy and these disorders? Reproductive health problems are becoming more and more serious globally. We are hopeful that progress in understanding the role of autophagy in reproduction will provide new ideas for the treatment of reproduction-related diseases.

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# Chapter 22
# Autophagy in Development and Differentiation


**Yang-Xi Hu, Xiao-Shuai Han and Qing Jing**

**Abstract** Autophagy is crucial in the differentiation and development of both mammals and invertebrates, as a rapid response to environmental and hormonal stimuli. Autophagy is also important for intracellular renewal, maintaining the health of terminally differentiated cells. Studies of *Drosophila*, *Caenorhabditis elegans*, and other species revealed abnormal autophagy lead to developmental and differential abnormality, including those in salivary glands and midgut development, protein aggregation, removal of apoptotic cell corpses, and development of dauer and synapse. Autophagy also participates in the development of mammalian embryos before implantation into the uterus, adaption to the nascent hunger environment, blood cells production, and cell differentiation in adipogenesis. Autophagy found in various stem cells, like hematopoietic stem cells, bone marrow mesenchymal stem cells and neural stem cells (NSCs), is tightly associated with their self-renewal, directed differentiation, and senescence.

**Keywords** Autophagy · Development · Differentiation · Invertebrates · Stem cells

## Abbreviations

| | |
|---|---|
| 3-MA | 3-methyladenine |
| Ambra1 | Activating molecule in Beclin-1-regulated autophagy |
| AMPK | Adenosine 5′-monophosphate (AMP)-activated protein kinase |
| ATG | Autophagy-associated gene |
| ATP | Adenosine triphosphate |
| CAV | Caveolin-1 |

Y.-X. Hu
Department of Cardiology, Shanghai Changhai Hospital, 168 Changhai Road, Shanghai 200433, China

X.-S. Han · Q. Jing (✉)
CAS Key Lab of Tissue Microenvironment and Tumor, Shanghai Institute of Nutrition and Health, Chinese Academy of Sciences, 320 Yueyang Road, Shanghai 200031, China
e-mail: qjing@sibs.ac.cn



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| | |
|---|---|
| CDK | Cycling-dependent kinase |
| CMA | Chaperone-mediated autophagy |
| CR | Calorie restriction |
| CSC | Cancer stem cell |
| DCIS | Ductal carcinoma in situ |
| DNA | Deoxyribonucleic acid |
| DP110 | Crosophila PI3K 110 subunit |
| EMT | Epithelial mesenchymal transition |
| ESCs | Embryonic stem cells |
| FGF | Fibroblast growth factor |
| GABARAP | Gamma-aminobutyric acid receptor-associated protein |
| GFER | Growth factor, augmenter of liver regeneration |
| GFP | Green fluorescent protein |
| GTP | Guanosine triphosphate |
| HDAC | Histone deacetylase |
| HSCs | Hematopoietic stem cells |
| LC3 | Microtubule-associated protein 1 light chain 3 |
| LIF | Leukemia inhibitory factor |
| LT-HSC | Long-term stem cell |
| MDS | Myelodysplastic syndrome |
| MEF | Mouse embryonic fibroblast |
| MHC | Major histocompatibility complex |
| MOs | Membranous organelles |
| mRNA | Messenger ribonucleic acid |
| MSCs | Bone marrow-derived mesenchymal stem cells |
| mtDNA | Mitochondrial deoxyribonucleic acid |
| mTOR | Mammalian target of rapamycin |
| NAC | N-acetyl cysteine |
| NADPH | Reduced form of nicotinamide adenine dinucleotide phosphate |
| NSCs | Neural stem cells |
| PRMT | Protein arginine methyltransferase |
| PS | Phosphatidyl serine |
| Rab32 | Ras-related protein rab-32 |
| RAS | Rat sarcoma viral oncogene |
| RNA | Ribonucleic acid |
| ROS | Reactive oxygen species |
| SEAP-1 | Sekretiertealkalische phosphatase-1 |
| siRNA | Small interfering RNA |
| SVZ | Subventricular zone |
| TOR | Target of rapamycin |
| TSCs | Tumor stem cells |
| UCP | Uncoupling protein |
| ULK1/2 | UNC-51-like kinase 1 and 2 |
| UPS | Ubiquitin-proteasome system |

Autophagy plays an important role in differentiation and development. As a dynamic, highly inducible catabolic process that responds to environmental and hormonal stimuli, autophagy can drive rapid changes in cells to ensure proper differentiation and/or development. The organisms deficient in autophagy, including fungi, protozoa, worms, and insects, exhibit a variety of abnormalities in differentiation and development. These abnormalities may be due to defects in overall autophagy and may also be due to the failure of specific components to degrade by selective autophagy. In mammals, autophagy is important for the development of embryos before implantation into the uterus, the nascent hunger environment, red blood cells and lymphocyte production, and cell differentiation in adipogenesis. In addition, autophagy is also important for intracellular renewal, and is particularly important for maintaining the health of terminally differentiated cells.

## 22.1 The Role of Autophagy in the Development and Differentiation of Invertebrates

Autophagy is a conserved catabolic pathway that is widespread from yeast to mammals and humans. Under normal growth conditions, organisms can maintain protein and organelle renewal through basal autophagy to provide energy for life activities. Under stress conditions, the organism can induce autophagy activation to maintain the life of the cells, so that cells can survive the adverse internal and external environment and maintain cell survival. *Drosophila* and *C. elegans* as model organisms provide a good platform for us to study autophagy. However, autophagy in invertebrates is different from that in mammals and humans. This section focuses on the role of autophagy in the development and differentiation of invertebrates.

### *22.1.1 Autophagy in the Development and Differentiation of* Drosophila

*Drosophila* is an ideal model for studying the role of autophagy in multicellular organisms, and its steroid and growth factor signaling pathways that regulate autophagy are similar to those in humans. Autophagy-related genes (Atg) and their regulatory factors are highly conserved in humans and *Drosophila* (Baehrecke 2003). Unlike mammals, fruit flies have almost no genetic redundancy, and most of their genes are single copies (Rusten et al. 2004). During *Drosophila* development, large cells and tissues, such as the giant larvae salivary glands, may be too large to be degraded by phagocytosis alone, and they require autophagy to degrade the cytoplasm in large amounts. Autophagy contributes to the redistribution of nutrient resources and the survival of multicellular organisms. *Drosophila* autophagic cell death occurs when

animals have no external nutrients and must rely on their nutrient storage to maintain and develop adult structures. In addition, most ATG mutants have lethality, suggesting that autophagy is required for metamorphosis. Moreover, autophagy in *Drosophila* tissue is usually induced by nutrient starvation or ecdysterone.

#### 22.1.1.1 Autophagy of *Drosophila* Fat Body

Autophagy plays a decisive role in the proper use of nutrients during the development of *Drosophila* larvae. The glycogen, lipids, and proteins of *Drosophila* are stored in the fat body. When the *Drosophila* larvae lack amino acids, the autophagy in the fat body is induced by starvation and regulated by the Tor signal. Tor is a key regulator of cell growth that inhibits autophagy by phosphorylating Atg1. Inactivation of Tor signal can induce autophagy in the larval fat body, while activation of Tor or expression of type I Pi3k can inhibit starvation-induced autophagy in the fat body, and reduce the viability of larvae during starvation (Britton et al. 2002). In the fat body of *Drosophila* larvae, overexpression of Atg1 is able to inhibit cell growth by negative feedback regulation of Tor. Autophagy is also important for lipid metabolism in *Drosophila* fat bodies. The lipid droplets are small in Atg7 nonfunctional mutant fat bodies suggesting a defect in lipid deposition. Members of the Rab small GTPase family are also involved in lipid droplet formation. The researchers found that 18 Rab proteins increase or decrease the size of lipid droplets. The lipid droplets in the Rab32 mutant are small and the autophagy function of the fat body is impaired. Since Rab32 is localized to autophagosomes rather than lipid droplets suggesting that its role in regulating lipid droplet size is achieved by regulation of autophagy (Wang et al. 2012).

#### 22.1.1.2 Autophagy in the Development of Salivary Glands and Midgut in *Drosophila*

Autophagy also plays a role in the development of other tissues, including the salivary glands and midgut. Autophagy mainly effects during the larval stage suggesting an important role for autophagy in survival and tissue growth during the non-feeding period. In the process of degradation of salivary glands, growth inhibition requires the participation of autophagy. This process is regulated by the Type I PI3K pathway. Expression of activated Ras, Akt, or Dp110 (a type I PI3K contact reaction subunit) can maintain salivary gland growth and inhibit autophagy and glandular degradation. In addition, co-expression of negative Tor and Ras or Dp110 can inhibit excessive growth and degradation of salivary glands, suggesting that TOR cell growth regulation signals can inhibit autophagy and prevent salivary gland degradation. Moreover, overexpression of Atg1 can inhibit the salivary gland Dp110 phenotype, while the loss of Atg function can cause the persistence of salivary glands, suggesting that growth inhibition and autophagy are essential for maintaining salivary gland degradation (Tracy and Baehrecke 2013).

In the midgut, growth inhibition occurs before programmed cell death is induced. When cell growth in the midgut is maintained by activation of Ras or Dp110 expression, autophagy is inhibited and degradation of the midgut is delayed. Conversely, inhibition of growth by expression of the negative regulator of type I PI3K signaling, Pten or Tsc1/Tsc2, can result in a decrease of midgut and premature autophagy induction. This growth inhibition can be inhibited by knockout of Atg1 or Atg18 in the context of Pten or Tsc1/Tsc2 expression (Denton et al. 2012). Knocking out the Atg gene alone in the midgut resulted in a sustained expression of the PI3K growth signal and a significant delay in midgut degradation suggesting that the correlation between midgut growth and autophagy is similar to that in the salivary glands (Tracy and Baehrecke 2013).

The evolutionarily conserved Warts (Wts)/Hippo (Hpo) signaling pathway is an important negative regulator of cell growth, which works by inactivating Yorkie (Yki) in Wts pathway dysfunction or Yki overexpression can lead to tissue overgrowth (Huang et al. 2005). Wts is necessary for the induction of growth arrest and autophagy in salivary gland degradation. Mutation of Wts and Hpo or knockout of sav and mats to block this pathway prevents the degradation of salivary glands. Overexpression of Yki did not inhibit the degradation of salivary glands, suggesting that Wts regulates the growth of salivary glands through a non-Yki-dependent pathway. Wts mutants can cause sustained expression of salivary gland type I PI3K signaling. Knockout of chico or expression of Tor can inhibit defects in Wts cell death, suggesting that Wts regulates salivary gland cell growth via a type I PI3K-dependent pathway (Dutta and Baehrecke 2008).

### *22.1.2 Autophagy in the Development and Differentiation of* C. elegans

During embryonic development of *C. elegans* (hereinafter referred to as nematode), many protein aggregates are selectively degraded by autophagy, including P particles that are specifically present in germ cells. The P particle is derived from the mother and is a specific protein–RNA collection that is interspersed in the cytoplasm in newly fertilized embryos. However, in the asymmetric division of cells, they are specifically localized to the P1, P2, P3, and P4 segregating lines, and are ultimately localized to the Z2 and Z4 embryonic early cells that divided from P4 (Strome 2005). Pgl-1 and Pgl-3 are the components of P particles, which enter the parietal cells separately during early asymmetric cell division and are degraded by autophagy. In autophagy mutants, Pgl-1 and Pgl-3 form various aggregates in body wall cells. This process, as well as the autophagic degradation of Pgl-1 and Pgl-3, requires the involvement of the protein oligomer Sepa-1 (Zhang et al. 2009). In the early embryos, Sepa-1 forms a polymer that is independent of Pgl-1 and Pgl-3 and self-clears by autophagy, resulting in the absence of Sepa-1 in late embryos. In autophagy mutants, Sepa-1 is

completely colocalized with Pgl-1 and Pgl-3 to form a polymer called Pgl particles, which persist throughout the embryo formation process (Yang and Zhang 2014).

Like mammalian and *Drosophila* p62/Sqstm1, the homolog of p62, Sqst-1, in *C. elegans* is also degraded by autophagy (Yang and Zhang 2014). The level of Sqst-1 in autophagic mutant embryos is significantly increased and aggregated into a large number of polymers different from Pgl particles. Other proteins such as the Sepa-1 family and W07G4.5 are also selectively cleared by autophagy during embryogenesis. Since the development of nematode embryos relies on the degradation of maternal material rather than external nutrients, many protein aggregates are selectively degraded by autophagy during embryogenesis. Autophagic degradation of P particles and other protein substrates provides nutrients for embryo formation. Besides, some protein factors only play a role in specific embryonic stages, and their persistence is detrimental to embryonic development (Yang and Zhang 2014).

In animals, children inherit the mitochondrial gene (mtDNA) only from the mother, which is called maternally inherited. However, the maternal inheritance of mtDNA of *C. elegans* is a passive process that selectively degrades the mitochondria and mtRNA of the paternal line by autophagy (Yang and Zhang 2014). In the diploid and tetraploid phases of nematode embryos, sperm-derived mitochondria are randomly segmented into the blastomere but are cleared before the 64-cell embryo. In fertilized eggs with autophagy mutations, the mitochondria and mtDNA of the paternal line persist in the late embryonic stage and even in the larval stage. The paternal mitochondria are associated with early embryonic LGG-1 labeled autophagosomes. Ubiquitination acts as a trigger mechanism in the process of selective autophagy to degrade multiple autophagy substrates, but the mitochondria of nematode sperm are not ubiquitinated. Thus, how they are recognized and degraded by autophagy mechanisms is still unclear.

The nematode sperm cells also contain membrane organelles (MOs), which have specialized vesicular structures. MOs are also engulfed by LGG-1 positive autophagosomes (Yang and Zhang 2014). Unlike mitochondria, MOs are ubiquitinated in newly fertilized embryos and triggers selective recognition and degradation of autophagy. During the spermatogenesis of the nematode, the residual body is produced during the second meiosis of the spermatocytes. All ribosomes and almost all actin and myosin, including most tubulin and some of the intima, are segmented into the residual. In the autophagy mutant, the residual body did not accumulate significantly. The residual body expresses a phosphatidylserine (PS) signal on the surface, which can be recognized and engulfed by gonadal epithelial cells. Efficient clearance of the residual body regulates the number of sperm cells and the efficient delivery of sperm cells in male mating.

## 22.2 Autophagy and Mammalian Embryonic Development

As a highly inducible dynamic metabolic process under the influence of the environment, hormones and other factors, autophagy can drive the rapid response of cells necessary for development. In fact, some developmental abnormalities can be exhibited in autophagy-deficient organisms. These abnormalities may be due to defects in overall autophagy, or maybe due to defects in selective autophagy degradation of certain specific components. In mammals, autophagy is important for early embryo development. This section focuses on the role of autophagy in mammalian embryo development.

### *22.2.1 Autophagy in Fertilized Egg Development and the Neonatal Period*

Autophagy during mammalian development occurs at the ear of fertilization. Atg5 is essential for the development of the embryo before implantation into the uterus. Oocytes are highly differentiated cells that rapidly transform into a highly undifferentiated state after fertilization. This "reprogramming" occurs simultaneously in the nucleus and cytoplasm. The maternal mRNA and protein rapidly degrade after the two cell phases of the embryo and begin to synthesize new mRNAs and proteins encoded by the fertilized egg genome, resulting in significant changes in the types of proteins synthesized after the tetraploid to octoploid cell stage (van Blerkom and Brockway 1975). Moreover, the degradation of maternal proteins and RNA may be required for activation of the fertilized egg genome. The level of autophagy in unfertilized egg cells was very low, but autophagy was induced in a large amount for 4 h after fertilization (Tsukamoto et al. 2008). At this point, the induction of autophagy is entirely determined by fertilization, not because of cell starvation after ovulation. Autophagy at this time is triggered by calcium oscillations since parthenogenesis also induces autophagy in egg cells. In particular, autophagy is transiently suppressed during the late haploid to mid-diploid and then activated again. Since inhibition of autophagy in mitotic cells can also be observed in artificially cultured mammalian cells, this may be a general mechanism by which cells prevent important nuclear factors from being degraded during cell division (Tsukamoto et al. 2008). The ability of $Atg5^{-/-}$ mice to survive early embryogenesis is mainly due to the presence of Atg5 protein inherited from the mother in the egg cells. After the egg cell-specific Atg5 knockout mice were used to clear the maternal Atg5 protein, the embryos were lethal in the tetraploid to the octoploid stage. Autophagy is also necessary to remove nonessential proteins and organelles that accumulate in the egg cells, or to promote "reconstruction" by degrading the parent material that inhibits the fertilized egg genome (Mizushima and Levine 2010).

## *22.2.2 Autophagy in Embryonic Development*

### 22.2.2.1 Autophagy of Embryonic Stem Cells in Early Embryonic Development

Embryonic stem cells (ESCs) are pluripotent stem cells in early embryonic stages that can regenerate and differentiate into major germ layers: ectoderm, endoderm, and mesoderm. Studies of ESCs can demonstrate the role of autophagy in the early development of mammals. The first study to explore the molecular mechanism of autophagy in mammalian cells is the use of mouse embryonic stem cells. This study revealed that protein structure conversion of 14C-labeled amino acids can be induced by amino acid starvation in mouse ESCs, which is significantly reduced (>50%) in $Atg5^{-/-}$ mouse ESCs, suggesting that autophagy is important in protein structure conversion (Guan et al. 2013). However, the deletion of Atg5 protein in these cells does not affect their growth rate or clonal morphology under complete culture conditions. Similarly, $bedlin1^{-/-}$ mouse ESCs do not express Vps30/Atg6 homologs, nor do they exhibit growth defects under non-starved conditions, but these cells do not form embryoid bodies (Qu et al. 2003).

The role of autophagy in the function of ESCs in late embryonic development is not well defined. $Atg3^{-/-}$, $Atg5^{-/-}$, $Atg7^{-/-}$, $Atg9^{-/-}$, or $Atg16L1^{-/-}$ mice do not have any anatomical abnormalities after birth, suggesting that autophagy does not play a central role in the timing and coordination of developmental embryo differentiation (Guan et al. 2013). These autophagy-deficient embryos can develop to the octoploid phase due to the presence of the Atg protein inherited from the mother in the cytoplasm of the egg. However, compared to wild-type species, autophagy-deficient nascent organisms are lighter and die 1–2 days after birth, which may be due to a sucking defect caused by neurodevelopmental defects (Guan et al. 2013).

Other pathways, such as the ubiquitin-proteasome system (UPS), may also compensate for the loss of autophagic activity of ESCs to some extent (Guan et al. 2013). The activity of the human ESCs proteasome is high, and this should be down-regulated during the differentiation process, suggesting that the high activity of the proteasome is an intrinsic property of human ESCs (Vilchez et al. 2012). Moreover, human ESCs lose their high proteasome activity during continuous differentiation, and differentiated cells also exhibit increased levels of polyubiquitin. However, another study showed that the terminal product produced by carboxylation or advanced glycosylation was reduced in the accumulation of murine ESCs, which may be due to the enhanced autophagy activity during the differentiation (Hernebring et al. 2006). Unlike other Atg genes, $beclin1^{-/-}$ mice showed delayed development at E6.5, amnion fold development failed, and died early in the embryo (Yue et al. 2003). Beclin 1 acts on membrane trafficking through its binding partners and regulation of PIK3C3 lipid kinase, rather than through autophagy, which can also explain the more severe phenotype of $beclin1^{-/-}$ mice (Funderburk et al. 2010).

#### 22.2.2.2 Autophagy and Embryoid Body Formation

Embryonic development of ESCs in Atg5$^{-/-}$ mouse can proceed normally, but autophagy in in vitro development models may be important only in specific circumstances. The behavior of autophagy-deficient ESCs cultured outside the blastocysts was altered compared to wild-type mouse ESCs (Qu et al. 2007). Wild-type mouse ESCs can form undifferentiated cell colonies in the absence of feeder cells and leukemia inhibitory factor (LIF), and then develop into a single embryoid body, in which the outer layer is the primitive endoderm cells, and the inner core is ectoderm cells. When the inner ectoderm cells show a programmed death, a saclike embryoid body is formed. These events mimic the formation of cavities in early embryonic development. In this model of early embryo development, autophagy of wild-type ESCs runs through the entire developmental process. Atg5$^{-/-}$ and Beclin1$^{-/-}$ ESCs cultured in vitro were unable to undergo vacuolation compared to wild-type ESCs. The embryoid bodies produced by these Atg5$^{-/-}$ and Beclin1$^{-/-}$ cells have defects in the production of ATP when the inversion of methyl pyruvate is required, cavity formation is restored, and apoptotic bodies are removed. Methyl pyruvate is a cell-permeable form of pyruvate that is capable of participating in the mitochondrial tricarboxylic acid cycle. These results suggest that embryoid body cells rely on autophagy to maintain energy homeostasis, which may act through the production of amino acids (Guan et al. 2013).

mTOR also plays an important role in regulating the pluripotency and self-renewal of human ESCs. Inhibition of mTOR by rapamycin or inhibition of mTOR transcription in human ESCs results in a significant decrease in pluripotency-regulated transcription factors (POU5F1/OCT4 and SOX2) levels, promotion of mesoderm and endoderm activity, and decreased proliferation (Zhou et al. 2009). Disrupting the kinase activity of mTOR in mouse ESCs can cause cells to become smaller and inhibit proliferation (Murakami et al. 2004). The regulation of mTOR differs depending on the differentiation pathway. Transcriptome analysis of the differentiation of human ESCs into neural cells suggests that the transcripts associated with mTOR is up-regulated (Fathi et al. 2011). Therefore, autophagy plays an indispensable role in the loss of ESCs pluripotency and self-renewal (Guan et al. 2013).

#### 22.2.2.3 Midbophagy During Embryonic Development

The presence of multiple forms of autophagy (including nonspecific autophagy) suggests that each form of autophagy is regulated by independent spatiotemporal activities. Intermediate clearance mediated by intermediate autophagy occurs when ESCs differentiate (Kuo et al. 2011). Intermediates are organelles formed between daughter cells during cytokinesis and are essential for their eventual division. The intermediate selectively accumulates in stem cells and pluripotent stem cells cultured in vivo or in vitro, thereby maintaining or enhancing the pluripotency of stem cells.

Correct regulation of intermediate autophagy is necessary to maintain the pluripotent state of ESCs. Autophagy induced by starvation or rapamycin may stimulate differentiation by clearance of intermediates (Guan et al. 2013).

## 22.3 Autophagy and Differentiation of Stem Cells

Adult stem cells, which are widely distributed in various organs and tissues of the body, play an irreplaceable role in organ development, tissue renewal, and related diseases because of their self-renewal and directed differentiation potential. Autophagy, as a very important cellular activity under normal physiological conditions and pathological processes, directly affects the homeostasis and directed differentiation of various adult stem cells, and determines the response and behavior of adult stem cells under pathological conditions. However, the maintenance and function of autophagy in adult stem cells is beginning to emerge relative to a large number of known studies on somatic cells or cell lines. Here, we will introduce and discuss the mechanism and regulation of autophagy in adult stem cells (mainly including hematopoietic stem cells, neural progenitor cells, cardiac stem cells, and several cancer stem cells).

### *22.3.1 Hematopoietic Stem Cells and Autophagy*

Hematopoietic stem cells have been one of the most well-recognized and well-studied stem cells in adult stem cells due to their availability and sophisticated in vitro culture methods. The development of the blood system is a well-defined developmental pattern. A small number of long-term stem cells (LT-HSCs) are relatively static for a long time, and the precursor cells produced by their differentiation rapidly proliferate and differentiate into different types of mature blood cell lines, this process provides millions of fresh blood cells every day for our body. This section focuses on the role of autophagy in the process of quiescent, self-renewal, and differentiation of hematopoietic stem cells during normal development.

In normal development and under physiological conditions, true hematopoietic stem cells maintain a very fine balance between quiescence, self-renewal, and directed differentiation. Although the current research on the role of autophagy in hematopoietic stem cells is not very rich, we know that autophagy is highly activated in human hematopoietic stem cells. Moreover, in mouse stem cells, studies have found that both autophagy can be significantly stimulated by Foxo3, whether it is a decrease in cytokines or a shortage of carbon sources (Warr et al. 2013). It is speculated that autophagy can be used as a key factor to break the delicate balance of hematopoietic stem cells in homeostasis maintenance, self-renewal, and differentiation.

#### 22.3.1.1 Autophagy and HSCs

Unlike ubiquitination degradation, autophagy can degrade damaged cytoplasmic organelles including mitochondria, endoplasmic reticulum, and ribosomes. The oxygen content of the bone marrow microenvironment in which hematopoietic stem cells are located is usually relatively low, and hematopoietic stem cells tend to maintain a low level of active oxygen ROS in the microenvironment, tending to avoid energy supply by oxidative phosphorylation. In accordance with this, only a small number of mitochondria are contained in normal hematopoietic stem cells, and increasing the rate of mitochondrial production will impair the maintenance of hematopoietic stem cell homeostasis. The transition of hematopoietic stem cells from a quiescent state to proliferation or differentiated state is accompanied by an increase in mTOR activity and consequent increase in reactive oxygen species ROS levels (Chen et al. 2008). Antioxidants and rapamycin restore the ability of cells with high ROS activity to self-renew. Interestingly, mitochondria of hematopoietic stem cell were found to be in a low metabolic state (lower mitochondrial inner membrane voltage difference) by Rhodamine 123 (a reactive dye showing mitochondrial membrane potential). In addition, the researchers found that if hematopoietic stem cells are transplanted from a hypoxic microenvironment to an environment rich in oxygen or high ROS activity, the cells tend to differentiate into myeloid cells (Owusu-Ansah and Banerjee 2009). Interestingly, differentiation toward the myeloid line is one of the characteristics of hematopoietic stem cells that are in senescence or lack of autophagy.

In fact, Atg7 knockout mouse hematopoietic stem cells can not normally regenerate due to mitochondria, the damage of which can cause ROS accumulation. It has been found that in the mouse myelodysplastic syndrome (MDS) model, it is due to the accumulation of ROS that causes hematopoietic stem cells to differentiate into myeloid cells. Similar characterization also occurred in mice deficient in the Rb1cc1/Fip200 gene, i.e., abnormally increased myeloid differentiation. However, it is unclear whether ROS are elevated in the microenvironment caused by osteoblasts, macrophages or hematopoietic stem cells. Researchers speculate that this may be similar to the metabolic relationship between breast cancer cells and stromal cells, in which ROS produced by stromal cell metabolism affect tumor growth (Pavlides et al. 2009).

Finally, more and more experiments have shown that mutation of mTOR upstream genes (including Akt, Pten, etc.) in mouse models can inhibit the autophagy pathway, resulting in a hematopoietic stem cell phenotype like that of Atg7 knockout mice. Ly6g and Itgam-positive myeloid cells differentiate. Conversely, activation of autophagy by knocking out the Raptor gene in the mTORC1 complex results in decreased differentiation of hematopoietic stem cell myeloid cells (Hoshii et al. 2012). However, further experimental evidence is needed to demonstrate that mTOR affects hematopoietic stem cell differentiation by regulating autophagy, as mTOR can also regulate important processes such as protein synthesis, mitochondrial production, cell growth and proliferation, and cell migration.

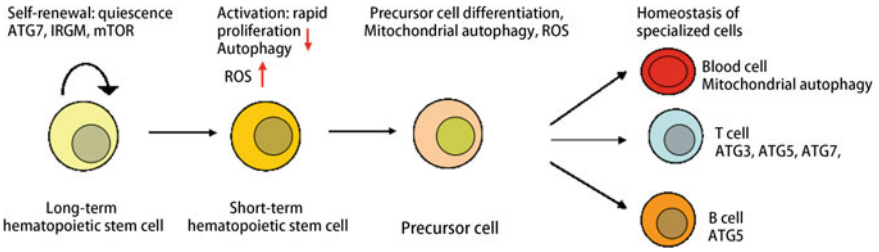


**Fig. 22.1** Autophagy function in hematopoietic stem cells. In the process of establishing hematopoiesis, various types of blood cells and lymphocytes are derived from hematopoietic stem cells. Long-term hematopoietic stem cells are first activated and transformed into short-term hematopoietic stem cells, then specialized into a variety of precursor cells, and finally differentiated into blood cells of different lineages. Autophagy plays an important role in the maintenance of hematopoietic stem cells in self-renewal and quiescent state, the maintenance of pluripotency of precursor cells, and the maintenance of terminal cell homeostasis and metabolism

In summary, there is enough evidence to prove that autophagy plays an important role in the process of hematopoietic stem differentiation and pluripotency maintenance (Fig. 22.1).

## *22.3.2 Autophagy in Other Tissue Stem Cells*

### 22.3.2.1 Autophagy in Bone Marrow Mesenchymal Stem Cells (MSCs)

MSCs are a series of multifunctional precursor cells with the ability to differentiate into mesenchymal cell populations including fat, bone, cartilage, and muscle. Although mesenchymal stem cells are originally found in the bone marrow, they are also present in organs and tissues such as muscle, fat, kidney, pancreas, brain, and liver. The role of autophagy in mesenchymal stem cells is still unclear. At present, the only knowledge is from a few articles on bone marrow mesenchymal stem cells. It has recently been reported that primary mesenchymal stem cells have sustained high levels of autophagy, and this high level of basal autophagy is diminished when they differentiate into osteoblasts (Oliver et al. 2012). Consistent with this observation, mesenchymal stem cells isolated in LC3-GFP transgenic mice showed a large amount of fluorescent protein aggregation during culture, and these fluorescent aggregates disappeared when these cells differentiated into osteoblasts. In addition, it has been reported that autophagy can protect rat mesenchymal stem cells from apoptosis under conditions of hypoxia and serum deficiency. The results showed that the experimental group rat mesenchymal stem cells treated with 3-MA showed a higher apoptosis ratio than the control group under the conditions of hypoxia or serum deficiency. The same results are also demonstrated in human mesenchymal stem cells, and these mesenchymal stem cells can affect the growth of solid tumors by secreting pro-apoptotic factors and inhibiting apoptotic factors (Sanchez et al. 2011).

In addition to the limited role of autophagy in mesenchymal stem cells, there is increasing evidence that autophagy plays an important role in mesenchymal stem cell-derived adipocytes, chondrocytes, and osteoblasts. It has been shown that fat cell-specific knockout of Atg7 can cause a decrease in lipid droplet volume and enhance sensitivity to insulin, demonstrating that autophagy plays an important role in lipid droplet formation (Singh et al. 2009). Autophagy activity is activated during the differentiation of primary embryonic fibroblasts into adipocytes, and the ability of Atg5 knockout fibroblasts to differentiate into adipocytes is greatly attenuated (Baerga et al. 2009). For chondrocytes, inhibition of beclin1 expression causes chondrocyte death. On the other hand, 3-MA treatment enhances the anti-apoptotic ability of the cells, demonstrating that sustained autophagy also causes chondrocyte cell death. Therefore, autophagy has both the effect of protecting cells from death and the induction of cell death in chondrocytes (Bohensky et al. 2007). Autophagy-related genes including Ulk1, beclin1 and Maplc3 are highly expressed in normal articular cartilage but are lowly expressed in cartilage and joints where osteoarthritis occurs, suggesting that autophagy may play a role in the pathogenesis of arthritis (Carames et al. 2010). Experiments have shown that bone cells also regulate cell stability through autophagy, which causes an increase in autophagy when stimulated by glucocorticoids, whereas bone cell survival is dose-dependent with glucocorticoids (Jia et al. 2011). Osteoblasts are precursors of bone cells and are responsible for the formation of the skeletal system. Since osteoblasts need to shape mineralized bones, it is conceivable that cells can meet high energy requirements by activating autophagy. In fact, it has been found that inhibition of autophagy by specifically knocking out Rb1cc1/Fip200 in osteoblasts severely affects the formation of the mouse bone system. In vitro experiments have shown that knocking out these two genes hinders the coagulation growth of osteoblasts, and autophagy inhibitors such as 3-MA and chloroquine can mimic the gene deletion effect.

In summary, although the known data show a higher level of autophagy activity in bone marrow-derived mesenchymal stem cells, the role of autophagy in the homeostasis maintenance, self-renewal, and differentiation of mesenchymal stem cells remains unclear. Recently, experiments have shown that autophagy plays an important role in the differentiation of mesenchymal stem cells into osteoblasts, chondrocytes, and adipocytes, but the specific mechanism in this process needs to be further studied.

#### 22.3.2.2 Autophagy in Neural Stem Cells (NSCs)

Although autophagy was initially thought to be only a self-protection method for cells to face hunger, more and more experiments have now demonstrated that basal levels of autophagy (in the absence of nutrient-deficient stress) can help terminally differentiated cells, such as nerve cells, maintain cell stability. Autophagy is thought to play a protective role in many neurodegenerative diseases like the ubiquitinated degradation system (Rubinsztein 2006). In fact, neuron-specific knockdown of autophagy-related genes such as Atg5, Atg7, or Rb1cc1 can lead to aggregation of ubiquitinated

proteins, p62 and impaired mitochondrial accumulation in neuronal cells, and aggravate apoptosis and neurodegeneration. These phenotypes provide direct evidence that basal levels of autophagy play a protective role in neurodegenerative diseases (Komatsu et al. 2006; Hara et al. 2006).

Researchers found that the expression of autophagy-related genes increased simultaneously during mouse olfactory bulb development suggesting that autophagy may play a role in this process. In fact, the researchers found that the autophagy key gene Ambra1 knockout mice developed neurodevelopmental disorders such as neural tube dysplasia and a marked decrease in the expression of various neuromarker genes. Consistent with the in vivo evidence, the level of autophagy was significantly increased in the differentiation of neural stem cells into neurons under in vitro conditions, and autophagy inhibitors such as 3-MA and wortmannin significantly inhibited the differentiation of neural stem cells. In addition, Ambra1 half-deficient and Atg5 knockout mouse olfactory bulb neural progenitors showed significant differentiation disorders. Another report claims that treatment of chicken embryos with 3-MA results in a change in the spatiotemporal specific expression profile of many neuronal marker genes and leads to a decrease in the volume of the auditory nerve vestibular ganglion, suggesting that autophagy is likely to affect the normal development of the auditory nervous system. In the above two experiments, by adding methyl pyruvate (a small molecule that can directly enter the cell to participate in the tricarboxylic acid cycle) to supplement the intracellular ATP content, the differentiation defect of neural stem cells can be repaired to some extent. One of the important roles of phagocytosis in the differentiation process is to provide the cells with the energy needed for differentiation. In addition, inhibition of mTOR activity by rapamycin can enhance dbcAMP-induced differentiation of NG108-15 neuroma cells, and 3-MA treatment or decreased Beclin1 gene expression can block this effect (Chin et al. 2010). This result further demonstrates that autophagy plays an indispensable role in the differentiation of neural stem cells.

### *22.3.3 The Role of Autophagy and Lysosomes in the Process of Stem Cell Senescence*

Cell senescence is a common physiological phenomenon, mainly characterized by cell cycle arrest, irreversible loss of proliferative potential, destruction of cell homeostasis, and impaired regenerative capacity. Although stem cells have a long life span, adult stem cells such as human hematopoietic stem cells and mesenchymal stem cells have a shortened telomere length with aging, and the number of cell divisions is about 50 times. Among the adult stem cells, the most relevant studies on the senescence of mesenchymal stem cells can be divided into three categories according to their aging characteristics: (1) Deteriorating mesenchymal stem cells, which are marked by a decrease in the ability of cells to self-renew, the cleavage limit of

Hayflick phenomenon, aging phenomenon such as telomerase inactivation; (2) persistent mesenchymal stem cells, which are not susceptible to aging, but eventually aging; (3) permanent mesenchymal stem cells, which are completely detached the effects of aging (Sethe et al. 2006).

Since many stress and lifespan related genes, such as FoxO3, NF-kappaB, Tp53, and Sirt1, can effectively regulate autophagy activity (Warr et al. 2013). In addition, mTOR acts as the most important growth and proliferation regulator of stem cells, and also regulates mitochondrial homeostasis and cellular ROS activity levels by autophagy, i.e., reduces cellular oxidative stress by reducing damaged mitochondria (Chen et al. 2008). This links the theory of mitochondrial free radicals to cell senescence and lifespan (Gottlieb and Carreira 2010).

Further experiments show that inhibition of mTOR can slow down the rate of cellular senescence, while knockdown of Tsc1 reduces autophagy levels leading to early maturation of mouse hematopoietic stem cells and many aging-related features, including lymphocyte reduction, decreased hematopoietic remodeling capacity, increased expression of aging-related genes such as CDK (cyclin-dependent kinase) inhibitors p16 (Ink4a), P19 (Arf), and p21 (Cip1). Treatment with rapamycin not only eliminates the above problems but also enhances the ability of hematopoietic stem cells to self-renew, reducing the expression of aging-related genes and significantly prolonging the life cycle of mice. Normally, bone marrow precursor cells and lymphoid precursor cells are maintained in a certain proportion in the blood cells of aged mice and humans, and the number of memory B cells and early childhood T cells is decreased. Treatment of mice with rapamycin allows older mice to produce more B lymphocytes, reduce the incidence of myelin, and exhibit a more youthful immune response to viral infections. Since mTOR regulates a variety of cell growth and metabolism activities, inhibition of mTOR may cause functions other than autophagy. These results only suggest that autophagy is likely to be involved in the anti-aging process. Consistent with the above experiments, the study found that the autophagy core protein Atg7 knockout mice significantly increased the number of myeloid cells compared to the control group, and the ability of hematopoietic stem cell reconstitution was weakened and higher levels of aging-related marker genes were expressed. Experiments have shown that congenital anemia found in the elderly is also partly due to defects in autophagy of hematopoietic stem cells (Phadwal et al. 2013).

In the process of human aging, it is often accompanied by the occurrence of tumors, and cell senescence is generally considered to have the effect of preventing tumorigenesis, so the process is very important to cells. Pten (tumor suppressor phosphatase and tensin homolog) acts as an upstream of mTOR to activate autophagy and inhibit cell senescence (Arico et al. 2001). Complete loss of Pten will cause premature aging of cells and inhibit tumorigenesis. More experiments have demonstrated that high levels of ROS activity in senescent or autophagy-deficient cells can inhibit Pten activity. Decreased levels of autophagy due to PI3K activation also promote cell senescence caused by Pten deletion. Therefore, we can infer that the expression and activity of Pten in aging stem cells determine the ability of cell proliferation.

In addition to senescence induced by Pten deletion, senescence induced by proto-oncogene expression is also an important measure to inhibit tumor formation. Autophagy was activated when overexpressing the oncogene ras, whereas ras overexpressing cells showed higher levels of autophagy at both basal and Baf-A treatment conditions compared to control IMR90 human dermal fibroblasts. Further studies have shown that the increase in the proportion of LC3II during proto-oncogene-induced senescence mainly occurs during the transition from the initial mitosis to the senescence and gradually decreases in the subsequent process (Young et al. 2009). Although the level of autophagy is not high in the final senescent cells, the outbreak of autophagy activity during the transition from mitosis to senescence may play an important role in maintaining cell homeostasis. As it accelerates protein turnover and provides a large number of the macromolecular intermediate metabolites, which can satisfy the need for the proliferation rate of cells caused by overexpression of oncogenes. The level of autophagy immediately decreases when there is no such requirement in the senescence period.

Overexpression of the p16 gene, a tumor suppressor gene that reduces the rate of cell proliferation, reduces the risk of tumors associated with aging. This provides us with an example of how cells balance the relationship between aging and tumorigenesis. Recently, it has been found that the caloric restriction method is likely to break the rule, and it uses autophagy to prolong life. Calorie-restricted feeding not only reduces the proliferation of stem cells in aging mice but also reduces the expression of p16, while reducing the risk of cancer.

During aging, the gradual increase of lipid droplets and protein oxidation in lysosomes leads to a larger and more fragile lysosome, affecting its ability to fuse with autophagosomes, and reducing the level of Lamp2a expression on the lysosomal membrane. This will lead to erroneous autophagy and chaperone-mediated autophagy. On the other hand, lysosomes remain an important target for oxygen-free radical attack during the aging process due to the accumulation of lipofuscin (aging pigment) and other nondegradable wastes. Decreasing the level of autophagy or chaperone-mediated autophagy will reduce the ability of the cell to catabolize, thereby weakening the ability of the cell to adapt to the microenvironment, which is very important for stem cells (Cuervo and Dice 2000). In addition, due to the gradual decline in the ability of stem cell proliferation during stem cell senescence, the ability of cells to dilute intracellular harmful waste by division is gradually weakened. During the aging process of bone marrow-derived mesenchymal stem cells, the autofluorescence caused by the accumulation of lipofuscin is continuously enhanced, the carbonyl group is increased, and the oxygen-free radicals in the cells are gradually increased (Stolzing and Scutt 2006). Including upstream Pten, Tp53, mTOR and downstream HDAC6, TFEB, and other genes can assist in the normal progression of autophagy by regulating lysosomes. In summary, we believe that lysosomes can influence the self-renewal, proliferation, and senescence of stem cells by regulating the metabolic efficiency of autophagosomes.

## 22.4 Summary

This chapter focuses on the role of autophagy in biological development and differentiation, including invertebrates, mammalian development, and stem cell differentiation. The developmental differentiation of organisms is a precisely regulated biological process. During the development and differentiation of organisms, autophagy can maintain the survival of cells by degrading misfolded and redundant proteins, cleaning up damaged organelles, providing raw materials, and energy for cell remodeling. Autophagy can also affect the direction of cell differentiation and development by specifically degrading certain proteins or cytokines in the cells. Autophagy plays an important role in the developmental differentiation of invertebrates, mammalian embryonic development and cell differentiation, and differentiation of tissue stem cells.

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# Chapter 23
# Autophagy in Normal Stem Cells and Specialized Cells


**Kun Liu, Liang Wang and Tongbiao Zhao**

**Abstract** Autophagy, the intracellular degradation and dynamic recycling system, plays critical roles in pluripotent and differentiated cells. It protects the homeostasis of the intracellular environment and maintains the functions of cells by degrading and recycling cytoplasmic components. Autophagy is associated with the entire life cycle of the body, from the totipotent fertilized egg to the terminally differentiated cell. However, autophagy also plays distinct roles in these different life stages and in specific cell types. Here, we summarize the functions of autophagy in normal stem cells and in specialized cells.

**Keywords** Autophagy · Stem cells · PSCs · HSCs · Specialized cells

## Abbreviations

| | |
|---|---|
| 3-MA | 3-methyladenine |
| Akt/PKB | Protein kinase B |
| AMPK | Adenosine 5′-monophosphate (AMP)-activated protein kinase |
| ASCs | Adult stem cells |
| Atg | Autophagy-associated gene |
| ATP | Adenosine triphosphate |
| BM-MSCs | Bone marrow-derived MSCs |
| CP-MSCs | Placental chorionic plate-derived MSCs |
| DNA | Deoxyribonucleic acid |
| EBs | Embryoid bodies |
| ERK | Extracellular signal-regulated kinase |
| ESCs | Embryonic stem cells |
| FGF12 | Fibroblast growth factor 12 |
| FIP200/RB1CC1 | RB1-inducible coiled-coil protein 1 |

K. Liu · L. Wang · T. Zhao (✉)
State Key Laboratory of Stem Cell and Reproductive Biology, Institute of Zoology, Chinese Academy of Sciences, Beijing 100101, China
e-mail: tbzhao@ioz.ac.cn



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| | |
|---|---|
| FOXO1 | Forkhead box O1 |
| GFP | Green fluorescent protein |
| GSK3 | Glycogen synthase kinase-3 |
| HPCs | Hepatic progenitor cells |
| HR | Homologous recombination |
| HSCs | Hematopoietic stem cells |
| ICM | Inner cell mass |
| iPSCs | Induced pluripotent stem cells |
| Klf4 | Krüppel-like factor family 4 |
| LC3 | Microtubule-associated protein 1 light chain 3 |
| LIF | Leukemia inhibitory factor |
| M-BMSCs | Mandible-derived BMSCs |
| MEK | Mitogen-activated protein kinase kinase |
| MPPs | Multipotent progenitor cells |
| mRNA | Messenger ribonucleic acid |
| MSCs | Mesenchymal stem cells |
| mTOR | Mammalian target of rapamycin |
| NHEJ | Nonhomologous end joining |
| NOD/SCID | Nonobese diabetes/severe combined immune deficiency |
| Nrf2 | Nuclear factor E2-related factor 2 |
| NSCs | Neural stem cells |
| Oct4 | Octamer-binding transcription factor 4 |
| PCR | Polymerase chain reaction |
| PSCs | Pluripotent stem cells |
| Rab5 | Ras-related protein Rab-5 |
| ROS | Reactive oxygen species |
| SATB2 | Special AT-rich sequence-binding protein 2 |
| SCs | Satellite cells |
| SIRT1 | Silence information regulator 1 |
| Sox2 | SRY related HMG box-2 |
| STAT3 | Signal transducer and activator of transcription 3 |
| SVZ | Subventricular zone |
| ULK1 | UNC-51-like kinase 1 |

## 23.1 Overview of Stem Cells

Stem cells have the ability to self-renew and indefinitely proliferate, and they are always in an undifferentiated cellular state. Differentiation of stem cells generates different cell types, and the autophagic level and rate are modulated during the differentiation process. First, for context, we give an overview of stem cells.

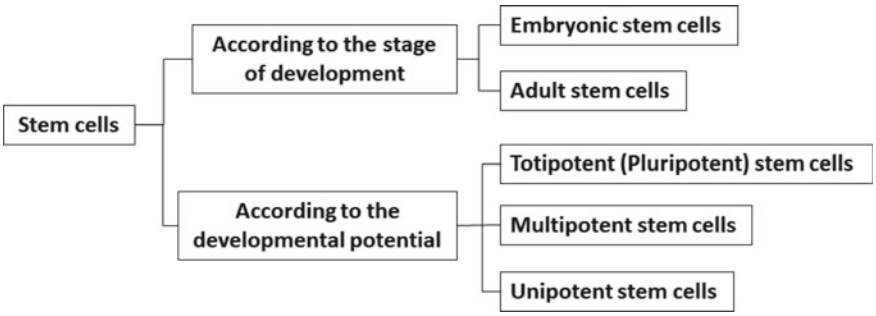


**Fig. 23.1** Classification of stem cells

Scientists catalog stem cells by different criteria (Dulak et al. 2015). According to the stage of development, stem cells can be divided into two groups, including embryonic stem cells (ESCs) and adult stem cells (ASCs) (Fig. 23.1). ESCs are derived from the inner cell mass (ICM) of blastocysts. They can differentiate into all kinds of cells in the human body and have great promise for use in regenerative medicine. The first identified ESC was from a mouse in 1981. Then, scientists revealed that the mouse ESCs could propagate indefinitely in culture and differentiate into all cell types of the body. Seventeen years later, the first human ESCs were isolated, which revolutionized regenerative medicine and disease remodeling. ASCs are from postnatal organisms and can generate one or more cell types and include hematopoietic stem cells (HSCs).

According to their developmental potential, stem cells can be divided into three groups, totipotent stem cells, multipotent stem cells, and unipotent stem cells (Fig. 23.1). Totipotent stem cells can differentiate into all kinds of cell types and include germ line cells, such as ESCs and induced pluripotent stem cells (iPSCs). ESCs together with iPSCs constitute the pluripotent stem cells (PSCs). A single totipotent stem cell has the potential to form an individual. The totipotent ability of mouse ESCs has been shown by tetraploid complementation experiments. Multipotent stem cells are found in specific organs, and they can produce more than one cell type, such as HSCs and neural stem cells (NSCs). Unipotent stem cells can generate only one cell type and includes the satellite cells of skeletal muscles.

### *23.1.1 The Self-renewal Capacity of Stem Cells*

Self-renewal is an important identifying feature of stem cells and indicates that the cells can proliferate indefinitely and retain pluripotency. There are small differences between the different types of stem cells. Here, we use ESCs as an example to describe this self-renewal ability. To maintain self-renewal in the cellular gene expression both in vivo and in vitro, the circumstances are critical. The complicated pluripotency gene network coordinates with the culture medium to maintain self-renewal of stem cells

(Martello and Smith 2014). There is a series of genes required for pluripotency, such as Oct4, Sox2, and Nanog. These genes are highly expressed in stem cells and coordinate to regulate gene networks in ESCs. Therefore, these genes are identified as markers for stem cells. To maintain the culture of ESCs in vitro, some inhibitors, such as LIF, MEK inhibitor, and GSK3 inhibitor, need to be used in the culture medium (He et al. 2009). LIF can promote the phosphorylation of STAT3 in mouse ESCs. Then, the phosphorylated STAT3 enters the nucleus and triggers the expression of target genes. Inhibition by MEK and GSK3 can enhance the viability and growth of ESCs and can suppress the differentiation of ESCs. The self-renewal ability of ESCs can be detected by colony formation assay. In theory, one single ESC can form a clone. By comparing their subclone formation ability, we can determine the self-renewal ability of the ESCs. For pluripotency detection, real-time PCR and western blotting are always used. By measuring the pluripotent gene expression, we can identify the quality of ESCs.

### *23.1.2 The Differentiation Potential of Stem Cells*

Stem cells have the ability to differentiate into different cell types that comprise the organism, including endoderm-, mesoderm-, and ectoderm-derived cells. In theory, once the tissues are injured, the corresponding stem cells are activated to move out of an undifferentiated state to differentiate into the specific cell type needed to restore the damaged tissues. Therefore, the differentiation potential of stem cells has great promise in regenerative medicine. For diverse types of stem cells, the differentiation potential largely differs. Totipotent stem cells can generate all elements of the human body, including teeth and eyes. ESCs injected into blastocysts can complement the blastocysts and contribute to chimeras. ESCs can also form embryoid bodies (EBs) and teratomas in vitro. Multipotent stem cells can differentiate into a series of cell types from one lineage, such as HSCs. HSCs can differentiate into T cells, B cells, and other kinds of blood cells. Unipotent stem cells give rise to only one cell type, such as satellite cells of skeletal muscle, which can differentiate only into skeletal muscle cells. Multipotent stem cells and unipotent stem cells usually exist in adult tissues and organs.

## 23.2 Autophagy of Pluripotent Stem Cells

Autophagy, a highly conserved cellular degradation pathway, can degrade and recycle intracellular molecules, proteins, and organelles. PSCs, including ESCs and iPSCs, have great promise in regenerative medicine and disease model building. In recent years, increasing research has focused on autophagy regulation in PSCs. As PSCs have the capability to self-renew and differentiate multi-directionally, they have been the focus of mechanistic studies on the metabolism of PSCs. PSCs have a short

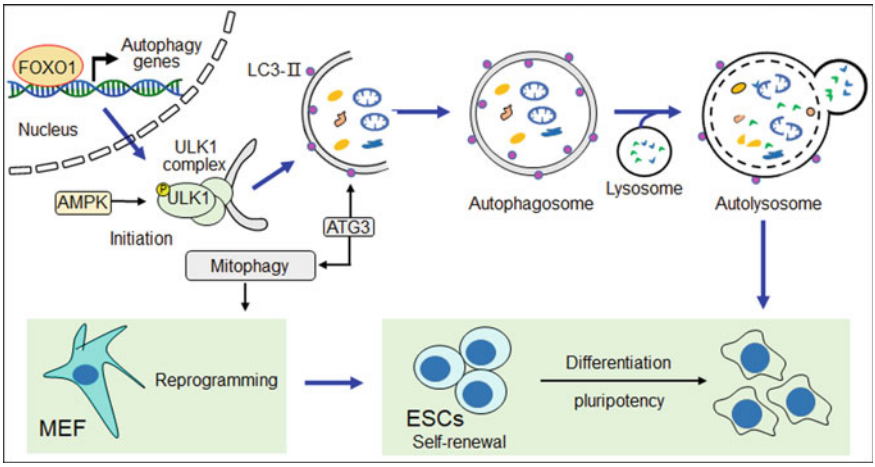


**Fig. 23.2** Autophagy regulates the identity of PSCs

cell cycle with rapid recycling and rebuilding processes that require autophagy to effect catabolism pathways. The basal level of autophagy is responsible for cellular homeostasis maintenance. It is not only recognized as a recycling system but is also known as a quality control system. Using ESCs, scientists have gradually revealed how autophagy regulates the activity of cellular components and influences stemness (Guan et al. 2013). Here, we summarize the features of autophagy in ESC identity maintenance (Fig. 23.2).

## *23.2.1 Autophagy in Stemness Maintenance of Embryonic Stem Cells*

Stemness refers to the potential to differentiate into various cell types or to self-renew without differentiation, and it refers to the balance between proliferation and differentiation. ESCs derived from the ICM of blastocysts at day 3.5 have a high proliferation rate and a short G1 cell cycle stage. ESCs require timely degradation of toxic proteins and dysfunctional organelles. Autophagy takes part in the course of early development described above to maintain cellular homeostasis.

### 23.2.1.1 Autophagy Regulates the Pluripotency of Embryonic Stem Cells

The expression of pluripotent genes is required for the stemness of ESCs. There are multiple pathways in autophagy regulation, and mTOR is one of the key regulators. Inhibition or depletion of mTOR in human ESCs can decrease the expression of

pluripotent genes such as Nanog, Oct4, and Sox2. In mouse ESCs, inhibition of autophagy by 3-MA has the same phenotype as mTOR. Furthermore, by using an Atg3 knockout model, researchers have revealed that autophagy plays important roles in stemness maintenance. They found that Atg3 null ESCs have low autophagy levels, and the degradation rate of Atg3 null ESCs is much lower than that of the wild-type ESCs. Defects in autophagy cause dysfunctional mitochondria accumulation, enhanced ROS production, and abnormal ATP content that significantly decreases the self-renewal ability of the Atg3 null ESCs. In addition, the pluripotent genes are significantly reduced in the transcription and translation levels in ESCs with autophagy defects (Liu et al. 2016). A study on the autophagy mechanism shows that AMPK can phosphorylate ULK1, which induces the autophagy-related (ATG) genes that lead to the formation of the ULK1 complex and initiates autophagy in ESCs (Gong et al. 2018).

Autophagy is responsible for protein turnover and organelle quality control. Early studies revealed that the bulk protein turnover of Atg5-knockout ESCs is significantly reduced compared with wide-type ESCs. A recent study found that dysfunctional mitochondria are significantly accumulated in Atg3 null ESCs. In addition, ATP production, ROS levels, and OCR of the Atg3 null ESCs are abnormal, demonstrating that the absence of autophagy disrupts the metabolism homeostasis of these ESCs (Liu et al. 2016). All of these studies indicate that protein and organelle turnover rely on autophagy in ESCs. Autophagy flux reflects the degradation and recycling rate, and it varies by cell type. A recent study showed that ESCs have higher autophagy flux for maintaining stemness compared with somatic cells. FOXO1, the core transcriptional factor, directly binds to autophagy-related genes to maintain their high expression in ESCs. Autophagy flux regulates self-renewal in a dosage-dependent manner. Self-renewal ability is successively decreased in Atg3 wild type and in heterozygous and knockout ESCs. A mechanistic study found that both protein and organelle turnover relies on autophagy in a dosage-dependent manner (Liu et al. 2017).

#### 23.2.1.2 Autophagy Regulates the Multipotential Differentiation of Embryonic Stem Cells

Autophagy takes part not only in stemness regulation but also in the regulation of differentiation in ESCs. To reveal the roles of autophagy in ESC differentiation, scientists have used Atg3- or ULK1-knockout ESCs to study EB differentiation. They found that the absence of Atg3 delayed the expression of endodermal and mesodermal genes. Furthermore, defects in ULK1 delayed endodermic and ectodermic gene expression. These findings indicate that autophagy regulates the formation and cell differentiation of the three germ layers in vitro. Furthermore, teratoma formation assays and chimeras have been used to reveal the roles of autophagy in differentiation in vivo. Researchers found that the absence of Atg3 or ULK1 impaired the weight and size of teratomas. To evaluate differentiation in vivo, they analyzed the contribution of Atg3-knockout ESCs to chimera formation and found that the absence

of Atg3 significantly decreased the contribution rates of the ESCs (Liu et al. 2016; Gong et al. 2018).

EBs are usually used for detecting the lineage differentiation of ESCs in vitro. EBs include cells from the three germ layers. Programmed cell death is accompanied by EB formation, and autophagy takes part in the course of apoptosis. Previous studies have shown that deficiency in Atg5 or beclin1 impairs autophagosome formation in ESCs. Autophagy levels gradually decrease during EB differentiation, and a lack of Atg5 or beclin1 will cause the failure of EB cavitation. Furthermore, these studies found that ATP production in Atg5- or beclin1-deficient ESCs is abnormal. This finding suggests that autophagy is essential for the differentiation of ESCs by modulating energy homeostasis.

To monitor autophagy in human ESCs, researchers established a GFP-LC3 knockin of human ESC lines. By detecting green fluorescence during the spontaneous differentiation of ESCs, they found that autophagy levels gradually increased. This finding indicates that autophagy levels change during development (Wang et al. 2018). Inhibition of mTOR by rapamycin upregulated the expression of mesoderm- and endoderm-related genes in the human ESCs. The proliferation of human ESCs also decreased under the inhibition of mTOR. Consistent with human ESCs, treatment with an mTOR inhibitor on mouse ESCs decreased the cell size and proliferation rate.

### *23.2.2 Autophagy of iPSCs*

Somatic cell reprogramming refers to cell reversion from a terminal, differentiated, matured or specialized state to a naïve pluripotent state. Yamanaka first reprogrammed mouse embryonic fibroblasts into ESC-like cells by using defined factors (Sox2, Oct4, Klf4, and c-Myc) and referred to the cells as iPSCs. Reprogramming efficiency is low, and only a small percentage of the somatic cells can be turned into iPSCs. Through reprogramming, the cellular membrane system, metabolic state, and organelles are remodeled to match the highly efficient proliferation of pluripotent cells. This course of reverting cells to a stem state is slow and complex and involves multiple cellular processes. Recent research has shown that inhibition of mTOR triggers autophagy and significantly improves reprogramming efficiency. The hypothesized explanation is that autophagy can degrade old materials and provide materials for building during reprogramming.

Studies have found that the critical point in autophagy occurs in the early stage of transcription factor-mediated reprogramming. The core regulator of autophagy, mTOR, is transiently downregulated after iPSC induction. Both mRNA and protein expression of mTOR significantly decreased. Adding the mTOR inhibitors at the early stage of reprogramming could significantly increase the reprogramming efficiency. These results indicate that mTOR is essential for autophagy regulation in reprogramming. A recent study found that both Atg3 and Atg5 are essential for

somatic cell reprogramming. Defects in Atg3 or Atg5 significantly impaired reprogramming. Further study revealed that Sox2 suppressed the expression of mTOR, and NuRD facilitated this suppression. The evidence has proven that autophagy plays critical roles in iPSC induction. Another study revealed that Atg5-independent autophagy (noncanonical autophagy) played crucial roles in establishing pluripotency and controlled the metabolic switch during reprogramming by modulating the AMPK signal pathway (Wang et al. 2015).

To investigate the course of reprogramming, scientists have found that both Atg3 and Atg5 deletion impaired the removal of mitochondria in pluripotency induction, and the iPSCs from Atg3-knockout fibroblasts were abnormal. Atg3-knockout iPSCs had dysfunctional mitochondria accumulation, enhanced ROS production, and low ATP content. All these findings reveal that autophagy-mediated mitochondrial remodeling during reprogramming (Liu et al. 2016). Recently, an interesting work showed that mitochondrial autophagy (mitophagy) receptors are required for Sox2-, Oct4-, and Klf4-mediated reprogramming. The researchers found that Bnip3l (also known as Nix) regulated removal of mitochondria through three factor-induced pluripotency, and the endosome-related gene Rab5 participated in mitophagosome formation in this reprogramming study (Xiang et al. 2017). Mitophagy regulates pluripotency acquisition by switching the status of the metabolic state. However, the underlying mechanism is still unclear.

## 23.3 Autophagy of Adult Stem Cells

### *23.3.1 Autophagy of Hemopoietic Stem Cells*

HSCs are probably the earliest and most thoroughly studied adult stem cells. Because they give rise to all blood and immune cells, they have been widely applied in bone marrow transplantation and other clinical therapies. HSCs are known to be contained in the LSK ($Lin^{-}Sca\text{-}1^{+}c\text{-}Kit^{+}$) cell population, which is heterogeneous. Only a small fraction of the LSK cell population consists of HSCs, while the larger portion consists of multipotent progenitor cells (MPPs). It has been shown that autophagy is highly activated in HSCs, indicating that autophagy plays critical roles in maintaining HSC identity.

#### 23.3.1.1 Autophagy Maintains the Quiescent State and Self-renewal Capacity of HSCs

HSCs are stem cells with rare populations; however, they must function throughout the entire lifetime of the organism, which makes their self-renewal capacity particularly important. In fact, more than 80% of HSCs exist in a quiescent state in the bone marrow microenvironment, which is thought to benefit stem cell longevity and

prevent exhaustion. The state of quiescence means HSCs have no need to require proteins or DNA synthesis for division, which confers a low metabolic rate to HSCs. Only a few HSCs enter the cell division process, which they do to either generate another HSC for self-renewal or to produce a progenitor cell by asymmetric division. The newly produced progenitor cells are responsible for daily blood cell supplementation.

Autophagy might have the following advantages for maintaining the HSC quiescent state (Guan et al. 2013): First, autophagy could boost the catabolic rate to remove damaged organelles and proteins, which cannot easily be diluted through passage to daughter cells by quiescent cells. Second, autophagy could facilitate metabolic changes by eliminating mitochondria. High levels of autophagy, combined with a hypoxic niche, directs the cells into glycolytic metabolism with low mitochondrial content. Indeed, glycolysis is the primary energy source for quiescent HSCs. Third, autophagy helps control ROS generation by regulating the quality and quantity of mitochondria. ROS are significant factors for DNA damage and negatively affect stem cell self-renewal and multipotency. Elevated ROS levels are a signature of disabled autophagy because they indicate that damaged mitochondria cannot be removed. Studies have shown that elevated ROS levels can disrupt HSC quiescence and can even lead to malignant transformation. For example, conditional deletion of Atg7 in the mouse hematopoietic system results in blocked mitochondrial clearance in hematopoietic stem and progenitor cells (HSPCs), which increase ROS levels and DNA damage, leading to dysfunctional self-renewal.

Indeed, the quiescent state is regulated by the PtdIns3K–mTOR pathway. Once the activity of mTOR is increased, the quiescence will be compromised with cell excessively proliferating, and the hematopoietic reconstruction ability is suppressed (Guan et al. 2013).

Hematopoietic reconstruction ability is the touchstone for testing HSC self-renewal capacity. The abovementioned Atg7-deficient HSCs have decreased colony formation ability in vitro, and in human HSCs, autophagy is inhibited by 3-methyladenine (3-MA) or ATG5 siRNA such that the HSCs fail to reconstruct the hematopoietic system when transplanted into lethally irradiated mice. In summary, autophagy deficiency compromises the self-renewal capacity of HSCs.

#### 23.3.1.2 Autophagy Regulates the Differentiation Potential of HSCs

Finely tuned adjustments among the quiescence, self-renewal, and differentiation processes guarantee HSC identity, as well as that of other adult stem cells. When the balance is disrupted, the multipotency of HSCs is impaired, as reflected as excessive cell proliferation, subdued multilineage differential potential, and even tumor transformation or emergence of other hematological diseases. It seems that changes in differential potential within the hemopoietic system are always revealed in elevated myeloproliferation but lowered lymphoid lineage differentiation. This phenomenon has been observed in Atg7- and FIP200-deficient mice as each mutant had superfluous mitochondria and increased ROS levels.

The increased ROS level in Atg7- or FIP200-deficient HSCs is expected to facilitate these phenotypes and thus result in increased myeloid cells and impaired erythropoiesis. Indeed, ROS are also proven to be accelerators of normal HSC differentiation, that is, HSCs from low-oxygen environments migrate to the oxygen-enriched niche, leading to ROS rising and medullary cell formation (Guan et al. 2013). Thus, we can conclude that the mitochondrial scavenging capacity of HSCs is impaired by autophagy deficiency, leading to ROS overproduction and differentiation abnormalities.

#### 23.3.1.3 Autophagy Prevents HSC Senescence

Autophagy is important for removing redundant mitochondria and keeping HSCs at a low metabolic rate. Moreover, autophagy is also conducive to the elimination of accumulated damaged proteins and other cellular contents. These functions help HSCs maintain a quiescent state, prevent HSC senescence, and reserve long-term HSCs for future use.

Autophagy was presumed to be suppressed in aging cells. However, it was found to be elevated in old HSCs. In fact, a high autophagy flux occurs only in approximately 30% old HSCs, while it remains at a low level in other cells (Ho et al. 2017). The high autophagy levels in the 30% of old HSCs may be responsible for removing healthy, activated mitochondria and reducing oxidative phosphorylation, thus maintaining the old HSCs at a low metabolic rate and conferring on them the strong regenerative capacity, similar to young HSCs. HSCs with low autophagy have overactive oxidative metabolism, driving aging blood phenotypes. Moreover, the conditional knockout of the autophagy gene Atg12 or Atg5 contributes to aging phenotypes in young mice, such as impaired engraftment, myeloid-biased lineage distribution, and reduced hematopoietic reconstruction ability (Ho et al. 2017). In more detail, it was proven that metabolic differences between old and young HSCs drive epigenetic reprograming, especially DNA methylation, to affect the aging phenotypes and drive the fate decisions of HSCs. However, other mechanisms related to HSC senescence need further exploration.

### *23.3.2 Autophagy of Mesenchymal Stem Cells*

Mesenchymal stem cells (MSCs) are highly heterogeneous populations. They can be isolated from various tissues, including bone marrow, adipose tissue, umbilical cord, muscles, kidney, placenta, and brain. MSCs from different sources inevitably exhibit discrepant stemness. For example, adipose tissue-derived MSCs present with seemingly better phenotypes than bone marrow-derived MSCs (BM-MSCs) in that they are more stable during long-term culture, have higher proliferative and differentiation potential, and age more slowly (Sbrana et al. 2016). However, it has been proven that MSCs from multiple sources are regulated by autophagy.

#### 23.3.2.1 Autophagy Maintains Self-renewal and Survival of MSCs

Autophagy is activated and important for MSC self-renewal and survival. It can be modified by several stimuli, such as hyperglycemia, senescence, ROS, hypoxia, and acidity (Sbrana et al. 2016). MSCs live in a hypoxic niche under physiological conditions, which is crucial for maintaining their stemness. Hypoxia can induce autophagy and regulate the self-renewal capacity of MSCs. It was found that the expression of stemness markers, e.g., Oct4, Nanog, and Sox2, are increased both in placental chorionic plate-derived MSCs (CP-MSCs) and BM-MSCs in hypoxia. Additionally, autophagy flux, measured by LC3 II expression and autophagosome formation, is more rapid in CP-MSCs compared to BM-MSCs. The elevated autophagy flux is caused by the augmented phosphorylation of ERK and mTOR during hypoxia (Lee et al. 2013).

Moreover, activated mTOR and autophagy also help to resist hypoxia-induced apoptosis, as shown by the level of apoptosis increasing by 3-MA but decreasing by rapamycin. The protective role of autophagy is also evident under serum starvation conditions. Thus, autophagy as a "self-eating" process can protect MSCs from hypoxia-induced apoptosis/serum deprivation and promote their survival. However, other studies obtained the opposite result, which might be due to the site-specific properties of the MSCs (Sbrana et al. 2016).

In addition, autophagy functions in the prevention of senescence in MSCs. Under hypoxic/serum deprivation conditions, special AT-rich sequence-binding protein 2 (SATB2) and stemness markers were found to be highly expressed in mandible-derived BMSCs (M-BMSCs).

Furthermore, the level of autophagy was increased, and the cells were more resistant to senescence (Chen et al. 2018).

#### 23.3.2.2 Autophagy Regulates the Multipotential Differentiation of MSCs

The heterogeneity and multipotency of MSCs give them the capacity to differentiate into various mesenchymal lineages, such as adipocytes, osteocytes, chondrocytes, muscles, and neurons. The differential capacity of MSCs is regulated by multiple factors involved in the tissue microenvironment and is also regulated by autophagy. In fact, it has been proven that autophagy functions in osteogenic, adipogenic, myogenic, and neuronal differentiation of MSCs.

During osteogenic differentiation, the accumulation of undegraded autophagosomes was reduced, indicating that autophagy flux was increased and the rate of autophagic cell turnover was increased. It is thought to be related to the Akt/mTOR pathway. In addition, SATB2 may function in upregulating pluripotency genes and autophagy-related genes to accelerate the process of osteogenic differentiation and reconstruction of bone defects (Sbrana et al. 2016).

Autophagy could also prevent the decreased differentiation potential of MSCs during senescence. The aging of stem cells is always accompanied by a decline in

multipotency and proliferation. For BM-MSCs, aging leads to imbalanced differentiation, which is reflected by the reduced osteogenesis and increased adipogenesis that gives rise to bone loss and fat accumulation, respectively. Inhibition of autophagy by 3-MA led to the conversion of young BM-MSCs into aged cells, an outcome similar to that of the young HSCs in which Atg12 or Atg5 was conditionally deleted. Fortunately, these phenotypes could be partially rescued by rapamycin-induced activation of autophagy. Experiments showed that bone mass was restored in vivo by rapamycin treatment in aged male mice with senile osteoporosis. The benefits were shown as elevated bone mineral density, trabecular bone volume, and trabecular number and in decreased trabecular space; however, the change in trabecular thickness was not significant. In addition, BM-MSCs contributed to the increase in bone-like tissue when transplanted to rapamycin-treated NOD/SCID mice. Therefore, autophagy regulators might be used as potential medicines for disease treatment.

### *23.3.3 Autophagy Influences Homeostasis of Neural Stem Cells by Regulating ROS*

Neural stem cells (NSCs), mainly located in the subventricular zone (SVZ) of the lateral ventricles and the subgranular zone of the hippocampal dentate gyrus in adult brains, can differentiate into neurons, astrocytes, or oligodendrocytes. Like HSCs, NSCs live in a hypoxic environment in a quiescent state to maintain their homeostasis; therefore, autophagy is required. Conditional deletion of the Atg gene FIP200 seriously affected the NSC pool and neuronal differentiation. NSC differentiation is presumably regulated by ROS and oxidative damage since it can be rescued by the antioxidant N-acetyl cysteine (Wang et al. 2013). Indeed, in consideration of the hypoxic niche of the NSCs, maintaining low ROS levels is important. Deficiency of FIP200 led to the accumulation of p62 aggregates in the NSCs. The increased p62 aggregates seemed to be the key regulators and their formation was accompanied by decreased SOD1, which increased the superoxide levels and impaired the homeostasis of the NSCs (Wang et al. 2016). Similarly, deficiencies in transcription factors of the FOXO family, such as FOXO1, FOXO3, and FOXO4, some of which are associated with autophagy induction, were harmful to NSC multipotency. Additionally, the harmful effect was accompanied by elevated ROS levels and could also be rescued by N-acetyl cysteine (Guan et al. 2013). In a word, maintaining a low level of ROS is crucial for maintaining the homeostasis of NSCs, and autophagy might be one of the important regulators of it.

### 23.3.4 Autophagy Contributes to the Activation of Quiescent Muscle Stem Cells

Autophagy is crucial for maintaining the quiescent state of ASCs, including muscle stem cells, namely, satellite cells (SCs). SCs are distributed between the basement membrane and the sarcolemma of multinucleated muscle cells and maintain a quiescent state until muscle injury. On the one hand, compromised autophagy disrupts the quiescent state of the SCs, leading to senescence. On the other hand, deficiency in autophagy inhibits SC activation because the activation process requires increased autophagy.

Indeed, stem cell activation is a process involving high energy consumption that is accompanied by a switch of metabolic conditions. Stem cells live in low metabolism conditions, with fewer metabolites and low enzymatic activity. Autophagy-mediated metabolic changes ensure that the activation process is on track by supplying enough energy and is building blocks via the degradation processes of autophagy. Tang et al. verified that autophagy is the driving force behind SC activation. Impaired autophagy delayed SC activation with a commensurate decrease in ATP. Further study found that enhanced autophagy during SC activation was induced by SIRT1, a nutrient sensor. In addition, the delayed activation process caused by impaired autophagy or SIRT1 deficiency could be partially rescued by exogenous energy sources, such as pyruvate (Tang and Rando 2014). In summary, these findings indicate that autophagy is an essential regulator of SC activation, as it can stimulate catabolism and meet the bioenergetic demands of the SCs.

### 23.3.5 Autophagy Inhibits the Neoplastic Transformation of Hepatic Progenitor Cells

Hepatic progenitor cells (HPCs) are ASCs with bidirectional differentiation ability. They can differentiate into mature hepatocytes or biliary epithelial cells during liver injury, thus endowing the liver with tremendous regenerative capacity. Similar to other ASCs, HPCs require autophagy to maintain their stemness.

Autophagy is critical for the self-renewal and proliferation of HPCs as shown by the loss of ATG genes, such as Atg5, beclin1, or ATG7, that impair the colony formation and proliferation capacity of the HPCs (Chen et al. 2018). Moreover, autophagy is negatively related to HPC differentiation. The level of autophagy is decreased during the biliary and hepatic differentiation of the HPCs, but that does not mean that autophagy is dispensable. A deficiency in autophagy also impairs the differential capacity.

In detail, one of the key effects of autophagy-regulated stemness is the change in ROS levels, which is discussed above. In HPCs, deficiency in autophagy elevates ROS levels. The elevated ROS levels induce DNA damage, including double-strand breaks. In addition, deficiency in autophagy impairs homologous recombination (HR) repair

(as the activity of some HR factors is decreased) but not nonhomologous end joining (NHEJ) in HPCs. HR is an accurate repair pathway; however, NHEJ introduces insertion, deletion, or other mutations such that reliance on NHEJ in autophagy-deficient HPCs might lead to genomic instability and tumorigenesis. Indeed, tumor formation was discovered in both carcinogen- and chronic liver damage-induced models (Xue et al. 2016). In conclusion, autophagy might inhibit the accumulation of DNA damage and resist the neoplastic transformation of HPCs.

### *23.3.6 Others*

In addition to the abovementioned ASCs, autophagy has been discovered in other stem cell types. It was found that autophagy is positively correlated with cardiac stem cell differentiation, and this effect could be inhibited by the FGF signaling axis via activated AKT and MAPK pathways (Guan et al. 2013). Furthermore, autophagy plays either an enhancement or suppressive role during the differentiation of adipose-derived stem cells, depending on the differentiation tendency. On the one hand, autophagy promoted adipose-derived stem cells differentiation toward neuronal-like cells, thus contributing to osteogenesis under oxidative stress. On the other hand, autophagy inhibited the adipogenic differentiation of adipose-derived stem cells, which might be mediated by DIRAS3 via downregulated Akt-mTOR signaling and activated autophagy (Chen et al. 2018).

Autophagy has also been found in both in epidermal and dermal stem cells. The inhibition of autophagy impaired the self-renewal and differentiation capacity of these skin cells, and their resistance to stress was also affected. Consequently, the regeneration and tissue repair ability of these cells were compromised (Salemi et al. 2012). In intestinal stem cells, autophagy was crucial for regeneration of damaged intestinal tissue. Atg5ΔIEC mice exhibited fewer intestinal stem cells and excessive ROS accumulation. However, these conditions could be rescued by antioxidants, suggesting that the increased ROS levels were among the potential mechanisms of autophagy-regulated intestinal regeneration.

In conclusion, autophagy seems to be a versatile regulator of all kinds of ASCs although the effects are discrepant. It is exactly through these subtleties that autophagy functions in unique ways to regulate stemness, depending on the cellular microenvironment or external stimulus.

## 23.4 Autophagy of Specialized Cells

Specialized cells refer to terminally differentiated tissue cells that are derived from the differentiation of stem cells. These cells have left the cell cycle and have lost the ability to divide, but they acquire new physiological characteristics. Autophagy not only guards the identity of stem cells but also takes part in maintaining the

homeostasis of specialized cells. Specialized cells exist in adult tissue and organs and play vital roles in physiological functions. Autophagy has distinct functions in specialized cells, such as neural cells, muscle cells, and lung cells.

### *23.4.1 Autophagy Participates in Material Degradation and Energy Metabolism of Neural Cells*

The nervous system is the command center of the entire body. It gives commands to each part of the organism and connects the distinct cell types in the body. The nervous system comprises different types of neural cells. Nerve cells include hypothalamic neurons, cortical neurons, Purkinje cells, and so on. Nutrients and energy are critical for the function of neural cells. Autophagy is responsible for material degradation and recycling. In neural cells, autophagy plays important roles in neural homeostasis. Early studies revealed that specific deletion of Atg5 or Atg7 in the neural system caused growth retardation in mice. Long-term observation revealed that these mice have behavioral defects, such as systemic tremor and abnormal limb-clasping reflexes (Mizushima and Komatsu 2011). Furthermore, Atg5 or Atg7 inactivation in the mouse nervous system could trigger the neuron death and axon swelling. Furthermore, the ubiquitin-positive aggregates had accumulated in autophagy-deficient neurons (Kulkarni and Maday 2018). All these findings show that autophagy is related to neurodegeneration. P62 and polyubiquitinated proteins had accumulated in nearly every neural region in the brains of autophagy-deficient mice. During aging, these proteins formed inclusion bodies that gradually grew in with size and number. Furthermore, neural cells, such as pyramidal neurons and Purkinje cells, were significantly decreased in the cerebellar cortex. Specifically, disruption of autophagy in Purkinje cells proved that autophagy kept Purkinje cells alive and healthy and could prevent degeneration of axon terminals and initiation of axonal dystrophy (Kim and Lee 2014).

### *23.4.2 Autophagy Regulates Glucose Utilization and Lipid Removal in Muscle Cells*

Muscle cells are important for maintaining the structure and function of the body, and they include cardiac muscle cells, skeletal muscle cells, vascular smooth muscle cells, and so on. Starvation can trigger autophagy in muscle cells. In starving GFP-LC3 transgenic mice, GFP-LC3 positive autophagosomes were significantly increased in muscles. This finding indicated that autophagy participated in the metabolism of muscles. Specifically, mice with disrupted Atg5 or Atg7 gradually displayed muscle atrophy during aging. The atrophic muscle cells exhibited accumulated p62, ubiquitinated proteins, and abnormal mitochondria, and their sarcomeres were disorganized.

In addition, the body and fat mass were significantly decreased in muscle, specifically in the Atg7-knockout mice, and the glucose clearance and energy expenditure was enhanced in these cells (Kim and Lee 2014).

The Pompe disease model mice, which is based on deletion of alpha-glucosidase, are manifested as accumulated glycogen in lysosomes. Inhibition of autophagy in the Pompe disease model can rescue glycogen consumption (Mizushima and Komatsu 2011), indicating that autophagy regulates glucose utilization in muscle cells. Furthermore, like a locomotive organ, skeletal muscle consumes large amounts of energy to maintain exercise capacity. Thus, exercise becomes a stimulator for autophagy activation specifically in skeletal muscle cells. In fact, inhibition of autophagy by either mutating the key loci of Bcl2, which is essential for dissociation of the Bcl-2–Beclin-1 complex or generating haploinsufficient beclin-1 ($Becn1^{+/-}$) impaired the exercise endurance and metabolic improvement of mice after exercise training (Kim and Lee 2014). This indicates that autophagy is not only important for the homeostasis of muscle cells but also essential for their normal functions.

### *23.4.3 Autophagy Participates in Homeostasis Regulation of Lung Epithelial Cells*

Lung consists of diverse cell types, such as lung epithelial cells, lung endothelial cells, and lung mesenchymal cells. The lungs are mainly responsible for the exchange of air. Currently, only a few studies have focused on autophagy function in the lungs. In their studies, they found that autophagy was essential for the homeostasis of lung epithelial cells. Knocking out Atg7 in bronchial epithelial cells caused p62 accumulation and Nrf2 activation. Nrf2 is an important factor for maintaining intracellular redox homeostasis. In addition, ER stress was observed in normal and transformed lung cells; cigarette smoke can induce transformation of lung cells. It was found that autophagy could be activated in response to ER stress and that it functioned in degrading aggregated proteins to reduce cytotoxicity (Ryter and Choi 2010). These results indicated that autophagy participated in the homeostatic regulation of lung epithelial cells. Many studies have focused on lung disease and lung cancer, and these works have proven that autophagy levels or autophagy flux is abnormal in lung disease models and cancer. By using electron microscopy, scientists observed more autophagosomes in lung tissues with the chronic obstructive pulmonary disease compared with normal human lung tissues. They also found that Atg genes (Atg4, Atg5, Atg7, and LC3B) were highly expressed in diseased lung tissues (Ryter and Choi 2010). Furthermore, to explore autophagy function in normal lung cells, disease models may be helpful for understanding autophagy regulation in lung metabolism.

### 23.4.4 Autophagy Participates in Bone Development and Diseases

Bone cells include chondrocytes, osteoclasts, osteocytes, osteoblasts, and so on. Autophagy levels differ in these bone cells, and the LC3 expression level in osteocytes is higher than that in osteoblasts. Under stress, such as starvation or hypoxia, autophagy activity significantly increases in bone cells. Recent studies have found that autophagy plays a vital role in maintaining bone homeostasis. Autophagy can protect bone cells from oxidative stress from apoptosis. Inhibition of autophagy by drugs increased oxidative stress and triggered apoptosis. In contrast, activation of autophagy can decrease oxidative stress and apoptosis.

Furthermore, autophagy regulates the development of bone. Deletion of Atg7 in chondrocytes reduces the length of femur and tibia. A study revealed that autophagy regulated type II collagen secretion in growth plate chondrocytes. Developmental regulation of autophagy was essential for bone growth, and FGF12 participated in autophagy regulation in chondrocytes (Cinque et al. 2015). Another study conducted in rats found that the absence of FIP200 in osteoblasts caused the loss of bone mass. Deletion of Atg genes (Atg5, Atg7, and LC3) affected osteoclast brush border formation. All these results reveal that autophagy is important in bone development. In many bone diseases, researchers found that dysfunctional autophagy occurred. Mutations in p62 were frequently identified in Paget disease of bone, and mutations in p62-enhanced osteoclastogenesis (Kim and Lee 2014).

### 23.4.5 Autophagy Regulates Pancreatic β-Cell Quality and Functions

Autophagy plays an important role in maintaining the quality and functions of pancreatic β-cells. Inhibition of autophagy in pancreatic β-cells leads to decreased cellular mass and accumulation of ubiquitinated proteins and abnormal organelles. With electron microscopy and staining, large inclusion bodies and abnormal organelle structures (mitochondria and endoplasmic reticulum) were observed in autophagy-deficient pancreatic β-cells, reflecting the vital role of autophagy in resisting ER stress (Kim and Lee 2014). Insulin secretion was impaired in autophagy-deficient pancreatic β-cells. The mice with pancreatic β-cell-specific knockout of Atg7 displayed glucose intolerance and hyperglycemia. Moreover, pancreatic β-cells displayed increased apoptosis and decreased proliferation. In the context of insulin resistance, the activity of autophagy was enhanced and was accompanied by increased insulin secretion from pancreatic β-cells (Mizushima and Komatsu 2011).

### *23.4.6 Others*

In adipose tissues, autophagy regulates adipose cell features. Adipose tissue-specific knockout of Atg7 decreased the mass of fat. In this case, the loss of autophagy impaired the differentiation of white adipocytes such that they presented with the same features as brown adipose tissues (Kim and Lee 2014). Knocking out Atg5 impaired the differentiation of white adipocyte tissue. Consistent with this, deletion of Atg7 in brown adipose tissue repressed brown adipose differentiation and enhanced the development of beige adipose tissue (Altshuler-Keylin and Kajimura 2017).

Autophagy participates in the lipid metabolism of the liver (Kim and Lee 2014). In mice, liver-specific knockout of Atg7 in the liver led to an increase in lipid content in the liver. Consistently, knocking out another important autophagy gene, Vsp34, also led to lipid accumulation in the liver. Furthermore, deletion of Atg7 in hepatic stellate cells triggered lipid accumulation and impaired liver fibrosis.

Autophagy is not only involved in the abovementioned specialized cells but it also plays critical roles in other cells and tissues. An increasing number of studies have found that autophagy can sustain cellular homeostasis. Disruption of autophagy is closely connected with disease occurrence. Therefore, exploring autophagy regulation in tissues and cells is helpful for exploring metabolic diseases.

## 23.5 Summary

Autophagy runs through the entire life process, including the development of fertilized eggs, the formation of stem cells, cell lineage differentiation, and organ formation. With the advancement of life science research, an increasing number of studies have revealed the physiological significance and molecular mechanisms of autophagy. Here, we review the regulation of autophagy in normal stem cells and in specialized cells. Since the study of autophagy is in the emergent stage, the regulatory mechanisms and physiological functions of autophagy are being revealed in different species, in different cells and at different developmental stages. In research on the autophagy of stem cells and specific cells, there are still many unknowns to explore and puzzles to solve.

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# Chapter 24
# Autophagy, Aging, and Longevity


**Li Luo and Zheng-Hong Qin**

**Abstract** Autophagy is a conserved process that degrades intracellular components through lysosomes, thereby maintaining energy homeostasis and renewal of organelles. Mounting evidence indicates that autophagy plays a key role in aging and aging-related diseases. Enhanced autophagy can delay aging and prolong life span. The absence of autophagy leads to the accumulation of mutant and misfolded proteins in the cell, which is the basis for the emergence and development of neurodegenerative diseases and other aging-related diseases. It will be of importance to develop approaches to extend the lifespan and improve the health of elderly individuals through the modulation of autophagy.

**Keywords** Autophagy · Aging · Longevity

## Abbreviations

| | |
|---|---|
| CMA | Chaperone-mediated autophagy |
| FOXO3a | Forkhead transcription factor |
| HSP70 | Heat shock protein 70 |
| IGF-1 | Insulin-like growth factor 1 |
| LAMP2a | Lysosome-associated membrane protein type 2a |
| LC3 | Microtubule-associated protein 1 light chain 3 |
| Nrf2 | NF-related nuclear factor |
| SAMP8 | Senescence-accelerated mouse P8 |
| TOR | Target of rapamycin |

L. Luo
School of Physical Education and Sports Science, Soochow University, Suzhou 215021, China

Z.-H. Qin (✉)
Jiangsu Key Laboratory of Neuropsychiatric Diseases, Department of Pharmacology and Laboratory of Aging and Nervous Diseases, College of Pharmaceutical Sciences of Soochow University, Suzhou, China
e-mail: qinzhenhong@suda.edu.cn



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TSC1 Tuberous sclerosis complex 1
ULK1 Unc-51-like autophagy activating kinase 1

Aging is defined as an irreversible deterioration of structure and function due to the increase of age. With the growing number of aging populations, great attention has been paid to the health, social, and economic problems caused by aging. Recently, some interesting results have been reported that can add some insights into how and why aging occurs. Autophagy has attracted much attention since it is a key mechanism related to aging and degenerative diseases.

## 24.1 Aging

Aging is a common phenomenon in eukaryotes. For example, in plants, leaf senescence and fruit senescence are very common phenomena. Single-cell yeast also shows an obvious senescence phenotype. The biological significance of aging is to maintain the stability and evolution of species.

### *24.1.1 Cellular and Molecular Characteristics of Aging*

In 2013, the journal "Cell" published a review summarizing the nine characteristics of aging: "genomic instability, telomere loss, epigenetic changes, loss of protein stability, disturbance of nutritional sensitivity, mitochondrial dysfunction, cell senescence, stem cell depletion and changes in intercellular communication" (Lopez-Otin et al. 2013). Among them, protein stability and mitochondrial function are closely related to the autophagy signaling pathway.

### *24.1.2 Signaling Pathways Related to Aging and Hypothesis About the Mechanism of Aging*

Aging requires the activation of specific signaling pathways, which are involved in the regulation of the cell cycle. These signaling pathways were first found in tumors and are associated with the occurrence and treatment of certain tumors, which is also the reason some researchers do not agree on the existence of aging.

#### 24.1.2.1 Signaling Pathways Related to Aging

p53/p21 Signaling Pathway

The tumor suppressor gene p53 is involved in the regulation of cell functions closely related to apoptosis, autophagy, and senescence. DNA damage is an important part of aging; severe damage directly causes apoptosis, and p53 levels increase gradually. In contrast, slight DNA damage can cause consenescence. After treatment with hydrogen peroxide or genotoxic drugs, DNA damage occurs and leads to the activation of the ATM/ATR signaling pathway; the damage signal is transmitted to p53, causing a transient increase in p53, activating p21, and eventually leading to cell senescence (Luo et al. 2014).

p16/Rb Signaling Pathway

In this signaling pathway, the damage signal causes a continuous increase in p16 expression, which leads to the dephosphorylation of Rb, blocking the cell in the G1 phase. Therefore, the high expression of p16 in normal diploid cells is one of the molecular markers of cellular aging. Although cell senescence can be induced by activation of a single signaling pathway, there is an interaction between the p16/Rb and p53/p21 signaling pathways that further refines the regulation of aging (Radpour et al. 2010).

Skp2/p27 Signaling Pathway

Skp2 is a member of the f-box protein family and forms a protein complex with skp1/cullin to specifically recognize target proteins in the ubiquitin-proteasome degradation pathway. In this signaling pathway, external injury signals are transmitted through Pten phosphatase, which inhibits Skp2 activity and leads to increased expression of p27, p53, and p16. These proteins were not activated, and their expression levels were not increased in senescent cells induced by this signaling pathway.

#### 24.1.2.2 Hypothesis on the Mechanisms of Aging

How does aging occur? There is still a lack of reliable and convincing theories. In recent years, the “free radical theory” and “telomere shortening theory” have been supported by increasing experimental evidence and may fuse into one unified hypothesis.

### The Free Radical Theory

In 1956, Harman first put forward this hypothesis. It is proposed that the excessive production of free radicals in the body causes damage to proteins, nucleic acids, and lipids, and the accumulation of the oxidation products of this damage leads to the appearance of aging phenotypes. Later, this hypothesis was developed further, and it was proposed that free radicals mainly come from mitochondrial metabolism. Fruit flies, mice, and other animal models of different ages were studied, and it was found that the content of free radicals increased continuously and reached its maximum at an older age. The most direct evidence is that the human catalase gene is expressed in the nucleus of mouse cells without any effect on longevity. Targeted expression in mitochondria can significantly extend the life span of mice by 4.5 months, suggesting that free radicals are closely related to aging. However, this hypothesis also has issues. An American medical association organized a large-scale randomized, double-blind, follow-up study on the effects of antioxidants with 14641 men over the age of 58 years. It was found that antioxidants (such as vitamin C and vitamin E) do not reduce the incidence of cardiovascular disease, prostate cancer or other cancers (Wang et al. 2014). In addition, a number of studies have shown that low concentrations of free radicals have signaling functions and are necessary for sperm development and elimination of pathogen invasion. Therefore, the free radical-induced aging hypothesis still lacks critical experimental evidence.

### The Hypothesis of Telomere Shortening

One theory proposed that aging is caused by the shortening of telomeres. Telomeres are short DNA repeat sequences (TTAGGG), with specialized structures at the end of chromatin that stabilize the chromosomal structure. Telomerase keeps their length stable by lengthening telomeres. Detection of telomere length in the leukocytes of different age groups showed that there is significant telomere shortening in elderly people. Through scanning human chromosomes, it was found that DNA damage occurred in the process of telomere shortening, and reactive oxygen species increased significantly, leading to genomic imbalance and initiating the process of cell senescence. By using $\gamma$ rays to cause telomere damage, it was found that the damage cannot be repaired, and the continuous activation of the DNA damage signaling pathway can eventually lead to cell senescence. However, this theory also has inconsistencies. For example, while telomeres shorten in humans with age, this phenomenon has not been observed in mice. Even when the telomerase was removed by gene knockout, the telomere was not shortened at the death of the first generation of mice, and the shortening was not obvious until the fourth generation of mice. The difference between humans and mice suggests that "telomere shortening" has limitations in explaining aging (Sahin and DePinho 2012).

In short, there is no theory of aging that can perfectly explain how and why aging occurs. There are still many unresolved issues to be explored.

## 24.2 Autophagy Is Downregulated in the Process of Aging, and Inhibiting Autophagy Accelerates Aging

The most obvious feature of aging skin is the appearance of senile plaques, in which oily skin is quite common. The formation of senile plaques is closely related to lipofuscin. Lipofuscin is a fluorescent dense substance that is observed in cell senescence. It is mainly composed of lipid residues and some other substances no degradable by lysosomes. Its formation is closely related to the damage of lysosome function, which is a key organelle in autophagy. Almost all tissues showed morphological and enzymatic changes in the lysosome system (Wang et al. 2014).

A decrease in LAMP2a (lysosome-associated membrane protein type 2a) has been observed in the livers of aging mice. During aging, the decline in chaperone-mediated autophagy (CMA) significantly weakens the binding of damaged proteins to lysosomal membranes and their transport into lysosomes. CMA is an autophagic activity mediated by molecular chaperones such as heat shock protein 70 (HSP70), which can specifically recognize and bind to degraded proteins and then transport them to lysosomes with the help of receptor LAMP2a. In addition, the progressive decline of LAMP2a expression on the lysosomal membrane can lead to a decrease in CMA efficiency. The expression of LAMP2a was also reduced in transgenic mice. LAMP2a transgenic mice are younger than wild-type mice at the same age. This phenomenon may be due to the increase in LAMP2a, which avoids the CMA decay related to aging, thus reducing the accumulation of ubiquitinated protein aggregates, oxidized products and apoptotic cells in the liver (Zhang and Cuervo 2008).

A large number of studies have shown that during aging, the expression of key proteins in some signaling pathways is downregulated and autophagy function is significantly reduced. The senescence-accelerated mouse P8 (SAMP8) model is a nongenetically modified animal model that has similar pathological characteristics to Alzheimer's disease. During the aging process of SAMP8 mice, some age-related autophagy functions decrease and ubiquitinated protein accumulated in the brain (Ma et al. 2011). Autophagy-related proteins such as Atg5, Atg7, and beclin1 were found to be expressed at lower levels in aging human brains than in young brains (Crews et al. 2010). In osteoarthritis, unc-51-like autophagy activating kinase 1 (ULK1), beclin1, microtubule-associated protein 1 light chain 3 (LC3) and other autophagy-related molecules are also downregulated.

Nematodes are some of the most commonly used model organisms in longevity research. In worms, studies have found that mutations in autophagy-related genes such as Atg1, Atg7, Atg8, and beclin1 can shorten the lifespan of nematodes. Similarly, in *Drosophila melanogaster (D. melanogaster)*, reducing the expression of Atg1, Atg8, and Estrin1 also shorten its life span. Inhibition of autophagy can also cause triglyceride accumulation, mitochondrial dysfunction, muscle degeneration, and cardiac dysfunction related to aging.

## 24.3 Enhanced Autophagy Can Delay Aging and Prolong Life Span

### *24.3.1 The Anti-aging Effects of Caloric Restriction Are Related to the Activation of Autophagy*

Caloric restriction has been proven to prolong life and resist aging in most animals. In rhesus monkeys, calorie restriction reduces the incidence of diabetes, cardiovascular disease, cancer, and brain atrophy. Epidemiological studies have also shown that caloric restriction is beneficial to human health. Caloric restriction can activate autophagy by activating the energy sensors AMPK and Sirtuin 1. In addition, caloric restriction can also inhibit the target of rapamycin (TOR) and activate autophagy by inhibiting the insulin/insulin-like growth factor signaling pathway. In yeasts, nematodes, fruit flies and mice, inhibiting TOR by drug (rapamycin) treatment or gene knockout can increase life span. When the TOR signaling pathway was inhibited, calorie restriction no longer increased life span. When an Atg gene is knocked out or knocked down, the life-prolonging effect of rapamycin disappears, suggesting that autophagy plays an important role in the life-prolonging effect of rapamycin (Harrison et al. 2009).

Sirtuin 1 (SIRT1) is a silencing factor in mammals. Its homologous analogues are Sir-2 in yeast and Drosophila and Sir-2.1 in worms. Overexpression of this gene can delay aging and prolong life span. In yeasts, worms, and Drosophila, caloric restriction can prolong life partly because of its upregulation of SIRT1 expression. A lack of Sir-2 can relieve the effect of caloric restriction on prolonging life span. Resveratrol can activate SIRT1 directly or indirectly to prolong life span, but when Sir-2 is absent in yeasts, worms, and fruit flies, this effect disappears. Similarly, when SIRT1 was knocked out, calorie restriction did not show a corresponding increase in life expectancy. In yeasts and nematodes, the presence of SIR-2 is required for calorie restriction and for resveratrol to enhance autophagy. When the Atg gene was knocked out or downregulated, the effects of calorie restriction, excessive expression of resveratrol and SIR-2 on longevity were eliminated (Guarente 2007). Therefore, the life-prolonging effect caused by the above factors requires SIRT1, and autophagy plays an important role in this process.

### *24.3.2 Enhancement of Mitophagy Can Prolong Life Span and Improve Health During Aging*

The accumulation of damaged mitochondria is a common marker of aging and related diseases. Although the underlying mechanism of mitochondrial dysfunction in aging is still far from clear, the decline in mitophagy may play a key role in it. In mammals, damaged mitochondria are degraded through the PINK1 and E3

ubiquitin-protein ligase Parkin pathways. Mitophagy plays an important role in many aging-related diseases, such as heart disease, retinopathy, kidney diseases, fatty liver, pulmonary hypertension, and neurodegenerative diseases. Mitophagy markers were found to decrease during aging in worms, *D. melanogaster*, mice and humans. This may be related to the loss of Pink1 or Parkin, which can shorten the life span of *D. melanogaster* and lead to earlier behavioral changes. Studies using the nematode model have also confirmed the importance of mitophagy for longevity. The DCT-1 gene is a homologue of the mammalian proteins BNIP3 and BNIP3L, which are receptors of mammalian mitophagy. Inhibition of DCT-1 or PINK1 shortened the lifespan of long-lived DAF-2 and Eat-1 mutants, as well as several long-lived nematode models.

Some studies have explored the effects of mitophagy on aging and longevity. Parkin was studied extensively or specifically in neurons and was proven to prolong the life span of *D. melanogaster*. In addition, it has been reported recently that the mitochondrial morphology of *D. melanogaster* becomes longer in middle age, which is related to the damage of mitophagy and mitochondrial dysfunction. Drp-1 was induced temporarily in middle age, which delayed the appearance of the aging phenotype and prolonged life span. Promotion of Drp-1-mediated mitochondrial division in middle age can make the mitochondrial morphology transit to a "young state", which contributes to mitophagy and improves mitochondrial respiration. This suggests that mitophagy, at least partially related to mitochondrial dynamics, is involved in the mitochondrial dysfunction associated with senescence in *D. melanogaster*.

Based on the above findings, some scholars suggest that pharmacological measures of promoting mitophagy may be an effective way to delay the decline of health during aging. This viewpoint was supported by some studies. Dietary administration of urolithin A can induce mitophagy and prolong life span. Urolithin A treatment restored the expression of a series of markers related to longevity in nematodes and protected mitochondrial respiratory function. At the same time, urolithin A prolongs life span in relation to the mitophagy genes pink-1 and dct-1. Urolithin A treatment is also beneficial to rodents and can improve aging-related skeletal muscle dysfunction (Ryu et al. 2016). This suggests that the beneficial effects of mitophagy on health are conserved across species.

## 24.4 Longevity and Anti-aging Effect

Longevity refers to the phenomenon that some individuals live longer than the average life span of the species. As far as humans are concerned, the average life expectancy is constantly changing. According to an independent survey, the incidence of Alzheimer's disease in people over 85 years old is 50%, which can be used as a reference for defining longevity. Therefore, people who are over 90 years old and still have no obvious diseases can be regarded as longevity elderly persons. As far as longevity is concerned, centenarians, also known as super longevity elderly persons, are the most studied and are suitable to explain the mechanism of longevity.

Research on centenarians can help people take measures to reduce the incidence of aging-related diseases.

Longevity is related to the progress of civilization and is a goal of mankind. However, for a long time, there has been limited progress in revealing the mechanism underlying longevity. This is mainly due to the complicated influencing factors underlying longevity, which makes it difficult to obtain reliable conclusions. According to an investigation on centenarians and their environment, the following five factors are concluded as to their health and longevity of centenarians: genetic factors, good environment, diet control, moderate exercise, and good psychological state. The latter three factors are self-regulated and are part of the theoretical basis of anti-aging (de Cabo et al. 2014; Table 24.1).

### *24.4.1 Longevity and Vitality Gene Groups*

To live over the age of 100, the genetic backgrounds from the parents play a decisive role including genes and epigenetic mechanisms. The most direct evidence is that there is a high probability of centenarians in families with long-lived members. It is the familial inheritance that has led some researchers to consider that there is a specific "longevity gene". To date, however, no genes responsible for longevity have been identified. At present, the insulin-like growth factor-1 (IGF-1) signaling pathway and fork head transcription factor (FOXO3a) have been found to regulate longevity. Many single nucleic acid polymorphism loci have been found to be associated with longevity in centenarians.

Detecting changes in vitagenes may explain the possible path to longevity from a genetic perspective. The vigorous gene group is a collection of genes related to anti-cell stress. At present, there are clear active genes: NF-related nuclear factor 2 (Nrf2), Sirtuin family, FOXO3a, heat shock protein family and so on. In normal cells, these genes are often expressed under heat stress, oxidative stress, and toxic stress, which are conducive to cell survival. In addition, these genes also activate autophagic signaling pathways to remove damaged organelles and denatured proteins and induce apoptosis to eliminate damaged cells.

### *24.4.2 The Mechanism of Longevity in Model Organisms*

Due to the great individual differences in human life span, it has been difficult to make breakthrough progress in the study of longevity mechanisms. In recent years, many achievements have been made on lower level model organisms such as nematodes that have provided many useful insights into the mechanisms of human longevity.

The average life span of *Caenorhabditis elegans* is 22 days, which is very useful for life-span research. The IGF-1 signaling pathway of mutant nematodes can prolong life span, mainly due to increased expression of DAF-16 (FOXO3A homologue).

**Table 24.1** Relationship between autophagy and aging

| Autophagy deficiency and genotype | Phenotype | Disease relevance | Reference (PubMed identification code) |
|---|---|---|---|
| $Bcn1^{+/-}$ | Enhanced inflammation, steatohepatitis, aged mice hepatocellular carcinoma, lung adenocarcinoma, and lymphoma frequency increases; neuron accumulation amyloid β after human amyloid precursor protein transgene expression | Aging-related malignant tumor; neurodegenerative disease | 19524509 18497889 |
| Knock out the central nervous system Atg5 or Atg7 | The accumulation of ubiquitinated proteins in neuronal cell bodies is accompanied by loss of cortical and cerebellar neurons | Neurodegenerative disease | 16625205 16625204 |
| Knock out Perkin's cell Atg5 or Atg7 | Axonal dystrophy and axonal degeneration; accompanied by Purkinje cell death and cerebellar ataxia | | 17726112 |
| Knock out the central nervous system FIP200 | Ubiquitinated protein accumulation, cerebellar cell loss and cortical cavernous vascular disease in Purkinje cell bodies | | 19940130 |
| Knock out HDAC6 | Accumulation of ubiquitin cations in brain cells at 6 months of age | Neurodegenerative disease | 20075865 |
| Knock out PINK1 | Selective mitochondrial defects in the striatum (complex I, II) | Parkinson's disease | 18687901 |

(continued)

**Table 24.1** (continued)

| Autophagy deficiency and genotype | Phenotype | Disease relevance | Reference (PubMed identification code) |
|---|---|---|---|
| Knock out skeletal muscle Atg7 | Muscle atrophy and age-dependent strength decline; abnormal mitochondrial volume accumulation, sarcoplasmic reticulum expansion, sarcomere disintegration | Skeletal muscle reduction | 19945408 |
| Knock out podocyte-specific constitutive Atg5 | Spontaneous age-dependent late-onset glomerulosclerosis, accompanied by accumulation of oxidative and ubiquitinated proteins, compensatory proteasome activation, lipofuscin, impaired mitochondria, ER stress, albuminuria, podocyte loss | Glomerulosclerosis | 20200449 |

It was also confirmed that interfering with this signaling pathway could prolong the life span of female mice. At first, it was thought that the increase in nematode longevity caused by low temperature was due to a decrease in chemical reactions, but in fact, it was not so. It was found that the increase in longevity caused by low temperature disappeared when the TRPA-1 channel was absent. TRPA-1 activates calcium influx and calcium-dependent PKC and then induces the expression of DAF-16, which is the mechanism of low-temperature-induced longevity. Genome analysis identified the regulator of nematode DAF-16. It was found that deletion of PQM-1, a widely regulated gene, could inhibit the longevity and development process caused by DAF-2. In addition, the expression of PQM-1 decreased with age. Removal of the gonads of nematodes can prolong longevity, which is related to the regulation of the let-7 microRNA family during L2–L3 transformation. These microRNAs prolong longevity by targeting the nuclear factor lin-14 and Akt-1, a kinase of early larvae and then activating DAF-16. Genetic analysis of mice and nematodes showed that mitochondrial ribosomal protein MRPs5 is a regulator of metabolism and longevity, which causes mitochondrial unfolded protein reactions and prolonged life span. By

using a variety of mutant models in mice it was confirmed that maternal mitochondrial DNA mutations significantly affected offspring' life span, accelerated the aging process of offspring mice, and randomly damaged brain function.

### *24.4.3 Longevity Signaling Pathways*

#### 24.4.3.1 Insulin/IGF-1 Signaling Pathway

Moderate inhibition of the insulin/IGF-1 signaling pathway is an evolutionarily conservative mechanism for the body to inhibit aging (Ziv and Hu 2011). DAF-2 is the receptor of insulin growth factor. When the DAF-2 gene was mutated, the life span of the nematode increased three times as much as that of the wild-type nematode. When cells are stimulated by nutrients, the activation of the insulin/IGF-1 pathway leads to the activation of the PI3K-AKT signaling pathway, and then the downstream transcription factor FOXO is phosphorylated and localized from the nucleus to the cytoplasm, which has adverse effects on DNA repair, cell proliferation, and antioxidant stress. Therefore, when ACE-1, the receptor of PI3K, is inhibited, the life span of *D. melanogaster* is prolonged, and FOXO in the nucleus directly binds to the promoter regions of antioxidant enzymes (such as catalase and mitochondrial superoxide dismutase), thereby increasing the expression of antioxidant enzymes, promoting the removal of reactive oxygen species, and enhancing the ability of antioxidant stress. Klotho participates in the metabolism of vitamin K, calcium, and phosphorus, protects the cardiovascular system and affects the immune function of the body. The Klotho gene is closely related to aging, which is related to inhibiting the activation of the insulin/IGF-1 signaling pathway (Owusu-Ansah et al. 2013). The mitogen-activated protein kinase superfamily pathway mimics the insulin/IGF-1 signaling pathway via the FOXO-related transcription factor DAF-16, which can induce DAF-16 to enter the nucleus. These results suggest that the insulin/IGF-1 signaling pathway or related pathways play a central role in regulating aging.

#### 24.4.3.2 TOR Signaling Pathway

TOR is a highly conserved serine/threonine-protein kinase that regulates cell growth and proliferation. TORC1 is sensitive to rapamycin and participates in intracellular transcription and translation. TORC2 is not sensitive to rapamycin and mainly regulates the remodeling of the cytoskeleton. TOR has been identified as another well-recognized signaling pathway regulating longevity. When the TOR signaling pathway is downregulated or inactivated in worms or fruit flies, the life span of yeasts is increased, and the life span of yeasts can be prolonged by injecting low doses of rapamycin. It is believed that TORC1 plays an important role in the anti-aging pathway and prolonging life span. S6K is a positive regulatory target downstream of TORC1. When S6K is knocked out in mice, the life span of mice is prolonged;

4E-BP is a negative regulatory target and is necessary for the normal life cycle. When 4E-BP is overexpressed, the life span of mice is prolonged (Weatherill et al. 2010). In addition, the TOR signaling pathway is directly involved in the regulation of autophagy. In yeast, TORC1 is involved in the regulation of the autophagy genes Atg1, Atg13, and Atg17, and when the TORC1 signaling pathway is blocked, life expectancy increases, which is related to the induction of autophagy (Kamada 2010).

## 24.5 Anti-aging Effect of Autophagy

### *24.5.1 Autophagy, Protein Homeostasis, and Neurodegenerative Diseases*

There is abundant evidence that autophagic dysfunction plays a central role in the occurrence and development of neurodegenerative diseases (Leidal et al. 2018). As a postmitotic cell, neurons cannot dilute misfolded proteins and damaged organelles through cell division, so the maintenance of neuronal homeostasis is highly dependent on autophagy. Dysfunction of autophagic lysosome degradation can lead to damage of neuroendocrine homeostasis and accumulation of lipofuscin, which will further inhibit autophagy and form a feedback cycle. It was found that autophages with decreased degradation ability accumulated in the hippocampus of aged mice, which may be related to cognitive impairment. When Atg5 or Atg7 is inactivated in mice, the mice exhibit neurobehavioral deficiencies, suggesting that autophagy plays an important role in maintaining the homeostasis of the intracellular environment. In addition, in autophagy-deficient neurons, the volume and quantity of polyubiquitinated proteins increased with aging. This suggests that autophagy can continuously remove abnormal proteins, thus playing an important role in maintaining the intracellular environment of nerve cells.

Many neurodegenerative diseases are characterized by abnormal folding and aggregation of cytotoxic proteins. These diseases include Alzheimer's disease, Parkinson's disease, tau protein lesions, and Huntington's disease. Many pathogenic proteins can only be degraded by proteasomes or CMA in soluble form. CMA specifically targets matrix proteins on the surface of lysosomes and digests protein structures one by one. When proteins are irreversibly degraded into oligomers or polymer complexes and the structure of these proteins cannot be fully unfolded, the protein structure can be degraded by large autophagy. Once macroautophagy begins to decrease and is accompanied by CMA, aggregated proteins can lead to neurological impairment and ultimately to neuronal death (Cuervo and Wong 2014).

Autophagy inducers, including rapamycin, rapamycin derivatives, valproic acid, and lithium, can alleviate abnormal Huntington protein accumulation and cell death in Huntington's disease models. In addition, rapamycin-activated autophagy can be used to treat diseases induced by mutations and wild-type tau proteins. Overexpression of tuberous sclerosis complex 1 (TSC1) and TSC2 in photoreceptor neurons of *D. melanogaster* can inhibit TOR and stimulate polyglutamine to boost Huntington protein clearance in vivo, which has the same effect as overexpression of Atg1. Cells overexpressing TSC1 and TSC2 can avoid aggregation of intracellular complexes caused by phospholipase C mutations induced by neurodegenerative diseases (Avet-Rochex et al. 2014). Many drugs can increase the clearance of abnormally aggregated proteins through autophagy, thus playing a role in Alzheimer's disease, Huntington's disease, and other neurodegenerative diseases.

### *24.5.2 Mitophagy and Mitochondrial Dysfunction in Aging*

Mitochondria are the main sites of energy metabolism in the cell. In addition, mitochondria also play an important role in the regulation of cell apoptosis, gene expression, redox potential, signal transduction, and electrolyte homeostasis. In 1972, Harman proposed the theory of mitochondrial aging, that is, the accumulation of free-radical damage in the mitochondria leads to cell aging. During aging, the function of the mitochondrial respiratory chain complex and energy production decrease progressively. In addition, the increase of reactive oxygen species (ROS) production results in oxidative damage to biological macromolecules such as proteins and lipids. In addition, the mitochondrial pathway can activate cell apoptosis. Mitochondrial DNA (mtDNA)-deficient mice showed premature aging. The mtDNA copy number in the long-lived elderly population is significantly higher than that in the general elderly population, suggesting that the maintenance of mitochondrial function may be the key to their health and longevity.

In the aging process, mitophagy usually plays a protective role, which can prevent the production of reactive oxygen species and the accumulation of toxic proteins and remove damaged mitochondria, which is essential for maintaining the normal function of neurons. It has been found that mitophagy is involved in the occurrence and development of various neurodegenerative diseases, including Parkinson's disease, Alzheimer's disease, and so on (Fig. 24.1). PINK1 is a protein kinase that is highly expressed in energy-intensive organs such as the heart, muscle, and brain. In the cell, it is mainly located in the intima of mitochondria. PINK1 scavenges damaged mitochondria by triggering mitophagy. Pink1 gene-deficient mice showed some symptoms of Parkinson's disease, resulting in inadequate phosphorylation of mitochondrial respiratory chain complex I (Complex I), resulting in reduced energy production. When the phosphorylation level of Complex I is normal, the phenotype of Parkinson's disease can be significantly reduced or eliminated (Liu and Lu 2010). Parkin is a multifunctional ubiquitin ligase. It is not only a housekeeping protein for protein quality control but is also related to mitochondrial homeostasis and

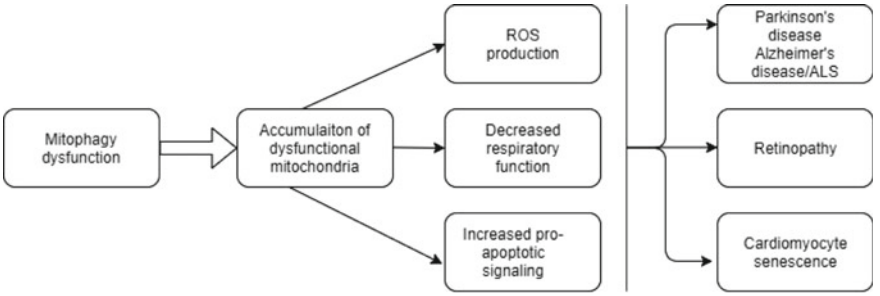


**Fig. 24.1** Mitophagy linked to aging. A decline in mitophagy is a hallmark of aging. The accumulation of dysfunctional mitochondria leads to ROS production, decreased respiratory function, and increased proapoptotic signaling, which is related to aging-related diseases, including Parkinson's disease, Alzheimer's disease/ALS, retinopathy, and cardiomyocyte senescence

stress-related signaling pathways. Parkin also plays an important role in the pathogenesis of Alzheimer's disease and amyotrophic lateral sclerosis. In 2008, Richard Youle and colleagues found that Parkin could selectively recruit mitochondria with reduced membrane potential, and the mediator mitochondria were encapsulated by autophages (Narendra et al. 2008). Since then, Parkin-dependent mitophagy has been extensively studied in many mammalian cells, *D. melanogaster,* mice, and the brains of PD/AD patients. Overexpression of Parkin by extensive or neuronal localization during Drosophila senescence can reduce protein toxicity, alter mitochondrial dynamics and prolong life span (Saini et al. 2010).

Mitochondrial membrane permeability is one of the reasons that restricts cell death. Exceeding the critical value of mitochondrial membrane permeability will cause some mitochondria to initiate the apoptotic pathway and cause cell death. If most of the damaged mitochondria can be selectively removed by autophagy, the remaining mitochondria will have a higher threshold of mitochondrial membrane permeability, a stronger inhibition of cytochrome C release, and a reduction in ROS production, thereby reducing cell death.

### *24.5.3 Autophagy, Cell Senescence, and Stem Cells*

A decreased number of tissue stem cells or their functional deficits may lead to aging. In older mice, rapamycin can prolong life span and stimulate the renewal of hematopoietic stem cells, thereby improving immune system function. When Tsc1 is knocked out in mouse hematopoietic stem cells, TOR activates the cells mimicking aging mice, which eventually leads to a decrease in lymphocyte proliferation and the remodeling of the hematopoietic system. It also increases the levels of CDK inhibitors p16p16 (lnk4a), P19 (Arf) and p21 (Cip1). Although other functions related to TOR also play a role, most of them are caused by inadequate autophagy, for example when Atg7 is knocked out, it eventually leads to severe depletion of hematopoietic

stem cells. There is also evidence that with aging, decreased autophagy may lead to decreased skeletal muscle stem cell function (Garcia-Prat et al. 2016). Autophagy also determines the properties of bone marrow mesenchymal stem cells and cell senescence during bone aging (Ma et al. 2018).

Similarly, inactivation of PTEN, a tumor suppressor gene, can produce a series of reactions different from the DNA damage induced by oncogenes, called PTEN-loss-induced cellular senescence. This process depends on the activation of TOR and can be inhibited by MDM2, leading to the expression of TP53 and ultimately inhibiting autophagy. Rapamycin can prevent cell senescence, which is induced by human expression of p21, without affecting the cell cycle (Italiano et al. 2012). Therefore, mTOR may restrict proliferation and regulate apoptosis of stem cells through aging, which can be inhibited by rapamycin.

## 24.6 Summary

There is growing evidence that autophagy plays an important role in longevity, especially in animal models such as yeast and mice, which confirms that multiple autophagy genes are necessary for longevity strategies. Long-lived animals have a high level of autophagy, which suggests that the enhancement of autophagy is involved in the prolongation of life span. In contrast, autophagy decreases in tissue specificity during normal aging. Autophagy is also involved in regulating the process of aging and the occurrence and development of aging-related diseases. Decreased or defective autophagy results in or accelerate aging; increased autophagy contributes to the elimination of mutant proteins and structural changes of toxic proteins, improves cell viability, and reduces apoptosis of nonrenewable cells, showing an anti-aging effect. There are still many problems to be solved in this field, such as why do autophagy levels in most tissues decrease during aging? What is the mechanism? In addition, what are the consequences of decreased autophagy? What is the precise process of autophagy regulating life? Different strategies for longevity require the involvement of autophagy. If the effects of these strategies on longevity can be superimposed, is there a synergistic effect in inducing autophagy when these strategies are combined? Studying the role of autophagy in aging and longevity will help to provide new ideas for the prevention and treatment of geriatric diseases.

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# Chapter 25
# Autophagy and Ubiquitin-Proteasome System


**Yan Wang and Wei-Dong Le**

**Abstract** Millions of protein molecules are synthesized per minute in each cell, and simultaneously, millions of protein molecules are degraded. Mutated and misfolded newly synthesized proteins are rapidly degraded to prevent the toxicity caused by the accumulation of these protein fragments. There are two main mechanisms of intracellular protein degradation: the ubiquitin-proteasome system (UPS) and the autophagy-lysosome pathway (ALP). There is a certain relationship between these two mechanisms, and there are some molecules that initiate compensatory effects to prevent disease progression.

**Keywords** Autophagy · Lysosome · Ubiquitin · Proteasome

## 25.1 Autophagy-Lysosome Pathway and Ubiquitin-Proteasome System

### *25.1.1 Autophagy-Lysosome Pathway*

Autophagy, an evolutionarily conserved process in eukaryotes, is involved in the turnover of intracellular substances. Macro-autophagy involves a unique membrane structure called phagophore. The bilayer membrane vesicles formed in the cytoplasm coat the damaged proteins or organelles to form autophagosomes. An autophagosome can be directly fused to a lysosome, or it might first fuse to an endocytic vesicle and then to a lysosome, where a lysosomal hydrolase eventually degrades the substrate. The degraded products are recycled back to the cytosol and are subsequently used

Y. Wang (✉)
Department of Pharmacology and Laboratory of Aging and Nervous Diseases, Jiangsu Key Laboratory of Neuropsychiatric Diseases, College of Pharmaceutical Sciences of Soochow University, 199 Ren Ai Road, Suzhou 215123, China
e-mail: yanwang@suda.edu.cn

W.-D. Le
Liaoning Provincial Center for Clinical Research on Neurological Diseases, The First Affiliated Hospital, Dalian Medical University, Dalian 116021, People's Republic of China
e-mail: wdle@sibs.ac.cn



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in new macromolecular structural units or in cell machinery that generates energy. Autophagy was initially considered a batch degradation system caused by starvation or nutrient deficiency. Autophagy is a common degradation process that is highly selective. Damaged organelles and proteins are targets for autophagic degradation. There are two important factors in selective autophagy: the autophagic receptor and the linking protein. These receptors and proteins can transport substrates in the autophagy pathway.

Autophagy is involved in the degradation and elimination of organelles that are damaged, denatured, aged or dysfunctional; denatured proteins; and other biological macromolecules in a process that provides the energy necessary for cell survival and repair. Autophagy can affect every aspect of the biological life process. Studies have demonstrated that multiple major human diseases, such as obesity, diabetes, neurodegenerative diseases, immune disorders and cancer, are associated with abnormal autophagic processes. The regulatory mechanism of autophagy is extremely complicated, and the targets of rapamycin (TOR), PI3K/PKB, ATP/AMPK, etc., play an important role in the occurrence and development of autophagy. In the study of Huntington's disease (HD), dynein was found to have a regulatory effect on autophagy. Decreased dynein function can inhibit the degradation of easily aggregated proteins, such as huntingtin, through autophagy. The activation of dynein may also be associated with other diseases related to intracellular aggregates including Parkinson's disease (PD). Inhibition of dynein activity by increased ATP levels might lead to a regulatory effect on the autophagic degradation of the easily aggregated α-syn in PD.

Over the past decade, approximately 40 conserved autophagy-related genes (*Atg*) have been identified, laying the foundation for understanding and studying the complicated processes of autophagy. The ULK1 complex includes Atg13, FIP200 and Atg101, which are essential elements in the process of phagophore nucleation. Class III phosphatidylinositol 3-kinase (PI3K) complex I and autophagy genes, including Beclin1 and Atg14L are additionally required in the nucleation process (Diao et al. 2015). The complete membrane protein mAtg9 also has a high likelihood of being adsorbed onto the nucleation site of the bacteriophage. When autophagosomes are formed, not only ubiquitin-like protein Atg12 and microtubule-associated protein 1 [MAP1 light chain 3 (LC3)] (the yeast Atg8 homologue) but also conjugated systems are required. The formation of autosomes is not much different from the process of ubiquitination. The E1-like enzyme (Atg7) activates Atg12 and LC3. Activated Atg12 and LC3 then bind with Atg 5 or phosphatidylethanolamine (PE), respectively, through E2-like enzyme (Atg10 and Atg3). The Atg12-Atg5 conjugate and the membrane-binding protein Atg16L are interconnected, and it is possible that they can form high-molecular-weight complexes. The Atg12-Atg5-Atg16L complex likely identifies where the LC3 is lipidated and promotes the interaction of LC3 and PE in an E3-like manner.

### *25.1.2 Ubiquitin-Proteasome System*

The ubiquitin-proteasome system (UPS) is an adenosine triphosphate (ATP)-dependent, highly selective protein degradation system in eukaryotic cells that is involved in the degradation of more than 80% of the cellular proteins. The UPS plays two main roles: on the one hand, the UPS can maintain the quality of cells by decomposing abnormal or damaged proteins; on the other hand, the UPS can control basic biological activities in cells by decomposing proteins with specific functions. The two mechanisms ultimately ensure the proper functions of tissues and organs. The UPS is involved in important physiological and biochemical processes, including cell growth and differentiation, DNA replication and repair, and cell metabolism and immune response. A variety of regulatory factors are involved in the whole UPS process. When these regulatory factors are in a normal state, the decomposition of various cellular proteins ensures the efficient functioning of the body so that dynamic equilibrium is maintained. However, an abnormal UPS can cause imbalanced cellular homeostasis, which leads to a variety of diseases such as tumour formation, neurodegenerative diseases, cardiovascular diseases and kidney diseases (Brown and Kaganovich 2016).

The UPS is an important regulator of a series of life processes in the cell, and is able to remove aged, damaged and misfolded proteins. The UPS is also involved in multiple important biological functions such as inflammatory regulation, immune response, those of the cell cycle and signal transduction, which are closely associated with the occurrence and development of various diseases. Studies have confirmed that the UPS also has important pathophysiological significance in cardiovascular diseases so that it can regulate the occurrence and progression of atherosclerosis, ischaemic-reperfusion injury, familial cardiomyopathy, cardiac hypertrophy, heart failure, etc. In addition, the important role of the UPS lies in its ability to metabolize human waste, such as toxins, fats and cancer cells, when it is fully utilized. Moreover, the energy produced by metabolism can stimulate cell self-replication to complete the self-metabolic repair function of the body.

The UPS functions as a multi-step reaction process that involves many different proteins. Proteins degraded by the UPS pathway are initially labeled with a homopolymer containing 76 amino acid residues of the ubiquitin protein and then undergo a series of enzymatic reactions until they are ultimately degraded by the UPS. Specific organelles (i.e. proteasomes) that are shuttled through specific mechanisms can degrade proteins of the UPS substrates that are labelled with multiple ubiquitin molecules on a single peptide. These monopeptides are released into the cytoplasm or nucleus and digested by soluble peptidases to become amino acids. The whole process of ubiquitination is specific and selective and is composed of three enzymes. The E1 enzyme activates ubiquitin and transports it to the E2 ubiquitin-binding molecule, thereby initiating the ubiquitination process. There are approximately 40 types of E2 ubiquitin-binding molecular codes in the mammalian genome. One of the hundreds of E3 ligases will likely bind to a ubiquitin-carrying E2 and will transfer the ubiquitin to specific lysine residues of the target substrate. Thus, ultimately, it is

expected that one or more sites of a particular substrate will be labelled by ubiquitin. However, these initial modifications are insufficient for transferring the substrates to proteasome sites. Because lysine residues, namely, 6, 11, 27, 31, 33, 48 and 63, are the sites of ubiquitination, each of these sites will likely become a receptor of multiple ubiquitin molecules through subsequent ubiquitination, and then, there is a high likelihood that various types of ubiquitin chains will appear. When at least four ubiquitin molecules are linked onto it, the K48 residue is suitable for the transport processes.

The proteasome is a tubular organelle of proteolytic enzymes. It consists of two parts: one is a 20S core complex, and the other comprises two 19S regulatory complexes. The 19S complex can be organically combined with the shuttle proteins containing the substrates, which enables the substrates to undergo de-ubiquitination, thus ensuring that they accurately reach one of the six protein hydrolysis sites of the 20S core subunits. The catalytic activity of the proteasomes has many specific targets, for example, trypsin, chymotrypsin, and glutamine trans-peptide, for which the function is the same: protein hydrolysis. The size of the proteasome-catalyzed pore is narrow, indicating that the protein substrate is partially expanded before entering the 20S subunit. Therefore, protein complexes and aggregates that are unfolded before being digested by the proteasome are not the preferred substrates for proteasome degradation.

## 25.2 Relationship Between ALP and UPS

The UPS and ALP have long been considered independent and parallel degradation systems. The UPS and ALP were once thought to target different types of proteins; that is, the proteasome system degraded short-lived proteins, while the autophagy pathway degraded long-lived, large protein complexes and damaged organelles. Endocytosis is an important process for many cellular functions (including lysosomal biosynthesis). The discovery that mono-ubiquitination plays a key role in endocytosis indicates that there is a complementary relationship between the ALP and UPS (Fig. 25.1).

### *25.2.1 Autophagy and UPS Compensate for Each Other*

One of the important manifestations of the association between the UPS and autophagy is the upregulated functions of autophagy caused by UPS damage, which is often considered a compensatory mechanism that enables cells to prevent the accumulation of UPS substrates. Studies have shown that the administration of rapamycin to upregulate autophagy in cells and mouse models can protect cells from the cell death caused by proteasome inhibitors (Guo et al. 2017; Russo et al. 2018). Additionally, it has been found that upregulated levels of autophagy protect cells from

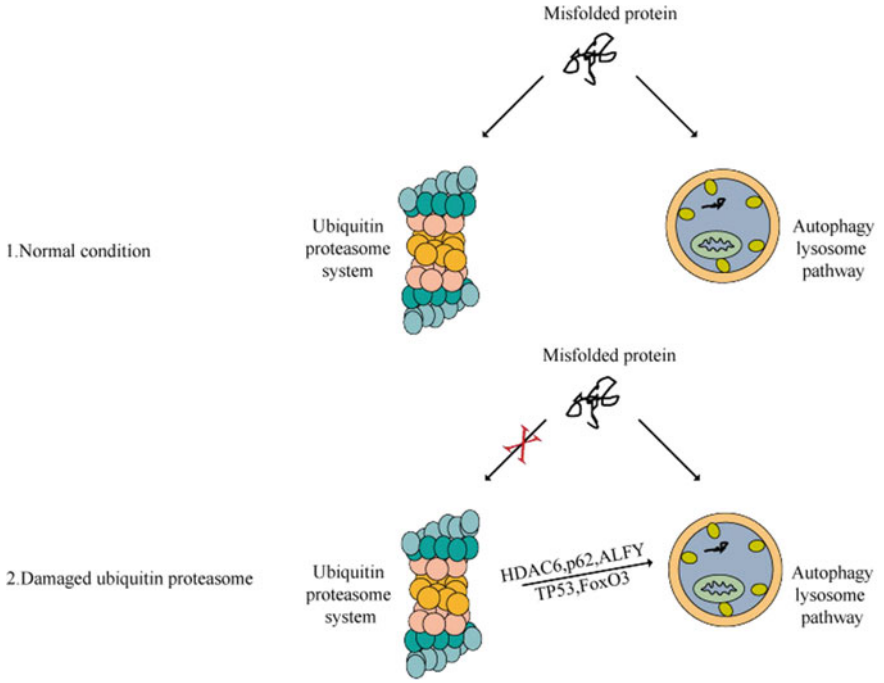


**Fig. 25.1** The association between the ubiquitin-proteasome system and the autophagy-lysosome pathway

cytotoxicity induced by deletions in proteasome-related genes in Drosophila. However, there has been no consensus about the exact mechanism for the interrelationship between the UPS and autophagy, although several possible explanations have been proposed. In one of the described mechanisms, misfolded proteins accumulate to activate an unfolded protein response (UPR), which causes endoplasmic reticulum (ER) stress. The UPR is an ER-nucleus signalling pathway that can lead to the transcription and activation of a variety of genes. It also includes a variety of genes such as those involved in the folding and degradation of ER proteins. The activation of the UPR is likely to cause the initiation of autophagy. The specific mechanisms of this phenomenon are different for different cell types and under different UPR stimuli. Bortezomib, a proteasome inhibitor, has been used to investigate the direct link between proteasome inhibition, UPS activation and autophagy. These studies have all shown that the transcription factor ATF4 plays an important role in the promotion of autophagy caused by proteasome inhibition. However, it has been pointed out that the mechanism of ATF4 upregulation is related to the activation of the PERK arm of the UPR and that eIF2α is needed to phosphorylate the PERK arm. There are also some studies that suggest that the main reason for the change in ATF4 protein level is due to a change in proteasome activity, which is not related to the upstream activity of PERK. These studies have also explored the downstream targets of ATF4,

either by upregulating Atg5 and Atg7 transcription or by upregulating LC3 expression. It has also been pointed out that the initiation of the compensatory autophagy induced by MG132 or bortezomib is mediated mainly by the IRE1 arm of the UPR and its downstream target, c-Jun $NH_2$-terminal kinase (JNK1). Some scholars have noted that JNK1 is likely to induce autophagy through the phosphorylation of Bcl-2, possibly by interfering with the autophagy inhibition induced by Beclin1 and JNK1. In neuronal cells, UPS impairment mediated by proteasome inhibition can activate the phosphorylated p38α-dependent apoptotic pathway and autophagy pathway. The inactivation of p38α greatly increased autophagy activity, reduced protein aggregation and reduced cell loss. Phosphorylated-p38α is a key mediator in the interaction between apoptosis and autophagy in response to UPS impairment (Guo et al. 2017). Inhibiting proteasomes in dopaminergic neurons is likely to induce autophagy through a p53-dependent mechanism, but this process has no relationship with the UPS (Du et al. 2009). It is also quite possible that proteasome inhibition can lead to an increase in p53 protein levels. In addition, the increase in the p53 protein level can potentially increase autophagy through a variety of ways, such as through the inhibition of mTOR. In summary, many researchers have studied and analysed the compensation effect of proteasome inhibition on autophagy and reached a well-established conclusion. However, the specific correlation mechanism requires further analysis and research. It is plausible that these different mechanisms are not mutually exclusive. It is also feasible that different cell types exert different effects at different time points after proteasome inhibition.

Another important manifestation of the relationship between the UPS and autophagy is the possible impact and role of autophagy on the UPS. Study results have shown that the autophagic process is inactivated by knockout of the autophagy gene *Atg5* or *Atg7* (Vuppalapati et al. 2015). There is a reasonable expectation that the ubiquitinated proteins will accumulate in a process that is also mediated by a variety of mechanisms. Some scholars have pointed out that ubiquitinated proteins can be degraded by autophagy, but this view raises some questions. For example, does the type of polyubiquitin chain that is aggregated in autophagy-deficient tissues coincide with the type of polyubiquitin chain associated with autophagy-specific K63? What specific proportion of ubiquitinated proteins degraded by autophagy in all ubiquitinated proteins in the cell? Is much of ubiquitin that accumulated in autophagy-deficient mice due to only the accumulation of ubiquitinated autophagic substrates? Could another mechanism in which the autophagic substrates do not initially undergo ubiquitination explain the reason that the proteins remain in autophagy-deficient cells for a specific period of time before being modified by ubiquitin? Another explanation suggests that autophagy disorders might affect the degradation flux of the UPS. Abnormal autophagy is likely to cause degradation of specific UPS substrates. In autophagy-damaged cells, the decrease in UPS flux was also largely independent of the impaired catalytic activity of the proteasomes. In these autophagy-deficient cells, the aggregated p62 can specifically and effectively mediate UPS dysfunction. When p62 is knocked out, the UPS substrates are restored to normal levels. Moreover, overexpression of p62 can specifically and effectively inhibit UPS, which depends on the p62 ubiquitin-associated (UBA) domains. Because p62 competes with other

ubiquitin-binding proteins involved in proteasome degradation, such as p97/valosin-containing protein (VCP), to bind ubiquitin proteins, the increase in p62 has the potential to prevent these shuttle proteins from turning to ubiquitinated UPS substrates. These findings could help scientists explain the upregulation of the soluble ubiquitinated proteins observed in autophagy-deficient tissues caused by p62 knockout. Therefore, p62 is involved in UPS and autophagy pathways. Under physiological conditions, autophagy operates at a normal rate, and p62 can transport ubiquitinated proteins into the proteasome degradation pathway. However, in the case of impaired autophagy (generally occurring in various pathological conditions, including certain neurodegenerative diseases), p62 may bind non-selectively to ubiquitinated proteins and prevent these ubiquitinated proteins from being delivered to the proteasome degradation pathway. Due to the accumulation of p62, it is too bulky to be a preferred substrate for the narrow catalytic pores in the proteasome pathway.

### *25.2.2 The Regulatory Factors of the Interactions Between ALP and UPS*

Studies have found that some regulatory factors play an important role in the interactions between ALP and UPS. These regulators include histone deacetylase 6 (HDAC6), p62/sequestosome1 (p62), autophagy-linked FYVE protein (ALFY), and the transcription factors Tp53 and FoxO3. In addition to their direct or indirect participation in the two major protein degradation pathways, these proteins are involved in the regulation of aggregate formation.

#### 25.2.2.1 HDAC6

HDAC6 is a cytoplasmic microtubule-associated deacetylase with targets that include α-tubulin, heat shock protein 90, and cortactin. HDAC6 interacts with polyubiquitinated proteins and motor proteins through a highly conserved zinc finger ubiquitin-binding domain to provide a connected pathway for ubiquitination substrates and transport mechanisms (Leyk et al. 2015). Studies have demonstrated that the activity of HDAC6 is critical for the regulation of ubiquitinated proteins and lysosomes in vitro. HDAC6 can regulate substrate transport for autophagy. Overexpression of HDAC6 in Drosophila inhibits the degeneration caused by impaired UPS activity and suppresses cell degeneration caused by toxic polyglutamine expression. This rescue effect induced by HDAC6 is dependent on autophagy. HDAC6 also plays an important role in connecting the UPS with compensatory autophagic processes. Activation of HDAC6 can induce the release and activation of the transcription factor HSF-1 via Hsp90 deacetylation, thereby regulating chaperone expression.

HDAC6 can regulate the fusion of autophagosomes and lysosomes to induce substrate degradation. Under conditions of a defective UPS, the occurrence of autophagic

degradation requires the involvement of HDAC6. In this case, HDAC6 promotes the aggregation of autophagy-specific proteins and affects lysosomal dynamics. The activated compensatory autophagy in UPS-deficient cells exerts a protective cellular effect that depends on HDAC6. However, the mechanism by which HDAC6 is involved in this process is terminated by the effective delivery of the substrate to the autophagy pathway rather than an increase in autophagic flux. Early studies found that HDAC6 can regulate the formation of perinuclear ubiquitinated aggregates. The concentration of misfolded proteins in these aggregates enables their efficient degradation by the ALP. Autophagy compensation is an important pathway in the response to proteasome mutations and UPS injury in Drosophila models of neurodegenerative diseases, and it is also dependent on HDAC6. These studies explain the association between autophagy and the UPS and provide important references for the pathogenesis of neurodegenerative diseases and potential therapeutic options.

#### 25.2.2.2 p62

A cytoplasmic protein, p62 is an important regulatory molecule that links ubiquitinated proteins to autophagy (Zaffagnini et al. 2018). The C-terminus of the p62 protein contains a ubiquitin-associated (UBA) domain (as its main function, this domain is responsible for the interaction of ubiquitinated proteins through non-covalent bonds) and an LC3-interacting region (LIR). Under cellular stress, the transcription and translation of the p62 can be effectively activated, which indicates that p62 has a very wide regulatory role under stress conditions. p62 is found in various ubiquitin-positive neuropathological inclusions such as Lewy bodies in PD, prolonged huntingtin aggregates with polyglutamine in HD, and mutant SOD1 aggregates in familial amyotrophic lateral sclerosis. RNAi-mediated p62 knockdown can exacerbate the toxic effects of polyglutamine, reduce the production of ubiquitin-positive inclusion bodies caused by misfolded proteins under stress, and attenuate the ability of LC3 to co-precipitate with ubiquitinated proteins in vitro. These studies suggest that p62 can cope with misfolded protein stress to protect cells.

p62 has an LC3-recognition sequence, and the mutation of this sequence has the potential to lead not only to ubiquitin-positive inclusions but also to the formation of p62-positive inclusions. Therefore, p62 can specifically and effectively promote the autophagic degradation of ubiquitinated proteins. Furthermore, p62 can function as a conjugate in autophagy and the UPS, which is of great significance. Under the condition of stress caused by misfolded protein, p62-deficient mice are less likely to have ubiquitin-positive protein aggregates, and the p62-deficient mice have the features of neurodegenerative diseases. Similar results were observed in Ref(2)p (the homologue of p62 in Drosophila)-deficient Drosophila. With similar functions, p62 and HDAC6 promote the autophagic degradation of proteins with specific multi-ubiquitin topology specifically and effectively. K63-linked multi-ubiquitin chains recruit p62 and HDAC6 to provide specific and effective autophagic degradation signals.

An important autophagic substrate, p62 recruits ubiquitin proteins through the UBA domain to form homo-oligomers; these complexes contribute to the recognition of ubiquitinated substrates via an autophagy mechanism (p62 directly interacts with LC3 via a specific LIR motif), followed by the engulfment and degradation of these substrates. The UBA domain of the p62 likely has more affinity for the single ubiquitin or multi-ubiquitin chains with open conformation (K63 linkage) than those with closed conformation (K48 linkage). Therefore, it can be concluded that autophagy has a high probability of degrading substrates labelled with a single ubiquitin on either short or long K63 chains. Moreover, studies have shown that under UPS damage, when the concentration of K48 polyubiquitinated protein can make the chain interact specifically, effectively and adequately with p62, the K48-labelled substrates can still be recruited. In p62-deficient mice, aggregation of K63-linked multi-ubiquitin-labelled proteins was observed. Because p62 also seems to be a regulator of the proteasome degradation pathway for certain ubiquitinated proteins and because p62 degrades certain autophagic substrates independent of ubiquitination, these proteins have extremely complicated mechanisms of action. These studies reveal a common conclusion that p62 is an important autophagic regulator of ubiquitinated protein degradation.

As an autophagy receptor, p62 is associated with protein aggregates in various diseases. Aggregates are toxic and require degradation by proteolytic pathways. Ubiquitin and p62 were discovered in inclusion bodies in autophagy-deficient cells. The defects in p62 and autophagy can specifically and effectively reduce the production of ubiquitinated inclusion bodies in mice and *Drosophila melanogaster*, which also indicates that p62 participates in the composition of protein aggregates. The analysis also indicated that p62 participated in the formation of stress-induced ubiquitinated protein polymers in vivo. It is worth noting that p62 can promote the assembly of aggregated proteomes into polymers through its N-terminal Phox and Bem1p (PB1) domains or its ubiquitin-bound UBA domain, which can be degraded by the ALP. The accumulation of p62 has been shown to inhibit UPS function, partly through the UBA domain of p62. It is noteworthy that p62 competes with ubiquitin-binding protein p97 to bind to ubiquitinated proteins. One of the functions of p97 is to deliver ubiquitinated substrates to the proteasome. Increasing the level of p62 can prevent p97 from approaching ubiquitinated UPS substrates.

#### 25.2.2.3 ALFY

ALFY (also called WDFY3, WD repeat, and FYVE domain protein 3) is a member of the FYVE domain protein family, which is also a molecular linker for autophagy and UPS. In the case of cell stress (such as starvation or UPS inhibition), ALFY is translocated from the nuclear membrane to the filamentous cytoplasmic structures that are closer to the autophagic membrane, ubiquitinated protein endosomes and autophagosomes (Isakson et al. 2013).

ALFY is a protein that contains 3526 residues and has a predicted molecular weight of 395 kDa. The gene encoding ALFY is located on chromosome 4q21 (human

gene map of the NCBI), which contains 68 exons. The existence of ALFY transcriptional variants has been predicted, but it is still uncertain whether they encoded proteins. Two-thirds of the ALFY N-terminus does not include predicted functional domains, but some regions and other protein domains have the same likelihood of homology. Region I (ALFY residues 247-351) is the most prominent region. To a large extent, it is very similar to the TBC domain of 'TBC1 family member 30'. The TBC domain has been found in many different proteins, including GTPase-activated proteins (GAPs). This observation leads to the conclusion that ALFY has the potential for GAP activity. The ALFY residue 842-923 (II) is very similar to a part of the 'original recognition complex subunit 2'. Moreover, the ALFY residue 2281-2397 (III) is also very similar to a domain in 'BCL-2-related transcription factor 1', which is also the conserved region of the protein most closely related to ALFY. The C-terminal region of ALFY consists of several membrane transport domains. The FYVE domain refers to a zinc finger structure that can interact with PI3P. Most of the elements in FYVE exist in early endosomes and multi-vesicular bodies, and they have many PI3P zinc fingers. The FYVE domain and PI3P-specific domain of the ALFY can interact with each other, but ALFY either does not share the same localization with the inner body or does not exist at all. However, ALFY, ubiquitin, and p62-positive protein aggregates co-localize and even co-localize at autophagic membranes. ALFY recruits to the protein aggregate in a manner likely independent of its binding to PI3P.

ALFY contains five WD40 repeats that have a significant impact on the interaction between ALFY and atg5. WD40 domains typically exist in eukaryotic proteins, and they often form a seven-bladed β-propeller structure, which can combine with proteins specifically and effectively. The WD40 ALFY domain has great potential to form a complete β-propeller structure. There are no seven WD40 repeats. However, it is feasible that the WD40 repeats contributed by other interacting partners form β-propellers. Moreover, ALFY has not only the PH-like domain but also the BEACH domain. In general, it is plausible that the PH domain mediates the interaction with inositol phosphate and/or the protein–protein interaction. The PH-like domain of the human BEACH protein factor associated with neutral sphingomyelinase (FAN)-related factors binds to PI(4,5)P2. However, the function of the PH-like domain of ALFY needs to be studied further. The acid-base domain and the WD40 domain of the human BEACH protein are moderately conserved, while the BEACH protein is highly conserved. However, the BEACH domain mainly exists in the wholly conserved protein family of eukaryotes, and its function needs to be further studied. Based on the function of FAN, the PH-like domain of BEACH and the conserved connection between them, these substances are indispensable, specific and effective. They exert their own influence and function as a single unit. It is concluded that the PH-BEACH domain of ALFY interacts with the autophagic receptor p62.

One-third of the C-terminus of ALFY contains not only several domains capable of transporting substrates to phagocytic mechanism but also the PH-BEACH domain that binds to p62, the WD40 domain that binds to Atg5 and the FYVE domain that binds to PI3P. ALFY downregulation likely prevents ATG5 and LC3 from effectively recruiting to the protein endosomes formed by Huntingtin (Htt)-polyQ. This

finding also shows that ALFY likely contributes to the stability of the interaction between LC3 and p62 and plausibly promotes the interaction between ALFY and LC3. Considering that the Atg5-Atg12 conjugate has E3 ligases activity which connects LC3 and PE, ALFY may transport Atg5 to inclusion bodies, thereby promoting the lipidation of LC3, thus contributing to the eventual formation of an autophagosome membrane. In the process of autophagy, each substrate can be targeted. Then, these substrates are cloaked in autophagic membranes and are separated from the cytoplasm.

Protein aggregation begins with the incorrect folding of proteins, which leads to the formation of oligomer intermediates and then the gradual development of small protein polymers. Finally, under certain specific conditions, they gradually become larger polymers or inclusions. The UPS system provides an important mechanism for degrading misfolded proteins. However, oligomers and relatively large protein aggregates have difficulty crossing the narrow cannular proteasome of the UPS, and therefore, they are degraded by the ALP. Usually, ALFY exists in the nucleus but shuttles continuously. Under cell stress and the gradual formation of protein polymers, ALFY moves from the nucleus to the cytoplasmic p62 body. Currently, scientists have not yet determined the signals that induce ALFY nucleation during the process of protein-polymer formation. However, they believe that p62 has the potential to participate in this process. In cells treated with puromycin or bafilomycin A1 (a lysosomal pump inhibitor), the loss of ALFY reduces the number of p62 bodies in the cytoplasm and the size of the p62 bodies, which leads to the diffusion and localization of ubiquitinated proteins. Although insoluble ubiquitin-protein disappeared in the p62-deficient cells, it was still detected in ALFY-deficient cells (although its level was significantly lower than that of the normal control cells). Moreover, Ref2p (p62 homologue in Drosophila) accumulated in the ubiquitin-positive inclusions of Drosophila brains with mutant ALFY homologue blue cheese (bchs). This finding suggests that ALFY is involved in the degradation of p62-related ubiquitinated proteins in vivo. Moreover, bchs did not accumulate in *Drosophila melanogaster*, and functional ref2p was absent. Moreover, these findings reveal that p62 can bind to ubiquitinated proteins that are easily aggregated and that the PB1 domain promotes the formation of micro-polymers. These tiny polymers can form larger polymers through ALFY binding. Therefore, ALFY in abundance may indicate its use as a scaffold protein to promote the formation of p62 bodies specifically and effectively, and the p62 body is subsequently subjected to autophagy and degraded. The degradation of ALFY by autophagy depends on p62, but the transformation of p62 by the autophagy pathway does not depend on ubiquitinated proteins or ALFY. It is feasible that the level of p62 is controlled specifically and effectively in this process.

#### 25.2.2.4 Tp53

As a transcription factor, Tp53 is activated under a variety of stress conditions and is also involved in the regulation of various signal pathways, including cell cycle arrest, DNA repair or apoptosis (Lazo 2017). Recent studies have found that

Tp53 also plays a role in the regulation of autophagy. Specifically, Tp53 inhibits the activity of mTOR by activating AMP-activated kinase (AMPK), thereby regulating phosphatidylinositol-3-kinase/Akt (PI3K/Akt), or phosphatase and tensin homology deleted on chromosome ten (PTEN), thereby activating autophagy. Tp53 has also been demonstrated to activate autophagy in a damage-regulated autophagy modulator (DRAM)-dependent manner in the human osteosarcoma cell line Saos-2.

JNK, an upstream molecule of p53, has been revealed to play an important role in the degeneration of DA neurons induced by proteasome inhibitors owing to the release of cytochrome c and activation of caspase 3 in mitochondria. Proteasome inhibitors can increase the protein level of Tp53, while inhibiting Tp53 expression can significantly decrease the neurotoxic effects induced by proteasome inhibitors, as shown in midbrain DA stem cells or mouse models. These results suggest that the JNK-Tp53 signalling pathway plays a very important role in the degeneration of the nigrostriatal pathway induced by proteasome inhibitors.

In the PD model established by proteasome inhibitors, elevated Tp53 is closely associated with enhanced autophagy activity. The administration of Tp53 inhibitors or enhancers in primary midbrain VM neurons or dopaminergic SH-SY5Y cells reveals that Tp53 plays a key regulatory role in enhancing autophagy function in the case of UPS dysfunction and is an important linker molecule between ALP and UPS (Du et al. 2009).

#### 25.2.2.5 FoxO3

The transcription factor FoxO3 belongs to the O subtype of the forkhead family, which is mainly characterized by the fork head DNA-binding domain. The function of FoxO3 is to induce gene transcription, which encodes proteins that generally upregulate proteasomal and autophagic degradation functions. FoxO3 in the heart initiates atrophy by activating an E3 ubiquitin ligase. FoxO3 can directly bind to promoters, including LC3, Gabarapl1, Atg121, and Bnip3, and thus control the transcription of autophagy-related genes in skeletal muscle. In contrast, downregulation of FoxO3 impedes starvation-induced autophagy in isolated muscle fibres. FoxO3-induced Bnip3 plays a major role in the formation of the autophagic bodies in muscle atrophy (Cao et al. 2013). The FoxO3-induced activation of the ubiquitin-proteasome and autophagy may be mediated by the transcriptional coordination of key genes in both pathways rather than mediation of the direct link between these two systems. Inhibition of either of these two degradation pathways does not alter the other pathway activated by FoxO3.

#### 25.2.2.6 Other Regulatory Factors

In addition to the five regulatory factors described above, several other factors which mediate the interaction between autophagy and the UPS have recently been discovered. For example, Cdc48/p97 plays an important role in the interaction

between autophagy and the ubiquitin-proteasome catabolic pathways. AAA-ATPase Cdc48/p97 controls multiple cellular functions such as protein degradation, cell division and cell fusion, by regulating the fate of the ubiquitinated proteins. Cdc48/p97 is also involved in autophagy. p97/VCP is also an important molecule that mediates the interaction between autophagy and the ubiquitin-proteasome system. Moreover, p97/VCP is one of the essential factors in the autophagy pathway that can bidirectionally regulate the protein degradation mediated by the UPS and autophagy and thus plays a key regulatory role in the intersection of these two protein-hydrolyzing pathways. Cyclic AMP phosphodiesterase-4A4 (PDE4A4) aggregates are involved in the p62/sequestosome-1 junction and participate in the regulation of autophagy and the proteasome system. Neighbour of BRCA1 gene 1 (NBR1) is similar to p62 and directly binds to ubiquitin and LC3. NBR1 provides an important link between autophagy and the UPS in a manner similar to that provided by p62. Tumour necrosis factor receptor (TNFR)-associated factor 6 (TRAF6) has E3 ubiquitin ligase activity that leads to polyubiquitination of the K63 site of the target protein. TRAF6 regulates autophagy and the ubiquitin-proteasome systems in atrophic skeletal muscle disease. Moreover, TRAF6 is an important adapter protein involved in the receptor-mediated activation of various signalling pathways. Blocking the activity of TRAF6 can preserve skeletal muscle mass and function for therapeutic benefit.

### *25.2.3 Autophagy and UPS Share Certain Substrates*

Autophagy and UPS share certain substrates and regulatory molecules that have a compensatory effect in some cases. Autophagy and UPS share certain ubiquitin recognition molecules or shuttling factors. For instance, p62 can transport ubiquitinated substrates to either the proteasome pathway or the autophagy pathway. Studies have shown that many proteins can be degraded by both pathways. In addition, the neuronal protein α-synuclein is likely degraded by UPS, macrophages, and chaperone-mediated autophagy. In addition, mutations in proteins degraded by the proteasome pathway may mean that the degradation of these proteins depends on the autophagy pathway. The oligomeric or high-level structure of certain proteins render these proteins incapable of entering the narrow protease pores; therefore, these highly aggregated mutant proteins are prone to be degraded by the autophagy pathway.

Ubiquitin is a common degradation determinant that regulates the substrate-targeting UPS or autophagy. In ubiquitination, ubiquitin binds to other protein lysine residues and labels them with degradation signals as a substrate that can be hydrolyzed by the proteasome or lysosome. In addition, ubiquitination is also involved in regulating non-proteolytic processes. In protein quality control (PQC), misfolded proteins can be recognized by molecular chaperones and ubiquitinated by E3 ligases, such as Parkin or CHIP. Substrates are targeted to proteasomes if their ubiquitin chains bind to the UBDs of proteasome-related adapters such as RPN 10 and RPN 13. Ubiquitous substrates that resist proteasome degradation, such as easily accumulated proteins, are directed towards macro-autophagy or are allowed to

exist as aggregates temporarily. Through macro-autophagy, the misfolded proteins are degraded by lysosomal hydrolase as mediated by p62 or NBR1. When the pathway is blocked, histone deacetylase 6 (HDAC6) can also isolate aggregates in the lysosomes by combining the ubiquitin chains of substrates (Ciechanover and Kwon 2017).

Misfolded proteins are mostly degraded by the UPS. Before the degradation of misfolded proteins, the E3 ligase binding chaperone system will try to restore the misfolded proteins by refolding them. For example, the E3 ligase CHIP interacts with HSPs to ubiquitinate chaperones binding substrates that failed to refold. This process leads to mild protein toxicity stress and stimulates proteasome activity. Sustained stress inhibits the proteolytic capacity of the UPS. When the primary repair pathway and the proteasome are overwhelmed, the compensatory activation of autophagy drives the accumulation of misfolded proteins into large aggregates that cannot be specifically recognized or effectively cleared by the UPS. In the interaction between the UPS and autophagy, p62 and HDAC6 play a vital role; they are involved in the process of the formation of aggregates by misfolded proteins and tend to convert them from the protein degradation pathway of the UPS to autophagy.

Many independent studies have demonstrated that when the refolding and the 26S proteasome functions are blocked, the disordered degradation of the misfolded proteins seems to be promptly sensed by cells so that compensatory autophagy is activated (Hetz et al. 2015; Livneh et al. 2016). For example, bortezomib and other proteasome inhibitors can directly induce compensatory autophagy; the mTOR inhibitor rapamycin is able to alleviate the accumulation of abnormal proteins caused by proteasome inhibition and thus ameliorates cytotoxicity; in Drosophila models of neurodegeneration induced by proteasome dysfunction, the HDAC6-dependent autophagy pathway described above confers protective effects.

The BAG protein family is involved in the coordination of proteasome and autophagy activity. These proteins are key regulators of the PQC system and are involved in age-related degenerative diseases. BAG1 transports incorrect or unfolded substrates that are identified by chaperones to proteasomes, where they can be effectively degraded by the UPS. In contrast, BAG3 promotes protein degradation through autophagy. In fact, BAG1 and BAG3 seem to compete for HSP-bound ubiquitinated substrates, and their expression levels are mutually regulated. Therefore, the BAG3/BAG1 ratio determines the balance between proteasome and autophagic degradation. This balance shifts from BAG1 to BAG3 during conditions of oxidative stress, proteasome inhibition or aging (Minoia et al. 2014).

## 25.3 Effects of UPS and ALP on Diseases

### *25.3.1 Abnormal UPS and Diseases*

The homeostasis of the intracellular environment depends on the balance between protein synthesis and degradation. Hindered degradation can lead to unwanted or toxic protein accumulation that triggers cell apoptosis or necrosis. The UPS is the main mechanism for cellular protein degradation, controlling the amount of proteins and maintaining homeostasis, which is of great significance. Thus, ubiquitin-proteasome damage is involved in the pathophysiology of many diseases such as neurodegenerative diseases, cardiovascular diseases, autoimmune diseases, cancer and viral infections.

A variety of neurodegenerative diseases are characterized by the accumulation of abnormal proteins in neurons such as Parkinson's disease (PD), Alzheimer's disease (AD), amyotrophic lateral sclerosis (ALS) and Huntington's disease (HD). To maintain brain function, an effective system is needed to maintain neuronal integrity. In addition to cell repair, harmful substances such as accumulated denatured proteins need to be eliminated. In many neurodegenerative diseases, misfolded and aggregated proteins are found to be ubiquitinated inclusions, suggesting that the ubiquitin-proteasome pathway may be important for the understanding of the molecular pathogenesis of these conditions. Previous studies have shown that loss of cellular protein balance may occur when substrate ubiquitination and proteasomal degradation are impaired; specifically, it is involved in neurotoxic effects and subsequent neuronal damage. Neurodegeneration is associated with gene mutations that determine important components of the ubiquitin-proteasome system. Mutant proteins in neurons may accumulate to toxic levels when proteasome activity is inhibited (Deger et al. 2015). It has been found that UPS activity is neither completely nor positively correlated with neural survival. In ubiquitin homeostasis studies, both ubiquitin loss and overexpression lead to a phenotype similar to that of neurodegenerative diseases. On the one hand, the overactive UPS leads to abated accumulative toxicity. On the other hand, the overactive UPS leads to a compensatory increase in the protein accumulation levels in the disease and results in increased neurotoxicity.

#### 25.3.1.1 PD

Parkinson's disease (PD) is a common neurodegenerative disease. The main pathological feature is the degenerative death of the dopaminergic (DA) neurons in the substantia nigra of the midbrain. In rat ventral mesencephalic cultures, inhibition of ubiquitin C-terminal hydrolase causes degeneration of dopaminergic neurons and formation of Lewis body-like inclusions. This evidence suggests that UPS damage is involved in the pathological formation of PD. Mutations in some genes such as PINK1, Parkin, α-synuclein (α-Syn), leucine-rich repeat kinase 2 (LRRK2) and DJ-1 have been suggested to cause familial Parkinson's disease. Among them, α-Syn and

LRRK2 mutations lead to autosomal dominance of PD through toxic effect from proteins and functional acquisition mechanisms, respectively, while PINK1 and Parkin mutations lead to PD through a functional loss mechanism.

UCH-L1 is a deubiquitinase that regulates ubiquitin expression and plays an important role in ubiquitin-dependent protein hydrolysis together with ubiquitin activating enzyme, ubiquitin-coupled enzyme and ubiquitin ligase. Genetic evidence suggests that its function is inhibited in several familial Parkinson's diseases. In a German family with PD, the Ile93Met mutation inhibits the catalytic activity of UCH-L1, which leads to UPS dysfunction and abnormal protein accumulation. The decrease in UCH-L1 activity hinders the degradation of the covalent bonds between ubiquitin and the substrates. It also reduces the level of free ubiquitin in the brain, which leads to further accumulation of the misfolded proteins.

Parkin is also involved in regulating UPS activity and is a specific E3 ubiquitin ligase. In the healthy human brain, the substrate of the Parkin ubiquitin ligase is α-synuclein, and decreased Parkin level leads to abnormal protein aggregation. Wild-type α-synuclein, as the substrate of 26S and 20S proteasomes, can be degraded preferentially in a manner that does not involve ubiquitination (Park et al. 2017). Studies have shown that poorly degraded α-synuclein tends to aggregate, inducing protein accumulation and destroy cell homeostasis in vivo and in vitro. Accumulated α-synuclein also causes UPS damage, which may be related to the accumulation of abnormal proteins.

Parkin can directly ubiquitinate the accumulated polyglutamine protein in vitro and bind to CHIP. CHIP is an E3 that relies on partners to identify certain misfolded proteins. PINK1 is a serine/threonine-protein kinase located in mitochondria upstream of Parkin. Wild-type PINK1 reverses mitochondrial dysfunction and neuronal damage induced by proteasome inhibitors in vitro, whereas mutations in PINK1 in the brains of PD patients cause this protective mechanism to malfunction. Moreover, the PINK1 mutation impairs mitochondrial function and reduces ATP production so that it is insufficient to maintain the normal function of the UPS.

DJ-1 is widely expressed in the central nervous system. DJ-1 has the function of antioxidant stress. In some PD mutants, the loss of DJ-1 activity leads to the abnormity in proteasome conformation and the decrease in proteolytic activity. DJ-1 can interact with Parkin and CHIP/HSP70, which confirms that it may be related to the protein hydrolysis system. Wild-type DJ-1 inhibits the aggregation of synuclein, and DJ-1 is mutated in PD.

LRRK2 is a tyrosine-like protein widely expressed in the brain and is anchored on the outer membrane of mitochondria. Tissue culture experiments showed that LRRK2 could interact with Parkin. In SH-SY5Y cells and primary neuron cultures, overexpression of wild-type LRRK2 increased the aggregation of pathogenic proteins and their neurotoxic effects.

In addition, AAV2-mediated GDNF gene therapy can significantly reduce the loss of dopaminergic neurons and the decrease in DA levels in the striatum that had been induced by lactomycin and protect dopaminergic neurons from UPS-induced cytotoxicity. Some studies confirm the potential value of proteasome-targeted drugs in the treatment of PD.

#### 25.3.1.2 AD

The characteristic pathological changes of Alzheimer's disease (AD) are plaques formed by β-amyloid protein and neurofibrillary tangles formed by excessive tau protein phosphorylation, together with neuronal loss and glial cell proliferation. In neuro-plaques and neurofibrillary tangles of AD patients, the frameshift form of ubiquitin accumulates in an age-dependent manner, which may be a main factor leading to proteasome dysfunction and may promote the progression of AD. Studies have shown that multi-ubiquitinated tau proteins are mainly located in the K48 chain and can be degraded in an ATP/ubiquitin-dependent manner by the 26S proteasome. Unfolded recombinant tau can also be degraded by UPS without ubiquitin modification in vitro. The proteasome activator Blm10 accelerates the renewal of proteasome substrates and increases the degradation of over-phosphorylated, unstructured tau proteins in vitro. FK506 binding protein 51 kDa (FKBP51) forms a mature chaperone complex with Hsp90, blocking the clearance of the tau protein by affecting proteasome and leading to the accumulation of tau protein (Sulistio and Heese 2016).

#### 25.3.1.3 Prion Disease

Prion disease is a fatal neurodegenerative disease characterized by the accumulation of misfolded prion protein (PrPSc) in the brain. Time-course studies of UPS activity in vivo have found that the occurrence of UPS dysfunction is closely related to PrPSc deposition, early behavioural deficits and neuron loss along with the accumulation of the poly-diazotization matrix and the decrease in neurons and astrocytes. All of these occur in the early stage of prion diseases. The small-molecule inhibitor IU1 can activate the UPS by eliminating the poly-diazotization matrix and poly-dicyandiamide coupling to reduce the abnormal prion protein and prevent the neurodegeneration caused by prion infection in vitro.

#### 25.3.1.4 Cardiovascular Diseases

Strict monitoring of protein quality is essential to maintain normal cardiac function, and protein turnover disorders are common in various cardiovascular diseases (Willis et al. 2014). The UPS is the core of protein control in cardiomyocytes. In recent years, increasing evidence has shown that UPS plays an important role in the physiology of cardiovascular diseases, and dysfunctional proteasome function can lead to cardiovascular diseases. UPS substrate fluorescence tracer assay showed that the ubiquitin-proteasome system of genetic cardiomyopathy is impaired and the E3 ubiquitin ligase gene is mutated. The COP9 signalosome (COPS) has been reported to degrade misfolded proteins through autophagy and the UPS, thus maintain the function of cardiomyocytes. In conclusion, the UPS plays an important role in many cardiovascular diseases, such as CDM, heart failure and AS (Liu et al. 2016).

#### 25.3.1.5 Other Diseases

Protein controlling by UPS plays a central role in some lung diseases. Proteasome inhibitors targeting E3 ligase and FBPs have been proven to have pharmacological activity in clinical trials. Skeletal muscle is affected by physical activity, hormones, and metabolism. Catabolic conditions can activate signalling pathways to regulate the process of muscle loss. When the protein degradation rate exceeds the level of protein synthesis, muscle atrophy occurs in adult tissues. UPS and ALP are activated during muscle atrophy and lead to loss of muscle weight. Myostatin mediates muscle catabolism through synergistic activation of autophagy and the ubiquitin-proteasome system in the muscular atrophy induced by chronic kidney disease (CKD) (Wang et al. 2015).

### *25.3.2 Abnormal Autophagy and Diseases*

Autophagy is the main pathway for degrading aggregated isoproteins and dysfunctional organelles in neurodegenerative diseases. In models of aging and various neurodegenerative diseases, autophagy plays a neuroprotective role. It has been widely accepted that abnormal autophagy promotes neuronal death in some diseases (Tan et al. 2014).

#### 25.3.2.1 PD

In Parkinson's disease, autophagy has a dual effect. Autophagy suppression or overactivation can damage dopaminergic neurons in the substantia nigra striatum. In cellular and rodent models of Parkinson's disease, oxidative stress, abnormal autophagy and reduced degradation of abnormal protein were found. According to genetic studies, some specific genes encode proteins that interfere with autophagy-lysosome pathways, and they are among genes that, when mutated, are associated with familial genetic PD. In addition to being degraded by ubiquitin proteasomes, α-synuclein can also be degraded by CMA and macrophages. Once autophagy activity is destabilized, oligomers assemble into large aggregates, and α-synuclein accumulates in cells. This destabilization also has a feedback effect on autophagy. The wild-type and mutant α-synuclein induces macrophage autophagy. The mutant α-synucleins A53T and A30P specifically bind lysosome-associated membrane protein 2A (LAMP-2A) to inhibit CMA, while in mammalian cells, wild-type α-synuclein affects different types of autophagy. Recent studies have confirmed the presence of oligomeric Syn, Syn-immuno-positive aggregates and changes in membrane potential in PD, all of which lead to subsequent dopaminergic cytotoxicity.

Among the pathogenic genes of familial Parkinson's disease, the alpha-synuclein mutation is relatively rare, while the autosomal dominant mutation to LRRK2 is the most common. A large number of reports support the view that LRRK2 plays a role

in autophagy. Although knockdown or kinase inhibition of LRRK2 seems to increase the autophagic flux, the phenotype associated with the pathogenic mutant LRRK2 is consistent with autophagic inhibition. That is, it is characterized by the accumulation of non-degradable substances, lysosomal-like structures and lipid droplets in cells and in animal models. The exact mechanism of LRRK2-mediated autophagy is still unclear and may involve changes in lysosomal function. In addition, LRRK2 can be degraded by CMA. The pathogenic mutant interferes with the CMA pathway, resulting in the accumulation of α-synuclein. This finding is consistent with the observation that LRRK2-dependent neurodegeneration relies on α-synuclein. Brain-specific deletion of autophagic genes leads to the accumulation of endogenous α-synuclein and LRRK2 and subsequent neurodegeneration. This finding suggests that the transformation of these two proteins depends on the appropriate autophagic clearance mechanism. Although the increase in the α-synuclein level is partly responsible for the increased cytotoxicity, the increase in the LRRK2 level, along with its kinase activity, may also affect the autophagic flux (Rivero-Rios et al. 2016).

Autophagy is also involved in mitochondrial renewal, and its dysfunction is an important pathogenic mechanism of cell death in PD. The products of Parkin and PINK-1 genes, serine-threonine kinase PINK-1 and Parkin, were recruited into damaged mitochondria to facilitate mitochondrial autophagy. Parkin and PINK-1 regulated mitochondrial elimination through mitophagy in this way. Although in total human Parkinson's disease cases, familial PD accounts for less than one-tenth of cases, 18 PARK genes that follow dominant or recessive genetic patterns have been discovered (Sarraf et al. 2013). PARK2, PARK6 and PARK7 have an autosomal recessive inheritance, while PARK1, PARK4 and PARK8 have autosomal dominant inheritance. In addition, in dominant and recessive genetic forms, overexpressed or aggregated toxic forms of the proteins are degraded and eliminated by autophagy. Because of the action of hydrolytic enzymes such as glycosidase, protease and sulfatase, the acidic environment of the lysosome can degrade redundant organelles and proteins. The main proteins that accumulate easily in neurons are α-nucleoprotein, ubiquitin and heat shock protein 70 (Hsp70). Mitochondria and the endoplasmic reticulum are the key organelles most prone to these abnormalities.

Drugs targeting autophagy pathways and reducing oxidative stress have been proven to be effective for the treatment of PD. In the rotenone-induced neurotoxicity model, the autophagy inducer rapamycin has a significant neuroprotective effect and can inhibit the formation of poly-quinolate aggregates. It triggers autophagy, to a certain extent, based on oxidative stress levels; for example, in primary cortical neurons, rapamycin- and trehalose-induced autophagy can be inhibited by sulfhydryl antioxidants. Therefore, the ideal drug for PD should eliminate ROS while inducing autophagy. Studies have shown that some plant polyphenols such as resveratrol, quercetin and curcumin have such potential.

Regulating autophagy through mTOR is another mechanism for the treatment of PD and other progressive neurodegenerative diseases. In some studies, activating mTOR and inhibiting autophagy prevents the damage to dopaminergic neurons during oxidative stress, suggesting that autophagy may be harmful to dopaminergic neurons. mTOR inhibits or regulates FoxO protein to prevent the death of substantia

nigra neurons when α-synuclein accumulates by reducing α-synuclein and promoting lipofuscin-containing autophagic vesicles. The growth factor EPO has been shown to prevent dopaminergic neurotoxicity by inducing autophagy and inhibiting mTOR (Maiese 2016). Autophagy may also protect neurons in PD by maintaining mitochondrial homeostasis and does not require mTOR suppression (Sasazawa et al. 2015).

DJ-1 is a ubiquitous redox protein with multiple functions. Deficiency in its function can lead to autophagic changes in mice and human cells, which may be related to an increase in autophagic flux. Some studies have shown that UCH-L1 may regulate CMA by binding with LAMP-2A to mediate ubiquitination-induced translocation to lysosomes. An abnormal increase in binding of UCH-L1 with LAMP-2A suppresses CMA of a-synuclein, which leads to the accumulation of a-synuclein. Another study reported that several pathogenic mutations of LRRK2 protein can bind lysosomes to interfere with the formation of CMA translocation complexes, leading to CMA defects. In conclusion, these deficiencies in autophagy activation and lysosome clearance may be involved in the pathogenesis of PD.

#### 25.3.2.2 ALS

Amyotrophic lateral sclerosis (ALS) is a fatal paralytic disease characterized by selective loss of motor neurons in the brain and spinal cord, which can lead to muscle weakness and atrophy. So far, there is no effective treatment for ALS. Most of ALS cases are sporadic, while about 10% are familial ones. Mutations of enzyme copper–zinc superoxide dismutase 1 (SOD1), TAR DNA-binding protein (TDP43) and fused in sarcoma/translated in lip sarcoma (FUS/TLS) are common types of familial ALS. Approximately 20% of familial ALS has mutations in the gene coding for the SOD1. Although the causes of most cases of ALS are not fully understood, however, a large number of aggregates of misfolded proteins in the lesion tissue are common features of all ALS patients.

Increasing evidence shows that autophagy plays a protective role in the pathogenesis of ALS. Autophagic activation was observed in the spinal motor neurons of $SOD1^{G93A}$ mutant transgenic mice, and the change was prior to the disease symptoms. At the same time, aggregated autophagosomes in the cytoplasm of degenerated motor neurons were also observed in ALS cases. P62 is a component of pathological inclusion bodies of ALS. Mutant SOD1 can be degraded by connecting p62 with LC3 into autophagosomes. The number of SOD1 mutants phagocytized by autolysosome decreased in p62 knockout cells.

Autophagy can be induced by mTOR-dependent and mTOR-independent pathways. It is noteworthy that rapamycin, an mTOR-dependent autophagic inducer, can significantly activate autophagy in $SOD1^{G93A}$ transgenic mice, but has no significant effect on clearing abnormal proteins. After rapamycin administration, the degeneration of motor neurons and mitochondrial damage were accelerated in $SOD1^{G93A}$ mouse model. In addition, rapamycin treatment can accelerate disease progression by activating apoptotic signalling pathway and shorten the life span of transgenic mice,

suggesting that ALS transgenic mice may have abnormal autophagic flow (Zhang et al. 2011). In view of cell growth and translation and other important cellular processes controlled by mTOR, mTOR-independent autophagy inducers may be more suitable for the treatment of ALS. For example, trehalose, an mTOR-independent autophagy inducer, can improve autophagy flux, significantly reduce SOD1 aggregation and delay the occurrence of disease, which provides a new idea for the treatment of ALS (Zhang et al. 2014).

#### 25.3.2.3 Cancer

Autophagy may have multiple effects on the occurrence, development and treatment of cancer. In mouse models, studies have shown that tumour inhibition is mediated by autophagy-related proteins (Uvrag and Bif1) and core autophagy proteins (Atg4C, Atg5 and Atg7). Antigen signalling pathways triggered by tumour suppressor proteins (PTEN, TSC1/2, LKB1 and p53) may also stimulate autophagy. In addition, Beclin1-dependent autophagy may be inhibited by activation of AKT in human cancer. The expression of other autophagic proteins was also changed in human cancer specimens, for example, upregulation of Atg5 expression has been observed in prostate cancer.

#### 25.3.2.4 Immune Diseases

Autophagy contributes to the regulation and function of innate and adaptive immune responses. Several important pathogenic bacteria are degraded by bacteriophages in vitro, such as *Streptococcus A*, *Mycobacterium tuberculosis*, *Shigella flexneri*, *Salmonella enterica*; herpes simplex virus type 1 (HSV-1); and Toxoplasma and other parasites. In mice with sepsis, autophagy seems to have protective effects on multiple organs when facing damage. To some extent, this protection is achieved by preventing apoptosis, maintaining the balance between pro-inflammatory and anti-inflammatory cytokines, and improving mitochondrial function.

#### 25.3.2.5 Cardiovascular Diseases

Autophagy is associated with cardiovascular diseases, including cardiomyopathy, ischaemic heart disease, heart failure, myocardial hypertrophy and ischaemia. The deletion of the lysosome-associated membrane protein 2 (LAMP 2) gene affects autophagosome–lysosome fusion and causes a cardiomyopathic condition called Danon disease. In patients with Danon disease, the number of autophagosomes and the mitochondrial dysfunction in cardiac myocytes increases as they do in heart tissue from patients with heart failure. In the mouse model of desmin-associated cardiomyopathy, autophagy has been shown to have cardioprotective effects.

#### 25.3.2.6 Pulmonary Diseases

In the lung tissues of chronic obstructive pulmonary disease (COPD) patients, LC3-II expression and autophagosomes were increased. In animals that inhaled cigarette smoke for a long time, the increased autophagic activity is related to an increase in epithelial cell apoptosis. Mutations of the α1-anti-tryptase gene can cause emphysema and liver dysfunction. The pathological accumulation of the mutant α1-anti-tryptase may be eliminated by autophagy (Zhou et al. 2016).

#### 25.3.2.7 Aging

Autophagy is considered to be an anti-ageing process in terms of its functions in long-lived protein turnover and damaged organelle and cell debris removal. Similar to neurodegeneration, the accumulation of unprocessed substances (lipofuscin pigments and ubiquitinated protein polymers) is accelerated, and the compensation mechanism is activated. Studies in mice and nematodes treated with sirolimus have shown that regulation of autophagy may affect longevity. In addition, the analysis of gene expression in the brains of the elderly has shown that autophagy genes (Atg5, Atg7 and BECN 1) were downregulated with age. Calorie restriction may be a mechanism to reverse the decline in age-dependent autophagy.

## 25.4 Summary

In the past few years, a deeper understanding of the relationship between autophagy and the UPS has been acquired. There are significant similarities between the two catabolic pathways of autophagy and the UPS, such as UBL modification and some overlapping functions in some cases, indicating that autophagy and UPS are likely to have the same biological origin. Moreover, the function of autophagy and that related to an uninterruptible power supply are very similar. For example, UPS deficiencies are likely to cause upregulated autophagy. Moreover, under certain conditions, autophagy activation is likely to compensates for impaired UPS function. However, considering that nearly no reagents can upregulate UPS, the following question emerges: is this compensatory relationship mutual? The answer to this question is not clear. UPS upregulation may exert a neuroprotective effect against the toxicity caused by disease proteins; nevertheless, no effects or roles of UPS upregulation have been detected in autophagy-deficient cells. An increasing number of studies have begun to elucidate the mechanisms and molecular regulators shared between autophagy and the UPS. The identification of UBL modifications is becoming a hot topic for research.

Exploring and analysing the relationship between the UPS and autophagy can promote breakthroughs in the history of human disease research. Therefore, this study has an important role. Elucidation of the mechanism of the interrelationship between these degradation systems would not only expand the understanding of the

regulatory mechanism of intracellular substance degradation but also significantly contribute to the understanding of the mechanisms of the diseases related to disorders in the cellular degradation system. Hopefully, the targets of these catabolic pathways will eventually be utilized to provide novel treatments for human diseases.

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# Chapter 26
# Immune Signaling and Autophagy Regulation


**Fang Hua, Ke Li, Shuang Shang, Feng Wang and Zhuowei Hu**

**Abstract** Autophagy is one of the key degradation systems in organisms. Starvation and nutrient deprivation induce autophagy activation, providing energy and anabolic substances to maintain energy homeostasis. A variety of signals participate in the induction of autophagy, including endoplasmic reticulum stress, oxidative stress, and activation of immune signals. Autophagy is closely related to immunity and inflammation. Autophagy-related gene mutations increase the risk of infectious diseases and malignancies. Autophagy can be regarded as an effector of the immune system to eliminate invading pathogens and is also involved in the immune system recognizing the invasion of pathogens. Autophagy plays important roles in regulating innate immunity and adaptive immunity. In terms of innate immunity, autophagy not only participates in the clearance of pathogens and cell debris after apoptosis but also plays a protective role against toxins, regulates cytokine production, and activates the inflammasome. In the adaptive immune response, autophagy plays an important regulatory role in thymic selection, T cell maturation, T cell polarization, T cell and B cell homeostasis, antigen processing, antigen presentation, and antibody response. On the other hand, autophagy is regulated by immunological and stress signals. The crosstalk between these signaling pathways helps maintain homeostasis and physiological functions. Dysfunction of these regulatory networks is the cause of several kinds of diseases.

**Keywords** Autophagy · Cytokines · TLRs

F. Hua · S. Shang · F. Wang · Z. Hu (✉)
State Key Laboratory of Bioactive Substance and Function of Natural Medicines, Institute of Materia Medica, Chinese Academy of Medical Sciences & Peking Union Medical College, Beijing 100050, People's Republic of China
e-mail: huzhuowei@imm.ac.cn

K. Li
NHC Key Laboratory of Biotechnology of Antibiotics, Institute of Medicinal Biotechnology, Chinese Academy of Medical Sciences & Peking Union Medical College, Beijing 100050, China



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## Abbreviations

| | |
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| AD | Acidic activation domain |
| ADAR1 | dsRNA-specific adenosine deaminase |
| AICD | Activation-induced cell death |
| ALIS | Aggresome-like induced structures |
| APCs | Antigen presenting cells |
| BCR | B cell receptor |
| CARDs | Caspase recruitment domains |
| CLRs | C-type lectin receptors |
| CRD | Carbohydrate recognition domain |
| CTLA-4 | Cytotoxic T lymphocyte-associated antigen-4 |
| DAPK | Death-associated protein kinase |
| DAMPs | Damage-associated molecular patterns |
| DC | Dendritic cell |
| DD | Death domain |
| GBP | Guanylate-binding protein |
| GRP78 | Glucose-regulated protein |
| HMGB1 | High mobility group box 1 |
| IPS-1 | IFN-β-promoter stimulator 1 |
| IRE1 | Inositol-requiring enzyme 1 |
| IRE1α | Inositol-requiring enzyme 1α |
| ITAM | Immunoreceptor tyrosine-based activation motif |
| JNK | c-Jun N-terminal kinase |
| LRR | Leucine-rich repeat |
| LPS | Lipopolysaccharide |
| MD2 | Myeloid differentiation factor 2 |
| MHC | Major histocompatibility complex antigen |
| NLRs | NOD-like receptors |
| p38IP | p38 interacting protein |
| PAMPs | Pathogen-associated molecular patterns |
| PI3P | Phosphatidylinositol-3-phosphate |
| PI3KC3 | Class III phosphatidylinositol 3-kinase |
| ROS | Reactive oxygen species |
| SOCS | Suppressor of cytokine Signaling |
| TCGF | T cell growth factor |
| TCR | T cell receptor |
| TLRs | Toll-like receptors |
| TIR | Toll-Interleukin 1 Receptor |
| TRAF6 | Tumor necrosis factor receptor (TNFR)-associated factor 6 |
| TNF-α | Tumor necrosis factor alpha |
| TGF-β | Transforming growth factor beta |
| UPR | Unfolded protein response |

## 26.1 Pattern Recognition Receptor Signaling and Autophagy Regulation

### *26.1.1 Overview of Pattern Recognition Receptors*

Inflammation is a part of the biological response to harmful stimuli such as pathogens, damaged cells, or irritants and is a protective response involving immune cells, blood vessels, and molecular mediators. In the traditional sense, inflammation is characterized as redness, swelling, heat, pain, and dysfunction. These macroscopic symptoms reflect an increase in the permeability of vascular endothelial cells, allowing serum components and immune cells to leak or penetrate into the lesion tissues. However, immune cells may oversecrete cytokines to fight against pathogens, which is seriously harmful to the body, even resulting in fatal damage. Noninfectious diseases, such as "graft versus host" disease, can also trigger the aforementioned "cytokine storm". The innate immune system is primarily responsible for the acute inflammatory response caused by microbial infections and tissue damage, as well as the activation of the acquired immune system. Innate immune cells mainly include macrophages and dendritic cells (DCs), which recognize pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs) through pattern recognition receptors. The pattern recognition receptors (PRRs) that have been discovered include transmembrane proteins and cytosolic proteins. PRRs located on the membrane include Toll-like receptors (TLRs) and C-type lectin receptors (CLRs). Formic acid-induced RIG-I-like receptors (RLRs) and NOD-like receptors (NLRs) are located in the cytoplasm. Receptor-related intracellular signaling cascade activation induces the transcriptional expression of a set of cytokines, including pro-inflammatory cytokines, type I interferons, chemokines, and antimicrobial proteins. Abnormal activation of these receptors results in immunodeficiency, septic shock, or autoimmune disease.

### *26.1.2 TLR Signaling and Autophagy Regulation*

#### 26.1.2.1 TLRs and Their Signaling Pathways

The TLR family is the most well-studied family of pattern recognition receptors, which are responsible for sensing extracellular or intracellular pathogens (Dolasia et al. 2018). TLRs are type I integrated transmembrane glycoproteins, consisting of a conserved intracellular Toll-Interleukin 1 receptor (TIR) homology domain, a single transmembrane helix domain, and an extracellular domain containing leucine-rich repeats (LRRs). Ten and twelve TLRs have been identified in humans and mice, respectively. Different TLRs recognize different molecular patterns, and their subcellular localization is also different, as seen in Table 26.1.

**Table 26.1** Pattern recognition receptors and their ligands

| Pattern recognition receptor | Ligand | Ligand | Ligand source |
|---|---|---|---|
| *TLR* | | | |
| TLR1 | Plasma membrane | Triacyl lipoprotein | Bacteria |
| TLR2 | Plasma membrane | Lipoprotein | Bacteria, viruses, parasites, the host itself |
| TLR3 | Endosome lysosome | dsRNA | Viruses |
| TLR4 | Plasma membrane | LPS | Viruses, bacteria, host itself |
| TLR5 | Plasma membrane | Flagellum | Bacteria |
| TLR6 | Plasma membrane | Diacyl lipoprotein | Viruses, bacteria |
| TLR7 (human TLR8) | Endosome lysosome | ssRNA | Viruses, bacteria, host itself |
| TLR9 | Endosome lysosome | CpG-DNA | Viruses, bacteria, protozoa, host itself |
| TLR10 | Endosome lysosome | Unknown | Unknown |
| Plasma membrane | Plasma membrane | Profibrin-like molecules | Protozoa |
| *RLR* | | | |
| RIG-I | Cytoplasm | Short dsRNA, 5′ triphosphate dsRNA | RNA viruses, DNA viruses |
| MDA5 | Cytoplasm | Long dsRNA | RNA viruses |
| LGP2 | Cytoplasm | Unknown | RNA viruses |
| *NLR* | | | |
| NOD1 | Cytoplasm | iE-DAP | Bacteria |
| NOD2 | Cytoplasm | MDP | Bacteria |
| NLRC3 | Cytoplasm | Toxins | Bacteria |
| NLRC4 | Cytoplasm | Flagellin | Bacteria |
| NAIP | Membrane | Flagellin | Bacteria |
| NLRP1 | Cytoplasm | MDP | Bacteria |
| *CLR* | | | |
| Dectin-1 | Plasma membrane | Dextran | Fungi |
| Dectin-2 | Plasma membrane | Dextran | Dextran fungi |
| MINCLE | Plasma membrane | SAP130 | Host itself, fungi |
| *DNA sensors* | | | |
| AIM2 | Nucleus | dsDNA | Bacteria, viruses |
| Cgas | Nucleus | dsDNA | Bacteria, viruses |
| IFI16 | Nucleus | dsDNA | Bacteria, viruses |

TLR2 forms heterodimers with TLR1, TLR6, and possibly TLR10, recognizing a broad range of components from bacteria, mycoplasma, fungi, and viruses. Activation of the TLR2 receptor by ligands, such as lipoproteins, induces the production of various pro-inflammatory cytokines in macrophages and dendritic cells (DCs). TLR4 mainly recognizes lipopolysaccharide (LPS) and myeloid differentiation factor 2 (MD2) on the surface of the cell membrane. TLR5 is highly expressed in DC cells and infiltrates in the lamina propria of the small intestine. TLR5 recognizes bacterial flagella and induces B cells to differentiate into plasma cells, producing IgA, subsequently triggering the differentiation of naive T cells into antigen-specific Th17 and Th1 cells. TLR3, TLR7, TLR8, and TLR9 recognize nucleic acids derived from bacteria and viruses or the endogenous nucleic acids released under pathogenic conditions. Activation of these TLR signals promotes the production of type I interferons and pro-inflammatory cytokines. TLR3 recognizes viral double-stranded RNA, TLR7/8 recognizes the single-stranded RNA of RNA viruses, and TLR7 also recognizes RNA from bacteria. TLR9 recognizes unmethylated DNA in bacterial and viral CpG motifs. The subcellular localization of TLRs is also closely related to their recognition of ligands. The nucleic acids from the host are also strong ligands for TLRs and may induce autoimmune diseases. Under normal conditions, TLRs distinguish nucleic acids from the host and avoid abnormal activation of signals.

TLRs can be divided into two distinct signaling pathways, the MyD88-dependent signaling pathway, and the TRIF-dependent signaling pathway. MyD88 contains a death domain (DD) and a TIR domain. In addition to TLR3, MyD88 is critical for downstream signaling of TLRs. TLR2 and TLR4 require TIRAP/Mal to connect it to MyD88. MyD88 then interacts with IRAK-4, which activates two other members of its family: IRAK1 and IRAK2. The activated IRAK dissociates from MyD88 and in turn interacts with TNF receptor-associated factor 6 (TRAF6). TRAF6 is an E3 ubiquitin ligase that catalyzes the formation of a K63 polyubiquitin chain and the production of free polyubiquitin chains. The free polyubiquitin chain activates TAK1, TAB1, TAB2, and TAB3 complexes, catalyzing the phosphorylation of the inhibitory kappa B kinase (IKK) subunit and MAPKK6. IKK complexes (including IKK-α, IKK-β and NEMO proteins) phosphorylate the NF-κB inhibitor protein IκBα, triggering its degradation via the ubiquitin-proteasome system. NF-κB, released from IκBα, enters the nucleus and activates the transcription of several pro-inflammatory cytokines. Activation of the MAPK signaling cascade also activates another transcription factor complex, AP-1, which also regulates transcription of cytokines.

TLR3 signaling is dependent on the TRIF signaling pathway. Double-stranded RNA stimulates TLR3 to recruit the adaptor protein TRIF. TLR4 activates both MyD88 and TRIF-dependent signaling pathways, but TLR4 requires the involvement of another adaptor protein, TRAM, when it activates the TRIF-dependent signaling pathway. TRIF interacts with TRAF3 and TRAF6 via the N-terminal TRAF binding motif and interacts with RIP1 and RIP3 via the C-terminal RIP isoform interaction motif. After activation by TLR3 signaling, TRAF3 acts as an E3 ubiquitin ligase to catalyze the self-ubiquitination of K63, while the MyD88-dependent signal can catalyze the K48-mediated ubiquitination of TRAF3. TRAF3 is degraded by the ubiquitin-proteasome pathway and activates the MAPK signaling cascade,

thus inducing the transcription of pro-inflammatory cytokines. TRAF3 is important for the activation of two IKK-related kinases: TBK1 and IKKε. TBK1 and IKKε catalyze the phosphorylation of IRF3 and IRF7 to mediate the translocation of the IRF3-IRF7 dimer to the nucleus, thereby inducing the expression of type I IFNs and the expression of IFN-inducible genes. IKKε also phosphorylates STAT1 and promotes the expression of a range of IFN-induced genes, including Adar1, Ifit3, and Irf7.

#### 26.1.2.2 Activation of TLRs Induces Autophagy

Autophagy is important in both innate and acquired immunity and is involved in the elimination of bacterial, viral, and parasitic infections. Therefore, inevitably, there is a close relationship between autophagy and TLR signals. Autophagy promotes the recognition of PAMPs by TLRs, which will be mentioned in other sections. In this section, the regulation of autophagy by TLR activation will be discussed.

Autophagy is considered to be an effector of immunity. Diverse members of the TLR family, including TLR2/TLR1 heterodimers, TLR3, TLR4, TLR5, TLR6, TLR7/8, and TLR9, can activate autophagy in a variety of cells, including macrophages, DCs, and neutrophils. However, the mechanisms of autophagy induction by different cellular TLR signaling is not the same. For example, in plasmacytoid DCs, TLR7 signaling cannot induce autophagy activation. LPS stimulation induces the formation of a large number of autophagosomes in primary human monocytes but not in primary murine macrophages. These phenomena indicate that the induction of autophagy by TLRs is a cell type-specific response. Abnormal TLR signal-induced autophagy inhibition is an important mechanism for the clearance of infectious pathogens from the body. For example, in a study of macrophage TLR7, TLR7-induced autophagy via single-stranded RNA and imiquimod ligand, and knockdown of TLR7, MyD88, or Beclin1 impaired the clearance of intracellular *Mycobacterium tuberculosis* variants. In addition, autophagy inhibition caused by abnormal TLR signaling also plays an important role in tumorigenesis. Studies in TLR2 and TLR4 knockout mice have found that knockout of TLR2 or TLR4 significantly increases the sensitivity of mice to (Diethylnitrosamine) DEN-induced hepatocellular carcinoma. In these mice, liver autophagy activity was significantly reduced and DEN-induced oxidative stress and accumulation of p62 aggregates could not be eliminated, aggravating the occurrence and progression of hepatocellular carcinoma (Lin et al. 2013; Wang et al. 2013).

Autophagy activation is a dynamic process. In the early stage of separation membrane formation, vesicle nucleation requires the PI3KC3 core complex to phosphorylate phosphatidylinositol and form phosphatidylinositol-3-phosphate (PI3P). Beclin 1 is a member of the core complex of the class III phosphatidylinositol 3-kinase (PI3KC3) proteins, which act as a molecular platform in the complex. In addition to binding PI3KC3, Beclin 1 also interacts with molecules, such as UVRAG, Ambra1, and Bif-1, which positively regulate the activity of PI3KC3 and promote autophagy. Beclin 1 also binds to Bcl2 family proteins. In contrast, the association between Bcl2

and Beclin 1 inhibits the formation of core complexes and inhibits autophagy activity. Therefore, the Bcl2-Beclin 1 interaction is considered as the "brake" for autophagy. TLR-induced autophagy depends on both MyD88 and TRIF. The key point in regulating autophagy activation by TLRs is the regulation of the Bcl2-Beclin 1 interaction. Both MyD88 and TRIF can interact with Beclin 1. TLR signaling activation recruits Beclin 1 to the TLR signaling complex, enhances the interaction between Beclin 1 and MyD88 or TRIF and reduces the binding of Bcl2 to Beclin 1 to induce autophagy activation. In addition, TRAF6 can mediate K63 ubiquitination of lysine on Beclin 1, and Lys117 ubiquitination promotes Beclin-1 multimerization, enhances the enzymatic activity of PI3KC3, and induces autophagy activation, as shown in Fig. 26.1. LPS activation of TLR4 signaling also increases the binding capacity of PI3KC3 to the membrane and enhances the expression of the immune-related GTPase LRG47, both of which are involved in the induction of autophagy.

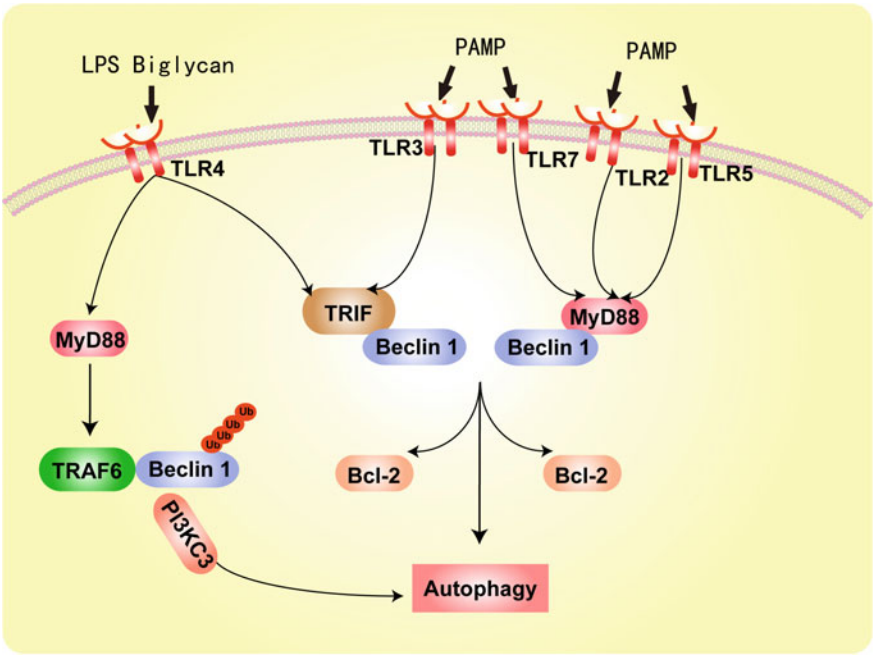


**Fig. 26.1** TLRs signaling and autophagy regulation. TLRs signalings are activated when a lipopolysaccharide or disaccharide binds to the TLR4 receptor, or the pathogen-associated molecular pattern binds to TLR2 or TLR5. Beclin 1 is recruited to the TLRs signaling complex, and the interaction between Beclin 1 and MyD88 or TRIF is enhanced, while Blocking the binding of Bcl2 to Beclin 1 induces autophagy activation. In addition, TRAF6 acts as an E3 ubiquitin ligase, which mediates K63 ubiquitination of lysine at position 117 of Beclin 1, and Lys117 ubiquitination promotes the multimerization of Beclin 1 and enhances the enzymatic activity of PI3KC3, thereby inducing autophagy activation

#### 26.1.2.3 Regulation of Autophagy by TLR2 Signaling and Its Biological Significance

Ligand-activated TLR2 and its downstream signal cascade are based on the formation of heterodimers of TLR2 and TLR1 or TLR2 and TLR6, whose dimerization does not require induction. The crystal structure shows that the LRR module forms a horseshoe-shaped structure, and the heterodimer of TLR2 binds to the ligand to form an "m" type complex, stabilizing the two receptors. The ligands for TLR2 include molecules containing diacyl triacylglycerol groups, lipoproteins, and lipopeptides, which are present in almost all bacteria, especially in the outer membrane of gram-negative bacteria. TLR2 also has many endogenous ligands, which are also known as "alarmins", representing tissue damage, necrosis, and tumor cells. Defensin 3, hyaluronic acid fragments, heat shock proteins, and high mobility group box 1 (HMGB1) are endogenous ligands for TLR2. TLR2-induced autophagy is involved in the clearance of infectious pathogens and self-damaging cells.

*Listeria monocytogenes* is a pathogen of zoonotic diseases. *L. monocytogenes* infection usually leads to several diseases, including sepsis, meningitis, and mononucleosis. Recent studies have found that macrophages lacking TLR2 and NOD2 are susceptible to infection by *L. monocytogenes* and cannot activate autophagy after infection (Anand et al. 2011). This evidence suggests that *L. monocytogenes* infection can mediate autophagy via two pattern recognition receptors: TLR2 and NOD2. Moreover, researchers further found that TLR2 and NOD2 play a role in the clearance of *L. monocytogenes* by affecting the ERK signaling pathway to activate autophagy.

*M. tuberculosis* infection is one of the main causes of infectious death worldwide. Mycobacteria are a special intracellular pathogen that can survive in host macrophages after infection and affect the fusion of phagosomes with lysosomes to form phagolysosomes. Autophagy activation allows mycobacteria-containing phagosomes to interact and fuse with autophagosomes to aid in the clearance of *M. tuberculosis*. *M. tuberculosis* lipoprotein LpqH is a ligand of TLR2/1 which strongly activates autophagy in human monocytes to exert anti-tuberculosis effects. The activation of autophagy by LpqH is dependent on the activation of vitamin D receptor signaling and the synthesis of antimicrobial peptides. Several key molecules are involved in the regulation of this process. LpqH activates TLR2/1 signaling, induces rapid phosphorylation of Tyr172 by Src kinase, and activates PLC-γ, which induces calcium influx. Increased intracellular calcium concentration induces Thr172 phosphorylation of AMPK and activates downstream p38 MAPK. The abovementioned activated signaling pathway upregulates the expression of the hydroxylase Cyp27b1 and converts the vitamin D prohormone (25-hydroxyvitamin D3) into an active 1,25-dihydroxy vitamin D3, thereby inducing expression of antimicrobial peptides. Antimicrobial peptides facilitate the fusion of autophagosomes with lysosomes and promote the clearance of intracellular *M. tuberculosis*.

Parkinson's disease (PD) is a degenerative nervous system disease associated with the accumulation of alpha-synuclein, and TLR-associated inflammation is closely related to its progression. In a large-scale meta-analysis of the largest PD gene research, genome-wide association study (GWAS), researchers analyzed data from

more than 19,000 PD patients and over 100,000 controls, identifying 24 gene sites related to PD in the relevant genomes. Interestingly, at least 11 of these 24 sites are involved in or disrupt various functions of the autophagolysosomal pathway. This finding suggests that abnormalities in autophagy activation may be closely related to the development of PD. TLR2 has higher expression in the brain tissue of PD patients compared with healthy individuals, and TLR2 is closely related to pathological alpha-synuclein accumulation. Increasing the level of endogenous alpha-synuclein leads to enhanced expression of P62, thus inhibiting the development of autophagy and promoting the development of PD (Dzamko et al. 2017).

HMGB1 is a typical DAMP molecule that is released extracellularly when tumor cells die or are treated with cytotoxic drugs. It has been reported in the literature that HMGB1 is an endogenous ligand of TLR2, and other pattern recognition receptors capable of recognizing HMGB1 include RAGE, TLR4, and TLR9. HMGB1 is capable of inducing autophagy activation at both the transcriptional and nontranscriptional levels. HMGB1 directly regulates the transcription of heat shock protein beta-1 (HSPB1), which is a key regulator of cytoskeletal actin kinetics and plays an important role in autophagy activation. Inhibition of HMGB1 affects the normal dynamic process of autophagy, affecting the clearance of damaged mitochondria by autophagy. On the other hand, under stress conditions, such as starvation, oxidative stress and chemotherapy, HMGB1 in the nucleus can be translocated from the nucleus to the cytoplasm and interacts with Beclin 1, and the interaction of HMGB1 with Beclin 1 inhibits the association between Beclin 1 and Bcl2, which is the initial signal for autophagosomes. However, HMGB1 plays a "double-edged sword" role in tumors and can both promote tumor proliferation and apoptosis. The detailed mechanism remains to be studied.

#### 26.1.2.4 Regulation of Autophagy by TLR4 Signaling and Its Biological Significance

LPS, a component on the surface of gram-negative bacteria, is a potent proinflammatory PAMP molecule and one of the ligands of TLR4. TLR4 acts as a "sensor" for autophagy activation through TRIF-dependent and MyD88-independent signaling pathways. LPS-activated autophagy can terminate the dormancy of *M. tuberculosis* in phagosomes, promote the fusion of phagosomes and autophagosomes, and induce the integration of PI3KC3 into the plasma membrane to promote the formation of autophagic vesicles.

In septic shock, cells face strong oxidative stress and metabolic demands, which may cause mitochondrial damage, protein accumulation with aggregation tendencies, energy depletion, and cell death, ultimately leading to multiple organ failure and death. To avoid this, cells need to activate autophagy and autophagy-related clearance processes to maintain cell homeostasis. It has been found that LPS can induce autophagy activation in cardiomyocytes. Autophagy activation was observed after LPS stimulation in the neonatal cardiomyocyte cell line HL-1 or in mCherry-LC3 transgenic mice. The researchers also found that LPS activates autophagy by

inducing TNF-production, a process mediated by the production of p38 MAPK, NOS, and ROS. Inhibition of p38 MAPK and NOS can reduce autophagy activity. LPS-activated autophagy exerts a protective effect on myocardial damage in sepsis. LPS stimulation activates p62-dependent selective autophagy in macrophages. LPS stimulation first activates the transcription factor Nrf2 and promotes p62 transcription, which in turn induces the formation of aggresome-like induced structures (ALIS) and autophagic degradation. MyD88, TRIF, IRAK4, TRAF6, ROS, and p38 are involved in the regulation of this process. LPS binds to TLR4 and activates downstream signaling, which increases the activity of the E3 ubiquitin ligase TRAF6 and enhances TRAF6-mediated ubiquitination of K63 at Beclin 1, thereby further activating autophagy. LPS can also induce autophagy activation in hepatocytes, a process that requires the involvement of p62. Thus, in septic shock, LPS maintains cellular homeostasis by activating TLR4 signaling to activate autophagy. Biglycan is a leucine-rich protein-polysaccharide. As a danger signal, it aggravates the ischemia-reperfusion injury by recruiting macrophages through TLR signaling. In the past, it was believed that disaccharides were mainly regulated by TLR2/4 and the coreceptor CD14, but recent studies have found that disaccharides can also promote autophagy activation of macrophages by binding to TLR4 and its coreceptor CD44. Targeting disaccharides and specific TLR coreceptors are potential therapeutic strategies for the treatment of ischemia-reperfusion-induced renal damage.

In fact, TLR2 and TLR4 signals have distinct regulatory effects on autophagy under certain conditions. Tissue fibrosis is the basic pathological change of many chronic diseases. TLR2 and TLR4 play different roles in regulating the development and outcome of tissue fibrosis. In the model of acute and chronic dilated cardiomyopathy induced by doxorubicin in mice, neutralizing antibodies blocking TLR4 can inhibit autophagy activity in myocardial tissue. The upstream autophagy activation signals MyD88-p38-MAPK and TRIF-IRF3 are in a low activity state, while the upstream autophagy inhibition signal PI3K-AKT-mTOR is in an activated state, resulting in the inhibition of autophagy. Unlike blocking TLR4, blocking TLR2 with neutralizing antibodies does not affect autophagy activity in doxorubicin-damaged myocardial tissue. The upstream autophagy activation signal MyD88-p38-MAPK and the inhibitory signal PI3K-AKT-mTOR were all in a low activity state, and there was no corresponding change in TRIF-IRF3. Based on the differential regulation of autophagy, genetic or pharmacological blockade of TLR2 signaling can attenuate pulmonary fibrosis, myocardial fibrosis, and myocardial remodeling, while blockade of TLR4 aggravates pulmonary fibrosis and heart muscle fibrosis. Consistently, activation of TLR4 results in rapid relief of acute inflammation in the lungs, reducing cardiovascular hypertrophy and cardiac fibrosis caused by stress overload. In addition to tissue fibrosis, TLR2 and TLR4 also play opposite roles in regulating the development and outcome of acute kidney injury (AKI). Acute kidney injury is a progressive, cumulative, and severe syndrome that often occurs in cancer patients receiving chemotherapy. TLR2 plays a protective role in renal injury, while TLR4 has the opposite effect. The difference in the role of TLR4 in regulating renal injury is due to its differential regulation of the downstream autophagy pathway (Andrade-Silva et al. 2018). In the cisplatin-induced mouse kidney injury model, TLR2 KO

mice showed severe kidney damage and decreased survival compared to wild-type mice, while TLR4 KO mice showed protective renal effects. Compared with TLR4 KO mice, TLR2 KO mice showed lower expression of the autophagy-related proteins LC3 and ATG5 in renal tissues. This difference may be due to the fact that TLR4 also regulates autophagy by regulating TRIF, in addition to sharing the same downstream regulatory molecule MyD88 with TLR2. These studies further demonstrate the differential effects of different TLR signals in regulating the homeostasis of autophagy signals and thereby maintaining cellular homeostasis.

#### 26.1.2.5 Regulation of Autophagy by Other TLRs and Their Biological Significance

In addition to TLR2 and TLR4, other TLR receptor signals are also involved in autophagy regulation. Single-stranded RNA (ssRNA) is the ligand of TLR7. ssRNA induced significant dot-like aggregation of LC3 and conversion of LC3-I to LC3-II in RAW264.7 cells. The formation of mature autophagosomes (autophagy lysosomes) could also be observed by electron microscopy under such conditions. Imiquimod is an imidazoquinoline compound and a novel agonist of TLR7. Imiquimod can induce RAW264.7 cells to form LC3 punctate aggregates and increase long-term protein degradation. Activation of autophagy by TLR7 ligands is dependent on Beclin 1, TLR7 and MyD88. Activation of autophagy by TLR7 signaling is also important for the clearance of *M. tuberculosis*. Stimulation with single-stranded RNA or imiquimod in *M. tuberculosis*-infected macrophages can lead to the elimination of 20–40% of intracellular fractions.

Flagellin (a ligand of TLR5) and CpG (a ligand of TLR9) did not induce significant autophagy activation in RAW264.7 cells. Poly (I:C) can induce autophagy activation. However, since poly (I:C) can activate both TLR3 and MDA5, it is not completely certain whether poly (I:C) activates autophagy by activating TLR3 or MDA5 or if both receptors are necessary for the activation of poly (I:C)-induced autophagy.

TLR3 plays an important role in regulating heart failure following myocardial infarction in mice. In vitro stimulation of TLR3 in cardiomyocytes can increase the expression of autophagy components, such as LC3-II and P62, through the TRIF signaling pathway, but TLR3 does not affect autophagic flow in cardiomyocytes, thereby promoting heart failure and increasing mortality.

#### 26.1.2.6 MicroRNAs Regulate the TLR Mediated Autophage and Its Biological Significance

MicroRNAs (miRNAs) are a class of noncoding RNAs that typically contain 18–25 nucleotides and promote degradation of target gene mRNA or inhibition of translation through binding to the 3′-nontranscribed domain (3′-UTR) of the mRNA. Previous studies have shown that miRNAs can participate in the development of

various diseases, such as tumors, blood diseases, bone diseases, and nervous system diseases, by affecting cell proliferation, apoptosis, aging, and metastasis. Recent studies have found that miRNAs can alleviate the pathogenesis of epilepsy by inhibiting the activation of autophagy signals associated with TLRs. IL-1, TLRs, etc., are important factors in the pathogenesis of epilepsy. TLR4 is involved in the promotion of astrocyte temporal lobe epilepsy. LPS stimulation can lead to the acute and long-term decline in the seizure threshold. In a rat model of epilepsy-induced by kainic acid injected into the lateral ventricle, miR-181b negatively regulated the TLR4 signaling pathway by inhibiting the P38/JNK signaling pathway. This effect downregulated the expression of autophagy-related proteins, such as LC3II/I and Beclin-1, exerting a neuroprotective effect by inhibiting autophagic cell death and apoptosis. In another report, the researchers found that miR-421 can negatively regulate TLR2/TLR4-mediated autophagic flow activation by inhibiting transcription of MYD88, ultimately inhibiting autophagy and apoptosis in hippocampal neurons and alleviating Piro Carcin-induced pathogenesis in mouse epilepsy (Wen et al. 2018). At present, few reports have focused on the effects of miRNAs on TLR-mediated autophagy, and the complex regulatory network and regulatory mechanisms used by miRNAs remain to be elucidated.

## *26.1.3 RLR Signaling and Autophagy Regulation*

### 26.1.3.1 RLRs and Their Signaling Pathways

RLRs are a newly discovered type of pattern recognition receptor belonging to the DExD/H-box RNA helicase family, which mainly recognizes viral RNA in the cytoplasm and induces the production of interferon and pro-inflammatory cytokines through the RLR cascade. RLRs play critical roles in the establishment of innate immunity against viral infection. RIG-I, MDA5, and LGP2 are currently identified as members of the RLR family. There are two caspase recruitment domains (CARDs) at the N-terminus, a DEAD-box helicase/ATPase domain in the middle and a regulatory domain at the C-terminus of RLRs. RLRs are located in the cytoplasm of cells and they recognize genomic RNA of double-stranded RNA viruses and double-stranded RNA produced by single-stranded RNA viruses during replication. Type I interferon stimulation or viral infection induces the expression of RLRs.

RIG-I and MDA5 recognize different viral RNAs, RIG-I recognizes short double-stranded RNAs with a tail of triphosphate (less than 1 kb in length), while MDA5 recognizes long double-stranded RNAs (more than 2 kb), such as poly I:C. When the ligand binds to the RLR, the CARD domain of the RLR interacts with the N-terminal linker molecule IPS-1, which also contains a CARD domain, and initiates a signal cascade through EYA4, TRAF3, NAP1/SINTBAD, TBK1/IKK-i, and IRF3/7, subsequently inducing the expression of the type I interferon gene. The RLR signal also induces NF-κB translocation to the nucleus via TRADD, FADD, and caspase-8/-10.

#### 26.1.3.2 Mutual Regulation of RIG-I and Autophagy

Although less is known about the relationship between RIG-I and autophagy, some evidence has shown that there is a close correlation between RIG-I and autophagy. The ATG12-ATG5 heterodimer complex interacts directly with RIG-I, MDA5, and IPS-1 to inhibit its dissociation and thus negatively regulates the RLR signal. When autophagy is defective, the damaged mitochondria accumulate, as they cannot be cleared. The expression of the mitochondrial membrane protein IPS-1 increases in the cells, thereby activating IFN and cytokine production. ROS accumulation caused by mitochondrial damage also enhances RLRs. Consistently, activation of the RIG-I signal induces autophagy activation. The inhibitor of caspase 8 can not only induce activation of the RIG-I signal in the leukemia cell line U937 but also promote the accumulation of autophagosomes in the cytoplasm, the conversion of LC3-I to LC3-II and the enhancement of dot-like aggregation of LC3. The activation of autophagy by RIG-I is involved in the death of acute myeloid leukemia cells caused by upregulation of RIG-I. Detailed mechanistic studies have demonstrated that RIG-I, which is not activated by exogenous RNA, interacts with Src to inhibit Akt-mTOR activation, thereby activating autophagy.

RLRs are essential for preventing RNA viral infection, and their activity must be strictly controlled to maintain immune homeostasis. Tetherin (BST2/CD317) is an interferon-inducible antiviral factor preventing the release of enveloped viruses by infected cells. Tetherin is a negative regulator of RLR-mediated type I interferon signaling. Type I interferon-induced Tetherin promotes autophagic degradation of MAVS by recruiting the E3 ubiquitin ligase MARCH8 to catalyze K27-linked ubiquitin chains on MAVS at lysine 7, which serves as a recognition signal for NDP52-dependent autophagic degradation. This study revealed a negative feedback loop for the RLR signal generated by the Tetherin-MARCH8-MAVS-NDP52 axis. Leucine-rich repeat-containing protein 25 (LRRC25) is also a key negative regulator of RLR-mediated type I interferon signaling. After RNA virus infection, LRRC25 specifically binds to RIG-I, promoting the interaction between RIG-I and the autophagy carrier receptor p62, thereby promoting the degradation of the RIG-I autophagy pathway. Infection with typical swine fever virus (CSFV) activates autophagy. CSFV induces autophagy and delays apoptosis by downregulating ROS-dependent RLR signaling, resulting in sustained infection of the virus in host cells. Dengue virus (DENV) is a single positive-stranded RNA virus that promotes viral replication by inhibiting innate cellular immunity. Antibody-dependent enhancement (ADE) of DENV infection is identified as the major risk of severe dengue fever. Both DENV and DENV-ADE infections induce early ISG (NOS2) expression via the RLR-MAVS signaling axis, thereby activating autophagy.

## *26.1.4 CLR Signaling and Autophagy Regulation*

### 26.1.4.1 CLRs and Their Signaling Pathways

Typical CLRs contain one or more carbohydrate recognition domains (CRDs), and there are two $Ca^{2+}$ binding sites on the loops on the surface of the protein that is necessary for CLRs to function. CLRs include both secreted and transmembrane types, and representatives of secreted CLRs are members of the collagen lectin family, such as MBL. Transmembrane CLRs are classified into type I and type II according to their protein N-terminal orientation. Type I CLRs have DEC205 and MMR, Type I CLRs belong to the mannose receptor family, and DC-expressed CLRs mostly belong to type II transmembrane proteins, including Dectin 1, Dectin 2, Mincle, DC-SIGN, and DNGR-1. Type II CLRs belong to the asialoglycoprotein family. The transmembrane CLRs primarily function as pattern recognition receptors. DC-expressed CLRs interact with pathogens by recognizing the carbohydrate structure of mannose, fucose, dextran, and so on. These CLRs recognize most types of human pathogens. Mannose allows receptors to specifically recognize viruses, fungi, and mycobacteria. Fucose structures are more likely to be expressed in certain bacteria and parasites. Glucan structures exist both in mycobacteria and fungi. CLRs recognize and internalize pathogens, causing them to degrade and undergo subsequent antigen presentation. Different pathogens bind to CLRs and can trigger different immune responses. DC-SIGN interacts with a variety of pathogens by recognizing mannose and fucose. DC-SIGN recognizes the N-mannan on Candida and plays a role in inducing T helper cell (TH) cellular responses. When the immune system encounters a mannose-expressing pathogen, e.g., *M. tuberculosis*, *Mycobacterium leprae*, HIV-1, measles virus and *Candida albicans*, mannose-binding to DC-SIGN can influence the TLR4-mediated response. DC-SIGN interaction with TLR4 can depend on TLR4 (including TLR3 and TLR5) signal activating NF-κB. DC-SIGN signaling activates the serine/threonine-protein kinase RAF1, which catalyzes the phosphorylation of the p65 subunit of NF-κB. The phosphorylated p65 subunit binds to CBP and p300 to undergo acetylation modification. Acetylation increases the affinity of p65 to DNA and enhances its transcriptional activity. Notably, activation of this pathway and recruitment of the RAF1 signaling body may depend on the nature of the identified carbohydrate ligand and affect the nature of the inflammatory response and subsequent adaptive immunity. For example, when DC-SIGN binds to a mannose-expressing pathogen such as *M. tuberculosis*, it is necessary to recruit upstream effector leukemia-associated RHo-GEF (1ARG; also known as ARHGEF12) and RHoA to activate the RAF1 signaling body. RAF1 signaling leads to the phosphorylation and acetylation of the p65 subunit of NF-κB, thereby increasing the pro-inflammatory response.

Dectin 1, Dectin 2, and Dectin 3 and Fc receptors of IgG (FcγRs) induce responses in a spleen tyrosine kinase (SYK)-dependent manner, and Dectin-1 is expressed in two major human DC subpopulations, bone marrow DCs (mDCs), and plasmacytoid DCs (pDCs), which have opposite effects in the control of Th2-type $CD4^+$ cells.

Dectin-1 expressed on mDCs reduces the Th2-type $CD4^+$ T cell response, while Dectin-1 expressed on pDCs promotes the Th2-type $CD4^+$ T cell response.

Dectin 1 can identify β-1,3-glucan, a molecule that is widely expressed in fungi, including *C. albicans*, *Aspergillus* and *Pneumocystis carinii*, and by insects. Fungi binding with Dectin 1 induces phosphorylation of the cytoplasmic domain YxxL, which Syk tyrosine kinase then recruits to the two phosphorylated receptors and assists in forming a CARD9, BCL-10 and MALT1 complex, which in turn induces activation of IKK complexes. Phosphorylation of the IKK catalytic subunit IκBα occurs, allowing it to enter the ubiquitin-proteasome degradation pathway and releasing NF-κB to translocate to the nucleus and thus regulate transcription. Dectin 1 also activates downstream signals independent of Syk, and activation of Dectin 1 leads to the activation of RAF1 by phosphorylation, which leads to Ser276 phosphorylation of p65. Ser276 phosphorylation of p65 provides a binding site for CBP and p300, multiple lysine sites of p65 are acetylated, and transcriptional regulatory activity is enhanced. In addition to directly inducing cell activation, several transmembrane TLRs, including TLR1, TLR2, TLR4, and TLR6, and Dectin-1 synergistically identify fungi, as these TLRs recognize *C. albicans* cells through mannan-containing structures.

Recent studies have reported that Dectin 1 has another important biological function. It prevents the uncontrolled release of neutrophil extracellular traps (NETs) during fungal infection by Dectin 1 signaling. Importantly, this activity prevents extensive tissue damage from occurring during the immune response to the fungus. However, not all strains of *C. albicans* were identified by Dectin 1, probably due to the glucan component of the cell wall structure; related strains have subtle differences, which may explain the susceptibility of different host Dectin 1 to defects.

Both Dectin 2 and Mincle can associate with the signal adaptor protein molecule Fc receptor γ chain (FcRγ) in the transmembrane region via a positively charged amino acid residue. CLR signal activation-induced tyrosine phosphorylation of the FcRγ immunoreceptor activation motif (immunoreceptor tyrosine-based activation motif, ITAM), further recruiting Syk kinase to activate downstream signaling cascades. Activation of these two receptor signals results in the production of cytokines independent of TLRs, such as tumor necrosis factor and IL-6.

#### 26.1.4.2 Dectin-1 Signaling and Autophagy Regulation

At present, research on the association between CLR signaling and autophagy regulation has just started. More studies have focused on the function of Dectin 1 signaling in autophagy regulation. β-dextran on fungal cell wall surfaces binding with Dectin 1 can trigger phagocytosis, production of ROS and inflammatory cytokines in response to fungal infections. In fact, key proteins in the activation of autophagy are also involved in the regulation of traditional phagocytosis. Both fungal and β-glucan particles induce the conversion between LC3-I and LC3-II in macrophages and DCs and are recruited to phagosomes. This process requires the involvement of

Syk kinase and NADPH oxidase. Glucan-activated Dectin 1 induces LC3 recruitment to phagosomes to enhance the recruitment of MHC-II molecules, promote antigen presentation to fungi, and regulate adaptive immune responses. In addition, the recognition of β-1,3-glucan by Dectin-1 also triggers the localization of TLR9 to phagosomes. Dectin-1 specifically regulates changes in TLR9-dependent gene expression.

LC3-related phagocytosis (LAP) is an emerging unconventional autophagy process that bridges signals from pattern recognition receptors to autophagy. The formation of LAP results in the incorporation of lipidated LC3 into the phagosome membrane (LAPosome). Studies have shown that LC3 incorporates into the phagosome membrane after phagocytosis by macrophages and activation of the Syk Dectin-1 interaction triggers a subsequent active oxygen-mediated NADPH oxidase species (ROS), which is involved in the induction of LAP; β-Dextran-activated Dectin-1 also functions to induce atherosclerosis by converting macrophages into the M1 phenotype by the NF-κB-autophagy-dependent pathway (Li et al. 2019). Large-scale transcriptome and secretory studies suggest that increased protein secretion is only partially associated with increased transcription. Bioinformatics analysis combined with functional research shows that the activation of Dectin 1/Syk signaling activates both traditional and nontraditional (vesicle-mediated) protein secretion. Among them, the nontraditional protein secretion process activated by Dectin 1 is dependent on inflammasome activity and autophagy activation. However, the effect of Dectin-1 in the autophagy signaling remains to be further studied.

## *26.1.5 NLR Signaling and Autophagy Regulation*

### 26.1.5.1 NLRs and Their Signaling Pathways

The NLR family is an important class of cytoplasmic pattern recognition receptors in the innate immune system that is capable of sensing exogenous invading microbial components and endogenous DAMP molecules, such as ATP, mitochondrial DNA and ROS. NLRs play an important role in the innate immune response and resistance to pathogen invasion (Dolasia et al. 2018). NLR molecules include three specific domains: an N-terminal effector domain that recruits downstream effector signaling molecules; a centrally located NACTH domain with ATPase activity and nucleotide-binding activity, which is critical for NLR oligomerization and activation; and the C-terminal leucine-rich repeat (LRR), which is responsible for the detection and recognition of ligands while mediating autoregulation and protein interactions. The NLR family can be divided into five subfamilies: NLRA, containing the AD domain; NLRB, containing the BID domain; NLRC, containing the CARD domain; NLRP, containing the PYD domain; and NLRX.

NOD1 and NOD2, which are currently well studied, are members of the NLRC subfamily. NOD1 recognizes peptidoglycans containing diaminopimelic acid structure present in gram-negative bacteria, while NOD2 recognizes muropeptide, the

common macromolecular structure of both gram-negative and gram-positive bacteria. After recognizing the ligands, NOD1 and NOD2 recruit and activate the downstream molecule RICK (RIP2) by self-oligomerization, thus activating the NF-κB and MAPK signaling pathways. RICK is a serine/threonine kinase that can be ubiquitinated when interacting with NOD1 or NOD2 via the CARD domain. K63-linked ubiquitination of RICK plays an important role in recruiting TAK1 to activate downstream signaling. RICK recruits IKKβ and induces its K63 ubiquitination. Such modification mediates the interaction between IKKβ and TAK1 and induces IKKβ phosphorylation, leading to IκB phosphorylation and degradation. The released NF-κB translocates into the nucleus to induce target gene transcription. In addition to the NF-κB pathway, NOD1, and NOD2 also activate the MAPK pathway including the P38, ERK, and JNK signaling pathways. However, the activation mechanism of the MAPK pathway is still not clear, and it is certain that the upstream molecules RICK and TAK-1 are involved.

There is also some research about the NLRP subfamily and the NLRX subfamily. Hypoxia increases inflammation by regulating the rapamycin (mTOR)/NLRP3 pathway during the development of colitis. NLRP4 inhibits autophagy via inactivating Beclin 1. Upon infection with *Streptococcus aureus*, NLRP4 is recruited onto the phagosome membrane and transiently dissociates from Beclin 1 to initiate autophagy. *Pseudomonas aeruginosa* activates the NLRP3 inflammasome to trigger autophagy, allowing bacteria to escape from phagocytic cells. During viral infection, NLRX1 interacts with the mitochondrial Tu translation elongation factor (TUFM), which in turn interacts with ATG-5, ATG-12, and ATG16L to initiate autophagy. This process indicates that the NLR-ATG signaling cascade plays an important role in inducing autophagy in both bacterial and viral infections.

#### 26.1.5.2 NOD Signaling Regulates Autophagy and Participates in the Development of Crohn's Disease and Bacillary Dysentery

Crohn's disease is a chronic autoinflammatory disease characterized by an excessive inflammatory response in the intestinal mucosa. Crohn's disease has long been recognized as an autoimmune disease controlled by a T cell-dependent immune response. Recent genetic studies have established a link between this inflammation and innate immune gene polymorphisms, including NOD2, the autophagy-associated protein ATG16L1, and the immune-related GTPase family protein M. Peptidoglycan binds to NOD2 to induce autophagy activation and bacterial clearance. Individuals with Crohn's disease-associated NOD2 and ATG16L1 variants exhibit a defect in the induction of an autophagic response and bacterial clearance. *Mycobacterium avium* subtype tuberculosis and invasive *Escherichia coli* (enteroinvasive *E. coli*), are considered to be microorganisms that cause Crohn's disease. The host's inherent defense mechanisms against bacteria that invade the intestinal mucosa are the recognition of bacteria by TLRs and intracellular NLRs and the subsequent production of antimicrobial peptides. Other innate immune defense mechanisms also include phagocytosis,

intracellular killing and secondary antigen processing, presentation, and T cell activation. NOD1 and NOD2 recognize peptidoglycans from gram-negative bacteria and muropeptides from gram-positive bacteria, respectively. Such recognition induces the production and release of chemokines and pro-inflammatory cytokines such as TNF-α, IL-1β and IL-6. Loss of function of NOD2 increased susceptibility to Crohn's disease. The autophagy protein ATG16L1 and IRGM gene polymorphisms also increase susceptibility to Crohn's disease, suggesting that the occurrence and development of this disease are closely connected with autophagy. Thus far, researchers believe that the effects of NOD2 signaling and autophagy on inflammation in Crohn's disease are irrelevant. A recent study found that the activation of NOD2 by the muramyl dipeptide component of peptidoglycan has a strong induction effect on autophagy. This activation also promotes bacterial transport for autophagic lysosomal clearance and subsequent antigen presentation. When bacteria enter the cell, NOD2 recruits ATG16L1 to the plasma membrane and initiates the formation of autophagosomes. Intracellular antigens are processed via macroautophagy for presentation on MHC Class II molecules (Munz 2016). This process promotes bacterial clearance, antigen presentation, and immune activation. Under hypoxic conditions, cells enhanced intestinal inflammation by reducing the binding of NLRP3 to mTOR and activating autophagy (Cosin-Roger et al. 2017).

*Shigella* is a pathogen that is highly adapted to the body, and infection causes bacillary dysentery. The most prominent pathogenic feature of *Shigella* is its ability to invade a variety of host cells, including epithelial cells, DCs, and macrophages. *Shigella* invades host cells and causes a serious inflammatory response in the intestine. If *Shigella* invades the resident macrophages of the lamina propria of the intestine, bacteria can escape from the phagosome into the cytosol and induce caspase-1 activation and macrophage death. The regulation of the activation of caspase-1 by *Shigella* and the processing of IL-1β are mediated by the NOD-like receptor IPAF. IPAF and caspase-1 are critical for *Shigella*-induced caspase-1-dependent macrophage death. However, IPAF and caspase-1 deficiency significantly induced autophagy activation and inhibited the death of *Shigella*-infected macrophages. The regulatory mechanism of this process is not yet clear.

#### 26.1.5.3 Loss of Mitochondrial Autophagy Induces NLRP3 Inflammatory Body Activation

Inflammatory corpuscle activation is an inflammation-associated caspase activation mediated by polyprotein complex assembly signals, interleukin-1β (IL-1β), and leukocyte-mediated maturation of inflammatory factors such as interleukin-18 (IL-18). Among them, NOD-like receptor (NLRP3) inflammasomes are the most thoroughly studied inflammasomes, which are involved in the development of many human diseases. A study found that autophagy can negatively regulate the activity of inflammasomes and that autophagy-related proteins can maintain mitochondrial integrity. Deletion of the autophagy-related gene 16L1 promoted endotoxin-induced inflammation, and deletion of the autophagy microtubule-associated protein LC3B

and the membrane-like protein BCL2-interacting protein Beclin1 leads to mitochondrial dysfunction and mitochondrial DNA release into the cytosol, promoting NLRP3 inflammasome activation. LPS-induced NF-κB activation promotes p62/SQSTM1 expression, mediates mitophagy, and inhibits NLRP3 inflammasome activation.

Mitochondria are the main source of intracellular ROS, which is involved in the regulation of NLRP3 inflammatory bodies, but their role has not yet been elucidated. On the one hand, ROS can mediate the activation of NLRP3 inflammasomes; on the other hand, ROS can promote the sensitivity of NLRP3 but have no effect on the activation of NLRP3 inflammasomes.

## 26.2 Cytokine Receptor Signaling and Autophagy Regulation

Cytokines are small secreted proteins that mediate and regulate immune, inflammatory, and hematopoietic processes. They play an important role in transmitting inflammatory signals during innate immune responses and adaptive immune responses. The biological function of most cytokines is to stimulate the proliferation and differentiation of immune cells. For example, IL-1 stimulates T cell differentiation; IL-2 stimulates antigen-activated T lymphocytes and B lymphocytes; IL-4, IL-5, and IL-6 stimulate B lymphocytes; and IFN-γ stimulates macrophages. IL-3, IL-7 and granulocyte monocyte colony-stimulating factor stimulates hematopoietic cells. Helper T lymphocytes (Th cells) are cells that produce most cytokines and play a central regulatory role in the adaptive immune system. The major surface marker of Th cells is CD4, and these cells secrete a variety of cytokines and regulate or "assist" other lymphocytes to function by reacting with MHC II-presented polypeptide antigens on the surface of antigen-presenting cells (APCs). Th cells are mainly classified into Th1, Th2, Th17, and Treg cells, depending on the type of cytokines produced.

The formation of autophagosomes is not only stimulated by a variety of external stimuli, including nutrient deficiencies under starvation conditions but also regulated by the host- and pathogen-derived molecules, including toll-like receptor ligands and cytokines. Cytokines regulate the autophagy signaling pathway; for example, IFN-γ, TNF-α, IL-1, IL-2, IL-6, and TGF-β can induce autophagy activation, while IL-4, IL-10, and IL-13 mainly play a role in inhibiting autophagy, as shown in Fig. 26.2. In addition, the autophagy signaling pathway regulates the production and secretion of cytokines, such as IL-1, IL-18, TNF-α, and type I interferons (Harris 2011). The first section of this chapter described in detail how the TLR family and its ligands regulate autophagy. This section focuses on the relationship between autophagy and Th1, Th2, Th17, and Treg cytokines.

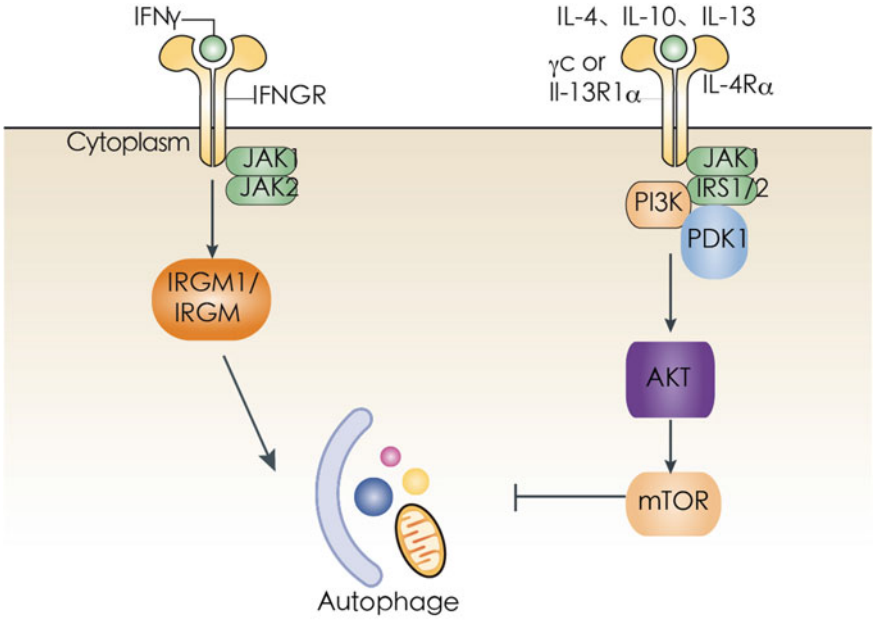


**Fig. 26.2** The regulation of cytokines on autophagy signaling pathways. The cytokine IFN-γ induces autophagy activation via the IFNGR/Irgm1/IRGM signaling pathway; the cytokines IL-4, IL-10 and IL-13 inhibit autophagy mainly through the PI3K/AKT/mTOR signaling pathway

## *26.2.1 Th1 Cytokine Signaling and Autophagy*

### 26.2.1.1 Overview of Th1 Cytokines and Their Functions

Th cells produce the most types of cytokines and can be classified as Th1 cells and Th2 cells, depending on the type of cytokines they produce. Under the stimulation of IFN-γ, Th0 cells can differentiate into Th1 cells, and Th1 cells mainly secrete Th1 cytokines, including IL-2, IFN-γ, IFN-α, and TNF-α. Th1 cells mainly mediate cytotoxicity and local inflammation-related immune responses, assist in antibody production, and participate in cellular immunity and delayed type hypersensitivity inflammation; therefore, they are called inflammatory T cells and can be regarded as delayed type hypersensitivity reaction-sensitized T cells. Thus, Th1 cells play an important role in host resistance to intracellular pathogen infection. Autophagy is involved in the infection of many pathogens. The immune system protects against pathogens through autophagy, but some pathogens can evade or utilize autophagy through specific mechanisms. Autophagy can be regulated by Th1 cytokines. For example, TNF-α can induce autophagy activation in Ewing's sarcoma cells when NF-κB activity is inhibited. The protective mechanism of IFN-γ against mycobacteria is related to the induction of autophagy, indicating that autophagy is an important

mechanism in the Th1-type immune response. This section focuses on how Th1 cytokines regulate autophagy involvement in pathogen infection and the host anti-infection process.

#### 26.2.1.2 IL-2 and Autophagy Regulation

Interleukin-2 (IL-2), also known as T cell growth factor (TCGF), is a cytokine with broad biological activity that is mainly produced by activated $CD4^+$ Th1 cells. In addition to promoting T cell proliferation, IL-2 can also promote the production of cytokines and the proliferation of B cells and NK cells, making it an important factor regulating the immune response. In addition, IL-2 is involved in important biological functions such as antibody response, hematopoiesis, and tumor surveillance. Studies have indicated that IL-2 can promote both cell proliferation and cell death, which depends on the type of cells and the dose of IL-2. In the treatment of melanoma and renal epithelial cell carcinoma, IL-2 can promote the recognition and clearance of cancer cells by the immune system. Currently, recombinant IL-2 has been approved by the US FDA for the treatment of melanoma and advanced kidney cancer.

In recent clinical reports, high-dose IL-2 is effective in 25% of patients with renal cell carcinoma, and 20% of patients have a survival period of more than 5 years. However, the side effects of high-dose IL-2 treatment are obvious, and the most serious side effects are hypertension and heart, intestine, central nervous system, lung, and liver toxicity. The above adverse reactions are a serious threat to patients, so high-dose IL-2 treatment must be limited to medical institutions, and this limitation often leads to interruption of treatment. Various clinical investigations, including the combined use of TNFα, iNOS, or VEGF to improve the efficacy of IL-2 and reduce the toxicity of IL-2, have failed, and these combinations have not significantly improved the therapeutic effect of IL-2. Other attempts include vaccination, adoptive cell therapy, or inhibition of CTLA-4 to increase the effects of IL-2 treatment while also increasing the toxicity of the treatment. So far, high-dose IL-2 is still considered to be the only drug with a definite clinical effect on metastatic renal cell carcinoma. The specific mechanism by which high-dose IL-2 triggers adverse reactions is unclear. Systemic autophagic syndrome triggered by IL-2 is thought to be the cause of adverse reactions, as using autophagy inhibitors (chloroquine) can reduce the adverse effects of IL-2 and improve its therapeutic effect. Given that IL-2 is an important antitumor immunoregulatory factor in the body, the IL-2 gene is transfected into tumor cells, tumor stromal cells and tumor-infiltrating lymphocytes, which can achieve effective antitumor concentrations in tumors and avoid the toxic side effects of systemic administration.

As an important "sentinel" cell in the immune system, fibroblasts play a vital role in wound healing, chronic inflammation, and tumor development. Both human and mouse fibroblasts express functional IL-2 receptors including IL-2Rα, β, and γ. IL-2-transduced fibroblasts have effects in treating neuroblastoma and colon cancer tumors. Autophagy acts as an evolutionarily conserved pathway responsible for the transport of intracellular degradation materials into lysosomal degradation, and


**Fig. 26.3** The role of IL-2 in autophagy modulation. IL-2 promotes translocation of HMGB1 in mouse embryonic fibroblasts, and HMGB1 interacts with Beclin1 to induce activation of autophagy and promotes fibroblast proliferation and survival. Autophagy inhibitors or knockout autophagy-related genes Atg5 and Beclin1 reduce the extent of IL-2-activated autophagy, inhibit the proliferative effect of IL-2, and induce apoptosis in fibroblasts

each step plays an important role in maintaining cell homeostasis. Autophagy activation is essential for IL-2 to induce fibroblast proliferation, and IL-2 also induces autophagy activation in mouse embryonic fibroblasts and primary lung fibroblasts (Kang et al. 2013). Studies have shown that IL-2 (6000 u/ml) stimulation of mouse embryonic fibroblasts and lung fibroblasts for 48 h significantly induced LC3-II expression and increased the number of autophagosomes; IL-2 promotes LC3-II puncta aggregation in a dose-dependent manner. IL-2 also activates autophagy by promoting the translocation of HMGB1 and enhancing the interaction between HMGB1 and Beclin1 in mouse embryonic fibroblasts. Inhibition of autophagy activity by autophagy inhibitors (3-MA and Bafilomycin A1) or knockdown of the autophagy genes Atg5 and Beclin1 can reduce IL-2-induced autophagy activation and inhibit IL-2-induced cell proliferation and apoptosis, as shown in Fig. 26.3.

#### 26.2.1.3 IFN and Autophagy Regulation

Interferons are a type of cytokine with antiviral, antitumor and immunomodulatory effects and include type I interferons (IFN-α, IFN-β, IFN-ω and IFNτ), type II interferons (IFN-γ) and type III interferons (IFN-λ). The immunomodulatory effects of interferons include the following aspects: (1) induction of the MHC antigen-presenting signaling pathway; (2) enhancement of Th cell effects; (3) improvement of the antitumor effect; (4) antimicrobial action; (5) regulation of leukocyte transport; and (6) enhanced LPS signaling. Although the activity of most interferons is associated with activation of the JAK-STAT signaling pathway, the MAPK signaling pathway also plays a key role in the activation of interferon-regulated genes. Studies have shown that type I interferons can regulate the process of autophagy involved in antiviral infection through the JAK-STAT or PI3K-AKT-mTORC1 signaling pathway. PI3K-AKT-mTOR is a classic signaling pathway involved in the regulation of autophagy activity. Type I interferons can induce autophagy activation in a variety of human cell lines (Schmeisser et al. 2013). For example, IFNA2c/IFNα2c and IFNB/IFNβ can induce autophagy in B lymphoma cells for 24 h, including increasing autophagy marker proteins LC3-II, the ATG12-ATG5 complex, and the autophagy truck protein SQSTM1. Moreover, IFNA2c stimulated the expression of

the autophagy marker protein LC3-II in HeLa S3, MDA-MB-231, T98G, and A549 cell lines for 48 h. A transmission electron microscopy assay also showed that type I interferons can increase the number of autophagosomes. Inhibition of type I interferons through mTOR or PI3K-AKT signaling can further increase the expression of LC3-II, indicating that the PI3K-AKT-mTORC1 signaling pathway is involved in the activation of type I interferon-induced autophagy. Recent studies have shown that interferon-activating gene 15 (ISG15) is in a class of ubiquitin-like protein molecules induced by infection, IFN-α/β stimulation, ischemia, and DNA damage. ISG15 can be conjugated to target proteins (ISGylation) via the stepwise action of E1, E2, and E3 enzymes. Studies have shown that IFN-I can induce ISGylation of signal proteins and increase cytokine expression to defend against infection; ISGylation can also prevent viral invasion, replication and release of intracellular pathogens to resist viral infection. Recent studies have shown that ISG15 interacts with P62 and histone deacetylase 6 (HDAC6) to promote ISGylation of P62 and HDAC6 proteins, increase the formation of autophagosomes, and promote the degradation of target proteins through the autophagy-lysosome pathway (Fan et al. 2015). Therefore, ISG does not induce autophagy pathways by conventional pathways but increases autophagy by inducing fusion of autophagosomes or lysosomal components with target proteins. Studies have also shown that short-term stimulation of IFN-I can induce autophagy, whereas long-term stimulation of IFN-I inhibits autophagy by inducing ISGylation of Beclin 1. The above studies indicate that IFN-I regulates autophagy by inducing ISGylation of the target protein.

IFN-α/β is the first line of defense against viral infections, and IFN signaling is critical for controlling intracellular bacterial infections. Secretion of IFN-γ by $CD4^+$ T cells plays an antimicrobial role by activating macrophages. IFN-γ can help resist certain specific viral infections by upregulating protein kinase (PKR), dsRNA-specific adenosine deaminase (ADAR1) and guanylate-binding protein (GB). IFN-induced clearance of intracellular microbes can also play an important role in cell signaling and the regulation of inflammatory responses through the production of nitric oxide and ROS molecules. In the case of *M. tuberculosis* infection, $CD8^+$ T cells and NK cells also secrete IFN-γ to help clear infected cells. Studies have shown that IFN-γ plays an important role in the host's resistance to *M. tuberculosis* infection and clearance of *M. tuberculosis* in macrophages by activating autophagy. IFN-γ can also induce the production of intracellular GTPase, which induces innate immunity against external infections. IFN-γ also has a direct antitumor effect, inhibits cell proliferation, increases the sensitivity of tumor cells to apoptosis, and upregulates the expression of MHC I and MHC II complexes to enhance the antitumor immune response. Moreover, the IFN-signaling pathway and the STAT1-dependent IFN-signaling pathway regulate $CD8^+$ T cell-mediated acute lung injury. IFNγ increases L-arginine-induced acute pancreatitis by upregulating autophagy, resulting in intracellular trypsin activation and inflammatory responses.

Autophagy was first considered to maintain cell homeostasis via the degradation of aggregated proteins and damaged organelles and the formation of autophagosomes, playing an important role in immune defense, apoptosis, and central nervous system degenerative diseases. Autophagy also participates in the recognition of intracellular

pathogens, IFN-γ effects, signaling pathways, and the presentation of MHC II complexes. Numerous studies have demonstrated that IFN-γ activates autophagy, which not only enhances intracellular microbial clearance and immune activation but also promotes downstream JAK2-STAT1 signaling and cellular inflammatory responses. Studies have shown that chemotherapy drugs Gemcitabine and Mitomycin can induce autophagy activation in the treatment of tumors, while blocking autophagy significantly reduces the expression of IFN-γ and the growth of tumor cells. Blocking the expression of IFN-γ can reduce JAK2 signaling and inhibit autophagy. Therefore, chemotherapeutic drugs combined with JAK2/autophagy inhibitors can significantly inhibit tumor cell proliferation and increase the sensitivity of tumor cells to chemotherapeutic drugs. IFN-γ also mediates antiviral responses by activating ULK1 and subsequently leads to activation of mitogen-activated protein kinase MLK3 and downstream kinase ERK5. IFN-γ activates autophagy by inducing Irgs (p47 GTPase), eIF-2α kinase, and protein kinase R, all of which are involved in the regulation of autophagy signaling pathways. More studies have shown that IFN-γ induces autophagy by activating JAK2-STAT1, which phosphorylates the STAT1 tyrosine site via JAK1 and JAK2 to initiate its signal transduction cascade; subsequently, the STAT1 dimer binding to IFN activates a response element and, in turn, induces the transcription of hundreds of IFN-activating genes. Deletion of the autophagy-related genes Atg5 and/or Atg7 not only inhibits IFN-γ-promoting LC3 aggregation and autophagosome formation but also blocks IFN-γ activation of the JAK2-STAT1 signaling pathway, while negatively regulating SHP2 expression via inhibiting the JAK2-STAT1 signaling pathway upregulates the expression of phosphorylated STAT1 and activates autophagy.

#### 26.2.1.4 TNF-α and Autophagy Regulation

Tumor necrosis factor α (TNF-α) is a kind of adipokine involved in systemic inflammation and is also an important cytokine involved in the acute inflammatory response of cytokine family members. TNF-α is mainly produced by M1-type macrophages and can also be produced by $CD4^+$ lymphocytes, NK cells, neutrophils, mast cells, basophils, and neurons. The main role of TNF-α is immunomodulatory, as it serves as an endogenous source of heat, capable of inducing fever, apoptosis, and inhibition of tumorigenesis and viral replication. TNF-α-promoting inflammatory responses can also cause many clinically relevant problems, such as rheumatoid arthritis, mandatory spondylitis, inflammatory bowel disease, psoriasis, and allergic asthma.

TNF-α mainly participates in rheumatoid arthritis via regulating autophagy activity. Rheumatoid arthritis (RA) is a systemic condition characterized by joint disease. There are other systems and important organs involved in addition to joints. In recent years, the role of the cytokine TNF-α in rheumatoid arthritis has received more attention. Clinical studies have indicated that serum TNF-α levels in the rheumatoid arthritis group are significantly higher than those in the normal control group. The involvement of TNF-α in the pathogenesis of rheumatoid arthritis may be related to changes in iron metabolism, inhibition of erythropoietin production, inhibition of

erythroid colony-forming units, and formation of red blast colony-forming units. The activation of the autophagy signaling pathway in rheumatoid arthritis is reflected by the upregulation of Beclin1 and Atg7 in osteoclasts. Overexpression of Beclin1 activates autophagy to induce osteoclastogenesis and enhances the ability of osteoclasts to absorb the material. Inhibition of phagocytosis can inhibit the differentiation ability of osteoclasts. Studies have shown that TNFα can activate autophagy by upregulating the expression of autophagy-related genes (Beclin1 and Atg7) in vivo and in vitro. By transplanting bone marrow cells deficient in the Atg7 gene, TNFα transgenic arthritis mice not only showed a decrease in the number of osteoclasts but also were protected against TNFα-induced bone erosion, loss of proteoglycans, and death of chondrocytes.

## *26.2.2 Th2 Cytokine Signaling and Autophagy*

### 26.2.2.1 Th2 Cytokines and Their Functions

Th2 cells, a subset of Th cells, specifically secrete Th2 cytokines such as IL-4, IL-5, IL-6, and IL-13. The main function of Th2 cytokines is to stimulate B cell proliferation and promote the production of antibodies such as IgG and IgE, enhancing antibody-mediating humoral immune responses. Unlike Th1-type cytokines, Th2-type cytokines play an important role in inhibiting autophagy under starvation or IFN-γ stimulation. However, studies have shown that IL-4 can activate autophagy in $CD4^+$ Th2 cells, dendritic cells and B cells. IL-13 also activates autophagy in bronchial epithelial cells. IL-6 plays a protective role in activating autophagy in pancreatic beta cells and mantle cell lymphoma cells (Harris et al. 2007). The results indicate that Th2-type cytokines have different effects on the regulation of autophagy in different cell types.

### 26.2.2.2 IL-4/IL-13 and Autophagy Regulation

IL-4 is mainly produced by Th2 cells. Activated mast cells and basophils can also produce IL-4. Its physiological function is to regulate IgE production and mast cell- or eosinophil-mediated immune responses. Specifically, it includes (1) inducing the growth and differentiation of Th2 cells; (2) inducing antibody class switching of B cells to produce IgE, while inhibiting the class conversion to IgG2a and IgG3; (3) stimulating endothelial cells to express the adhesion molecule VCAM-1, which increases the binding ability of lymphocytes, monocytes, and eosinophils to bind to them and to secrete CC family chemokines such as MCP-1, which results in increased local IL-4 concentration, inducing a large number of monocytes and eosinophils to participate in the inflammatory response; and (4) functioning as a mast cell growth factor and acting synergistically with IL-3 to stimulate mast cell proliferation.

IL-13 is produced primarily by activated T cells, and anti-CD28 antibodies can induce the transcriptional activation of IL-13. The role of IL-13 includes the following biological activities: (1) It prolongs the survival time of monocytes in vitro and inhibits LPS-induced production of IL-1, IL-6, IL-8, TNF-α and other inflammatory factors by monocytes and macrophages. (2) Through synergy with anti-IgM, it activates B cell proliferation, induction, and upregulation of B cell MHC II antigen, CD23, and CD72 expression, and induces B cell production of IgM, IgG, and IgE. (3) It induces large granule lymphocytes (LGL) to produce IFN-γ, stimulates synergistically with IL-2 to produce IFN-γ by LGL and plays an important role in inducing LAK activity and Th1-type cell immunity.

As classic Th2 cytokines, IL-4 interacts with two receptors consisting of IL-4Rα/γ chain receptor (γC) and IL-4Rα/IL-13Rα1. In contrast, IL-13 not only binds to IL-13Rα2 but also shares the receptor complex containing IL-4Rα/IL-13Rα1. Activation of IL-4 and IL-13 receptor complex signaling requires the insulin receptor substrates IRS-1/2 and STAT-6 signaling pathways. Although STAT-6 is involved in IL-4- and IL-13-induced gene expression, IRS-1/2 activates the PI3K and AKT signaling pathways. IL-4 and IL-13 can inhibit the function of macrophages to eliminate mycobacteria via autophagy activation. Furthermore, IL-4 and IL-13 in mouse and human macrophages can not only inhibit autophagy activation induced by starvation but also inhibit IFN-γ-induced autophagy activation. The autophagy inhibition of IL-4 and IL-13 is mainly reflected in the inhibition of mycobacteria entering lysosomes. In addition, they inhibit the autophagy activity of macrophages and enhance the viability of mycobacteria. IL-4 and IL-13 also activate the PI3K-AKT signaling pathway, which in turn activates mTOR to inhibit autophagy. The expression of phosphorylated PI3K and mTOR is decreased in IL-4 knockout mice, while the expression of the autophagy marker protein LC3-B is increased, indicating that IL-4 inhibits autophagy activation.

As a multifunctional cytokine, the binding of IL-4 to the IL-4-receptor activates JAK signaling, which regulates B cell function through the induction of B cell autophagy via mTOR-independent and PI3K-dependent pathways. IL-4-induced autophagy enhances B cell-mediated T cell proliferation and promotes B cell antigen presentation. IL-4 also induces autophagy flow through rapamycin target protein complex 1 (mTORC1) and RUFY4, thereby accelerating the degradation of LC3. IL-4 promotes autophagosome formation and its fusion with lysosomes and promotes endogenous antigen presentation by MHC II, suggesting that IL-4 promotes autophagy activation in special cell types.

#### 26.2.2.3 IL-6 and Autophagy Regulation

IL-6, a type of Th2 cytokine, can be secreted by lymphocytes such as T cells and B cells, as well as fibroblasts, macrophages, endothelial cells, osteoblasts, and tumor cells. The production of IL-6 in different tissues requires the induction of different stimulating factors, including various antigens and non-antigenic substances, such as DNA or RNA virus infection, bacterial endotoxin, and cytokines such as TNF-α,

IL-1, IL-8, platelet-derived growth factor, and IFN. IL-6 binds to the IL-6 receptor (IL-6R), which is widely expressed on the surface of a variety of cells, thus producing a variety of biological effects. The interaction of IL-6 with IL-6R mainly regulates the expression of target cell genes through two intracellular signal transduction pathways, JAK-STAT and Ras-MAPK, leading to corresponding biological effects. After IL-6 and IL-6R interaction, dimerized gpl30 activates nonreceptor tyrosine-protein kinase JAKs that bind to the inner cytoplasm, and activated JAKs catalyze the phosphorylation of the tyrosine residue on the cytoplasmic side of gpl30; phosphorylation of tyrosine residues at the distal end from the membrane can recruit STAT3 and phosphorylate it. Phosphorylated STAT3 forms a complex and then transfers to the nucleus to activate the transcriptional activity of the regulatory gene. This is the known JAK-STAT signal transduction pathway.

The C-fos oncogene and the JunB tumor suppressor are both targets of activated STAT. Phosphorylation of two tyrosine residues near the membrane end can recruit the adaptor protein SHP2 and activate the Ras-Raf-MAPK signaling pathway. SHP2 also plays a negative role in IL-6 signaling. As a tyrosine-protein phosphatase, SHP2 binds to SOCS3 of the IL-6 signaling pathway and attenuates IL-6 by inhibiting JAK and promoting protein degradation. As a multifunctional cytokine, IL-6 is a "double-edged sword". IL-6 not only regulates a variety of important physiological functions and participates in many pathological injuries but also plays an important role in the development of many diseases.

JAK/STAT, PI3K/Akt and Ras/Erk, the downstream pathways of IL-6/IL-6R/gp130, play important regulatory roles in autophagy. The regulation of IL-6 in autophagy is manifested by inhibition of starvation-induced autophagy activation of monocytes. IL-6 inhibits autophagy by downregulating the protein levels of LC3, Beclin1 and PI3KC3 and the phosphorylation of tyrosine at position 705 of STAT3. The inhibitory effect of IL-6 on autophagy was reversed by the inhibitor LLL12, which inhibits STAT3 phosphorylation. This study indicates that IL-6 inhibits starvation-induced autophagy mainly through the STAT3/Bcl2/Beclin1 signaling pathway. In addition, IFN-γ-induced autophagy activation is involved in mycobacterial infection of monocytes. IL-6 inhibits IFN-γ-induced autophagy activation by reducing the expression of the Atg12–Atg5 complex, which in turn activates the mTOR, p-38, and JNK signaling pathways. Studies have shown that IL-6 also activates autophagy through multiple mechanisms. IL-6 inhibits the activation of mTORC1 and Akt, thereby activating beta cells of the pancreas to resist apoptosis induced by pro-inflammatory cytokines. Moreover, IL-6 can activate STAT3 and the autophagy-related protein GABARAPL1 in human islet cells to protect cells. In summary, IL-6 participates in the maintenance of physiological functions of cells through the dual regulation of autophagy.

## *26.2.3 Th17 Cytokine Signaling and Autophagy*

### 26.2.3.1 Overview of Th17 Cytokines and Their Functions

Th17 cytokines are a subtype of cytokines secreted by specific helper T cells (T helper cell 17, Th17). Interleukin 17 has significant effects on autoimmune diseases and body defense responses. Transforming growth factor β (TGF-β), IL-6, IL-23, and IL-21 play an active role in the differentiation and formation of Th17 cells, while IFN-γ, IL-4, SOCS3, and IL-2 inhibit their differentiation.

By binding to its receptor, IL-17 can induce effector cells to secrete chemokines, colony-stimulating factors, etc., and promote the production and recruitment of neutrophils and macrophages. In addition, there is a complex inter regulatory network between Th17 cells and other $CD4^+$ T cell subsets. In normal organisms, Treg cells, which mediate immune tolerance, and Th1 cells, which mediate inflammatory responses, antagonize each other and maintain a balance. When the body is abnormal, the Th17/Treg function is unbalanced, causing a series of inflammatory immune responses which damage the body. Studies have reported that Th17 cells and their cytokines are associated with a variety of autoimmune diseases, such as systemic sclerosis, rheumatoid arthritis, systemic lupus erythematosus, chronic lymphocytic thyroiditis, and organ transplant rejection.

Systemic lupus erythematosus (SLE) is an autoimmune disease caused by immune cell dysfunction. The abnormal function of Th17 cells and Treg cells promotes the development of SLE, which is accompanied by abnormalities in autophagy. IL-17A, IL-17F, and IL-22 secreted by Th17 cells promote autoimmune responses and the formation of germinal centers, resulting in the secretion of a large number of pathogenic autoantibodies by B cells. Treg cells maintain immune homeostasis by releasing the immunosuppressive cytokines IL-10 and TGF-β and suppress abnormal immune responses in a cytokine-dependent manner. Studies have indicated that autophagy maintains T lymphocyte homeostasis and Treg-mediated autoimmune responses in a lysosomal-dependent manner. Abnormal autophagy is the key to Th17/Treg immune imbalance in the progression of SLE, and the autophagy inhibitor chloroquine (CQ) can inhibit the response of Th17 cells, promote the immune regulation of Treg cells, and reduce the secretion of pro-inflammatory cytokines and anti-dsDNA antibodies. Autophagy inhibitors have been applied to the clinical treatment of SLE.

### 26.2.3.2 IL-17A and Autophagy Regulation

The IL-17 family includes 6 members (A–F), of which IL-17A was first discovered and studied, and other members have high homology with IL-17A. Since its discovery, IL-17A has been extensively studied for its biological functions. Mesenchymal nuclear myeloid cells are the main target cells of IL-17A, and its main target genes include inflammatory cytokines, hematopoietic factors, chemokines, antimicrobial peptides, and tissue remodeling substances. The amplification and chemotaxis of

neutrophils by IL-17A is considered to be a characteristic effect. Studies have shown that IL-17A induces tissue inflammation mainly by stimulating pro-inflammatory cytokines including IL-6, TNF-α and G-CSF. IL-6 is the first identified IL-17A target gene, and this mechanism has also been used to identify the biological activity of IL-17A. IL-17A also induces the production of other pro-inflammatory cytokines, such as TNF-α and IL-1β, and increases prostaglandins and nitric oxide in various cell types by stimulating COX-2 and inducible nitric oxide synthase. Chemokines are also important target genes for IL-17A. CXC-like chemokines CXCL1, CXCL2, CXCL5, CXCL8, and CXCL10 may mediate the biological function of IL-17A by recruiting granulocytes.

IL-17A also promotes the expression of a variety of antimicrobial peptides, such as defensin and S100-proteins, which are natural antibiotics in the lungs, skin, and intestines. IL-17A induces epithelial cells to produce these antibiotics, which provides a broad spectrum of antimicrobial protection. IL-17A also induces the production of associated molecules in certain tissue remodeling. Matrix metalloproteinases (MMPs), including MMP1, MMP3, MMP9, and MMP13, are also target genes of IL-17A, which play an important role in extracellular matrix destruction, tissue damage and tumorigenesis. Recently, IL-17A has been found to regulate the biological functions of lymphocytes. IL-17A alone or in combination with B lymphocyte activators can promote the survival, proliferation, and differentiation of B lymphocytes. The process relies on the transcription factor Twist-1. On the other hand, T cells are not only the source cells of IL-17A but also the target cells of IL-17A. At the cellular and global levels, IL-17A inhibits TGF-β expression by directly acting on $CD4^+$ T cells, thereby modulating the polarization of Th0 cells toward Th1.

Numerous studies have shown that IL-17A is involved in the pathogenesis of various diseases, such as arthritis, tumors, chronic obstructive pulmonary disease, asthma, and autoimmune diseases. Studies have reported that autophagy plays an important role in IL-17A-related diseases. IL-17A is involved in the regulation of tissue fibrosis by activating the STAT3 signaling pathway to regulate the expression and activation of autophagy-related core complex components. IL-17A also participates in the inhibition of autophagy activity and the process of extracellular collagen degradation, which involves autophagy-related proteins. Blocking IL-17A in a variety of tissue fibrosis models can significantly restore damaged autophagy activity and improve the development of fibrosis. These studies indicate that IL-17A can participate in the development of tissue fibrosis by inhibiting autophagy activity. Activated autophagy and autophagy-associated cell death have important implications in lung tissue inflammation and fibrosis in acute lung injury. Therefore, the involvement of IL-17A in autophagy is reflected in the progression of pulmonary fibrosis. IL-17A can significantly inhibit starvation-induced epithelial autophagy activation, reduce the number of autophagosomes, downregulate the expression of autophagy core complexes Vps34 and Beclin1, and inhibit autophagy activation independent of TGF-β1 signaling (Liu et al. 2013; Mi et al. 2011).

Studies have shown that IL-17 promotes rheumatoid arthritis (RA) disease progression by affecting the proliferation of fibroblast-like synoviocytes (FLS) and regulating autophagy levels in a STAT3-related manner. IL-17 can improve autophagy

and proliferation of FLS cells, while the autophagy inhibitor 3-MA can inhibit autophagy and IL-17-mediated proliferation of FLS cells. Furthermore, inhibition of STAT3 activation reduces autophagy activation in FLS cells. IL-17A also regulates the level of autophagy in endometrial (EM) cells in chronic endometritis (CE) patients and affects embryo acceptance in transplants. The pathological process of chronic endometritis is accompanied by lymphocyte dysregulation and abnormal expression of cytokines, chemokines, and regulatory molecules. In addition, autophagy is essential for maintaining the survival and function of T lymphocytes. Women with CE have an abnormal expression of IL-10, TGF-β, and IL-17, and their level of autophagy is decreased. The pro-inflammatory response of the endometrium leads to a decreased pregnancy rate. Autophagy can regulate the local immune response to improve the success rate of endometrial implantation in patients.

### *26.2.4 IL-22 and Autophagy Regulation*

IL-22 is produced by active T cells (mainly Th17 and Th1 cells) and was previously named IL-10-associated T cell-derived inducible factor. IL-22 has 23% homology with IL-10 and is identified as an IL-10 family member. IL-22 exerts its biological function by binding to the IL-22 receptor, which is composed of two type II cytokine receptor chains: IL-22R1 and IL-10R2. Between them, IL-22R1 specifically binds with IL-22, and IL-10R2 is a shared chain between the IL-10 receptor complex and the IL-22 receptor complex. Upon binding to the receptor complex, IL-22 transduces activation signals primarily through the Jak/STAT pathway. Activation of Jakl and Tvk2 leads to phosphorylation of tyrosine residues on STAT1, STAT3, and STAT5. In addition, IL-22 also activates three major MAPK pathways: the MEK-ERK, JNK/SAPK, and p38 kinase pathways. As a novel cytokine, IL-22 participates in multiple signaling pathways in cells and plays various biological functions, such as inflammatory response, activation of the innate immune system, cell proliferation, cell differentiation, and gene expression. IL-22 also affects the occurrence and development of diseases such as atherosclerosis, sclerotherapy, and tumors.

IL-22 affecting the progression of pancreatitis by regulating autophagy has been reported. Several studies have suggested that IL-22 has a certain therapeutic effect on acute/chronic pancreatitis. Compared with wild-type mice, IL-22 knockout mice have a significantly increased risk of pancreatitis, while liver-specific IL-22 transgenic mice have a very low incidence of pancreatitis. Further studies suggest that IL-22 mediates the protective effect in pancreatitis by inhibiting autophagy activity, and IL-22 increases anti-apoptosis-related proteins Bcl-2/Bcl-$X_L$ expression, increases the interaction of Bcl-2/Bcl-$X_L$ and Beclin, inhibits the activity of Beclin, and blocks the formation of autophagosomes to inhibit autophagy activity (Feng et al. 2012).

Acute or chronic liver failure is the leading cause of death from liver disease, and IL-22 may play a therapeutic role in severe alcoholic hepatitis. Studies have shown that IL-22 upregulates autophagy to protect acetaminophen (APAP)-induced liver damage in an AMPK-dependent manner. In an in vivo APAP-induced acute liver

injury model, IL-22 significantly reduced the production of hepatic reactive oxygen species induced by APAP and inhibited hepatic necrosis. Further studies have found that IL-22 can increase the production of autophagosomes, upregulate the phosphorylation of LC3-II and AMPK in mice, and attenuate APAP-induced cytotoxicity. When AMPK inhibitors were added to block the AMPK signaling pathway, IL-22-mediated autophagy activity was reduced, and the protective effect on hepatocytes was diminished.

However, IL-22 plays a role in promoting the progression of intestinal fibrosis. Intestinal fibrosis is a chronic disease caused by hyperproliferation of myofibroblasts and collagen deposition. Studies have shown that the induction of monocyte autophagy can inhibit IL-23/IL-22-mediated intestinal fibrosis. Knocking out the mTOR gene or using the mTOR inhibitor rapamycin can reduce the production of IL-23 and IL-22 in the 2,4,6-trinitrobenzene sulfonic acid-induced fibrosis model and reduce liver fibrosis, which is closely related to the increase in autophagy activity. Knockout of autophagy-associated protein ATG-7 can inhibit autophagy and lead to increased expression of IL-22 and IL-23, thus aggravating the progression of fibrosis. IL-22 also promotes the conversion of fibroblasts to myofibroblasts, and neutralizing IL-22 with antibodies can reduce the fibrotic response. These findings suggest that IL-23 and IL-22 may be new targets for the treatment of intestinal fibrosis.

## *26.2.5 Treg Cytokines and Autophagy*

### 26.2.5.1 Overview of Treg Cytokines and Their Functions

Treg cells are a subset of $CD4^+$ T cells with immunosuppressive activity. Treg cells inhibit the function of T cells and antigen-presenting cells by releasing the cytokines IL-10 and TGF-β and reduce the production of inflammatory cytokines and antibody secretion to exert immunosuppressive effects. FOXP3 is an important transcription factor in Treg cells, and its sustained expression is a key factor in maintaining the inhibitory activity of Treg cells. Tregs exert immunomodulatory effects by upregulating the expression of inhibitory immune cell surface molecules and downregulating the genes involved in activated T cells. The naive $CD4^+$ T cells are activated by the costimulatory signal after stimulation by a foreign antigen and differentiate into different subtypes of effector T cells. Naive $CD4^+$ T cells can differentiate into Treg cells under the induction of TGF-β alone. Treg cells can effectively control autoimmune diseases and maintain autoimmune tolerance. The number and functional changes of Treg cells are closely related to tumors, infections, autoimmune diseases, and so on.

#### 26.2.5.2 IL-10 and Autophagy Regulation

IL-10 is a cytokine that occurs after the production of pro-inflammatory mediators and has an inhibitory effect on a variety of immune cell functions. This cytokine plays an important role in limiting and preventing excessive immune responses and the resulting damage. At the same time, it enhances the function of the immune system "scavengers" and helps induce peripheral tolerance in the presence of antigens. Numerous studies have shown that IL-10 plays an important role in the pathogenesis of various diseases. Development of lupus erythematosus, EB virus-associated lymphoma, and melanoma are associated with overexpression of IL-10. In addition, in the early stage of viral infection, elevated IL-10 can enhance the activity of NK cells and have a positive effect on viral clearance. However, the immunosuppressive effects of IL-10 cause the persistence of the virus and worsen the condition. IL-10 also plays a decisive role in the establishment of immune paralysis. For example, after trauma, major surgery, burns or shock, IL-10 overexpression can cause bacterial infection and can even cause sepsis in severe cases.

IL-10 mainly utilizes the Janus kinase family members and STAT transcription factors to exert its inhibitory effects. IL-10 binding to its receptor stimulates two members of the Janus kinase family, Jakl and Tyk2, to phosphorylate two tyrosine residues (Tyr446 and Tyr496) on IL-10R1, and the transcription factor STAT3 binds to IL10R1 through its SH2 domain and is phosphorylated; STAT3 then transfers into the nucleus and binds to different promoter binding elements, inducing transcription of the corresponding gene. In addition, intracellular STAT1 and STAT5 can also be activated in certain cells after stimulation with IL-10. IL-10 also induces the production of SOCS3, which binds to Janus kinase and inhibits the activity of Janus kinase. The role of SOCS3 is related to the inhibitory effect of terminating IL-10.

Autophagy activation is involved in the early infection process of human cytomegalovirus (HCMV). As an important cytokine that regulates the immune response, IL-10 inhibits viral replication by inhibiting HCMV-induced autophagy activation (Wang et al. 2014). IL-10 inhibits autophagy and activates an antiviral response by activating the PI3K/Akt signaling pathway. The use of the autophagy activator rapamycin or overexpression of Beclin 1 reverses the effect of IL-10 on autophagy. The IL-10/IL-10R signaling pathway also activates the phosphorylation of p70S6K by activating the type I PI3K/AKT signaling pathway, which activates mTORC1 and inhibits starvation-induced autophagy activation in macrophages.

#### 26.2.5.3 TGF-β and Autophagy Regulation

Transforming growth factor β (TGF-β) is a type of multifunctional cytokine that affects cell proliferation and differentiation and plays an important role in embryonic development, extracellular matrix formation, bone formation, and reconstruction. TGF-β is widely present in various tissues of different organisms and functions by binding to receptors of two different types (type I and type II), of which type I is a serine (Ser) kinase receptor, and type II is a threonine (Thr) kinase type receptor.

After the binding of TGF-β to the TGF-β type II receptor (TGF-βRII), the TGF-β type I receptor (TGF-βRI) is recruited to create a dimeric form of the receptor complex. TGF-βRII then phosphorylates the glycine-serine-rich region (GS sequence) of TGF-βRI and activates the serine/threonine activity of TGF-βRI. Activated TGF-βRI, in turn, phosphorylates Smad proteins. The phosphorylation-dependent shuttling of Smad between the cytoplasm and nucleus is important for the dynamic regulation of TGF-β signaling. In addition, non-Smad-dependent signaling pathways are also involved in the activation of TGF-β downstream signaling. For example, c-Jun N-terminal kinase (JNK) is a member of the MAPK kinase family and is involved in TGF-β-promoting collagen generation.

TGF-β is involved in the regulation of renal fibrosis as a multifunctional cytokine. A large number of studies have shown that TGF-β participates in the development of renal fibrosis by activating autophagy. TGF-β can increase the expression of the autophagy-associated protein LC3 and the number of autophagosomes in a time-dependent/dose-dependent manner and subsequently upregulate the expression of autophagy-related genes *Atg5*, *Atg7,* and *BECN1*. In addition, TGF-β can increase the amount of reactive oxygen species (ROS) and NADPH oxidase in renal epithelial cells and induce autophagy-related death, and the autophagy inhibitor 3-MA can inhibit the pro-apoptotic effect of TGF-β (Ding and Choi 2014).

TGF-β participates in the progression of liver cancer by activating autophagy in liver cancer cells. For example, TGF-β increases the number of autophagosomes in liver cancer cells and increases the rate of degradation of a series of long-acting proteins. TGF-β can also upregulate the mRNA levels of Beclin1, ATG5, ATG7, and death-associated protein kinase (DAPK). Knockdown of the TGF-β downstream signaling pathway Smad2/3/4 or inhibition of JNK kinase activity can inhibit TGF-β-induced autophagy activation, suggesting that TGF-β-induced autophagy activation can occur through either the Smad signaling pathway or through the non-Smad signaling pathway. TGF-β is not only involved in the regulation of autophagy signaling, but autophagy signaling pathways can also influence TGF-β-related biological functions; i.e., inhibition of autophagy-related gene expression can inhibit TGF-β-mediated growth inhibition and apoptosis promotion.

### *26.2.6 Other Cytokine Signaling and Autophagy Regulation*

#### 26.2.6.1 IL-1β and Autophagy

IL-1β is a member of the IL-1 cytokine family and is produced by activated macrophages. IL-1β binds to the IL-1 receptor (IL-1R) to produce a biological effect. IL-1β is a very important pro-inflammatory cytokine and plays a major regulatory role in the inflammatory immune injury. IL-1β is also involved in a variety of cellular biological activities, including cell proliferation, differentiation, and apoptosis. IL-1β can participate in the regulation of inflammation and infectious diseases by activating autophagy. For example, IL-1β activates autophagy by affecting intracellular

calcium balance and trypsinogen activity in pancreatic acinar cells. IL-1β expression is upregulated during acute pancreatitis, and IL-1β upregulates endoplasmic reticulum stress markers. The expression of glucose-regulated protein (GRP78) and inositol-requiring enzyme (IRE1) induces the release of calcium ions, promotes the conversion of trypsinogen to trypsin, and activates autophagy to reduce pancreatic acinar cells.

#### 26.2.6.2 IL-27 and Autophagy Regulation

IL-27 is an immunoregulatory cytokine that is primarily produced by APCs in response to stimulation with microbial products or inflammatory mediators. Tregs, IFN-γ, and statins can also induce IL-27 production by APCs. The binding of IL-27 to its receptor (IL-27R) induces STAT1 and STAT3 activation and has dual roles of pro-inflammatory and anti-inflammatory immune regulation. Studies have shown that IL-27 downregulates IFN-induced autophagosome production and phagosome maturation in *M. tuberculosis* H37Rv-infected macrophages and inhibits nutrient-deficient autophagy in these cells. As a result, IL-27 effectively promotes the survival of intracellular mycobacteria. The effect of IL-27 on lysosomal degradation during this process remains unclear. Given the activation of STAT1 and STAT3 by IL-27, the potential role of these signaling proteins in the regulation of autophagy by IL-27 requires further investigation.

#### 26.2.6.3 PD1/PD-L1 and Autophagy Regulation

Many tumor cells and tumor-infiltrating immune cells express high levels of PD-L1 to escape immune surveillance. Under normal conditions, the PD-L1 signaling pathway plays an important role in maintaining immune homeostasis. In tumors, PD-L1 can protect tumor cells from cytotoxic T cells in two ways. The first is that in lymph nodes, PD-L1-overexpressing tumor-infiltrating immune cells can prevent the activation of cytotoxic T cells and subsequently recruit them into tumors. The second is that upregulation of PD-L1 on dendritic cells results in inactivation of cytotoxic T cells in the tumor microenvironment. In both cases, the interaction of PD-L1 with PD1 inhibits T cell function, leads to T cell tolerance, inhibits T cell proliferation, reduces cytokine production, and blocks tumor cell recognition. In addition to its immunosuppressive effects, the PD1/PD-L1 signaling pathway is thought to play a key role in intrinsic tumor function and survival. Recent studies have shown that murine melanoma cells and human ovarian cancer cells that highly express the PD-L1 receptor are more sensitive to autophagy inhibitors than those that weakly express PD-L1. Although autophagy inhibitors in combination with anti-PD-L1 drugs enhance the antitumor activity, autophagy is only one component of a complex network that affects immune system-mediated tumor cell death, and further research is needed to demonstrate whether autophagy blockade combined with PD-L1 inhibitors can be a new approach to tumor therapy.

## 26.3 T Cell Receptor Signaling, B Cell Receptor Signaling and Autophagy Regulation

The T cell receptor (TCR) and the B cell receptor (BCR) are antigen-specific receptors located on the surface of T lymphocytes and B lymphocytes, respectively. The TCR is a specific receptor for T cell recognition of protein antigens, and the molecular structure of the antigen recognition receptors on different T cell clones is different. The TCR molecule on most mature T cells (approximately 95%) is a TCRαβ molecule composed of two α and β heterodimeric peptide chains. Under normal circumstances, the heterologous protein antigen molecule must bind to the self-MHC molecule on the cell surface to be recognized by the TCR. Like the TCR, the BCR is a complex composed of molecules that specifically recognize antigens and signaling molecules. The BCR can directly recognize free antigen molecules, and the recognized epitope is the conformational determinant of the antigen. TCRs and BCRs regulate the development, survival, differentiation, and function of T cells or B cells by binding antigens.

The important role of autophagy in the immune system is undoubted. For lymphocytes, autophagy regulates the homeostasis of naive T lymphocytes, especially in the regulation of mitochondrial mass and renewal processes. In addition, autophagy is also important for the proliferation of mature T lymphocytes. Autophagy is also an important way to regulate lymphocyte death. Long-term cytokine withdrawal and acute antigen receptor stimulation (such as HIV infection) can lead to high activation of autophagy, resulting in a large number of T cell deaths. Autophagy is also involved in the regulation of positive and negative selection of developmental thymocytes. For B cells, autophagy promotes B lymphocyte survival and B lymphocyte precursor development. Stimulation of B cell antigen receptors induces strong autophagic cell death in the absence of costimulatory molecules. In summary, autophagy, as an important cellular process, is involved in the regulation of lymphocyte development, differentiation, and function, which has been covered in other chapters. In this section, we will focus on the regulation of autophagy by TCR and BCR signaling activation.

### *26.3.1 Autophagy Induction in T Cells Participates in the Regulation of T Cell Proliferation and Survival*

The number of T cells is associated with T cell proliferative capacity and the regulation of death. The occurrence of T cell death is tightly regulated by two pathways: activation-induced cell death (AICD) and the spontaneous death of activated T cells. AICD is mainly mediated by Fas ligand and TNF-triggered death signals and is an important mechanism for preventing excessive activation of T cells. In the absence of survival signals, activated T cells undergo spontaneous death, a process that is independent of external death signals and is primarily mediated by pro-apoptotic

members of the Bcl-2 family. Autophagy regulated by TCR signaling is involved in the regulation of T cell proliferation and survival.

#### 26.3.1.1 Autophagy Induction in CD4$^+$ T Cells

The number of T cell subsets is strictly regulated. When T cells are stimulated by specific antigens, the number of T cells is greatly expanded; when the antigen is cleared, the number of T cells gradually decreases. Both apoptosis and autophagy-associated cell death are involved in the regulation of this process. In resting naive CD4$^+$ T cells isolated from mice, the presence of autophagosomes was not detected, suggesting low autophagy activity. The anti-CD3 antibody specifically recognizes the CD3 molecule on the surface of the T cell and activates and induces proliferation of the T cell by binding the TCR-CD3 complex on the surface of the T cell to the MHC class II molecule-antigen peptide on the surface of the APC. However, during full T cell activation, T lymphocytes require not only the first signal of the T lymphocyte receptor (TCR)/CD3 complex but also the second signal to enhance the activation and proliferation of T lymphocytes. The interaction of the costimulatory molecule CD28 on the surface of T lymphocytes with its ligand B7 is one of the main ways to provide a second signal for T lymphocyte activation. T lymphocytes can be fully activated by in vitro stimulation with anti-CD3 antibody to provide a first signal, while costimulation with anti-CD28 antibody provides a second signal. When treating resting T cells with CD3 and CD28 antibody stimulation and culturing under Th2 polarization conditions, the presence of autophagosomes and LC3 punctate aggregation were observed in 20% of cells, suggesting that TCR signal activation can induce autophagy activation. Both rapamycin and the caspase inhibitor zVAD stimulation of T cells enhance autophagy activity. In addition, type III PI3K inhibitors, JNK inhibitors or knockout of JNK can inhibit T cell autophagy activation, suggesting that the induction and maintenance of autophagy in T cells require the participation of JNK and type III PI3K signaling pathways. Activation of CD4$^+$ T cells upregulates a range of autophagy signaling proteins to activate autophagy. For example, mouse primary CD4$^+$ T cell activation can upregulate Beclin 1 protein expression by NK-κB activation of transcription of the Beclin 1 promoter. Reactivation of effector CD4$^+$ T cells after activation or differentiation of naive CD4$^+$ T cells upregulates LC3 protein levels.

Autophagy activated by T cell signaling activation is critical for maintaining CD4$^+$ T cell homeostasis, proliferation, differentiation, and function. The inhibition of timely removal of mitochondria and organelles caused by autophagy defects in T cells and the accumulation of reactive oxygen species in the cells caused chronic damage, which eventually led to the decrease in thymocytes, the extreme proportion change in CD4$^+$ and CD8$^+$ T cell populations in peripheral lymphoid organs, and the decrease in cell viability and increased apoptosis of CD4$^+$ T cells. Autophagy participates in maintaining CD4$^+$ T cell homeostasis and peripheral organ survival, and TCR-induced autophagy can also promote TCR signaling-driven CD4$^+$ T cell proliferation. T cell proliferation was monitored by carboxyfluorescein N(5)-carboxyfluorescein

N-hydroxysuccinimidyl ester (CFSE). The proliferation of $CD4^+$ T cells that had Atg5 deficiency was significantly impaired after in vitro TCR activation with anti-CD3 antibody, anti-CD28 antibody, and IL-2. However, autophagy inhibition did not affect TCR-driven $CD4^+$ T cell activation. Autophagy activated by T cell signaling activation is in fact associated with energy metabolism requirements that satisfy T cell activation. In Th1 cells, inhibition of autophagy, whether by pharmacology or genetics, impairs the production of ATP, interferon-gamma (IFN-γ), and IL-2 after TCR activation. These defects in the function of autophagy-deficient Th1 cells can be reversed by the addition of methyl pyruvate, resulting in the recovery of the electron transport chain and oxidative phosphorylation activity.

Autophagy is critical for maintaining the functional integrity of inhibitory $CD4^+$ $Foxp3^+$ Treg cells. Specific deletion of Atg7 in $CD4^+$ $Foxp3^+$ Treg cells resulted in a decrease in the proportion of $Foxp3^+$ $CD4^+$ T cells in peripheral organs and increased staining of caspase-3, indicating that autophagy defects impaired the viability of Treg cells. However, Atg7-deficient Treg cells were cultured in vitro with anti-CD3 antibody, anti-CD28 antibody, and IL-2 and transplanted into $Rag1^{-/-}$ mice, where they proliferated normally. These phenomena suggest that Atg7 is critical for Treg cell survival but is not an essential condition for TCR-induced Treg cell proliferation. Treg cells lacking Atg7 showed decreased expression of Foxp3, Foxo, and Bach2 and enrichment of the effector T cell differentiation pathway, and inhibition of mTORC1 by rapamycin restored Foxp3, Foxo, and Bach2 expression. This observation suggests that autophagy is involved in maintaining the negative regulatory effect of Tregs.

Peripheral tolerance mechanisms exist in the organism to control T cells that express autoreactive T cell receptors (TCRs) but escape thymic deletion. Among them, T cell incompetence is very important for the prevention of autoimmune reactions and participates in the production of peripheral Treg cells. Autophagy is involved in the maintenance of T cell tolerance-evasion mechanisms and determines the fate of $CD4^+$ T cells (Mocholi et al. 2018). Inhibition of autophagy during $CD4^+$ T cell activation induces a sustained T cell hyporesponsive state with unresponsive characteristic gene expression. Cells that are unable to induce autophagy after TCR engagement have reduced mitochondrial respiration and protein tyrosine phosphatase PTPN1 turnover, resulting in abnormal TCR-mediated signaling. This finding also means that inhibition of autophagy may help restore the tolerance characteristics of autoimmune T cells, thereby inhibiting the autoimmune response.

#### 26.3.1.2 Autophagy Induction in $CD8^+$ T Cells

In $CD8^+$ T cells, autophagy is also involved in the regulation of T cell survival. In $CD8^+$ $CD28^+$ T cells, TCR-mediated activation produces strong autophagy induction. In contrast, TCR activation in $CD8^+$ $CD28^-$ T cells induced only low levels of autophagy activation. Autophagy induced by TCR activation is important for $CD8^+$ T cells to form memory T cells (Xu et al. 2014). Studies in a viral infection model have shown that clonal expansion of naive $CD8^+$ T cells in the acute phase of viral infection is not dependent on autophagy. After pathogen infection, antigen-specific

T cells rapidly proliferate to produce an effector cell population; after the infection is cleared, only a small number of T cell populations with long-lasting memory remain to respond quickly to a secondary infection. Autophagy is crucial for the formation of memory $CD8^+$ T cells, and autophagy may play a regulatory role in this process mainly by regulating T cell energy metabolism. A mouse model of CD4 promoter-specific knockout of the autophagy gene Atg7 showed that the effector type of $CD8^+$ T cells can perform normally in the absence of autophagy. However, during the reduction phase of effector $CD8^+$ cells, the population of $Atg7^{-/-}$ $CD8^+$ T effector cells ($CD8^+$ Teff) experienced a catastrophic collapse, resulting in the inability to form a memory $CD8^+$ T cell population. $Atg7^{-/-}$ $CD8^+$ T effector cells did not show significant abnormalities in transcriptional and cell surface marker phenotypes. However, these cells had a high mitochondrial load and increased mitochondrial reactive oxygen species in the cells compared to wild-type antigen-specific $CD8^+$ T cells. As cells began to turn to mitochondrial respiration, $CD8^+$ T cells underwent mitochondrial biogenesis during the effector phase, an important event in the formation of memory $CD8^+$ T cells. Prior to memory formation, high mitochondrial loads associated with oxidative phosphorylation initiation can lead to the escape of electrons from the electron transport chain, which can interact with molecular oxygen, resulting in the production of superoxide. Therefore, mitochondria may need to be tightly regulated during the late effector phase of $CD8^+$ T cell responses. Upon activation of $CD8^+$ T cells, cellular metabolism is converted to glycolysis to support cell proliferation. Subsequent to the reduction in the effector cell population, memory precursor cells turn to oxidative phosphorylation and fatty acid oxidation, a change that promotes memory $CD8^+$ T cell differentiation. In wild-type $CD8^+$ T cells, GLUT-1 is upregulated after activation to support glycolysis, and late GLUT-1 is downregulated in $CD8^+$ Teffs. Autophagy-deficient $CD8^+$ Teffs showed higher GLUT-1 expression at the peak of the effector phase, and no GLUT-1 downregulation occurred in the late effector phase compared with that in wild-type mice. Glucose uptake was also significantly increased in early and late $CD8^+$ Teff cells in $Atg7^{-/-}$ mice relative to wild-type controls. These results imply that $Atg7^{-/-}$ $CD8^+$ Teff cells continue to maintain glycolysis and fail to shift to mitochondrial respiration. In addition, autophagy is also a necessary step for the delivery of lipids for lysosomal decomposition, which in turn provides fatty acid metabolism for fatty acid oxidation. In general, autophagy helps antigen-specific $CD8^+$ T cells resist oxidative damage and helps T cells maintain the necessary energy conversion to prevent T cell apoptosis.

There is a close relationship between autophagy and aging. Autophagy activity decreases with age. In this regard, the immune system is similar to other organs in the organism. During aging, the T cell response is reduced. For example, both aged mice and humans show increased susceptibility to disease. When mice were treated with the autophagy-inducing compound spermidine, the response of the aged mice to influenza vaccination and infected $CD8^+$ T cells was significantly improved. This effect is lost in elderly T-$Atg7^{-/-}$ mice. This suggests that the decrease in autophagy is an inherent driving force behind the cellular immune aging of T cells. As the world's population ages, it is important to improve the immunity and vaccine efficacy of

elderly people. Autophagy pathways targeting compounds such as spermidine may provide a good opportunity to achieve this goal.

#### 26.3.1.3 Arginine-Regulated TCR Signaling and Autophagy Induction

Arginine deficiency leads to T cell proliferation disorders and dysfunction. In cultured Jurkat cells and human peripheral blood mitogen-activated T cells, arginine deprivation inhibits the expression of a variety of membrane antigen molecules, including the TCR receptor complex signaling mediator CD247, and induces the endoplasmic reticulum stress response and activation of autophagy but does not lead to apoptosis. Arginine deprivation can induce the phosphorylation of eIF2, JNK, and Bcl-2 and reduce the binding of Beclin 1 to Bcl-2, thereby promoting autophagosome formation. Under conditions of deprivation of arginine, knockdown of the endoplasmic reticulum stress signal molecule IRE1 can inhibit autophagy activation and lead to T lymphocyte apoptosis. These studies suggest that autophagy activity is beneficial for maintaining T cell survival in the absence of arginine. Rescue of arginine in a cell culture environment restores T cell proliferation and inhibits autophagy. These results suggest that the signal activation of the TCR plays an inhibitory effect on autophagy. This finding also suggests that TCR signal activation may have different regulatory effects on autophagy for different T cell subsets under different environmental conditions.

### *26.3.2 Autophagy Connects BCR Signals with Costimulatory Signals*

In B cells, antigen stimulation induces apoptosis by activation of BCR signaling. Costimulatory factors (such as CD40L and TLR ligands) inhibit B cell apoptosis and induce self-activation and proliferation. Activation of BCR signaling induces the formation of a large number of autophagosomes in B cells. In addition, autophagy plays an important role in antigen processing, presentation, MHC II coexpression, and T cell recognition by B cells and other antigen-presenting cells (e.g., dendritic cells). The B cell antigen presentation process is involved in the interaction of B cells with CD40L-positive T helper cells, BCR ligandization, and receptor internalization. The interaction between the autophagosome and the costimulatory factor receptor TLR9 is involved in the process of TLR9 binding to its nucleic acid ligand; therefore, autophagy may act as a bridge to connect BCR signals and costimulatory signals.

#### 26.3.2.1 The BCR Signaling Pathway Induces Autophagy Activation

The BCR signaling pathway plays an important role in B cell apoptosis, activation, and induction of autophagosome formation. BCR ligandization (the ligand antigen or antibody forming a cross-linking reaction with BCR) induces the formation of LC3 punctate and autophagic small body-like structures in B cells, and the formation of excessive autophagosomes eventually induces apoptosis. Although BCR ligand-induced apoptosis occurs only in certain B lymphocyte lineages and antigen-mediated BCR ligandization induces apoptosis in primary B lymphocytes, autophagosome formation is a key step in the induction of apoptosis. Activation of the CD40 costimulatory signaling pathway reverses autophagic apoptosis induced by BCR ligandization. A variety of external stimuli induced autophagy and apoptosis in B cells, while inhibition of apoptosis (via caspase inhibitor or overexpression of Bcl-2) did not affect autophagy, indicating that activation of autophagy is not a necessary step to induce apoptosis. BCR ligand induces autophagy activation in the early stage and induces B cell apoptosis in the late stage. This kind of apoptosis is different from classic apoptosis, suggesting that autophagy-induced apoptosis may be a new apoptotic signaling pathway.

The relationship between the BCR signaling pathway and autophagy has been reported in tumors. Melanoma is a malignant tumor of skin melanocytes characterized by high malignancy, rapid progression, and high mortality, and its pathogenesis is inconclusive. The expression of Lyn kinase, an important protein in the BCR signaling pathway, is upregulated in melanoma. Knockdown of Lyn leads to downregulation of P62, upregulation of caspase 3 and LC3-II/I, and induction of melanoma cell apoptosis and autophagy, inhibiting melanoma cell proliferation, migration, and invasion. In addition, drug-resistant recurrence of lung cancer and lung infection are also related to autophagy induced by the BCR signaling pathway. Lyn and Src are overexpressed and activated in small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC) cells, and autophagy-induced apoptosis is also inhibited when dasatinib is used to inhibit BCR signaling. During lung infection, Lyn is an essential protein that induces autophagy to clear pathogens after gram-negative infection. Lyn can mediate alveolar macrophage phagocytosis and autophagosome maturation. When lysosomes encapsulate bacteria, TLR2 initiates phagocytosis to activate Lyn, promoting early phagosome formation, autophagosome maturation, and autophagy-mediated bacterial degradation. When the autophagy inhibitor 3-MA was used to block autophagy, the phagocytic ability of macrophages was weakened, and bacterial clearance was decreased, suggesting that the BCR signaling pathway is involved in the process of tumor and infection by regulating autophagy.

#### 26.3.2.2 B Cell Antigen Presentation and Autophagy

Autophagy plays an important role in B cell antigen presentation. The expression of MHC I and MHC II molecules on the surface of B cells is involved in B cell antigen presentation. T helper cells specifically recognize antigenic peptides presented by

MHC II molecules, and antigenic peptides presented by MHC II molecules on the surface of B cells play a key role in the interaction between B cells and T helper cells. Autophagy participates in starvation-induced B cell presentation of antigens. Under starvation-induced conditions, intracellular antigens are efficiently presented to MHC II molecules on the surface of B cells, suggesting that intracellular antigens are processed through autophagy in vivo, which is more favorable for antigen presentation. After binding to BCRs, B cell antigens are internalized, processed, and loaded on the surface of MHC II molecules and presented to T helper cells, which is the most effective path for B cell antigen presentation. B cells are maximally activated by BCR-mediated antigen processing and T helper cell interactions. Although antigen processing and loading occur in the MHC II compartment, the fusion of the MHC II compartment with autophagosomes further facilitates antigen processing. Recent studies have shown that BCR ligands are first translocated to the MHC II compartment and then translocated to autophagosomes via fusion of the MHC II compartment with autophagosomes. Thus, autophagy plays an important role in antigen processing and presentation via BCR internalization.

#### 26.3.2.3 Autophagy Is Linked to BCR Signaling Pathways and Costimulatory Factors

CD40L (a T cell–B cell activating molecule and tumor necrosis factor-related protein) is a costimulatory signal derived from T cells. CD40L inhibits B cell apoptosis by antigen stimulation and induces B cell activation and proliferation. BCR-mediated antigen processing is essential for the interaction between B cells and CD40L-positive T helper cells, which forms a costimulatory signal with CD40 on the surface of B cells that occurs later than BCR-mediated antigen treatment. The BCR transmits apoptotic signals prior to the binding of B cells to CD40L, and the apoptotic signal induces the formation of autophagosomes. Activation of autophagy induced by BCR signaling increases antigen processing by B cells and enhances the interaction between B cells and CD40L-expressing T cells. In addition, autophagy linked the costimulatory factor receptor TLR9 to the BCR signal. As a receptor for recognizing nucleic acid antigens, TLR9 binds to antigens and then translocates to autophagosomes, while BCR ligands internalize and translocate to autophagosomes to form interactions with TLR9 and participate in antigen processing and presentation. In summary, autophagy acts as a bridge to connect the BCR signaling pathway and the costimulatory factor signaling pathway.

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# Chapter 27
# Autophagy and the Immune Response


**Bing Cui, Heng Lin, Jinmei Yu, Jiaojiao Yu and Zhuowei Hu**

**Abstract** Innate immunity and adaptive immunity play critical roles in maintaining normal physiological functions and the development of diseases. In innate immune responses, heterogeneous autophagy can directly remove intracellular pathogens while activating PRRs, including TLRs and NLRs, to trigger their signal transduction pathways and promote NKT cell activation, cytokine secretion, and phagocytosis. In adaptive immune responses, the autophagy reaction has an important effect on the homeostasis, function, and differentiation of T lymphocytes, the survival, and development of B lymphocytes and the survival of plasma cells. This review highlights the key role that autophagy plays in the innate immune system and the acquired immune system. Further clarifying the mechanism by which autophagy regulates the immune system is essential for elucidating the precise mechanisms of various diseases and for developing new treatment methods.

**Keywords** Autophagy · Innate immunity · Adaptive immunity

## Abbreviation

| | |
|---|---|
| GAS | Group A streptococcus |
| GcAVs | GAS-containing autophagosome-like vacuoles |
| IRGs | Immunity-related p47 GTPases |
| SLRs | Sequestosome 1-like receptors |
| TLRs | Toll-like receptors |
| NLRs | NOD-like receptors |
| RLRs | RIG-I like receptors |

B. Cui and H. Lin contributed equally to this work.

B. Cui · H. Lin · J. Yu · J. Yu · Z. Hu (✉)
State Key Laboratory of Bioactive Substance and Function of Natural Medicines, Institute of Materia Medica, Chinese Academy of Medical Sciences & Peking Union Medical College, Beijing 100050, People's Republic of China
e-mail: huzhuowei@imm.ac.cn



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|---|---|
| MAMPs | Microbe-associated molecular patterns |
| DAMPs | Damage-associated molecular pattern molecules |
| OPTN | Optineurin |
| NDP52 | Nuclear domain 10 protein 52 |
| GABARAP | GABA A receptor-associated protein |
| 3-MA | 3-methyladenine |
| SCVs | Salmonella-containing vacuoles |
| T3SS | Type III Secretion System |
| TBK1 | TANK binding kinase 1 |
| LIR motif | LC3 interacting motif |
| DAG | Diacylglycerol |
| Tecpr1 | Tachylectin-II-like beta-propeller domain |
| AIEC | Adherent-invasive *E. coli* |
| LRSAM1 | Leucine-Rich Repeat and Sterile α Motif-Containing-1 |
| LAP | LC3-Associated Phagocytosis |
| TIMD4 | T-cell immunoglobulin and mucin domain-containing 4 |
| CLEC7A | C-type lectin domain family 7 member A, Dectin 1 |
| NETs | Neutrophil extracellular traps |
| NOX2 | NADPH oxidase 2 |
| WIPI2 | WD Repeat Domain Phosphoinositide-Interacting Protein 2 |
| IRGM | Immunity-related GTPases |
| IFN-γ | Interferon gamma |
| HSV-1 | Herpes simplex virus 1 |
| KSHV | Kaposi's sarcoma-associated herpesvirus |
| ActA | Actin assembly-inducing protein |
| ARP2/3 | Actin-Related Proteins 2/3 |
| InlK | Internalin K |
| MVP | Major Vault Protein |
| SLAP | Spacious Listeria-Containing Phagosome |
| LLO | Listeriolysin O |
| GAPR1 | Golgi-associated plant pathogenesis-related protein 1 |
| FLIP | FLICE-like inhibitory protein |
| MHC | Major Histocompatibility Complex |
| AGO | Argonaute |
| eIF2α | Eukaryotic Translation Initiation Factor 2α |
| IRESs | Internal Ribosome Entry Sites |
| mTORC1 | Mammalian Target of Rapamycin Complex 1 |
| eIF4EBP1 | Eukaryotic Translation Initiation Factor 4E binding protein 1 |
| $p70^{S6K}$ | Ribosomal Protein S6 kinase 70 kDa polypeptide 1 |
| miRNA | MicroRNAs |
| DRiPs | Defective Ribosomal Initiation Products |
| ALISs | Aggresome-Like-Induced Structures |
| TAP | Transporter-Associated with Antigen Processing |
| LPC | Lysophosphatidylcholine |

| | |
|---|---|
| CRT | Calreticulin |
| PS | Phosphatidylserine |
| HSPs | Heat-Shock Proteins |
| HMGB1 | High-Mobility Group Box 1 |
| CLEC9AL | C-Type Lectin Domain Family 9 A Ligand |
| MIICs | MHC class II Compartments |
| OVA | Ovalbumin |
| UBA | The ubiquitin-associated domain |
| pDCs | Plasmacytoid Dendritic Cells |
| IRF7 | Interferon regulatory factor 7 |
| STK11, or LKB1 | Serine Threonine Kinase 11, or Liver Kinase B1 |
| AMPK | AMP-Activated Protein Kinase |
| DALISs | DC Aggresome-Like Lipopolysaccharide-Induced Structures |
| HSCs | Hematopoietic stem cells |
| iNKT cells | Invariant Natural Killer T cells |
| Vps34 | Vacuolar protein sorting 34 |
| CFLIP, or CFLAR | Cellular FLICE-like inhibitory protein |
| TCR | T cell receptor |

With a more profound understanding of the molecular mechanisms of cellular autophagy and the immune system, the close relationship between the autophagy system and the immune system has been gradually revealed (Deretic et al. 2013; Jiang et al. 2019). In mammalian cells, autophagy involves multiple steps of innate and adaptive immune responses. Autophagy provides a primitive innate immune form of defense for eukaryotic cells against invading microorganisms. With the help of autophagy adaptor proteins, autophagy triggers a series of immune responses through pattern recognition receptors (PRRs). These steps constitute an essential process for eliminating intracellular microorganisms. Autophagy interferes with immune mediators such as cytokines by eliminating the endogenous inflammasome, thereby regulating innate immune signaling pathways (Levine and Kroemer 2019). Moreover, autophagy is also involved in the antigen-presentation process and further influences the adaptive immune response through the regulation of antigen-presenting cell homeostasis (Puleston and Simon 2014). This chapter focuses on the molecular immunological mechanisms underlying how autophagy regulates immune functions and the immune response.

## 27.1 The Restriction and Scavenging Effect of Autophagy on Pathogens

### *27.1.1 Xenophagy: The Direct Scavenging Effect of Autophagy on Intracellular Pathogens*

Classified on the basis of their localization with respect to the host cell, extracellular bacteria and intracellular bacteria constitute the main types of bacteria. Most pathogenic bacteria are intracellular, which is strictly related to the chronic onset and recurrence of the disease. The pathogenicity of extracellular bacteria is related to virulence factors. The immune defense processes for fighting against these two types of bacterial infections are different. For infection with intracellular bacteria, the primary process consists of pathogen invasion, colonization of the tissue, amplification within the cell, and eventually, illness of the host. For example, *Salmonella typhimurium* can utilize a type III secretion system (T3SS) to secrete a large number of virulence proteins into the host cell. These virulence proteins are integrated into the host cytoskeleton to induce erythrocyte membrane shrinkage, facilitate the bacteria invading the cells, and form a vesicle called SCV containing the bacteria, thereby allowing the long-term survival of latent bacteria. Evidence suggests that some *Salmonella typhimurium* infections can block the formation of SCV and initiate mitochondrial division and autophagy. Unlike intracellular bacteria, extracellular bacteria cannot invade host cells. For example, *Pseudomonas aeruginosa* mainly relies on the secretion of virulence factors to infect the host and destroy cell structures to activate autophagy. It is still unclear what the molecular mechanism of autophagy induction by extracellular bacterial infection is. The exotoxin A (PEA) of the opportunistic pathogen *Pseudomonas aeruginosa* can induce oxidative stress damage in MLE-12 cells and activate autophagy. Vacuolating cytotoxin A (VacA) of *Helicobacter pylori* (Hp) interferes with endocytic pathways, lysosomal pathways, and host immune responses via cellular vacuolation and induces stress responses.

Approximately 30 years ago, the initial evidence suggested that inflammation can induce autophagy. In the last decade, studies have shown that autophagy plays a crucial role in the host defense system against pathogen invasion. The bacteria can be ubiquitinated after invading the cells and degraded through the autophagy pathway. This autophagic process is named “xenophagy”. Currently, autophagy has been found to be involved in the direct clearance of a variety of pathogens, including *Listeria*, *Shigella flexneri*, *Salmonella*, *Toxoplasma*, and Sindbis virus. However, parasites have acquired ways to circumvent host cell autophagic clearance during the evolutionary process. Parasites ultimately utilize autophagy of the host cell to proliferate. These phenomena reflect the multiple roles of autophagy in microbial infection.

The occurrence of xenophagy involves almost all molecules of the classical autophagy pathway. These molecules coordinate with each other to recognize, capture, and remove intracellular pathogens. Group A *Streptococcus* (GAS) was the

first bacterium found to be cleared by autophagy. GAS infects cells by endocytosis and then forms GAS-containing autophagosome-like vacuoles (GAS-containing autophagic small body-like vesicles) in the cytoplasm. The size of a common autophagosome is approximately 1 μm. However, the diameter of GcAV can reach 10 μm. The formation of GcAV depends on the autophagy core protein complex and the small GTP binding protein RAB7. After fusion of GcAVs with lysosomes to form autophagosomes, GAS is degraded and inactivated by lysosomes. GAS is inactivated in most cells by the xenophagy pathway described above.

Autophagy maintains intracellular metabolic homeostasis and is closely associated with microbial infections (Gomes and Dikic 2014). On the one hand, research evidence suggests that autophagy is involved in the direct clearance of multiple pathogens. On the other hand, parasites have evolved ways to circumvent autophagic clearance. When parasites start to proliferate, they rely on the autophagy of the host cells. This evidence suggests that autophagy has dual roles in microbial infection. Infectious diseases have become increasingly severe in recent years. Moreover, new infectious diseases continue to emerge. For example, super bacteria, SARS, Ebola virus, avian influenza virus, Middle East respiratory syndrome (MERS), and malaria, which have been afflicting people in the tropical regions, bring health threats and severe panic to the public. Antibiotics, interferons, and other medications have had essential roles in combating infectious diseases. However, with antibiotic overuse, bacterial resistance has become a serious problem. Viruses have also been shown to exhibit trends with increasing new mutations and drug resistance. According to WHO reports, the rates of drugs becoming ineffective are currently comparable to the speed of discovering new drugs. Targeting the intracellular autophagy process has been proven to be an effective way against intracellular infection. Studies of the molecular mechanisms between autophagy and pathogen-induced signaling pathways will continue to contribute to the discovery of new antibacterial methods with high efficiency and low drug resistance.

#### 27.1.1.1 The Role and Molecular Mechanism of Xenophagy

Research has suggested that autophagy plays a key role during the clearance of pathogens such as bacteria and viruses. The host cells identify and clear the pathogens through autophagic degradation. This is similar to other types of selective autophagy, such as aggregate autophagy (aggrephagy) or mitochondrial autophagy (mitophagy). Autophagy receptors selectively recognize ubiquitinated pathogens in xenophagy. After an autophagy receptor interacts with LC3 or GABARAP, the pathogen is transported to autophagosomes. Therefore, the clearance of invading pathogens by xenophagy is ubiquitination dependent. The modification with ubiquitin provides "eat-me" signals during xenophagy. *Salmonella* can be modified inside the host cells by linear and K63-linked ubiquitin chains. K48-linked and K63-linked ubiquitination can modify *Mycobacterium marinum* in macrophages. In epithelial cells, the residual membranes of *Shigella* can be identified and modified with K48-linked ubiquitination. During bacterial and viral infection, the signals mediated by the host cell

receptors further trigger xenophagy. These receptors include Sequestosome 1-like receptors, pattern recognition receptors such as TLRs (Toll-like receptors) and NOD-like receptors, RLRs (RIG-I-like receptors), pathogen receptor CD46, and RAGE (receptor for advanced glycation end products, or AGEs). These receptors trigger xenophagy by recognizing a large number of MAMPs (microbe-associated molecular patterns) and DAMPs (damage-associated molecular patterns). The autophagy receptors SLRs play a key role in controlling pathogen infection. SLRs contain one or more LIR domains that are capable of linking autophagic adaptor proteins. SLRs mainly include p62 (sequestosome-1, SQSTM1), OPTN (Optineurin), NBR1 (neighbor of BRCA1 gene) and NDP52 (nuclear dot protein 52 kDa). SLRs can recognize ubiquitin-, galectin-, and membrane phospholipid-modified proteins. These modified proteins bind to the surface of invading microorganisms and damaged host cell surfaces. Moreover, SLRs assist in the connection between the modified proteins and ATG8 homologous proteins such as LC3 and GABARAP (GABAA receptor-associated protein) and then promote the autophagy receptor loaded with foreign microorganisms to enter newly formed autophagosomes. SLRs have different affinities for diverse types of ubiquitin chain-modified proteins, non-ubiquitin-modified proteins, or other ATG8 homologous proteins. These different affinities might be the reasons for the different abilities of pathogenic microorganisms to be recognized. For example, both NDP52 and p62 inhibit the proliferation of *Salmonella*, but only p62 can eliminate Sindbis virus.

Phagosome vesicles containing *M. tuberculosis*, *Salmonella* or *Helicobacter pylori* can be fused with autophagosomes. It is also possible that these pathogens are directly phagocytosed into autophagosomes. These bacteria have evolved the ability to interfere with autophagic degradation, which becomes a crucial part of their pathogenesis. For example, the fusion of autophagosomes with lysosomes is inhibited in macrophages infected with *Mycobacterium tuberculosis*, an intracellular pathogen. Macrophages cannot effectively induce mature autophagosomes to degrade and eliminate *Mycobacterium tuberculosis*. Autophagy inhibition provides a pivotal mechanism to explain the pathogenesis of *M. tuberculosis* infection. Gutierrez et al. have shown that activated autophagy can inhibit the intracellular survival efficiency of *M. tuberculosis*. Starvation- or rapamycin-induced autophagy promotes the fusion of phagosomes containing *M. tuberculosis* with lysosomes and delivers more *M. tuberculosis* into lysosomes for degradation. In an *M. tuberculosis*-infected mouse model, it was found that myeloid cells from *ATG5*-deficient mice can accumulate more *M. tuberculosis* and cause more severe necrosis in lung tissue.

It was found that the ESX-1 secretion system increases the phagosome membrane permeability of host cells during macrophage infection by *M. tuberculosis*. This system promotes the exposure of bacterial DNA in host cells. The adaptor protein STING then recognizes ubiquitinated bacteria and degrades these bacteria through NDP52- or p62-dependent autophagic degradation. *Pseudomonas aeruginosa* can induce autophagy in MH-S alveolar macrophages. Silencing *BECN1* or treating with 3-MA (3-methyladenine) can increase the infection load of *P. aeruginosa* in MH-S cells. Rapamycin, an autophagy inducer, promotes the clearance of *P. aeruginosa* infection in MH-S cells.

The process in which individual viral components are degraded via autophagy is termed "viral phagocytosis" (virophagy). Unlike xenophagy, the targets of virophagy are newly synthesized viral components rather than the entire viral particle. Different autophagy receptors can recognize viral proteins and RNA-protein complexes in the host and then undergo autophagic degradation. For example, p62 recognizes the Sindbis virus capsid protein, but this effect is not dependent on ubiquitin modification.

#### 27.1.1.2 The Role and Mechanism of Xenophagy in *Salmonella* Infection

*Salmonella* infection is typically associated with xenophagy. After infection of epithelial cells, most of the *Salmonella* hide in the host SCV (*Salmonella*-containing vesicle). However, due to damaged type III secretion system (T3SS), 15–20% of bacteria are modified with ubiquitin and then encapsulated by the autophagosome membrane. The autophagy receptors p62, NDP52, and OPTN are involved in the recognition of ubiquitin-modified *Salmonella* and fusion with autophagosomes (Fig. 27.1). Silencing the previously mentioned autophagy receptors leads to the proliferation of *Salmonella*, indicating the essential roles of these autophagy receptors in the inhibition of *Salmonella* infection. It was recently found that NDP52 binds LC3 and TBK1 (TANK-binding kinase 1) to promote the phosphorylation of serine residues at OPTN 177, thereby enhancing the binding ability of LC3B.

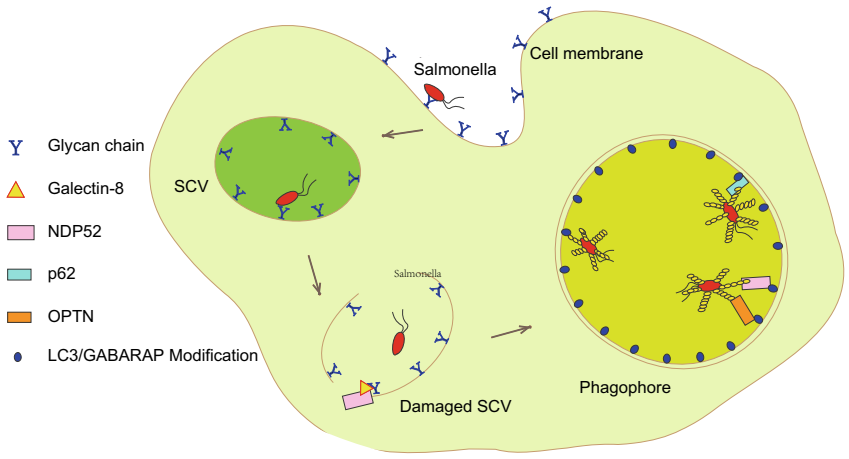


**Fig. 27.1** Clearance of *Salmonella* by autophagy. When SCVs are damaged, the glycan chains of the host cells are exposed and recognized by Galectin-8. At this time, *Salmonella* is rapidly modified by ubiquitination. Autophagy receptors p62, NDP52, and OPTN recognize ubiquitin-modified *Salmonella* and transport them into the autophagosome

Galectin-8 is involved in maintaining the integrity of endosomes and lysosomes and recognizes bacterial infections by interacting with protein-sugar complexes. Galectin-8 recognizes the glycan chains of host cells exposed to the surface of damaged SCVs. It was found that host cells infected with *Listeria*, *Shigella flexneri*, and *Streptococcus pyogenes* can produce a large number of glycan chains. In addition, Galectin-8 can recognize damaged endosomes or lysosomes in the host cell. The glycan chain, the damaged endosomes or the damaged lysosomes are transiently bound to Galectin-8 and are then coupled to the NDP52 protein in a ubiquitination-dependent manner. Therefore, Galectin-8 plays essential roles in host defense in the early phase of pathogen infection.

The second messenger diacylglycerol (DAG), which mediates autophagic elimination of bacteria, does not depend on ubiquitination modification. DAG can directly localize to SCVs, which provides the possibility for the induction of autophagy pathways to clear *Salmonella*. DAG can cause damage to the SCV membrane, recruit protein kinase PKCδ, and then trigger autophagy via the JNK and NADPH oxidase pathways. It was found that bacterial autophagosomes independently colocalized with DAG or ubiquitin, suggesting two independent autophagy degradation pathways. Consistent with the ideas of Noda and colleagues, DAG can trigger autophagy on the membrane of an SCV surrounding *Salmonella*. Noda et al. suggested that the recruitment of LC3 does not depend on the formation of barrier membranes, but instead requires at least three levels of ATG recruitment that involve the ULK1 complex, ATG9L1, and the ATG16L complex. Therefore, ATG recruitment is most likely at the site of pathogen invasion in the host cells with the contribution of non-LC3/GABARAP autophagy receptor proteins. It was found that the Tecpr1 (tachylectin-II-like beta-propeller domain) receptor interacts with ATG5 and clears *Shigella* by xenophagy. ATG16L1 was then recruited to ubiquitin-modified *Salmonella* by interacting with ubiquitin chains to complete autophagy clearance.

Studying the clearance of *Salmonella* helps to understand the mechanism of xenophagy. However, there are still many unresolved questions. Ubiquitin can colocalize to invading bacteria. It is still not clear whether the ubiquitin-modified components are from bacteria or host cells. During infection with *Salmonella* or AIEC (adherent-invasive *E. coli*), LRR (leucine-rich repeat, E3 ligase-rich leucine-rich repeat) and LRSAM1 (sterile alpha motif-containing-1) are required for ubiquitination modification. The LRR of the LRSAM1 domain recognizes the bacterial protein, and the RING domain is involved in ubiquitination. However, the bacterial ligands recognized by LRSAM1 and their ubiquitinated substrates are still unknown. The E3 ligase Parkin was found to be involved in the clearance of *Mycobacterium tuberculosis* by the host cell through the ubiquitination autophagy pathway. Parkin-deficient mice are more susceptible to *Mycobacterium tuberculosis* infection, and Parkin-deficient flies are more susceptible to *Salmonella* and mycobacterial infections. However, it is still not clear what the binding target for Parkin is. Different autophagy receptors may recognize different “eat-me” signals. NDP52 is the only autophagy receptor that recognizes Galectin-8 binding-exposed polysaccharides. Different autophagy receptors have different affinities for polyubiquitin chains. *Salmonella* in the cytoplasm can be modified by at least two types of ubiquitin chains,

the linear chain and the K63-linked ubiquitin chain. Compared to the K48-linked ubiquitin chain, p62 and OPTN preferentially bind to K63-linked and linear ubiquitin chains. The posttranslational modifications close to the ubiquitin-binding region can affect the binding properties of autophagy receptors. The advantage of using multiple autophagy receptors in xenophagy is the recruitment of different effector proteins. For example, NDP52 recruits TBK1 to bind *Salmonella* in the host cell. TBK1 increases LC3 binding by phosphorylating OPTN and p62 and promotes autophagy by regulating autophagosome maturation.

## *27.1.2 The Restrictive Effect of Autophagy on Pathogens*

### 27.1.2.1 LC3-Mediated Phagocytosis Inhibits the Propagation of Bacteria

After phagocytosis, LC3 rapidly modifies phagocytic vesicles with a monolayer membrane structure. This process is named LC3-associated phagocytosis (LAP). LAP relies on PI3K complexes and LC3 to form a conjugated system. LAP is independent of the ULK1 complex and does not form double-membrane vesicles. LAP is engaged in the macrophage clearance of *Rhizoctonia solani* and dead cell degradation and is also involved in the macrophage antigen-presentation process for fungi via TIMD4 (T-cell immunoglobulin and mucin domain-containing 4, T cell immunoglobulin mucin 4) or CLEC7A (C-type lectin domain family 7 member A, also known as Dectin 1) proteins.

### 27.1.2.2 Other Nonclassical Pathways of Autophagy Restriction on Pathogens

It was found that autophagy plays an essential role in the formation of neutrophil extracellular traps (NETs). Neutrophils are stimulated by exogenous microorganisms or chemicals to release extracellular fibrous structures composed of chromatin and granule proteins. These fibrous structures capture and kill a variety of extracellular microorganisms, including bacteria, fungi, and protozoa. Evidence has shown that similar events are also present in eosinophils and mast cells. NETosis is a cell death program in which chromatin release leads to host cell death. NETosis differs from apoptosis and necrosis. It requires three processes: oxidative stress, citrullinated histones, and autophagy. The importance of autophagy for NET formation has been supported by evidence that pharmacological autophagy interventions can regulate NET formation and affect the delivery of NET functional proteins. It was found that PMA relies on autophagy to induce NETosis. The autophagy inhibitor Wortmannin can inhibit NET formation in neutrophils.

Moreover, the autolysosome contains many active antibacterial molecules, including reactive oxygen radicals, antimicrobial peptides, proteolytic enzymes, thiol

reductases, and proton pumps. After triggering by inflammatory signals, phagocytic cells produce many active oxygen-free radicals to kill pathogenic microorganisms in phagolysosomes via NOX2 (NADPH oxidase 2). Additionally, autophagosomes contain defensin-like antimicrobial peptides that antagonize the action of microorganisms. These antibacterial peptides are mostly derived from products of cytoplasmic protein degradation such as ubiquitin and ribosomal precursor proteins, which can fuse with and kill bacteria in phagosomes. In *M. tuberculosis*-infected macrophages, it was found that the autophagy receptor p62 transports cytoplasmic proteins into autophagosomes, where these proteins are processed to kill *M. tuberculosis*. Moreover, acidic conditions activate cathepsin and chymase in autophagosomes, thereby enhancing the ability of autophagosomes to kill microorganisms and antigen processability.

It was found that some autophagy-related proteins have the ability to target the microorganism directly. For example, ATG5 can directly bind to the *Shigella* surface protein VirG. However, the interaction between ATG5 and VirG occurs on the presupposition that the Shigella protein IcsB does not occupy the VirG recognition epitope. It is dependent on the interaction between two ATG5 binding accessory proteins, TECPR1 and WIPI2 (WD-repeat domain phosphoinositide-interacting protein 2). WIPI2, involved in the formation of phagocytic vacuoles, is a paralog of mammalian phosphatidylinositol PtdIns3P-binding protein ATG8.

Other autophagy antibacterial pathways also involve the direct binding of the autophagy proteins Beclin-1, IRGM (immunity-related GTPases, immune-related GTPase family proteins) and bisphosphatidylglycerol (cardiolipin). Cardiolipin is a lipid found only in bacteria and mitochondria. IRGM, a genetic susceptibility factor for Crohn's disease, is partially localized to mitochondria. The E3 ubiquitin ligase Smurf-1 is a critical factor in host cell defenses against Sindbis virus and herpes simplex virus. Smurf-1 is involved in mitophagy, which in some respects resembles xenophagy.

IFN-γ-induced IRGs (IFN gamma-inducible immunity-related p47 GTPases) and Gbps (guanylate binding proteins) are involved in host cell xenophagy of *Chlamydia trachomatis* and toxoplasmosis. IRGs and Gbps can accumulate on the surface of PV (pathogen-containing vacuole) membranes, changing the electron density of the membranes and destroying them. Cell membrane deformation, perforation, and rupture lead to the death of the pathogens and eventually necrosis of the infected cells. This restriction of pathogens by IRGs and Gbps is independent of the formation of autophagosomes but is dependent on the autophagy proteins ATG3 and ATG5. It was found that silencing the *ATG3* or *ATG5* gene in host cells results in the inability of IRGs and Gbps to localize to PV membranes.

### 27.1.3 Pathogens Escape from Autophagy and Manipulate Autophagy

Bacteria employ various mechanisms to survive in the host cytoplasm and evade autophagic degradation. *Salmonella* and *Mycobacterium tuberculosis* inhibit phagosome maturation to survive and multiply in phagosomes. *Shigella flexneri*, *Legionella pneumophila*, herpes simplex virus (HSV-1) and Kaposi's sarcoma-associated herpesvirus (KSHV) can destroy autophagosomes or use the phagosome as a proliferation site. Studies have shown that *Shigella flexneri* quickly escapes to the cytoplasm and replicates after invading nonphagocytic cells. At the initial stage of infection, the autophagy core protein ATG16L1 is rapidly recruited by NOD1 and NOD2 to the invasion site of *Shigella flexneri*, activating autophagy by the RICK but not the NF-κB pathway. After *Shigella flexneri* enters the cytoplasm, it utilizes the virulence protein IcsA to promote actin polymerization, thereby avoiding autophagy. The binding of the autophagy protein ATG5 to the IcsA protein isolates *Shigella flexneri* and promotes autophagy-mediated degradation. However, the T3SS (type III secretion system) of *Shigella flexneri* secretes another *Shigella* protein, IcsB. IcsB competes to inhibit protein binding of ATG5 to IcsA. After IcsB binds to the IcsA protein, the IcsA protein of *Shigella flexneri* is protected from ATG5 binding, which in turn causes *Shigella flexneri* to escape autophagic degradation. In addition to ATG5, other host factors also limit the effects of *Shigella flexneri*. For example, septin can capture *Shigella flexneri* and promote autophagic degradation. It was found that *Shigella flexneri* in the Septin cage colocalized with p62, NDP52, and LC3. Moreover, most *Shigella flexneri* and their vacuolar membrane residues that have escaped from autophagy can be recognized by Galectin-8, modified by ubiquitin and bound to autophagy receptor p62, and then isolated into autophagosomes. This isolation and degradation of *Shigella flexneri* residues help control the extent of downstream inflammatory reactions that would otherwise cause excessive damage to the host.

Similar to *Shigella*, *Listeria* monocytogenes can evade autophagic degradation after infecting the host. *Listeria* monocytogenes enters the cytoplasm from the vacuole and rapidly replicates in the cytoplasm. Birmingham et al. found that the colocalization of *Listeria* and LC3 was observed in the early phase of the infection. However, the bacteria were still able to avoid autophagic degradation. After the cytoplasmic *Listeria* is modified by ubiquitin, the bacteria can be recognized by p62, which binds to LC3, and then *Listeria* undergoes autophagosome phagocytosis. *Listeria* monocytogenes, phospholipase C and ActA (actin assembly-inducing protein) are three Listeria-derived proteins that inhibit autophagy in host cells and prolong the survival of *Listeria monocytogenes*. Once in the cytoplasm, ActA on the surface of *Listeria* cells interacts with actin monomer and ARP2/3 (actin-related protein 2/3) complex in the host cell to induce actin polymerization and drive motility. The above effects not only prevent *Listeria* from evading autophagic degradation but also inhibit the modification of the pathogen by ubiquitin. It was found that *Listeria* virulence factor K (Internalin K, InlK) can recruit host MVP (major vault protein). MVP is a major

component of the cytoplasm that makes up the nucleus of ribonucleoprotein particles. The interaction between InlK and MVP helps *Listeria* to camouflage and evade host autophagy recognition. *Listeria* replication occurs primarily in the cytoplasm of host cells. *Listeria* is also found in SLAP (spacious Listeria-containing phagosome) of the host cell that has a monolayer membrane structure.

LC3-positive SLAP, a nonacid degrading chamber, has a spacer-like autophagosome characteristic. *Listeria* isolated in SLAP grows more slowly than in the cytoplasm. However, it is believed that the presence of SLAP causes a long-term confrontation between *Listeria* and the host immune system. The formation of SLAP is also considered to be a cause of persistent bacterial infection. Evidence has shown that LLO (Listeriolysin O, cholesterol-dependent Listeria lysin O) is involved in the formation of SLAP and inhibits the fusion of phagosomes with lysosomes. Autophagy is involved in the formation of SLAP in the early stages of *Listeria* infection. Autophagy inhibitors inhibit SLAP formation.

On the other hand, autophagy degrades *Listeria* in SLAP vacuoles. This effect becomes more apparent when LLO activity is impaired. Inhibition of autophagy promotes excessive proliferation of *Listeria*. However, regardless of the mechanism by which *Listeria* evades autophagy, conditional knockout of *ATG5* in mouse macrophages and granulocytes increases the susceptibility of mice to *Listeria*. This evidence also indicates that autophagy plays a crucial role in antimicrobial responses.

*Burkholderia pseudomallei* is the causative agent of melioidosis. It is a common infectious disease with a high mortality rate in the tropics, especially in southeast Asia and northern Australia. *B. pyogenes* can adhere to nonphagocytic cells through bacterial surface type 4 pili. A small number of intracellular bacteria are recognized by the host cell through autophagy and are directly recruited into endosomes via LC3 rather than classical autophagosomes. However, most bacteria can evade autophagy and other defense mechanisms of the host cell, such as iNOS (inducible nitric oxide synthase), and replicate in the cytoplasm. These bacteria invade adjacent cells by fusion with cell membranes, resulting in the formation of multinucleated giant cells. Invasion of host cells by *Burkholderia pseudomallei* involves one or several proteins encoded by the TTSS3 (type III secretion system cluster 3) gene, which is partially involved in the escape of the bacterium from the endosome to the cytoplasm. For example, the Pseudomonas serotype III secreted protein BopA plays a crucial role in the escape process. In studies of mouse macrophage infection by *Burkholderia pseudomallei*, the bacterial bopA gene mutation enhances the colocalization of LC3, and the lysosomal marker protein LAMP1 reduced the number of intracellular viable bacteria. A large number of bacteria are killed in phagolysosomes. It was found that the *Burkholderia* VI-type cluster-related gene bpss0180 can induce autophagy in phagocytic and nonphagocytic host cells. In macrophage infection, the colocalization of bpss0180-mutated *Burkholderia pseudomallei* strains and LC3 is decreased, and the number of viable bacteria in the cell is reduced. Introducing the wide-type bpss0180 gene can reverse the above phenomenon. These results indicated that the bpss0180 gene product supports the intracellular survival of *Burkholderia pseudomallei* in host cells.

Similar to bacteria, viruses have evolved a variety of mechanisms against autophagy. The ICP34.5 protein of type I herpes simplex virus (HSV), the M2 protein of influenza virus and the HIV-encoded protein Nef can block autophagy by inhibiting Beclin-1, which is the core protein for the whole autophagy system. Nef binds to the conserved domain of Beclin-1, which is the same binding region for the endogenous autophagy inhibitor GAPR1 (Golgi-associated plant pathogenesis-related protein 1). The mouse herpesvirus 68-encoded protein M11 (a homolog of the B-cell lymphoma 2 protein) inhibits the autophagy protein Beclin-1 via the BH3 domain. FLIP (FLICE-like inhibitory protein) of Kaposi's sarcoma-associated herpesvirus (KSHV) inhibits the E2-like enzyme ATG3 in the membrane of the LC3 binding region. The HIV Nef protein, the hepatitis C virus NS3 protein, and the measles virus Mev3 protein interact with the autophagy-related GTPase family protein IRGM. The effect of this interaction remains to be further studied. HBV can chronically infect human liver cells. The latent replication of HBV-infected hepatocytes is the primary cause of liver cancer development and drug resistance. Recent studies have indicated that the R-224 Smad-mediated noncanonical autophagy pathway helps HBV replication in host cells and promotes the establishment of chronic HBV infection.

## 27.2 Autophagy, Antigen Presentation, and Antigen-Presenting Cells

Antigen presentation refers to the process of efficiently delivering antigen via major histocompatibility complex (MHC) molecules. MHC molecules are classified into MHC class I molecules and MHC class II molecules. Almost all karyocytes express MHC class I on their surface. $CD8^+$ T lymphocytes mainly recognize antigen peptides presented by MHC class I molecules. Upon infection, $CD4^+$ T cells can recognize MHC II on the surface of antigen-presenting cells and antigenic peptides presented by MHC class II molecules of epithelial cells.

### *27.2.1 Autophagy and MHC Molecule Antigen Presentation*

MHC class I molecules mainly present antigens derived from the cell, such as viral proteins, endogenous tumor antigens, and autoantigens from the cytoplasm or nucleus. Intracellular antigens are processed into immunogenic polypeptide fragments as antigenic peptides in the proteasome. The antigenic peptide is transported to the lumen of the endoplasmic reticulum with the help of a peptide chain transporter and forms a complex with MHC I. The MHC I/antigen peptide complex is expressed on the cell surface after passing through the Golgi. Autophagy affects the presentation of MHC I molecules in various aspects, such as restriction of antigen

peptide production, antigen peptide membrane localization, and the antigen presentation process.

#### 27.2.1.1 Autophagy Regulates the Expression of MHC Class I Molecules on the Cell Membrane Surface

Autophagy can affect the production of MHC I-restricted antigen peptides and the expression of MHC I on the cell membrane surface by regulating protein translation (Van Kaer et al. 2017). Autophagy activation is often accompanied by eIF2α (Eukaryotic Translation Initiation Factor 2α) phosphorylation. Phosphorylated eIF2α not only inhibits conventional 5′-cap ribosome transcription but also promotes binding of mRNA to IRESs (internal ribosome entry sites). Inhibition of mTORC1 (mammalian target of rapamycin complex 1) can inhibit eIF4EBP1 (eukaryotic translation initiation factor 4E binding protein 1) and $p70^{S6K}$ (ribosomal protein S6 kinase 70 kDa polypeptide 1, RPS6KB1). Evidence has indicated that both eIF4EBP1 and $p70^{S6K}$ are involved in 5′-cap general ribosome transcription. Rapamycin treatment can increase the diversity of the immunopolypeptides and produce more new antigens on the cell surface. Immunization of mice with these new antigenic peptides induced cytotoxic T lymphocytes to specifically kill rapamycin-treated lymphoma cells without killing untreated lymphoma cells. It is still not clear whether there is indirect activation between eIF2α and the mTORC1 signal transduction pathway.

Moreover, the internalization and recycling of the plasma membrane affect the expression of MHC class I molecules on the cell membrane surface. MHC class I molecules can be internalized and degraded in the lysosome or recycled back to the cell membrane. In primary mouse DCs, silencing *ATG5*, *ATG7* or *Vps34* upregulates MHC class I expression on the cell surface. It was found that silencing *ATG5* and *ATG7* mainly reduced the internalization and degradation of MHC class I molecules. Enhanced MHC class I expression is not related to the protein recycling of the plasma membrane. Clathrin-mediated endocytosis and lipidated LC3 promote the binding of AP2-associated protein kinase 1 and MHC class I molecules to promote the internalization and degradation of MHC class I molecules. The above evidence suggests that autophagy can reduce the presentation of MHC class I antigens. Indeed, *ATG5*-deficient DCs are more potent at activating virus-specific T lymphocytes.

#### 27.2.1.2 Autophagy Regulation of Antigen Presentation

Autophagy regulates the antigen presentation process of MHC class I molecules. The antigenic peptides presented by MHC class I molecules are mainly derived from newly synthesized antigenic peptides or DRiPs (defective ribosomal initiation products). The autophagy inhibitor 3-MA can inhibit the autophagic degradation of DRiPs in HeLa cells, promote its degradation by the proteasome pathway, and enhance the presentation of MHC class I antigens. After macrophage infection by HSV-1 (herpes

simplex virus-1), activation of autophagy contributes to the presentation of HSV-1 glycoprotein B-derived peptides to $CD8^+$ T cells. Similarly, the presentation of the human cytomegalovirus latency-associated antigen UL138 requires autophagy. This process relies on endosomes but not proteasomes and antigen processing transporters. Autophagy plays a role in antigen presentation by DCs. The ability of *ATG5*-deficient DCs to present influenza virus and lymphocytic choriomeningitis virus was significantly reduced. *Vps34* deletion promoted the ability of DCs to present chicken ovalbumin, influenza virus, and lymphocytic choroiditis virus.

#### 27.2.1.3 Autophagy Regulates the Steady-State Effects of miRNAs (microRNAs) on Immune Peptides

The abundant MHC class I molecular antigen peptides are mainly derived from the transcription products of miRNA response elements. RISC (RNA-induced signaling complex) recognizes complementary mRNA with miRNA as a template activates Argonaute and cleaves the RNA. It is easy to generate DRiPs from mRNAs under the action of RISC. Compared to full-length polypeptides, DRiPs are more readily loaded onto MHC class I molecules and become a significant source of MHC class I antigen peptides. The miRNA processing mechanism consists of two core components, Dicer and AGO2 (Argonaute RISC Catalytic Component 2). Dicer is a processing enzyme for miRNAs. AGO2 is the main effector protein of RISC. Dicer and AGO2 are critical for the formation of DRiPs, which in turn affects the diversity of immune polypeptides. The autophagy receptor NDP52 captures Dicer and AGO2 into the autophagy degradation pathway and affects the diversity of the immune polypeptides by altering miRNA homeostasis.

It was found that autophagy can directly affect DRiPs. DRiPs are modified by ubiquitin and processed in proteasomes. Otherwise, DRiPs can be captured by the autophagy receptor NBR1 and degraded by autophagy. Upon autophagy inhibition, a large number of ubiquitinated DRiPs accumulate in ALISs (aggresome-like-induced structures), are proteasome degraded via the p62-dependent pathway, and then enter the classic MHC class-like molecule TAP (transporter associated with antigen processing)-dependent pathway. Autophagy activation does not accumulate DRiPs in ALISs. Ubiquitinated DRiPs are recognized by the autophagy receptor NBR1 for autophagic degradation. Therefore, inhibition of autophagy in the clearance of DRiPs may promote MHC class I antigen presentation.

## *27.2.2 Autophagy and MHC II Antigen Presentation*

### 27.2.2.1 Overview

MHC class II molecular antigen presentation involves extracellular antigen capture by antigen-presenting cells and transportation to autophagosomes. Proteolytic peptides (such as cathepsins) derived from endosomes are used to produce immunogenic peptides. The peptides are then loaded onto the MHC class II molecules and presented on the surface of the antigen-presenting cells, thereby activating $CD4^+$ T lymphocytes. MHC class II molecules are critical for the acquired immune response. These molecules present processed antigens to stimulate lymphocytes, thereby triggering an adaptive immune response. During infection, $CD4^+$ T lymphocytes recognize antigenic peptides presented by antigen-presenting cells and MHC class II molecules that are also on the surface of epithelial cells. Antigen-presenting cells, such as DCs and macrophages, link innate immunity with adaptive immunity. These antigen-presenting cells phagocytose pathogenic microorganisms such as bacteria, fungi, parasites, and other particulate antigens into the phagocytic bodies by endocytosis. While clearing microorganisms in the phagocytic body, the cells proteolytically process and produce immunogenic polypeptides. These peptides bind to MHC class II molecules to form a stable complex, avoiding further degradation. MHC class I antigen peptide processing mainly occurs in the cytoplasmic proteasome. However, MHC class II exogenous antigen processing mainly occurs in endosomes. Microbial antigens are degraded into peptides by lysosomal proteases. These peptides are loaded onto MHC class II molecules and enter the MIICs (MHC class II compartments) with an acid endosome containing cathepsin via the endoplasmic reticulum. With the roles of the autophagic-lysosomal system in the presentation of MHC class II antigens, most researchers believe that autophagy is involved in the MHC class II antigen-presentation process.

### 27.2.2.2 The Effect of Autophagy on the Presentation Process of MHC II Antigens

Autophagy plays an essential role in the presentation of MHC class II-restricted antigenic peptides (Hayward and Dinesh-Kumar 2010; Munz 2016). Schmid et al. found that autophagosomes and MIICs have a high frequency of fusion in primary monocyte-derived DCs. More than 50% of MIICs receive proteins derived from autophagosomes. Inhibition of autophagosome formation by siRNAs significantly attenuates the antigen presentation ability of MHC class II molecules in eliciting acquired immune responses. This evidence indicates that autophagy is involved in the MHC class II molecular antigen presentation process. Studies in human B lymphoblastoid cells have revealed that some of the epitopes presented by MHC class II molecules are derived from host cells. Starvation-induced autophagy activation enhances the presentation of intracellularly derived antigens by MHC class II

molecules. In macrophages or DCs that had phagocytosed BCG (Bacillus Calmette-Guerin), autophagy induction by starvation or rapamycin increased the potency of MHC class II molecules to present *M. tuberculosis* antigens. The secretory antigen Ag85B of *Mycobacterium tuberculosis* colocalizes with LC3-positive autophagosomes, suggesting that autophagy can capture the antigens that have escaped the phagosome and deliver them to lysosomes for expression by MHC class II molecules. In studies of experimental mice injected with DCs infected with *M. tuberculosis,* rapamycin-pretreated DCs promoted their ability to initiate *M. tuberculosis*-specific $CD4^+$ T lymphocyte responses. Blanchet et al. found LC3-positive autophagosome structures in DCs. These results suggest that autophagy participates in the activation of TLR receptor signaling and the antigen presentation process, thereby eliminating HIV-1. The HIV-1 Env protein activates mTOR to inhibit autophagy in DCs, thereby abolishing immune signals and enhancing the infection of $CD4^+$ T lymphocytes. It was found that the interaction of influenza matrix proteins and LC3 elicits antigen-specific $CD4^+$ T lymphocyte responses. Lee et al. found that autophagy is necessary for DCs to phagocytose extracellular antigens for presentation to $CD4^+$ T lymphocytes in *ATG5*-silenced cells. During HSV-2 infection, the ability of ATG5-silenced DCs to activate $CD4^+$ T lymphocytes and induce IFN-$\gamma$ release was significantly reduced. In an HSV-2 virus lethal challenge study, *ATG5*-deficient mice were more likely to die. *ATG5* knockout mice failed to initiate the proliferative response of T lymphocytes with cytosolic antigen OVA (ovalbumin) immunization.

Moreover, phagosomes play an essential role in the processing of MHC class II antigens. It was found that the phagocytic bodies of macrophages can phagocytose antigenic substances by endocytosis and decompose the enzymes to produce MHC class II-related peptides in the phagocytic bodies. In *Mycobacterium tuberculosis*-infected DCs, MHC II and lipid antigen CDlb molecules are recruited at phagosomes, and MHC II molecules are rapidly transferred from phagosomes to the plasma membrane with DC maturation.

### *27.2.3 Autophagy and Cross-Presentation*

It was found that MHC molecules have a cross-presentation function. MHC class I molecules can also present exogenous antigens, mainly by internalizing extracellular antigens into cells or loading antigen peptides onto endosomes expressing MHC I. The above process is named CP1 (type 1 cross-presentation). Similarly, intracellular antigens can also be presented by MHC class II molecules, a process known as CP2 (type 2 cross-presentation). Cross-presentation occurs primarily in $XCR1^+$ subtype DCs. $XCR1^+$ subtype DCs show more active autophagy than other subtypes of DCs. These results suggest that autophagy is highly activated in the cross-presenting cells.

Autophagy involves transferring microbes into the cells and processing microbial components. This indicates that autophagy can regulate the cross-presentation of MHC class I molecules. For example, DCs can control *Chlamydia trachomatis* infection in inclusion bodies. After the activation of DC, the inclusion bodies release

the bacteria into the cytoplasm. Cytoplasmic bacteria are captured into the autophagosome and degraded by cathepsin. The antigens are initially degraded by the protease inside the proteasome for further processing. The antigens are then loaded onto the MHC class I molecules via the endosomal pathway. Therefore, microbial peptide loading of MHC class I molecules is dependent on autophagy. This process requires neither TAP (transporter associated with antigen processing) nor N-terminal modification of the antigenic peptide. However, the transfer of the newly assembled MHC class I peptide complex to the cell surface by endocytosis is a necessary process. It is worth noting that the control of antigen presentation by autophagy is not limited to DCs but also involves other antigen-presenting cells, such as B lymphocytes and macrophages.

Autophagy transports multiple antigenic species into lysosomes, which constitute a major source of cell-derived MHC class II antigens. Autophagy activation promotes the production of antigenic peptides derived from the cytoplasm, mitochondria, and nucleus, indicating that autophagy facilitates the transport of these components into MIICs. It was found that MIICs from DCs contain autophagosome-like structures. These structures include not only molecules involved in antigen processing but also the autophagy marker proteins LC3 and ATG16L1. These findings suggest that autophagy is a major source of MHC class II antigens. Autophagy is primarily classified as an antigen processing pathway because autophagosomes are fused to antigen-loaded MIICs. Therefore, by participating in the MHC class II epitope presentation process, autophagy plays a crucial role in initiating the T lymphocyte immune response, thereby regulating the characteristics and intensity of acquired immune responses mediated by T lymphocytes.

### *27.2.4 Autophagy Affects the Immune Response of Antigen Donor Cells*

Autophagy is a critical switch that determines the survival or death of ADCs (antigen donor cells). Autophagy is the primary mechanism of cell survival when cells are exposed to stress conditions such as nutrition deprivation, growth factor withdrawal, pathogenic microbial invasion, mechanical damage, malignant transformation, chemotherapy, or radiation therapy. Under long-lasting and robust stress conditions beyond the ability of autophagy to respond to maintain cell homeostasis, cell death will occur. Dead cells become new antigen donors and then induce inflammation and immune responses.

Autophagy not only participates in the direct killing of pathogenic microorganisms by host cells but also is also involved in the immune response against pathogenic microorganisms or tumor-associated antigens. In the absence of dead ADCs, autophagy tends to be a spontaneous and self-limiting defense mechanism in ADCs. However, when ADCs are unable to reestablish a steady state by autophagy, dead ADCs generated by autophagy stimulate immune responses through multiple

mechanisms. First, autophagy-enhanced ADCs include ATP and LPC (Lysophosphatidylcholine) and other "discover me" chemotactic signals. Autophagy is involved in the transport of ATP from a specific lysosomal chamber to a secretory chamber in a process that has not been defined to date. Autophagy is also involved in a caspase-dependent ATP extracellular secretion process at the end of apoptosis. Autophagy causes tumor cells to release more ATP chemotactic signals during cell death, thereby recruiting antigen-presenting cells into the tumor and promoting antigen uptake by these cells. Autophagy-deficient malignant cells cannot induce an effective antitumor immune response in response to chemotherapy because they cannot release enough ATP to recruit myeloid cells and lymphocytes into the tumor. Exogenous administration of ATPase inhibitors can reverse this defect and drive myeloid cells and lymphocytes into the tumor by maintaining extracellular ATP concentration, thereby producing an effective antitumor immune response to dead tumor cells. The above ATP-driven chemotactic response to immune cells involves the metabotropic P2Y2 receptor. It was found that autophagy can induce phagocytic "eat-me" signals such as CRT (calreticulin) and PS (phosphatidylserine). PS exposure plays a crucial role in activating phagocytosis. As a ligand for a variety of receptors, PS not only participates in the recognition of dead cells by phagocytic cells but also activates the phagocytic uptake.

On the other hand, the ATP released by the dead ADCs not only promotes the differentiation of myeloid-derived granulocyte-like precursor cells into DCs but also binds to the ion receptor P2RX7 on DCs and activates inflammatory vesicles, thereby promoting the secretion of IL-1β by these cells. Dead ADCs can release intact autophagosomes, which contain not only many antigenic substances but also HSPs (heat-shock proteins), CRT, HMGB1 (high-mobility group box 1), and DRiPs. By expressing CLEC9AL (C-type lectin domain family 9 a ligand) on the surface of autophagosomes, antigen-presenting cells can use autophagosomes to cross-present a variety of antigens. Autophagosomes carrying antigenic material can be directly transferred from the dead ADCs to DCs for cross-presentation. Immunization with autophagosomes obtained by the generation of ADCs with proteasome inhibitor treatment can effectively stimulate specific immune responses in mice. During this process, proteasome inhibitor treatment of ADCs enhances the uptake of p62 compared to other autophagosome substrates. Autophagosomes obtained by purification and separation not only carry long-lived proteins but also load a variety of short-lived polypeptides, including DRiPs, CRT, and DAMPs. During cross-presentation by DCs, the nature of the antigen in the autophagosome depends on the interaction between its surface CLEC9AL and the DC CLEC9A. The antigenic substances carried by the autophagosome of ADCs by caveolae-mediated endocytosis can be transferred to a nonacid chamber. It was found that purified autophagosomes can directly induce B lymphocyte activation in a TLR2-MyD88-dependent manner. Although purified autophagosomes may be more effective in stimulating immune responses than whole-cell lysate-derived vaccines, it is still not clear that cross-presenting the autophagosome content of dead ADCs to antigen-presenting cells can occur in humans. In general, autophagy of ADCs under viral infection or malignant transformation can significantly promote the antigen phagocytosis and presentation ability

of antigen-presenting cells, suggesting that pharmacological induction of autophagy may be a strategy to activate anticancer immune responses.

## *27.2.5 Effect of Autophagy on Antigen-Presenting Cells*

### 27.2.5.1 Autophagy and the TLRs of Antigen-Presenting Cells

Autophagy affects the antigen presentation process through TLR-mediated signaling pathways. Autophagy helps antigen-presenting cells to recognize danger signals such as microbe-associated molecular patterns (MAMPs), which in turn activates the antigen presentation process (Into et al. 2012). In the cytoplasm, MAMPs shuttle into the endosomal cavity in an autophagy-dependent manner and interact with the ligand binding domains of TLRs such as TLR7/9. Natural or synthetic TLR ligands can be recognized by autophagy receptors and sequestered in autophagosomes, which in turn bind to the same internalized TLRs. This event then triggers downstream signaling to promote the secretion of type I IFNs, thereby enhancing the antigen-presentation ability of APCs. Vesicular stomatitis virus could not activate the plasma cell-like DCs (plasmacytoid dendritic cells, pDCs) in which the *ATG5* gene was deleted. The TLR7 and TLR9 of these cells could not respond to stimulation by herpes simplex virus 1 and DNA-containing immune complexes, and the IRF7 (interferon regulatory factor 7) could not be further activated to produce IFN-α. The absence of ULK1 does not affect the above effects, indicating that this effect is primarily associated with LAP rather than the traditional autophagy pathway.

### 27.2.5.2 Autophagy and Immune Synapses

Autophagy may affect immune synapse formation and binding properties between antigen-presenting cells and T lymphocytes, which in turn affects the T lymphocyte response. The immune synapse formed between antigen-presenting cells and T lymphocytes activates STK11 (serine threonine kinase 11, or liver kinase B1, LKB1) and AMPK (AMP-activated protein kinase), thereby inhibiting mTOR and activating autophagy. Once DCs form an immune synapse with T lymphocytes, the primary role of autophagy is to degrade immune synapses and synaptic components, thereby activating STK11 and AMPK. The resulting reduced synaptic stability terminates T cell activation in a time-dependent manner. It was found that silencing *ATG16L1* or *IRGM* inhibits autophagy in DCs, increases the duration of the immune synapses between DCs and T lymphocytes, prolongs the activation time of T lymphocytes and results in Th17-type responses.

#### 27.2.5.3 Autophagy and DCs

DCs have higher basal autophagy activity than other cells. Autophagy activity facilitates the processing of intracellular and extracellular antigens presented by MHC class I or class II molecules. In DCs, autophagy is mainly mediated by endosomes. The autophagosomes in MIICs have both molecular markers of antigen-presenting molecules and autophagosome molecular markers such as LC3 and ATG16L1. Endosomal-mediated autophagy involves direct phagocytosis of DALISs (DC aggresome-like lipopolysaccharide-induced structures). DALISs are p62- and polyubiquitin-labeled structures that are strictly related to protein synthesis. It was found that DRiPs entering DALISs can avoid degradation. Autophagy can affect the antigen presentation ability of DCs by direct phagocytosis of DALISs.

It was found that DCs deficient in the *ATG5* gene have impaired MHC class II molecular solubility and cell-associated antigen presentation. When a herpes simplex virus type 2 component is encountered, the $CD4^+$ T lymphocyte response cannot be stimulated. The presentation by MHC class II molecules of citrulline peptides is not only restricted by the expression level of ATG5 but also by the PI3K inhibitor 3-MA. This mechanism may be associated with the onset of rheumatoid arthritis characterized by citrulline autoantigens. The results of a study in which the thymus of *ATG5* gene deletion mice was transplanted into wild-type mice indicated that the autophagy-mediated antigen presentation by MHC class II molecules involved the differentiation of $CD4^+$ T lymphocytes. Autophagy plays a role in the selection of thymus T lymphocytes. This effect is more pronounced in response to rare antigens and some antigens entering the autophagic cavity. However, it is not clear that the above mechanism is true for the antigens presented by peripheral DCs.

## 27.3 The Role of Autophagy in Innate Immunity

### *27.3.1 Overview*

With the recent development of molecular biology, cell biology, and immunology techniques, the crosstalk between autophagy and innate immunity has been gradually revealed. Autophagy is essential for innate immunity and is involved in mediating a variety of innate immune pathways. Various stimuli, including hypoxia, bacterial infection, and organelle damage, induce autophagy, degrade intracellular damaged proteins and organelles, and eliminate bacteria and viruses in infected cells.

After recognition by pathogen recognition receptors (PPRs), pathogenic microbial components such as LPS, lipoprotein, flagellin, and nucleic acids activate transcription factors, causing upregulation of inflammatory cytokines, chemokines, type I interferons, and multiple anti-pathogenic genes, ultimately triggering innate immune responses. Abnormal activation of the innate immune response can cause inflammatory diseases such as autoimmune diseases and septic shock. The host has an

elegant innate immune regulatory system to prevent excessive or weak immune responses. Regarding the removal of pathogens, autophagy plays an essential role in innate immunity and adaptive immunity (Table 27.1). For example, nonphagocytic cells rely on the formation of Rab-7 bacterial autophagosomes during the clearance of *S. pyogenes* and *Q. rickettsia*. The transport proteins p62 and NBR1 bind to ubiquitin and LC3 through their UBA (ubiquitin-associated region) and LIR (LC3-interacting region) domains, respectively. Then, ubiquitinated proteins, damaged organelles, and intracellular pathogens are degraded by selective autophagy. It has been found that autophagy can selectively eliminate *Salmonella typhimurium*, *Shigella flexneri*, *Escherichia coli*, and *Streptococcus pyogenes* infection. *Listeria monocytogenes* secrete ActA on the cell surface of bacteria and then recruit Arp2/3

**Table 27.1** Interaction between pathogens and autophagy pathways

| Pathogen | Autophagy function |
|---|---|
| **Bacterial** | |
| *Mycobacterium tuberculosis* | Fusion of bacteria-containing phagosomes with autophagosomes |
| *Salmonella typhimurium* | Targeting damaged phagosomes |
| *Helicobacter pylori* | Targeting autophagosomes containing bacteria |
| *Pseudomonas aeruginosa* | Induce autophagy |
| *E. coli* | Invading strain and activate autophagy |
| *Bacillus anthracis* | Degradation of anthrax lethal toxin by autophagy |
| *Listeria* | Listeria, phospholipase C, and actin polyprotein A inhibit autophagy; autophagy clears intracellular bacteria |
| *Shigella flexneri* | P62 and NDP52 target bacterial and induce phagocytosis |
| *Vibrio cholerae* | Cholera toxin inhibits autophagy |
| **Virus** | |
| Sindbis virus | Degraded viral capsids induce autophagy |
| Vesicular stomatitis virus | During viral infection process of Plasma cell dendritic cells, autophagy transfers viral ligands to TLR7 |
| Human immunodeficiency virus | HIV can induce autophagy-dependent cell death by gp41 in bystander T cells. The virus also inhibits autophagy in dendritic cells |
| Herpes simplex virus 1 | HSV-1 protein ICP34.5 inhibits autophagy by interacting with Beclin-1 |
| Human cytomegalovirus | hCMV virus protein TRS1 inhibits autophagy through interaction with Beclin-1 |
| Measles virus | Viral infection induces autophagy through CD46 and GOPC proteins function |
| **Protozoa** | |
| *Toxoplasma gondii* | CD40-dependent activation of macrophages induces autophagy to kill Toxoplasma gondii |

and Ena/rasp proteins to mimic host cell organelles, thereby avoiding ubiquitination and autophagic degradation. The effector of the *Shigella* type III secretion system damages the phagosome membrane. The damaged phagosome membrane is ubiquitinated, encapsulated, and degraded via the p62 pathway. Damaged phagosome membranes bind to proteins such as Galectin3, NOD1, Ipaf, ASC, Caspase-1, TRAF6, and NEMO, which are specifically related to NF-κB-dependent cytokine storm. Therefore, degradation of bacteria by selective autophagy may have adverse effects on infection control.

Autophagy-related proteins (ATG) cooperate with each other and cause cell membrane changes during autophagosome formation. ATGs were found to be involved in the formation of autophagosomes and are responsible for Irga6 (immune-associated GTPase) transport between the endoplasmic reticulum-Golgi and vesicles. This effect contributes to the clearance of *Toxoplasma gondii* by macrophages. Moreover, autophagy plays a crucial role in the antigen presentation process. Autophagy promotes the binding of MHC II molecules located on the surface of autophagosomes to viruses or autoantigens and then presents them to $CD4^+$ T cells. During the infection process of human herpes simplex virus l, autophagy controls MHC I to present viral antigens to $CD8^+$ T cells. In addition, autophagy plays an essential role in tissue barrier regulation, innate immune receptor-mediated immune responses, and immune effector regulation.

### *27.3.2 The Regulation of Autophagy in the Intestinal Mucosa*

The intestinal mucosa is composed of intestinal epithelial cells, mucus layer, immune cells, microbial population, and endocrine and other defense systems (Fig. 27.2). Intestinal epithelial cells (IECs) constitute the initial physical and immune barrier of the intestinal mucosa, preventing the invasion of foreign pathogens and commensal bacteria in vivo (Goto and Kiyono 2012; Haq et al. 2019). The IEC maintains the homeostasis of the gut through various functions, such as apoptosis, autophagy, and endoplasmic reticulum stress. This protection plays a vital role under rapidly changing conditions in the environment. IECs are mainly composed of absorption cells, goblet cells, endocrine cells, Paneth cells, M cells, and undifferentiated cells. Intestinal inflammation may be associated with autophagy. However, the role of autophagy in intestinal epithelial cells remains unclear.

As the largest absorption organ, the gut is closely related to obtaining nutrients. Studies have found that amino acids play an essential role in regulating autophagy in intestinal epithelial cells. Glutamine can enhance autophagy under heat and oxidative stress. This upregulation of autophagy is directly related to cell survival. After stress occurs, glutamine deficiency induces apoptosis and increases protease and ADP ribosylase activity. Inhibition of mTOR or p38MAP kinase reduces the above enzyme activities, suggesting that autophagy protects cells and reduces apoptosis by enhancing glutamine activity. The endoplasmic reticulum is abundant in Paneth

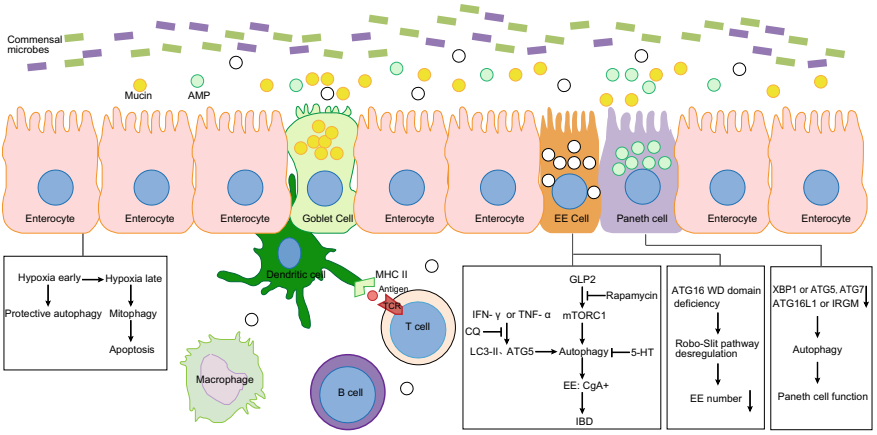


**Fig. 27.2** Autophagy is involved in the regulation of the normal function of the intestinal mucosa and the pathogenesis of enteritis. The intestinal mucosa is a defense system consisting of intestinal epithelial cells, mucus layer, immune cells, microbial population, and endocrine cells. In the early stage of hypoxia, protective autophagy is activated in intestinal epithelial cells; in the late stage of hypoxia, mitophagy is activated and results in apoptosis. Endocrine cells and autophagy are mutually regulated, 5-HT inhibits autophagy, and GLP2 activates the mTORC1 signaling pathway to activate autophagy. Under the stimulation of IFN-γ and TNF-α, autophagy is activated, and the number of $CgA^+$ EE cells increases, resulting in enteritis. ATG5 or ATG7 activates autophagy and is involved in maintaining the survival and function of Paneth cells

cells compared to other intestinal epithelial cells. Deletion of key autophagic proteins may disrupt organelle renewal and destroy endoplasmic reticulum homeostasis. XBP1 is a crucial transcription factor that maintains endoplasmic reticulum homeostasis. Paneth cells are abnormally sensitive to endoplasmic reticulum stress. However, the effect of autophagy deficiency is not fully understood by XBP1 deletion because Paneth cells die before they can exhibit autophagy deficiency. In vivo studies indicated that intestinal dysfunction is associated with autophagy deficiency.

In ATG16L1-deficient Paneth cells, electron microscopy results showed degradation of mitochondria and replacement of endoplasmic reticulum by many vesicular structures. In the *ATG5*- or *ATG7*-deficient mouse models, it was shown that the apical segmentation of the Paneth cell was separated from the basal part, and the Paneth cells disappeared. These effects result in a decrease in intestinal antimicrobial peptides and other secreted proteins. Clinical studies of patients with Crohn's disease showed that autophagy deficiency is related to the expression changes of various genes associated with intestinal mucosa homeostasis, injury healing, and pro-inflammatory response, suggesting that autophagy deficiency is closely related to human diseases. ATG16L1 and IRGM regulate the effects of autophagy. When ATG16L1 or IRGM is mutated, Paneth cells appear to be dysfunctional, leading to an impaired intestinal mucosal barrier. In the presence of hypoxic stress, secretory cells such as Paneth cells and goblet cells exhibited severe endoplasmic reticulum stress and reduced secretion, which may be an essential mechanism for endoplasmic

reticulum protein accumulation. The effects of autophagy on endoplasmic reticulum stress and exocytosis revealed that autophagy deficiency has various effects on intestinal secretory cells, affecting intestinal function.

Autophagy participates in the intestinal inflammatory process. It was found that in the *ATG5*- or *ATG7*-deletion mice, the overall morphology of intestinal epithelial cells of the colon and ileum appeared normal, but there were significant differences in intestinal Paneth cells. Compared with normal small intestinal epithelium, the expression of TNF-α and IL-1β mRNAs were significantly increased in the intestinal epithelial cells of mice lacking *ATG7*, and NF-κB pathway activation was significantly increased. This evidence suggests that autophagy regulates intestinal inflammation, reduces intestinal toxins, and maintains intestinal homeostasis via the NF-κB signaling pathway. Neonatal necrotizing enterocolitis (NEC) studies have shown that autophagy-associated protein expression is increased and autophagy regulatory proteins Beclin-1 and LC3II are activated, accompanied by extensive degradation of p62 protein, indicative of autophagy activation in intestinal epithelial cells. Inhibition of autophagy can increase the inflammatory response through endoplasmic reticulum stress. In contrast, inflammatory gene expression was significantly reduced when autophagy was activated, or endoplasmic reticulum stress was inhibited. A large number of studies have shown that autophagy at the initial stage can remove damaged organelles and protect the intestinal mucosa, but excessive activation of autophagy produces a large number of autophagic vacuoles, which have different degrees of adverse effects on organelles, eventually leading to cell damage and even cell death.

Enteroendocrine (EE) cells reside in the largest endocrine organ of the human body. The enterosteroids 5-hydroxytryptamine (5-HT) and glucagon-like peptides 1 and 2 (GLP-1, GLP-2) secreted by EE cells are closely associated with intestinal physiology and pathological function. The number of EE cells and the mode of secretion are related to enteritis. Clinical observations found that the density of polypeptide YY and chromogranin A-expressing ($CgA^+$) cells increased in patients with lymphatic enteritis, and the number of GLP-secreting cells and 5-HT-secreting cells (intestinal chromaffin cells) in patients with inflammatory bowel disease (IBD) increased. Research evidence continues to show that autophagy is involved in the regulation of EE cell function. In the mouse colonic IBD model induced by IFN-γ and TNF-α stimulation, the number of $CgA^+$ EE cells increased, while the colonic mucosa highly expressed the autophagy-associated proteins LC3-II and ATG5. It is suggested that autophagy is involved in regulating the production and differentiation of $CgA^+$ cells. Deletion of the WD-40 domain of ATG16 in the Drosophila intestinal mucosa leads to dysregulation of the Robo-Slit signaling pathway, resulting in a decrease in the number of mature EE cells. This evidence suggests that Drosophila ATG16 promotes the differentiation of intestinal stem cells into EE cells. Hormones secreted by EE cells are also involved in the regulation of autophagy. In liver cancer cells and lacrimal gland cells, 5-HT is capable of inhibiting autophagy activity. C57BL/6 mice were injected with GLP2 to promote phosphorylation of the S6 ribosomal protein and the mTORC1 signaling pathway downstream of eukaryotic translation initiation factor 4E (eIF4e)-binding protein 1 (4E-BP1). Pretreatment with the mTORC1 inhibitor rapamycin blocked the phosphorylation of 4E-BP1 and

the S6 ribosomal protein. These results suggest that GLP2 can significantly activate the autophagic pathway in the mouse gut. The function and molecular mechanism of autophagy regulation in various types of cells during the development of enteritis need to be elucidated.

### *27.3.3 The Effect of Autophagy Regulation Mediated by Innate Immune Receptors on the Immune Response*

#### 27.3.3.1 Autophagy Is Necessary for TLR-Mediated Immune Effects

When the cells are under stress, autophagy often plays a cytoprotective role and inhibits the inflammatory response. Similarly, the inflammatory response associated with the TLR signaling pathway is also regulated by autophagy. This section will focus on the regulation of the TLR-mediated signaling pathway by autophagy, mainly its mitochondria-related functions.

Normally, autophagy inhibits the TLR signaling pathway. Several autophagy-related proteins have been found to negatively regulate TLR-induced responses. After stimulation of TLR4 with LPS, *ATG16L1*-deficient macrophages produce a large number of IL-1β and IL-18 due to excessive activation of caspase-1. The recruitment of TLRs does not directly activate caspase-1, whereas the maturation of pro-IL-1β/pro-IL-18 requires caspase-1. Extracellular ATP activates the P2X7 receptor, induces caspase-1 activation, and produces IL-1β/IL-18 via the NOD-like receptor family pyrin domain-containing 3 (NLRP3) inflammasome. The above activation stress is associated with $K^+$ efflux and impaired lysosomal function. Caspase-1 activity is high in ATG16L1-deficient cells, and ROS production can be promoted without the need for extracellular ATP. Thus, TRIF-mediated signaling pathways produce ROS that activates the NLRP3 inflammasome, whereas ATG16L1-mediated autophagy significantly inhibits ROS production.

Studies have shown that inhibition of autophagy by deletion of LC3II or Beclin-1 leads to abnormal mitochondrial aggregation while producing large amounts of ROS in macrophages. In cells with abnormal mitochondrial structure, TLR4 becomes more sensitive to LPS stimulation. Mitochondrial ROS production causes the NLRP3 inflammasome to be recruited to the mitochondria-linked ER membranes, where NLRP3 interacts with ASC (a key inflammatory body formation regulator) to promote the formation of the NLRP3 inflammasome. In addition, the NLRP3 inflammasome and overproduction of ROS promote the release of mitochondrial DNA into the cytoplasm, which is a coactivator of Caspase-1. Therefore, autophagy can negatively regulate the inflammatory response by protecting mitochondrial homeostasis. The TLR signaling pathway may be involved in mitochondrial autophagy, and TRAF6-mediated ECSIT polyubiquitination plays a vital role in this process.

Immune-associated p47 GTPases (IRGs) act to protect against intracellular pathogens. Murine Irgm1 (LRG47) GTPase abolishes intracellular *Mycobacterium*

*tuberculosis* by forming a sizeable autophagic lysosome that induces autophagy. Stimulation of TLR4 induces Irgm1 expression, which in turn inhibits TLR4-triggered pro-inflammatory cytokine responses. The human Irgm1 homolog, IRGM, also exerts an autophagy-mediated clearance effect of intracellular mycobacteria. IRGM has an affinity for mitochondrial cardiolipin lipids that localize to mitochondria and thereby affect mitochondrial division, mitochondrial membrane depolarization, and the induction of autophagy.

Interestingly, mitochondrial division is necessary for the control of intracellular mycobacteria by IRGM autophagy. IRGM also plays an essential role in the process of autophagosome maturation. Therefore, IRGM may regulate mitochondrial function downstream of TLRs. Proteins with a tendency to aggregate tend to be associated with cytotoxicity. For example, a mutated huntingtin protein contains a large amount of repetitive polyglutamine, which is cytotoxic. Aggregated Huntingtin proteins are recognized by autophagy receptors (such as p62 and HDAC6) and are considered markers of Huntington's disease. Polyglutamine muteins are usually present in a variety of forms, such as monomers, water-soluble oligomers, and insoluble inclusion bodies. Studies have found that soluble or small monomeric mutant Huntingtin proteins are more toxic, through forming a polymer or inclusion body escape cell scavenging effects. MyD88 exists in different forms, such as monomers, oligomers and inclusion bodies, and has similar characteristics to mutant Huntingtin. Although MyD88 can be incorporated into inclusion bodies and polymers via p62 and HDAC6, it is still unclear whether such polymeric structures affect the TLR signaling pathway. Silencing of p62 or HDAC6 revealed that p62 and HDAC6 are involved in the inhibition of TLR-mediated activation of p38 and JNK signaling pathways, but the effect on the NF-κB signaling pathway is not yet apparent. The presence of these molecules impairs the recruitment of TRAF6 to the MyD88 signaling complex and promotes the recruitment of CYLD to the complex. Additionally, NDP52 can also affect the TLR signaling pathway. NDP52 mediates TRIF-TRAF6 complex polymerization and degradation, thereby inhibiting TLR3/4-induced activation of NF-κB and IRF3 signaling pathways.

OPTN is involved in the regulation of the TLR signaling pathway, and OPTN competitively antagonizes the RIP1-NEMO interaction by binding to polyubiquitinated RIP1. OPTN interacts with the TRAF3-TBK1 complex to reduce the production of type I interferon. UBQLN1 inhibits TRIF signaling by degrading TRIF. These results indicate that the autophagy receptor negatively regulates TLR signaling, but it is not clear whether this negative regulation is involved in the protein aggregation process.

#### 27.3.3.2 The Critical Roles of Autophagy in NLR-Mediated Immune Responses

The intracellular receptors NOD1 (nucleotide-binding oligomerization domain-containing protein 1) and NOD2 recognize the bacterial-specific polypeptides iE-DA (diaminopimelate) and MDP (cell wall phenol dipeptide), respectively, and play a vital role in the production of cytokines and antimicrobial peptides (Carneiro and

Travassos 2013). A study found that MDP activation of NOD2 can induce autophagy, thus promoting the binding of antigenic peptides to MHC II molecules by dendritic cells and ultimately eliminating intracellular bacteria. This effect requires the simultaneous involvement of NOD2 signaling, the downstream regulatory molecule RIPK2 and autophagy-related proteins, including ATG5, ATG7, and ATG16L1. The activation of NOD1 and NOD2 can stimulate the formation of autophagosomes in mouse embryonic fibroblasts (MEF), macrophages, and human lymphocytes. The above process does not depend on the adapter protein RIP2 and the transcription factor NF-κB. The study found that NOD1 and NOD2, together with ATG16L1, were recruited to the bacterial invasion site on the membrane, induced autophagy, and eventually cleared intracellular bacteria. Although the downstream signaling pathways of NOD1 and NOD2 remain to be further studied, the above results suggest that autophagy plays an essential role in the antibacterial immune response induced by NOD1 and NOD2.

Agrewala et al. found that NOD-2 combined with the TLR-4 signaling pathway activates autophagy, enhances the ability of DCs to kill bacteria, and activates killer T cells. AngII (Angiotensin II) promotes NLRP3 inflammasome activation, IL-1β secretion, and collagen production by activating ROS, which triggers pulmonary fibrosis. Administration of the autophagy agonist rapamycin attenuates the progression of pulmonary fibrosis described above. Autophagy eliminates ROS and inhibits the activation of the NLRP3 inflammasome, while the autophagy effector p62/SQSTM1 degrades ubiquitin-labeled IL-1β, ultimately decreasing pulmonary fibrosis injury.

#### 27.3.3.3 Autophagy Affects the RLR-Mediated Immune Response

RIG-I-like receptors (RLRs), including RIG-I, MDA-5, and LGP-2, are located in the cytoplasm and are responsible for recognizing viral dsRNAs. The viral dsRNA interacts with the linker molecule IPS-1 (IFN-β promoter stimulator l) on the mitochondrial membrane through the CARD (caspase activation and recruitment domain), activating TBKl/IKK-i and IRF3/IRF7, which induces the production of inflammatory cytokines and type I interferons; it also produces a large number of cytokines via the NF-κB activation pathway, which stimulates the host antiviral responses.

The ATG12 and ATG5 complexes directly bind to the CARDs of RLR and IPS-1, thereby inhibiting the production of type I interferon in the RLR-mediated antiviral process. In contrast, *ATG5*-deficient MEF cells showed enhanced expression and secretion of type I interferon after vesicular stomatitis virus (VSV) infection or dsRNA treatment and limited VSV proliferation. The study found that deletion of ATG7, which is required for the ATG12 and ATG5 complexes, promotes the expression of type I interferon-induced by dsRNA.

Autophagy deficiency disrupts cell homeostasis and affects the RLR-IPS-1 signaling pathway. *ATG5*-deficient MEF cells are deficient in autophagy, leading to dysfunctional mitochondrial aggregation, causing an increase in intracellular ROS.

The accumulation of ROS in the cell leads to an increase in the level of type I interferon after activation of the RLR, which ultimately enhances the clearance of VSV. Recent studies have found that HEK293T cells activate the RIG-I-IPS-1-TRAF6 signaling pathway during viral infection, resulting in the K63-linked polyubiquitination of Beclin-1 protein, which in turn activates autophagy to provide a defense against viral infection.

## *27.3.4 The Regulation of Immune Effector Molecules by Autophagy*

### 27.3.4.1 Autophagy Regulates the Activation of Inflammasomes

The inflammasome is a cytosolic polyprotein complex that belongs to a new class of inflammatory signaling pathways. The inflammasome controls the maturation and secretion of various inflammatory factors, such as interleukin (IL) IL-1β, IL-18, and IL-33. Members of the cytosolic NLR family (such as NLRP3 and NLRP1) interact with the autophagy receptor to form inflammasomes. In wild-type macrophages, the TLR agonist LPS is unable to induce inflammasome activation and secretion of IL-1β. When the autophagy regulatory gene *ATG16L1* or *ATG7* is knocked out or chemical agents are used to inhibit autophagy, the LPS-dependent inflammasome is reactivated. These findings suggest that autophagy regulates the activation of the inflammasome while limiting the production of the inflammatory cytokines IL-1β and IL-18.

Thus far, the mechanism by which autophagy inhibits the inflammasome is unclear. A possible molecular mechanism is direct degradation of the inflammasome by autophagy, as autophagy downregulates ROS production, which inhibits the inflammasome. Deficiencies in autophagy-related genes, such as *ATG5*, *ATG7*, *ATG12*, and *ATG16L1*, result in abnormal accumulation of mitochondria and a massive increase in ROS. ROS can be recognized by the inflammasome in innate immune cells, releasing pro-inflammatory factors, which in turn cause inflammation. Autophagy can reduce mitochondrial aggregation and apparent leakage of mitochondria and peroxisomes, thus negatively regulating intracellular ROS. These findings suggest that ROS-activated autophagy may be a negative feedback regulatory mechanism.

Recent studies have found that trimethylamine N-oxide produced by intestinal microbial metabolism inhibits the expression of ATG16L1, LC3-II, and p62 proteins to reduce autophagy and upregulate the nucleotide-binding domain of intestinal epithelial cells, the activity of alanine-rich family proteins and the NLRP3 inflammasome. The GCN2 (generally controlled nonrepressed kinase) knockout mouse model showed increased expression of IL-1β, enhanced Th17 cell responses, and increased production of reactive oxygen species, resulting in a severe enteritis response. Ravindran et al. found that enteritis was strictly related to the inhibition of autophagy in

IECs and APCs. Short-chain fatty acids (SCFAs) produced by the anaerobic glycolysis of carbohydrates, such as food residues, are the primary source of energy for IECs and maintain the normal function of the intestinal barrier. As HDAC inhibitors, SCFAs block the activity of the NLRP3 inflammasome and reduce autophagy, thereby alleviating LPS injury in the intestinal barrier.

#### 27.3.4.2 The Effect of Autophagy on the Production and Release of Cytokines

Studies have found that autophagy can directly affect the transcription, processing, and secretion of some cytokines. It is worth mentioning that the disruption of the normal autophagy pathway increases the secretion of the pro-inflammatory cytokines IL-1α, IL-1β, and IL-18. Regulation of IL-1β processing and secretion is mainly dependent on the activation of caspase-1, which subsequently affects the formation of the inflammasome (Piccioli and Rubartelli 2013). The secretion of IL-1β is usually divided into two phases: first, IL-1β precursor transcription is induced by LPS, and then inflammatory body assembly and caspase-1 activation are induced by ROS, uric acid crystals or ATP stimulation. Autophagy regulates the secretion of IL-1β by at least two different mechanisms. Knockout of *ATG7*, *ATG16L1* or the autophagy gene *BECN1*, or treatment with the autophagy inhibitor 3-MA, can inhibit autophagy in macrophages or dendritic cells. Inhibition of autophagy enhances the processing and secretion of IL-1β by TLR agonists. The study found that the above effects in murine macrophages and dendritic cells are dependent on TRIF (TIR-domain-containing adaptor inducing interferon-β), mitochondrial ROS and/or mitochondrial DNA and are partially dependent on NLRP3. However, the effects mentioned above in human peripheral blood mononuclear cells may be dependent on the p38-MAPK signaling pathway but not TRIF. TNF-α production is dependent on mitochondrial ROS and then induces oxidative stress. In the absence of *ATG7* in mouse intestinal epithelial cells, LPS can promote large-scale transcription of IL-1β. In LC3B-deficient mice, LPS can produce higher levels of IL-1β and IL-18. Clinical studies have found that the ATG16L1 polymorphism is closely related to the risk of Crohn's disease. Sodium dextran sulfate (DSS) is more likely to induce colitis in mice lacking ATG16L1. These results indicate that autophagy may be an essential mechanism for controlling inflammatory responses in vivo.

It was found that autophagy can control inflammasome activation and IL-1β production. Under the stimulation of LPS and ATP or alum, rapamycin-induced autophagy can inhibit the secretion of IL-1β by mouse dendritic cells. Furthermore, after treatment with rapamycin and LPS or PAM3Cys, the level of IL-1β precursor in macrophages is reduced. These findings indicate that autophagy can specifically target IL-1β precursors to undergo lysosomal degradation and to regulate the expression of IL-1β precursors inside the cell. Rapamycin-induced autophagy can also effectively reduce the increase of serum IL-1β induced by LPS in mice. Both IL-1α and IL-1β induce autophagy, which may be a negative feedback loop that regulates IL-1-induced inflammatory responses. For example, IL-1 and IL-23 can promote the

secretion of IL-17 by lymphocytes, and autophagy plays a vital role in regulating Th17 cell responses and participates in the development of autoimmune diseases such as multiple sclerosis.

Similar to IL-1β, IL-18 also binds to the complex (inflammasome) through NALP1, NALP3, and PAF, hydrolyzes procaspase-1 after recognition of PAMPs or DAMPs, and procaspase-1 hydrolyzes pro-IL-18 to produce IL-18, which then triggers an inflammatory response. Recent studies have found that the inflammatory cytokine IL-6 is involved in autophagy. Thorburn et al. found that autophagy activation promotes the secretion of IL6 in $CD44^{+}/CD24^{low/-}$ breast cancer stem cells, thereby maintaining breast cancer stemness. In glioma cells, hypoxia activates protective autophagy. Highly expressed IL-6 inhibits autophagy by regulating the STAT3-miR155-CREBRF-CREB3-ATG5 signaling pathway, thereby promoting the development of glioma.

## 27.4 Autophagy Participates in the Adaptive Immune Response

### *27.4.1 Autophagy Participates in T Lymphocyte Activation and Responses*

#### 27.4.1.1 Overview

T lymphocytes have been widely demonstrated to express autophagy-related genes and have autophagic activity. The constitutive autophagic activity of mouse and human $CD4^{+}$ and $CD8^{+}$ T lymphocytes is low. Stimulation of T lymphocyte receptors or HIV infection induces autophagy activation in vitro. The study found that thymic epithelial cells in the thymic cortex of mice have high levels of autophagic activity, suggesting that autophagy plays a role in the development and selection of T lymphocytes (Merkley et al. 2018).

#### 27.4.1.2 The Effect of Autophagy in the Homeostasis and Function of T Lymphocytes

It was found that the timely removal and reduction of mitochondrial load by autophagy are essential for maintaining the function of normal hematopoietic stem cells (HSCs) and are essential to produce myeloid and lymphoid progenitor cells. After naive T lymphocytes leave the thymus, their maturation is dependent on autophagy-mediated mitochondrial content reduction. After knocking out the thymic stroma via *ATG5*, the selection of $CD4^{+}$ T lymphocytes specific for multiple organ inflammatory responses changed, indicating that autophagy plays a role in T lymphocyte selection and central tolerance. In contrast, the selection of $CD8^{+}$ T lymphocytes

did not change. A series of gene knockout models have been established to study the effects of autophagy on T lymphocytes in vivo. In fetal liver chimeric *ATG5*$^{-/-}$, *ATG7*$^{flox/flox}$ Lck-Cre, *Vps34* $^{flox/flox}$ (Vacuolar Protein Sorting 34) CD4-Cre mice, it was found that autophagy-deficient T lymphocytes can develop normally in the thymus but peripheral T lymphocytes are affected. The number of T lymphocytes in the spleen and lymph nodes of *ATG5*$^{-/-}$, *ATG7*$^{-/-}$, *ATG3*$^{-/-}$, or *Vps34*$^{-/-}$ mice was significantly reduced compared to wild-type mice. These T lymphocytes are unable to achieve efficient proliferation under specific activation stimuli. Autophagy-deficient T lymphocytes do not adequately regulate and control intracellular organelle quality, while the mitochondrial burden of T lymphocytes increases, which leads to positive feedback with increased oxidative stress and cell death.

It was found that autophagy is involved in regulating the energy metabolism of T lymphocytes. Typically, ATP production is increased when T lymphocytes are activated, and lysosome inhibitors can inhibit the increase of ATP. Autophagy-deficient T lymphocytes supplemented with exogenous energy with methyl pyruvate restores some of their functions. Autophagy is involved in the development of iNKT (invariant Natural Killer T) cells. In transgenic mice with the T lymphocyte gene *vps34* knocked out, thymic iNKT cells were arrested at the G0 phase. A similar study found that autophagy also plays a crucial role in maintaining the homeostasis and function of Foxp3$^+$ regulatory T lymphocytes. A growing body of evidence suggests that autophagy regulates the homeostasis and function of T lymphocytes at multiple levels.

#### 27.4.1.3 The Effects of Autophagy in the Homeostasis and Function of T Lymphocytes

Autophagy affects the survival of T lymphocytes, and the autophagy of naive T lymphocytes is low. The autophagic activity of T lymphocytes is mainly inhibited by CFLIP protein (cellular FLICE-like inhibitory protein, also known as CFLAR, cell type FLICE-like inhibitory protein). The autophagy of activated T lymphocytes increases upon the induction of TCR (T cell receptor) signals and CD28 costimulatory signals. T lymphocytes maintain their activation status with the help of autophagy: on the one hand, autophagy counteracts the proapoptotic effects produced by upregulated CD95 (also known as FAS) and CD95L (also known as FasL) on TCR stimulation; on the other hand, autophagy eliminates impaired mitochondria in T lymphocytes and maintains normal oxidative stress levels to inhibit apoptosis. Compared with wild-type mice, the number of T lymphocytes in the spleen and lymph nodes of T lymphocyte autophagy-deficient mice was significantly higher.

Naive T lymphocytes develop into mature T lymphocytes in the thymus, which is accompanied by a series of changes in surface marker proteins. MHC molecules play an essential role in the differentiation and development of T lymphocytes. Naive T lymphocytes must come into contact with thymic epithelial cells expressing MHC I or MHC II antigens to differentiate into CD8$^+$ or CD4$^+$ T lymphocytes, respectively.

Autophagy selectively determines the maturation of T lymphocytes by controlling the MHC I or MHC II antigen peptides presented on the cell surface.

Autophagy is involved in T lymphocyte activation by maintaining homeostasis of endoplasmic reticulum calcium flux. T lymphocyte activation is dependent on a stable calcium ion flux of the endoplasmic reticulum, and autophagy promotes the homeostasis of the endoplasmic reticulum calcium ion flux. After TCR stimulates T lymphocyte activation, the calcium influx of the endoplasmic reticulum increases. However, calcium influx is impeded in *ATG7*-deficient T lymphocytes, and calcium ions bind to and accumulate on an endoplasmic reticulum-like structure and fail to respond to TCR stimulation.

#### 27.4.1.4 The Effect of Autophagy in T Lymphocyte Differentiation

Autophagy affects T lymphocyte differentiation. Part of this effect is achieved by controlling innate immune cells. For example, autophagy-deficient macrophages secrete IL-1α and IL-1β in large amounts and induce Th17-type T lymphocyte immune responses under the synergistic effect of IL-6 and TGF-β. Marrow-like cells in ATG5-deficient mice have higher levels of IL-17 after infection with *M. tuberculosis*. The use of *M. tuberculosis* antigen to stimulate the lymphocytes of lung cells of ATG5-deficient mice in vitro also showed a similar phenomenon of increased IL-17 expression in $CD4^+$ T lymphocytes. Additionally, autophagy defects lead to a prolonged duration of binding of DCs to T lymphocyte immune synapses, as well as tilting the T lymphocytes toward the Th17 type.

On the other hand, different cytokines present in the immune environment can induce the corresponding differentiation direction of T lymphocytes. As mentioned above, autophagy has a regulatory effect on cytokine production and secretion, and it is bound to affect the direction of T lymphocyte differentiation. For example, rapamycin induces autophagy to inhibit IL-1 secretion, and IL-1 itself activates autophagy, so autophagy itself constitutes a negative feedback mechanism that regulates IL-1-induced inflammation. IL-1 synergizes with IL-23 to drive Th17 polarized differentiation, so autophagy plays an essential role in regulating Th17-type lymphocyte responses.

Recent studies have found that the number and short-term activation of $CD4^+$ T cells in mice knocked out for ATG5 are not significantly different, but the ability to produce antibodies is significantly reduced. T cell-specific knockout of ATG16l1 in mouse enteritis animals showed a type II immune response and a lack of $Foxp3^+$ regulatory T (Treg) cells. Knocking out ATG16l1 specifically in $Foxp3^+$ Treg cells reduced the activity of intestinal Foxp3(+) Treg cells. Autophagy inhibits the ability of Th2 cells to expand. During selective autophagy, p62 inhibits the secretion of IL-9 by Th9 cells by degrading the transcription factor PU.1 of Th9 cells. The autophagy inhibitor chloroquine reverses the above effects and considerably promotes the secretion of IL-9, thereby exerting an antitumor effect.

#### 27.4.1.5 Autophagy and CD8$^+$ T Lymphocytes

CD8$^+$ T lymphocytes are essential cells for the body to eliminate viral infections. Scientists at the Emory University Vaccine Center in the United States vaccinated volunteers to detect the genes change in the blood. It was found that the *gcn2* gene was rapidly activated, producing a large number of CD8$^+$ T lymphocytes involved in the immune response (Ravindran et al. 2014). The protein encoded by gcn2 is a sensor for detecting amino acid levels in the cell and is involved in the regulation of autophagy. When DCs are infected by yellow fever virus, the amino acids in the cells are consumed in large quantities, and the autophagy of DCs is activated by GCN2, which enhances the antigen presentation ability of DCs to CD8$^+$ T lymphocytes. The absence of the gcn2 gene impairs the ability of DCs to activate CD8$^+$ T lymphocytes, and mice deficient in the gcn2 gene are unable to produce an effective immune response to yellow fever vaccine and influenza vaccine. Recent studies have found that herpes simplex virus 1 can disrupt the function of activated CD8$^+$ T cells by interfering with macroautophagy in mouse DCs.

### *27.4.2 The Effects of Autophagy on B Lymphocytes and Antibody Immune Response*

#### 27.4.2.1 Overview

Autophagy is involved in maintaining the survival of B lymphocytes and B1 lymphocytes in the precursor phase and is critical for the function, survival, and homeostasis of plasma cells. Highly secreting plasma cells require autophagy to maintain their endoplasmic reticulum function. Autophagy has a significant protective effect on the bone marrow plasma cell bank and participates in and maintains long-term humoral immune memory.

#### 27.4.2.2 Autophagy Is Required for the Survival and Development of B Lymphocytes

Autophagy is not essential for the survival of most mature B lymphocytes. Autophagy defects mainly affect the survival of B lymphocytes and B1 lymphocytes in the precursor phase (Miller et al. 2008). B1 lymphocytes are a self-renewing B lymphocyte subset, which is different from traditional bone marrow-derived B lymphocytes (B2 lymphocytes), which are non-bone marrow-derived and T lymphocyte-independent cells. B1 lymphocytes are capable of secreting many types of autoantibodies. Studies have found that the survival of B1 lymphocytes depends on autophagy involvement. Transplantation of fetal liver progenitor cells from ATG5-deficient mice to lethal dose-radiated mice increased B lymphocyte death in bone marrow-reconstructed

mice and impaired the pro-B lymphocyte transition to pre-B lymphocytes, eventually leading to a significant reduction in the number of peritoneal and peripheral antibody-secreting B lymphocytes B-1a cells. Another study on the specific knockout of mouse *ATG5* in $CD19^+$ (mature B lymphocyte marker protein) B lymphocytes found that, although there were normal numbers of mature B lymphocytes and normal marginal zone B lymphocytes/follicles B in the mouse, the number of B-1a cells of antibody-secreting B lymphocytes was significantly reduced, suggesting that autophagy plays a crucial role in maintaining secreting B-1a B lymphocytes. Recent studies on pro-B cells, found that in the pre-B cell-specific knockout of ATG5 transgenic animals, autophagy does not affect the transformation between the two cells but plays an essential role in maintaining the basal level of surrounding mature B cells. Autophagy of B cells in the kidney promotes the secretion of antinuclear antibodies and increases the number of long-lived plasma cells, thereby maintaining normal humoral immune responses.

#### 27.4.2.3 The Roles of Autophagy in Plasma Cell Differentiation and Antibody Response

Plasma cells are B lymphocytes in the terminal activation phase, which are the primary effector cells for acquired humoral immune responses. B lymphocytes present in the secondary lymphoid organs, such as the spleen and lymph nodes, differentiate into short-lived plasma cells after encountering an antigen. Repeated stimulation of T lymphocytes can produce memory B lymphocytes and long-lived plasma cells, which can survive for the lifetime of an individual in a specialized bone marrow niche, maintain basal antibody levels and retain immune memory against specific antigens, thereby enabling the timely generation of antibodies that protect against pathogens and toxic substances.

Plasma cells are specialized antibody-secreting cells in which a large number of antibodies are synthesized, assembled, and secreted. The differentiation from B lymphocytes to plasma cells is an intrinsic remodeling process mediated by cellular stress that is related to proteomic plasticity, involving the complicated relationships between cell pressure, metabolism, and cell renewal. The regulation of antibody production involves the study of protein folding and assembly in this specialized secretory cell, as well as the analysis of the components of the bone marrow long-lived plasma cell and its surrounding environment. It helps to understand the mechanism of the generation of antibody memory and the formation of plasma cell degenerative diseases such as monoclonal gamma globulin disease and multiple myeloma. Autophagy plays an essential role in plasma cell differentiation, which involves the balance between the endoplasmic reticulum, differentiation, and antibody production. Autophagy is an intrinsic factor that determines the fate of long-lived plasma cells and the long-term immunity of the body.

As professional antibody-secreting cells, plasma cells are capable of synthesizing, assembling, and secreting large amounts of antibodies. B lymphocytes must remodel their proteome structure, rapidly silence B lymphocyte functions in a genetically

encoded manner, inhibit the expression of the transcription factors PAX5 and BCL-6, and regulate IRF4 and PRDM1/Blimp-1 expression by inducing transcription, resulting in the establishment of plasma cell function. In the early stage of plasma cell differentiation, the critical misfolding reaction protein XBP-1 can increase the protein secretion load by driving the expansion and folding ability of the endoplasmic reticulum. The process of assembling and processing a large number of antibodies through the endoplasmic reticulum is also closely related to metabolic stress and oxidative stress, and the antioxidant response is one of the adaptive mechanisms to address these stressors. Plasma cells undergo proteasome pressure, and their strong secretion capacity also means degradation of a large number of antibody production byproducts via the proteasome degradation pathway. However, the proteasome component in short-lived plasma cells is significantly reduced, resulting in excessive consumption of free ubiquitin and accumulation of polyubiquitinated proteins. In addition, proapoptotic factors are stabilized, thereby establishing an intrinsic mechanism to reduce the death threshold and a temporary restriction on the antibody response. For the above reasons, plasma cells are particularly sensitive to apoptosis induced by proteasome inhibition. Even malignant transformed tumor cells, such as multiple myeloma cells, are equally sensitive. The proteasome inhibitor bortezomib can be used to reduce antibody-mediated autoimmune diseases.

Autophagy is a conservative self-digestive physiological process. As a major recovery pathway, autophagy is a possible resource for cell remodeling. Moreover, the differentiation process of various cells, including adipocytes, red blood cells, and lymphocytes, is dependent on autophagy. Pengo et al. found a robust autophagy-inducing effect during plasma cell differentiation. LPS was used to stimulate primary cultured B lymphocytes, and the number of LC3-positive autophagosomes and the acidic lysosomal fraction increased as the stimulation time was prolonged (Pengo et al. 2013). Bafilomycin or lysosomal inhibitor $NH_4Cl$ could further increase the number of LC3-positive autophagosomes. These results indicate that the autophagic flow is in an activated state in the early B lymphocyte activation process. The expression of LC3, ATG7, ATG9, and ATG4a increased, and the expression of the autophagy receptor p62 increased rapidly at the beginning of B lymphocyte activation and then gradually decreased. Compared with $CD19^+$ B lymphocytes, $CD138^+$ (specific markers of plasma cells) lymphocytes isolated from spleen have more LC3-positive fluorescent autophagic plaques, suggesting that autophagy participates in the differentiation of B lymphocytes into plasma cells.

Pengo et al. found that knocking out *ATG5* specifically in B lymphocytes inhibits autophagy (Conway et al. 2013). LPS can stimulate the differentiation of B lymphocytes into plasma cells. By comparing the changes in the *ATG5*-deficient and wild-type B lymphocyte proteomes, the authors found that *ATG5*-deficient B lymphocytes contained a large accumulation of antibody and unfolded protein in the endoplasmic reticulum during plasma cell differentiation, while mitochondria and ribosomal components did not show a significant change. The endoplasmic reticulum showed significant enlargement and increased activity in *ATG5*-deficient B lymphocytes, which was reflected in the accumulation of mRNAs encoding spliced XBP-1, total XBP-1, and BiP. Treatment with the lysosomal protease inhibitor leupeptin or

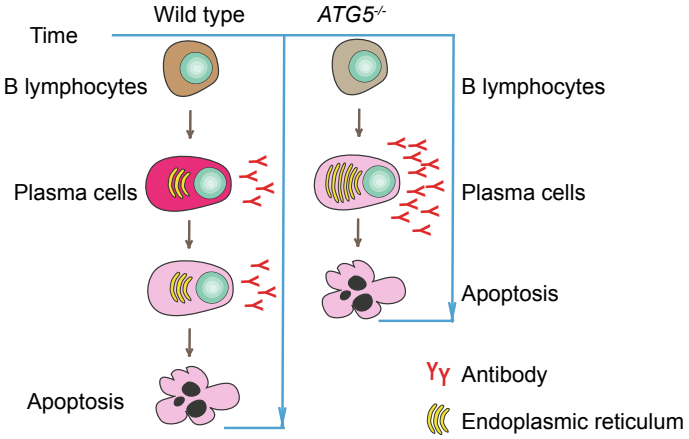


**Fig. 27.3** Effects of autophagy on plasma cell biology and antibody secretion. Knocking out *ATG5* in plasma cells causes autophagy deficiency, increased endoplasmic reticulum, and rapid secretion of large amounts of antibodies, ultimately resulting in premature plasma cell apoptosis

E-64d can also cause an accumulation of unfolded protein in the endoplasmic reticulum of B lymphocytes. Blimp-1, a key transcriptional regulator of plasma cells, is induced by endoplasmic reticulum stress and drives antibody expression. However, during increased endoplasmic reticulum stress responses, *ATG5*-deficient B lymphocytes have higher Blimp-1 mRNA and IgM μ chain levels. Treatment with the endoplasmic reticulum stress inducer tunicamycin of wild-type B lymphocytes also caused increased expression of Blimp-1 and IgM μ chain. ATG5-deficient B lymphocytes are capable of synthesizing and secreting a large amount of antibody (Fig. 27.3), which also indicates that autophagy has a certain limiting effect on the synthesis of antibody proteins.

Significant energy changes occur during plasma cell differentiation. Autophagy needs to meet the high metabolic requirements required for plasma cell differentiation and antibody secretion. In resting B lymphocytes, autophagy is not critical for ATP production. After three days of LPS stimulation, the ATP produced by *ATG5*-deficient B lymphocytes could not reach 50% of the level in wild-type B lymphocytes, suggesting that more *ATG5*-deficient B lymphocytes have undergone apoptosis. These results indicate that autophagy is involved in maintaining the energy and survival of B lymphocytes during plasma cell differentiation.

#### 27.4.2.4 Autophagy Prolongs Antibody Secretion

It was found that specifically knocking out *ATG5* in B lymphocytes produced lower levels of IgM and IgG antibodies only in mice immunized with T lymphocyte-dependent antigens and non-T lymphocyte-dependent antigens. In a mouse model of antigen-specific immunity, parasitic infection, and mucosal inflammation, knockout

of *ATG5* in mouse B lymphocytes can significantly reduce the antibody response. Although in vitro experiments have shown that the synthesis and secretion of antibodies by *ATG5*-deficient B lymphocytes are increased due to the lack of protection from autophagy, more B lymphocytes undergo apoptosis, which attenuates the effect of enhanced antibody secretion. To validate the above hypothesis, the researchers immunized mice with hapten NP-Ficoll, which is capable of continuously activating B lymphocytes. Two weeks after NP-Ficoll immunization, the number of plasma cells in B lymphocyte-specific *ATG5* knockout mice did not change compared with wild-type mice, but more NP antibodies were produced. This finding suggests that a single *ATG5*-deficient plasma cell has higher antibody secretion activity than a wild-type plasma cell. Batista et al. found that under viral infection, autophagy is highly activated in B cells and germinal center cells. Unlike the classical autophagy signaling pathway activated by rapamycin, GC B cells mainly undergo nonclassical autophagy signaling pathways. This indicates that autophagy regulates the activation state of B cells.

#### 27.4.2.5 Effects of Autophagy on Long-Lived Plasma Cells of the Bone Marrow

Autophagy reduces the expression of the transcriptional repressor Blimp-1 and immunoglobulin by inhibiting the endoplasmic reticulum stress response and its signaling, reducing the excessive consumption of energy. This mechanism allows the in vivo antibody response to persist and is an essential intrinsic mechanism to maintain the niche of bone marrow-derived long-lived plasma cells. Bone marrow-derived $CD19^{-}CD138^{hi}$ plasma cells have more LC3-positive fluorescent plaques. However, compared to wild-type mice, the total amount of antibody in the circulation and the number of bone marrow-derived plasma cells did not change in B lymphocyte-specific *ATG5* knockout mice. This phenomenon may be due to the reemergence of many ATG5 transcripts and LC3-positive fluorescent plaques in bone marrow-derived plasma cells B lymphocyte-specific *ATG5* knockout mice compared with the $B220^{hi}CD138^{lo}$ cells derived from spleen, suggesting a partial recovery of autophagy in the bone marrow. However, 11 months after immunization with NP-CGG antigen, NP antigen-specific, bone marrow-derived, long-lived plasma cells in B lymphocyte-specific *ATG5* knockout mice were reduced by 90% compared to wild-type mice. The reason for the decrease in long-lived plasma cells in the bone marrow is due to the increase in plasma cell death because of impaired autophagy function and the decrease in MHC II-restricted antigen-presenting ability dependent on autophagy. Finally, the interactions between B lymphocytes and T lymphocytes are blocked.

Long-lived, donor-reactive memory B cells (Bmems) generate homologous antibodies that regulate the posttransplant immune response. Knocking out *ATG7* to block B cell autophagy can inhibit the type II allergic reaction, does not affect the type I allergic reaction, and inhibits the antibody production frequency in Bmems. This finding suggests that targeted autophagy has the potential to clear the Bmems

response. The Epstein–Barr virus (EBV) tumor protein EBNA3C is an essential protein for the transformation of primitive B cells and the maintenance of lymphocyte expansion. EBNA3C affects the cell cycle and apoptosis by regulating ubiquitination-mediated protein degradation and gene transcription. Recent studies have found that tumor viruses (such as EBV) promote cell survival by tampering with autophagy. EBNA3C upregulates ATG3, ATG5, and ATG7 gene transcription to activate autophagy (Bhattacharjee et al. 2018). Under conditions of nutrient deprivation, EBNA3C up-regulates tumor suppressor genes such as p27Kip1 to activate autophagy. These findings suggest that EBNA3C acts as a survival mechanism by regulating autophagy and provides a potential therapeutic approach for EBV-induced B-cell lymphoma.

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# Chapter 28
# Autophagy and Immune Tolerance


**Yao-Xing Wu, Shou-Heng Jin and Jun Cui**

**Abstract** The immune system plays a critical role in defense against invading pathogens, and its function must be strictly controlled to maintain intracellular homeostasis. Once suffering microbial invasion or receiving danger signals, the immune system initiates the responses timely. After the threat removal, the immune system should be shut down to avoid the harm caused by excessive immune activation. Additionally, the immune system needs to be internally adjusted so that it does not respond to self-antigens to avoid autoimmune diseases. The states of nonresponse in immunity are termed as immune tolerance. Numerous studies indicated that macroautophagy (hereafter named as autophagy) is involved in T cells and B cells related immune tolerance. Recently, more and more researches demonstrated that autophagy is not only capable of nonselective degradation of cellular macromolecular components but also responsible for sorting and transporting autophagic substrates through a group of cargo receptors for selective degradation, which is called as selective autophagy. Recent studies indicated that selective autophagy can effectively regulate the immune tolerance and avoid over-activation of immune response by targeting multiple receptors and effectors of immune cells. In this chapter, we will focus on how autophagy participates explicitly in the adaptive and innate immune tolerance.

**Keywords** Autophagy · Immune tolerance · Selective autophagic degradation · Regulation

## Abbreviations

| | |
|---|---|
| AIM2 | Absent in melanoma 2 |
| AMPK | Adenine monophosphate activated protein kinase |
| APC | Antigen-presenting cell |
| ASC | Apoptosis-associated speck-like protein containing a CARD |

Y.-X. Wu · S.-H. Jin · J. Cui (✉)
MOE Key Laboratory of Gene Function and Regulation, State Key Laboratory of Biocontrol, School of Life Sciences, Sun Yat-Sen University, Guangzhou, China
e-mail: cuij5@mail.sysu.edu.cn



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| | |
|---|---|
| BMDM | Bone marrow derived macrophages |
| caspase | Cysteinyl aspartate specific proteinase |
| CCL5 | CC chemokine ligand 5 |
| CD4 | Cluster of differentiation 4 |
| cGAMP | Cyclic guanosine monophosphate–adenosine monophosphate |
| cGAS | Cyclic GMP-AMP synthase |
| cTEC | Thymic cortical epithelial cell |
| CTLA-4 | Cytotoxic T-lymphocyte-associated protein 4 |
| DAMP | Danger-associated molecular pattern |
| DC | Dendritic cell |
| DP | Double-positive cell |
| EGR | Early growth response protein |
| FAO | Fatty acid oxidation |
| FAS | Fatty acid biosynthesis |
| GSDMD | Gasdermin D |
| GWAS | Genome-wide association study |
| GZMB | Granzyme B |
| HFD | High-fat diet |
| HSV-1 | Herpes simplex virus type 1 |
| IFN | Interferon |
| IKK | IκB kinase |
| IL | Interleukin |
| ILC | Innate lymphoid cell |
| IRF3 | Interferon regulatory factor 3 |
| ISG15 | Interferon-stimulated gene 15 |
| JAK2 | Janus kinase 2 |
| JIA | Juvenile idiopathic arthritis |
| LC3 | Microtubule-associated proteins light chain 3 |
| LPS | Lipopolysaccharide |
| LRRC25 | Leucine-rich repeat-containing protein 25 |
| MAVS | Mitochondrial antiviral-signaling protein |
| MHC | Major histocompatibility complex |
| mTOR | Mammalian target of rapamycin |
| MyD88 | Myeloid differentiation primary response 88 |
| NBR1 | Neighbor of BRCA1 gene 1 |
| NDP52 | Nuclear dot protein 52 kDa |
| NF-κB | Nuclear factor kappa-B |
| NK | Natural killer cell |
| NLRP3 | NACHT, LRR and PYD domains-containing protein 3 |
| NOD2 | Nucleotide-binding oligomerization domain-containing protein 2 |
| OPTN | Optineurin |
| PAI-2 | Plasminogen activator inhibitor type 2 |
| PAMP | Pathogen-associated molecular pattern |
| PD1 | Programmed cell death protein 1 |
| PRR | Pattern recognition receptor |

| | |
|---|---|
| PTPN1 | Non receptor type 1 |
| RIG-I | Retinoic acid-inducible gene I |
| ROS | Reactive oxygen species |
| SKP2 | S-phase kinase-associated protein 2 |
| STAT1 | Signal transducer and activator of transcription 1 |
| STING | Stimulator of interferon genes protein |
| TAK1 | Transforming growth factor activated kinase-1 |
| TBK1 | TANK-binding kinase 1 |
| TCR | T cell receptor |
| TGF-β | Transforming growth factor-β |
| Th | Helper T cell |
| TLR | Toll-like receptor |
| TNF | Tumor necrosis factor |
| Treg | Regulatory T cell |
| TRIF | TIR domain-containing adapter molecule 1 |
| TRIM | Tripartite motif containing |
| ULK1 | UNC-51-like kinase 1 |
| VPS34 | Vacuolar protein sorting 34 |

## 28.1 Immune Tolerance and Autophagy

### *28.1.1 Introduction of Immune Tolerance*

#### 28.1.1.1 The Formation and Manifestation of Immune Tolerance

One of the major functions of the immune system is to distinguish the "self" and "non-self" components and generate the effective responses to eliminate the "non-self" substances (usually named as antigens). After recognition of "non-self" antigens, the immune cells can be activated and go through a set of cellular processes including proliferation and differentiation to generate proper immune responses. However, when faced with "self" antigens, immune cells display low responses or even no responses. These states of unresponsiveness of the immune system under certain conditions are considered as immune tolerance. As a particular form of an immune response, immune tolerance generally shows no reaction to a specific allogeneic antigen, while maintains a normal response to other antigens. Immune tolerance can be induced by prior exposure to specific antigens, which are called as tolerogens.

Although it has long been well accepted that the immune system does not respond to the host normal components (which is known as self-tolerance), it has not built up a theory before the studies from Medawar and Owen in the 1950s. In their studies, allogeneic antigens exposed at embryonic stage could induce immune tolerance. On this basis, Burnet has put forward the clonal selection hypothesis and expounded the

mechanism of the formation of acquired immune tolerance in 1957. This hypothesis states that the individual immune system is immature at the embryonic stage, and when the immune cells are exposed to the allogeneic antigens, such as host antigens at this stage. The immune cell clones that can respond accurately to these antigens will be suppressed or eliminated. Therefore, these specific antigens will not induce effective immune responses subsequently.

The formation and maintenance of acquired immune tolerance are affected by multiple factors, which can be divided into two major parts: antigen factors and host factors. In detail, the formation of immune tolerance can be affected by several antigen factors, including the physiochemical properties, dose, duration, and the epitope characteristics of the antigens. On the other hand, the formation and regulation of acquired immune tolerance can also be affected by the degree of development of the host's immune system and susceptibility to different stimuli.

It is traditionally believed that the formation of immune tolerance is mainly due to the regulation of the adaptive immune system such as the regulation of T cells and B cells. However, recent studies have revealed that the immune system can not only distinguish between "self" and "non-self" components, but also discriminate whether the relevant stimulus is harmful or not. For nearly 30 years study of the innate immune system, researchers have identified a series of receptors, termed as pattern recognition receptors (PRRs). PRRs recognize various danger signals in innate immune cells from both inside and outside the host cells, thus promoting the activation of the immune cells (especially the innate immune cells) and further directing the adaptive immunity to generate the corresponding immune responses. These danger signals are usually the fragments of both intracellular and extracellular biomacromolecules, called pathogen-associated molecule patterns (PAMPs) or danger-associated molecular patterns (DAMPs). Once recognized by PRRs, they activate an inflammatory response, interferon signaling, and other immune signaling pathways, which subsequently coordinate the response between innate immunity and adaptive immunity. To avoid the damage of excessive immune response induced by "danger signal" in cells, the host has evolved plenty of negative regulators and numerous mechanisms to orchestrate the innate immune response. Through regulating the tolerance of the innate immune cells to relevant stimuli in a specific period, the host maintains the balance of the immune system.

#### 28.1.1.2 The Regulatory Mechanism of Immune Tolerance

Based on when and where the formation of immune tolerance is originally induced, the acquired immune tolerance can be divided into central immune tolerance and peripheral immune tolerance. Central immune tolerance occurs mainly in the central immune organs and tissues of the host (thymus and bone marrow). During embryonic and newborn stages, immature T cells and B cells acquired tolerance to self-antigens through a series of processes including positive selection and negative selection during their maturation. In this process, cells with receptors specific for self-antigens are removed. In the thymus, T cells which are not capable of recognizing MHC-I or

MHC-II will be removed by apoptosis, which is called positive selection. In addition, T cells which interact with MHC-I/II alone or MHC-I/II-self-peptide complex with high affinity will also be induced to apoptosis, which is called negative selection. The process similar to negative selection occurs in the bone marrow during the development of B cells. B cells with autoreactive membrane antibodies will die by apoptosis, leading to clonal deletion. In the process of central tolerance, the immune system learns to discriminate self from non-self by eliminating the autoreactive T cells or B cells clones before the lymphocyte clones develop into fully functional immune cells.

Central immune tolerance could not completely remove autoreactive antigen-specific T cells and B cells, as many self-antigens do not exist in central lymphoid organs and cannot be presented during the embryonic period. In general, these "concealed" self-antigens won't induce immune response even though exposing to peripheral lymphocytes. Moreover, B cells with different receptor specificity due to the mutation of antibody gene (mainly in peripheral lymphoid organs or tissues) will also be removed or suppressed by peripheral tolerance. The mechanisms of regulating peripheral immune tolerance are various, including clonal ignorance, clonal deletion, clonal anergy, etc. In recent years, mounting studies have revealed that peripheral immune tolerance can be regulated by a variety of immunosuppressive cells (such as regulatory T cells and myeloid inhibitory cells) and diverse inhibitory immunomodulatory molecules (including cytokines like IL-10 and inhibitory receptors like CTLA-4 and PD1) under different conditions.

During the innate immune responses, a large amount of PAMPs and DAMPs can be recognized by PRRs, which in turn activate the NF-κB signaling network, inflammasomes, and type I/III interferon (IFN) signaling pathways, thus leading to inflammatory and antiviral responses in innate immunity and subsequent adaptive immunity. The continuous activation of inflammation and IFN can cause damage in the host cells, so their activity must be tightly regulated. A lot of downstream genes of NF-κB signaling and type I IFN signaling can negatively regulate these pathways through negative feedback loops, thus mediating cell tolerance for constant stimulation. Also, other cellular physiological processes such as endocytosis, autophagy, and proteasome degradation pathway can also alter the molecular localization and concentration of key receptors and regulators in the innate immune signal network, which subsequently regulates the tolerance of innate immune responses.

## *28.1.2 Autophagy Regulation of Immune Tolerance*

### 28.1.2.1 Autophagy and Immune Tolerance

Autophagy is a fundamental physiological process in eukaryotes. Initially, intracellular substances, including dysfunctional and aging organelles, harmful protein aggregates, and microbes that infect the host, are wrapped into the double-layer membrane and thus autophagosomes are formed. Then, autophagosomes fuse with

lysosomes to form autolysosomes, which results in the degradation of substrates. The main processes of autophagy include the initiation of autophagy, extension of the membrane, formation of autophagosome, fusion of autophagosome and lysosome, and the degradation of substrates.

The stimulation of autophagy, such as hunger, can initiate autophagy by suppressing the mTOR signaling pathway. mTORC1 complex, the principal receptor in the mTOR signaling pathway, inhibits the activity of ULK1 complex composed of UNC-51-like kinase 1 (ULK1), ATG13, ATG101, and FIP200. The activation signaling of autophagy can activate the ULK1 complex by inhibiting the activity of mTORC1, leading to the initiation of autophagy and the activation of the downstream Beclin-1 complex. The Beclin-1 complex is composed of Beclin-1, Phosphatidylinositol 3-phosphokinase VPS34, VPS15, and ATG14L. After the activation of Beclin-1 complex, phosphatidylinositol-3-phosphoric acid is produced, which can promote the nucleation of the autophagosome. The extension of autophagosome requires two ubiquitin-like conjunction systems, ATG5-ATG12 conjunction system and microtubule-associated protein light chain 3 (LC3/ATG8) conjunction system. The transformation of cytosolic soluble LC3-I to phosphatidylethanolamine-modified LC3-II is essential for autophagy. Subsequently, the expanding autophagosome fuses with lysosome with the help of SNARE-like proteins. In the lysosome, the substrates are further degraded by acid hydrolases, releasing amino acids and lipids, which can be reused as energy material. Autophagy can not only regulate the degradation of intracellular substances but also ensure the survival of cells by circulating nutrients during starvation. It plays an indispensable role in regulating the homeostasis of various immune cells and inducing immune tolerance.

Autophagy occurs in almost all of the immune cells. In T and B cells, it plays an essential role in central tolerance and peripheral tolerance. By generating a series of autophagy-deficient mouse models via knocking out related genes, including *Atg5* and *Atg16L*, several studies have shown that deficiency of autophagy leads to significant inhibition of the positive and negative selection of T cells and severe autoimmune diseases, which suggests that autophagy plays a vital role in the central tolerance. In addition, autophagy can affect peripheral tolerance by influencing the development of B cells and memory lymphocytes and regulating the activity of regulatory T cells. Moreover, autophagy is also involved in regulating the immune tolerance of nearly all kinds of innate immune cells including dendritic cells, macrophages, and natural killer cells, by regulating their function, survival, and differentiation. Autophagy can inhibit the maturation of immune dendritic cells and regulate their antigen presentation activity. Autophagy also participates in macrophage differentiation, and defect of autophagy leads to excessive activation of inflammatory macrophages. In addition, autophagy can affect survival and tumor-related immune tolerance of natural killer cells and mast cells. In summary, autophagy is indispensable for both adaptive and innate immune tolerance at multiple levels.

In addition to directly affecting immune tolerance through autophagy flux, autophagy can also affect inflammation, IFN, and other crucial immune signal pathways through ULK1, Beclin-1, ATG5, and other autophagy-related proteins in an

autophagy-independent manner, thereby negatively regulating immune response and inducing immune tolerance.

#### 28.1.2.2 Selective Autophagy and Immune Tolerance

Besides delivering intracellular substances to lysosomes for degradation and updating the energy materials required for metabolism upon starvation, autophagy can also specifically target proteins aggregates and damaged organelles or invading pathogens, mediating a process known as selective autophagy. The occurrence of selective autophagy depends on the participation of a series of cargo receptors, including p62/SQSTM1, NBR1, OPTN, NDP52, etc. In selective autophagy, cargo receptors determine the specificity of degradation substrates and are consumed together with the cargos. Recent studies have revealed that key receptors (RIG-I, cGAS, AIM2, etc.), the adaptor proteins (MAVS, TRIF, STING, etc.), kinases (IKK complex), and downstream transcription factors (p65) can be delivered to selective autophagic degradation by cargo receptors, thus avoiding the sustained activation of corresponding signaling and mediated stimulation-induced immune tolerance. Previous studies have indicated that substrate recognition of cargo receptors depends on ubiquitination, so the ubiquitin system is not only critical for intracellular proteasome degradation pathway, but also plays a crucial role in selective autophagy-mediated immunosuppression. Recent researches have revealed that cargo receptors such as p62 can also manipulate selective autophagy degradation through non-ubiquitin-dependent signaling, and such new regulatory mechanisms underlying immune response and tolerance remain to be clarified by further studies.

## 28.2 Adaptive Immune Tolerance and Autophagy

### *28.2.1 Regulation of Adaptive Immune Tolerance by Autophagy*

#### 28.2.1.1 Overview

Adaptive immune response refers to the process that antigen-specific T cells and B cells are activated under the stimulation of specific self or exogenous antigens and proliferate and differentiate into effector cells to generate a series of specific immune responses. It is generally accepted that the adaptive immune response can be divided into three stages including the antigen recognition stage, the proliferation and differentiation stage, and the effect stage as follows. In the first antigen recognition stage, antigen-specific T/B lymphocytes recognize antigens presented by antigen-presenting cells (APC) and initiate the activation stage. In the second

proliferation and differentiation stage, the T/B lymphocytes will be activated by specific antigen and proliferate/differentiate into effector cells under the coordination of co-stimulatory molecules and cytokines. In the final effect stage, T cells will release cytokines and cytotoxins, and plasma cells will secrete antibodies. In the process of the adaptive immune response, the appropriate immune response is based on the trait of lymphocytes to recognize "self" and "non-self" substances. In other words, antigen-specific T cells and B cells display immune tolerance to host normal tissues and cells but immune rejection to exogenous antigens. Adaptive immune tolerance is a phenomenon in which antigen-specific lymphocytes cannot be activated or do not generate immune responses under specific self-antigen stimulation. The adaptive immune tolerance can be divided into central tolerance and peripheral tolerance based on the period of generation, the cause of induction, and the mechanism of generation. Central tolerance is generally induced in the embryonic phase, in which adaptive immune cells such as T cells and B cells develop in the central immune organs. The cell clones which recognize self-antigens will be removed or in the state of unresponsiveness. Peripheral tolerance refers that mature T cells, and B cells do not generate positive immune responses when they enter peripheral immune organs and face self or exogenous antigens. Peripheral tolerance is the main mechanism of immune tolerance in adults. Immune tolerance is of great significance to the maintenance of normal physiological function of the body. In detail, central tolerance is the main way for the adaptive immune system to distinguish enemies from selves while peripheral tolerance is the key to prevent the immune system from overreacting to environmental entities. Central tolerance or peripheral tolerance deficiency may lead to a variety of autoimmune diseases including systemic lupus erythematosus, rheumatoid arthritis, and type I diabetes and may lead to asthma, allergy, and inflammatory bowel diseases. However, excessive adaptive immune tolerance can result in a series of serious consequences, including the escape of pathogenic microorganisms or tumors from the host's immune responses. Therefore, adaptive immune tolerance needs to be strictly regulated.

As a highly conserved degradation system, autophagy can clear damaged cellular structures, aging organelles, and useless macromolecules as well as provide energy and nutrients for reuse of cellular structures, and thus maintains living activities and normal metabolism of cells. Therefore, autophagy plays a key role in coordinating adaptive immune responses mediated by intracellular pathogens and autoantigens. On the one hand, autophagy can directly engulf pathogenic microorganisms and present pathogenic microbial antigens to the adaptive immune system, and thus regulates adaptive immune responses mediated by lymphocytes. On the other hand, by regulating the metabolism and longevity of immune cells, autophagy can maintain lymphocytes homeostasis and the proportion of different immune cells subsets and inhibit the excessive immune response to induce adaptive immune tolerance. Therefore, understanding the effect of autophagy on adaptive immune tolerance is of great significance in the study of adaptive immune responses, autoimmune diseases, tumor immunity, and transplantation immunity. In this section, the regulatory mechanism of autophagy in coordinating adaptive immune response and autoimmune tolerance is discussed.

#### 28.2.1.2 Autophagy Regulates Adaptive Immune Tolerance Through Multilayer Mechanisms

With the growing understanding of autophagy and adaptive immune tolerance, accumulating studies have revealed the pivotal role of autophagy in adaptive immune tolerance (Kabat et al. 2016b). Negative selection is the primary mechanism of the formation of central tolerance in T cells. Antigen presentation in central immune organs depends on autophagy in thymocytes. While in peripheral immune organs, autophagy not only affects the formation, development, and homeostasis of T/B lymphocytes but also regulates the distribution of lymphocyte subsets. During the activation of lymphocytes, autophagy controls the anergic state of lymphocytes and affects the proliferation of effector lymphocytes. In addition, autophagy also plays a vital role in the formation of memory lymphocytes. Thus, autophagy plays a direct and indispensable role in both central and peripheral tolerance. Moreover, the negative regulation of immunomodulatory cells and immune molecules also affects the formation of adaptive immune tolerance. Autophagy can not only affect the differentiation and function of immunomodulatory cells like regulatory T cells and regulatory dendritic cells but also regulate the production and clearance of regulatory cytokines such as TGF-β and IL-10. In summary, autophagy can regulate adaptive immune tolerance through multilayer mechanisms: autophagy controls the homeostasis, development, proliferation, and activation of lymphocytes and directly regulates adaptive immune response and immune tolerance; moreover, autophagy also indirectly restricts the excessive activation of adaptive immune signaling pathway by influencing immunomodulatory molecules and negative regulatory immune molecules, and thus monitors cells' adaptive immune tolerance (Fig. 28.1).

### *28.2.2 Autophagy Regulation of Central Tolerance*

#### 28.2.2.1 Overview

During T/B lymphocytes development in the primary lymphoid organs such as thymus and bone marrow, any self-reactive T/B lymphocytes are deleted or induced to be anergic, which ensures that immune system does not respond to self-antigens. Immune tolerance induced in primary lymphoid organs is considered as central tolerance. In the case of the development of T cells, double-positive $CD4^+CD8^+$ T cells (DP) migrate to the thymic cortex and react with cortical thymic epithelial cells (cTECs). Meanwhile, double-positive T cells go through a positive selection and negative selection to acquire MHC-restriction and self-tolerance, respectively. During positive selection, T cells with proper affinity to MHC class I or MHC class II compartments on the surface of TECs will separately differentiate to $CD8^+$ and $CD4^+$ single-positive cells, while those with low affinity will be induced to apoptotic cell death. Subsequently,

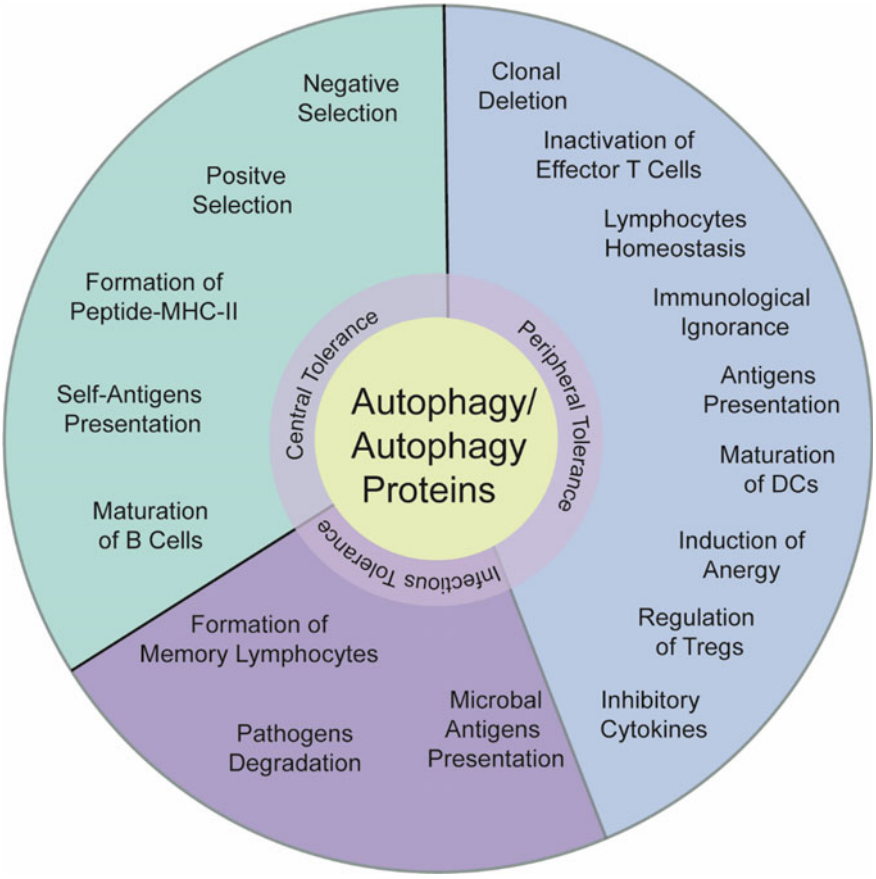


**Fig. 28.1** Autophagy regulates adaptive immune tolerance at multiple levels. In the generation of central tolerance, autophagy is involved in the formation of antigen presentation and its own antigen peptide-MHC-II molecular complex in central immune organs and plays a key role in both negative and positive selection of central tolerance. For peripheral tolerance, autophagy not only affects the formation and homeostasis of T/B lymphocytes, the proportion and distribution of lymphocyte subsets, and the incompetence of lymphocytes, but also controls the function of immunomodulatory cells such as regulatory T cells and regulatory dendritic cells, as well as the production of regulatory cytokines. During the formation of infection tolerance, autophagy is also involved in antigen presentation, memory lymphocyte formation, and protein degradation of pathogenic microorganisms. By participating in the regulation of adaptive immune response at multiple levels, autophagy plays a multifunctional role in adaptive immune tolerance

T cells with high affinity to self-peptide-MHC compartments will also undergo apoptosis, which is known as negative selection. As the only non-hematopoietic cells continuously expressing MHC molecules, TECs display a high basal level of autophagy even without stimulation such as starvation, indicating the potential functions of autophagy in TECs and the selection in the thymus (Nedjic et al. 2008).

#### 28.2.2.2 Autophagy Affects the Positive Selection

cTECs undergo positive selection through the self-antigens-MHC complex on their surfaces. As autophagy plays central roles in the formation of MHC-II complex, it is clear that autophagy is indispensable for positive selection. By using the *Atg5*$^{-/-}$ thymus transplantation mouse model, Nedjic and his colleagues showed that autophagy defect not only dramatically decreased MHC-II density on cTECs but also reduced the frequency of CD4$^{+}$ SP cells expressing MHC-II restricted T-cell receptors (TCRs). Moreover, deletion of autophagy also led to multi-organ inflammation in athymic mice grafted with *Atg5*$^{-/-}$ thymus, such as severe colitis (Nedjic et al. 2008). In conclusion, autophagy regulates the formation of MHC complexes on the surface of cTECs to participate in the positive selection of T cells, thus ensuring the generation of a functional and self-tolerant CD4 T-cell repertoire.

#### 28.2.2.3 Autophagy Affects the Negative Selection

Although the study above emphasizes the importance of autophagy in T cell positive selection, it is hard to distinguish whether the inflammation induced by autophagy deficiency is caused by the disorder of cTECs' positive selection or the lack of mTECs' negative selection. The evidence including LC3 puncta in both cTECs and mTECs under normal conditions and colocalization of LC3 puncta and MHC-II loading vesicles in these cells indicate the importance of autophagy in both positive and negative selections. Study from Aichinger and his colleagues shows that autophagy was dispensable in mTECs when the level of the antigen are high enough. Because APCs can compensate antigens peptide presentation and negative selection could be induced indirectly by APCs. However, at lower doses of antigen levels, autophagy-dependent direct antigen presentation in mTECs became indispensable (Aichinger et al. 2013). The importance of autophagy in negative selection can be supported by the infiltration of activated CD4$^{+}$ T cells into tissues in the athymic mice transferring *Atg5*-deficient thymus (Nedjic et al. 2008). However, as TECs had a normal proportion of T-cell subpopulations and did not exhibit significant autoimmune disease in *Atg7* conditional knockout mice, the association between autophagy and negative selection remains controversial (Sukseree et al. 2012). Collectively, autophagy may play a role in negative selection and removal of autoreactive lymphocytes through assisting the formation of MHC-II-self-antigens on mTECs.

## *28.2.3 Autophagy Regulation of Peripheral Tolerance*

### 28.2.3.1 Overview

Potential self-reactive T/B cells can be eliminated during negative selection. However, since negative selection is not perfect, there will be a fraction of lymphocytes with low affinity to self-antigens entering the immune periphery. It is necessary for peripheral tolerance to exist as a secondary safeguard to ensure that peripheral self-reactive lymphocytes do not cause the autoimmune disorder. Compared with central tolerance, mechanisms of peripheral tolerance are much more diverse, including direct inactivation of effector T cells by clonal deletion and induction of anergy, immunological ignorance, immunosuppressive function of regulatory T cells (Tregs), and regulatory roles of dendritic cells.

### 28.2.3.2 Autophagy Regulates the Survival and Homeostasis of T Cells and B Cells

Autophagy has dual functions in regulating cell survival. On the one hand, autophagy controls intracellular homeostasis and maintains cell survival. On the other hand, autophagy induces type II programmed cell death in specific situations. The dual functions of autophagy are also reflected in the regulation of the survival of T/B lymphocytes. It has been reported that although *Atg5*-deficient $CD4^+$ and $CD8^+$ T cells undergo full maturation in the thymus, $Atg5^{-/-}$ lymphocytes displayed remarkably increased cell death and failed to go through efficient proliferation under TCR stimulation. Subsequent studies further investigated the role of autophagy in T cell development and homeostasis by generating different mouse knockout models (Pua et al. 2007). Consistently, in a series of autophagy gene deficient T cells, including *Atg3*, *Atg5*, *Atg7*, *Atg16L*, *Vps34*, and *BECN1* knockout T cells, $CD4^+$ and $CD8^+$ T cells not only dramatically declined in number and proportion in the secondary lymphatic organ, but also failed to undergo active proliferation under the activation of TCR or CD28 (Kabat et al. 2016b).

In addition to the influence on T cell development and homeostasis, autophagy is also necessary for the efficient development of B cell in peripheral immune organs. Deletion of *Atg5* and *Atg7* in B lymphocytes exhibited a dramatical decrease in B cell development at the pro- to pre-B cell transition and insufficient B cell development in the bone marrow leads to increased cell death, which indicates the importance of autophagy in B cell development and survival. Moreover, autophagy was also required for the maintenance of B-1a B cells in the periphery (Miller et al. 2008). Collectively, the nutrients mobilization capacity of autophagy is critical for the development, survival, and proliferation of lymphocytes in the periphery, which ensures lymphocytes activity during immune responses.

Autophagy also plays essential roles in generation and maintenance of memory lymphocytes. For memory $CD8^+$ T cells, during the invasion of pathogens, naïve

$CD8^+$ cells will be activated and proliferated to remove the pathogens. After the elimination of pathogens, most activated $CD8^+$ T cells undergo apoptotic cell death, while a small proportion of remaining $CD8^+$ T cells will differentiate into memory $CD8^+$ cells. During the early phase of viral infection, deletion of autophagy-related genes failed to significantly affect the immune responses of primary $CD8^+$ cells as well as the release of antiviral cytokines and the control of viral titer. However, when $CD8^+$ T cells stopped dividing and entered the contraction phase, the autophagic activity enhanced rapidly in $CD8^+$ T cells, which implied the essential roles of autophagy in the formation of memory T cells (Puleston et al. 2014). Mice with *Atg5* or *Atg7*-deficient $CD8^+$ T cells failed to generate enough memory $CD8^+$ T cell responses in response to secondary infection and deletion of autophagy more likely led to tolerance toward chronic virus infection. Similar to the generation of memory $CD8^+$ cells, the maintenance and the function of memory B cells are mainly dependent on autophagy. Although autophagy did not influence the differentiation and proliferation of B cells, it maintained long-term survival of B cells, which might be due to the autophagic regulation of endoplasmic reticulum (ER) stress response in B cells.

Moreover, although autophagy is dispensable for primary antibody responses and the formation of initial memory B cells, it plays an indispensable role in the maintenance of memory B cells and secondary antibody responses during pathogens rechallenging. Mice with *Atg7*-deficient B cells could produce antibody generally during the first time of influenza virus infection. However, $Atg7^{-/-}$ B cells showed a drastic reduction of antibody, higher replication level of virus, and enhanced lung damage and death rates during secondary infection (Chen et al. 2014). Above all, in the late stages of viral infection, autophagy maintains the survival of immune cells and assists in the formation of memory immune cells, which protects the host from pathogens secondary infection.

#### 28.2.3.3 Autophagy Regulates T-Cell Anergy

The activation of $CD4^+$ T cells requires two signals. The first signal is derived by the engagement between TCR and its cognate peptide presented by MHC-II on APC. Co-stimulatory molecules of APCs provide the second signal. Lacking the costimulatory signals, T cells remain inactivated even after binding with antigens, which is considered as clonal anergy. In the peripheral immune organs, the clonal anergy of self-reactive T cells is the primary mechanism to maintain peripheral tolerance and prevent autoimmune diseases. A recent study shows that activation of autophagy could suppress clonal anergy of T cells to interrupt immune tolerance (Mocholi et al. 2018). Inhibition of autophagy, including autophagy inhibitors or knockout of key molecules of autophagy such as ATG7, resulted in increased expression of a set of anergic genes, including EGR2, EGR3, TLE4, and GRAIL, accompanied with decreased expression of activation signaling such as IL-2, and therefore leading to hyporesponsive state of T cells even upon stimulation. For further mechanism study, autophagy could promote the degradation of tyrosine kinase PTPN1, which

is considered to participate in the tolerance process of T cells and B cells. In an experimental autoimmune encephalomyelitis mouse model, inhibition of autophagy resulted in T cell anergy and decreased the severity of encephalomyelitis. Moreover, T cells from the juvenile idiopathic arthritis (JIA) patients, which displayed resistant to the regulation of Treg, had a low expression level of PTPN1. Autophagy inhibitor treatment for $CD4^+$ isolated from the synovial fluid of JIA patients could restore T cell anergy and enhance the expression of anergic genes. In summary, autophagy regulates T cell peripheral immune tolerance by inhibiting T cell anergy.

#### 28.2.3.4 Autophagy Regulates Regulatory T Cells and the Balance of T Cell Subsets

Tregs are a subset of immunosuppressive T cells. By direct interaction or secretion of inhibitory cytokines, Tregs can suppress induction and proliferation of effector T cells and inhibit the maturation of dendritic cells, subsequently modulating the immune system and tolerance to self-antigens. For this reason, Tregs play an essential role in maintaining peripheral tolerance and avoiding excessive immune responses. Autophagy is active in Tregs even without stimulation, which indicates the possible functions of autophagy in Tregs. Treg-specific deletion of *Atg7* or *Atg5* resulted in severe autoinflammatory disease in multiple organs. *Atg7*-deficient Tregs also appeared to have an increased level of apoptosis and decreased expression of cytokines such as Foxp3 and IFN-γ. Consistent with other T cells, autophagy deficiency also led to the disruption of metabolic balance in Tregs with significant upregulation of glycolysis, which contributed to defective Tregs function. Another study showed that autophagy deficiency not only enhanced glycolysis but also promoted fatty acid synthesis (FAS) and fatty acid oxidation (FAO) metabolism in Tregs, which subsequently reduced the survival of Treg (Wei et al. 2016). In summary, autophagy can maintain the stability of Tregs in peripheral immunity by regulating the metabolic balance, thus ensuring the functional integrity of Tregs in regulating peripheral immune signals and immune tolerance.

In addition to restricting the survival of Tregs, autophagy deficiency affects the proportion of T cell subsets. T cell-specific deletion of *Atg16L* led to a rapid reduction of Tregs as well as Th1 and Th17 effector cells, while ablation of autophagy facilitated the expansion of Th2 cells. It is believed that autophagy deficiency results in the enhancement of glycolysis of different cells. Activation of glycolysis promoted the apoptosis of Tregs, while Th2 cells are resistant to metabolic perturbations due to their high basal level of glycolysis. Imbalance of T cell subsets and aberrant activation of Th2 cells to antigens led to several allergic diseases, including asthma and food allergies (Kabat et al. 2016a). In conclusion, autophagy not only maintains the stability of Tregs to ensure their immunosuppressive capability but also alters the balance of the T cell subsets to avoid aberrant responses to antigens of Th cells. By modulating the stability of Tregs and the balance of Th1/Th2, autophagy plays a critical part in maintaining immune tolerance in peripheral immune organs.

#### 28.2.3.5 Autophagy Regulates Dendritic Cells

Dendritic cells (DCs) is one of the most well-known specific APCs. Without stimulation, most of the DCs stay in an immature state which expresses low levels of adhesion and co-stimulatory factors but have a strong ability of particle uptake. After receiving the signals from specific ligands, such as Toll-like receptor (TLR) ligands, DCs develop into a mature state which acquires a strong antigen-presenting cell capacity and the migratory potential. Mature DCs migrate to secondary lymphoid tissues and contact with naïve T cell to induce effector T-cell responses. When lacking inflammation or TLR stimulation, DCs are blocked in the semi-mature state. In this state, DCs secrete IL-10 instead of IL-12 and trigger Treg responses to maintain immune homeostasis and tolerance. Autophagy regulates the functions of DCs at multiple levels, including DCs' maturational program, antigen presentation capacity, migratory potential, and DC-mediated differentiation of T cells (Ghislat and Lawrence 2018).

DCs have dual functions in activating the immune response and mediating immune tolerance, and the function of DCs is determined by their degree of maturation. Autophagy plays an essential role in the maturation program of DCs. It has been reported that the proportion of mature DCs and the expression of co-stimulatory molecules (such as CD80 and CD86) were enhanced in *Atg16L1* knockout mice, which led to the proliferation of T cells and inflammatory disease, by using the mouse model of allogeneic hematopoietic stem cell transplantation (Hubbard-Lucey et al. 2014). Moreover, treatment of autophagy activators such as rapamycin could induce the tolerogenic DCs, and consequently, reduce the co-stimulation signal and increase IL-10 secretion to trigger Treg response (Ghislat and Lawrence 2018). Overall, the activation of autophagy inhibits the maturation of immune DCs by reducing the expression of CD86 and CD80 co-stimulatory signals and promotes the maturation of tolerant DCs by inducing the release of IL-10, thereby maintaining immune homeostasis and tolerance.

As antigen-presenting cells, the fundamental function of DCs is to deliver antigen-derived peptides to T cells. However, DCs' antigen-presenting process depends on the antigen's internalization and the formation of MHC-I and MHC-II molecules, which is primarily determined by autophagy. Autophagic machinery is required for MHC-II mediated presentation for both self and non-self antigens. Autophagic machinery is essential for the presentation of self-citrullinated peptides to $CD4^+$ T cells, as well as the processing of non-self-antigens, such as tumor or microbial antigens, and the formation of MHC-II-antigens peptide complex (Ireland and Unanue 2011). The MHC-II molecule is subsequently presented to $CD4^+$ T cell and triggers T cells immune response. Since autophagy is indispensable for the antigen presentation ability of DCs, plenty of pathogens inhibit DCs' antigen presentation by antagonizing autophagy, thus evading host immune response. For example, PE_PGRS47 protein from *Mycobacterium tuberculosis* could inhibit the activity of autophagy to suppress the MHC-II meditated antigens presentation to $CD4^+$ T cells in DCs, thereby escaping the T cell-mediated immune response (Ghislat and Lawrence 2018). Moreover, autophagy also participates in MHC-I mediated antigens presentation under certain

circumstance. Although most of the antigens presented by MHC-I are dependent on the ubiquitin–proteasome system (UPS), several autophagic substrates are derived by UPS-mediated MHC-I processing when autophagy is restricted. Autophagy can retain DCs' MHC-I internalization and restrict its induction of $CD8^+$ T cell responses. Collectively, autophagic machinery participates in antigens presentation process mediated by both MHC-II and MHC-I in DCs (Wenger et al. 2012). By regulating the antigen-presenting capacity of DCs, autophagy controls the differentiation and activation of T cells, thus moderating T cell responses after pathogens invasion. In addition, autophagy can also regulate DC migration, activation signaling of DCs, DCs-mediated differentiation of T cells, and the microenvironment of DCs. By controlling the physiological functions of DCs, autophagy plays a critical role in mediating immune responses and maintaining immune tolerance.

#### 28.2.3.6 Autophagy Regulates Cytokines in Immune Responses and Tolerance

Cytokines are a broad range of soluble small proteins produced by a broad category of cells under certain circumstances. Cytokines include interleukins, interferons, and tumor necrosis factors, which are closely relative with innate and adaptive immunity. It is generally believed that autophagy has multiple regulatory functions on cytokines. On one hand, autophagy can recycle energy and nutrients for the synthesis of cytokines; on the other hand, autophagy can mediate the degradation of cytokines. Recently, it provides a new direction for revealing the mechanism of immune tolerance to investigate the relationship between autophagy and cytokines.

Autophagy affects the release of several inflammatory cytokines and regulates the adaptive immune responses mediated by these cytokines. For instance, autophagy inhibits the release of IL-17 in $CD4^+$ T cells, thereby regulating Th1/Th17 cell balance and Th17-mediated immune response. As for IL-1β, autophagy not only inhibits NLRP3-mediated activation of IL-1β, but also specifically targets pro-IL-1β and facilitates its autophagic lysosomal degradation, thereby inhibiting the release of IL-1β. For IL-18, autophagy defect promotes the release of IL-18 and IL-1β upon stimulation of TLR activators by using autophagic inhibitors and autophagy genes deficient cells. In short, autophagy restricts the release of several interleukins, which may be associated with the mutation of autophagic genes such as *Atg16L*, which is often detected in autoinflammatory diseases (Saitoh et al. 2008). Autophagy can induce the production of IFN-γ and promote the inflammatory response mediated by IFN-γ. Autophagy is reported to participate in the JAK2-STAT1 pathway and inhibit the function of SHP2, an inhibitor of the IFN-γ pathway, thereby promoting the IFN-γ-induced downstream signaling.

Inhibitory cytokines, such as IL-10 and TGF-β, play an important role in immune defense. Inhibitory cytokines not only assist the removal of invading pathogens, but also induce the immune tolerance toward self-antigens. IL-10 has pleiotropic effects in immunoregulation, including antagonism against IFN-γ-induced immune response, inhibition of TLR-mediated inflammatory response, and activation of

Tregs. Several studies demonstrated that autophagy could promote the release of IL-10. For example, autophagic activators such as amyloid-β (25–35) could promote the level of IL-10, while blocking autophagy by knockout of autophagic key molecules or autophagic inhibitors such as 3-MA resulted in significant decrease of IL-10. However, under specific stimulation including grow factor VEGF165, the activation of autophagy suppressed the level of IL-10 (Wu et al. 2016). The association between autophagy and IL-10 remains largely unknown, the detailed mechanism employed by autophagy to affect the release of IL-10 still needs to be investigated. Growth factor TGF-β plays a critical role in regulating inflammatory responses and maintaining immune balance. TGF-β not only inhibits TCR-induced T cell activation and Th1 cell polarization, but also promotes the generation of Tregs. It is believed that autophagy inhibits TGF-β signaling through autophagic degradation of TGF-β. When autophagy was blocked, enhanced levels of TGF-β are observed. It has been reported that accumulation of p62 led to the stabilization of Smad4, the activator of TGF-β, which indicates that decrease of p62 during the activation of autophagy may also affect the level of TGF-β (Wu et al. 2016). In addition to two inhibitory cytokines described above, autophagy is also related to several novel inhibitory cytokines such as IL-27, IL-35, and IL-37. For instance, IL-27 inhibited IFN-induced autophagosome formation and promoted the survival of mycobacteria. In addition, autophagy participates in the IL-35-mediated immunosuppression process (Wu et al. 2016).

In adaptive immunity, autophagy can be induced by multiple inflammatory cytokines and suppressed by Th2-related cytokines. Although autophagy plays several roles in the release and degradation of different cytokines, the relationship between autophagy and cytokines remains to be elucidated. As the important role of inhibitory cytokines is to maintain immune tolerance, more works are needed to reveal the association between autophagy and inhibitory cytokines.

## 28.3 Autophagy Regulates Innate Immune Tolerance

### *28.3.1 Innate Immune Tolerance and Autophagy*

#### 28.3.1.1 Overview

Innate immunity, the first line of host defense, plays an important role in mediating the resistance and tolerance against invading pathogens. The innate immune response is provoked by PRRs, including TLRs, the RIG-I like receptors (RLRs), Nod-like receptors (NLRs), C-type lectin receptors (CLRs), and several RNA and DNA sensors which recognize the nucleic acids of pathogens. After pathogen invasion, these PRRs induce NF-κB and IFN signaling and mediate the assembly and activation of inflammasomes, leading to the production of type I/III IFN and proinflammatory cytokines, which subsequently turns on the host immune response. The innate immune response must be strictly controlled. The insufficient innate immune

response fails to activate the adaptive immunity or eliminate the invading pathogenic microorganisms effectively. However, persistent innate immune responses cause damage to cells and the body, leading to autoimmune diseases. Take endotoxin-induced innate immune response as an example. After recognition of lipopolysaccharide (LPS, also called endotoxin), a component of gram-negative bacterial cell wall enters the body and TLR4 triggers inflammation. To avoid excessive production of inflammatory cytokines, TLR4 mediates the internalization of LPS by recognizing the free LPS, leading to the formation of vesicles via clathrin-pulled plasma membrane invagination in the cytoplasmic side, which then forms the endosomes fused with lysosomes to eliminate the LPS, resulting in endotoxin tolerance. In addition, when endotoxin tolerance occurs, the body will further reduce the transcription of *TLR4* gene and downregulates the LPS reaction through decreasing the protein abundance of TLR4, thus abrogating the expression of inflammatory cytokines to improve the tolerance of endotoxin in host cells.

Although autophagy has been regarded as a highly conservative degradation system, its role in innate immune response and tolerance should not be ignored. First, autophagy can be activated by PRRs and provides a platform for removing the invading pathogens. Second, autophagy-related proteins participate in the regulation of the innate immune response and the autophagy process is highly integrated with the innate immune signaling pathway. Finally, besides promotion and the elimination of invading pathogens, autophagy can also selectively degrade the key receptors and positive regulatory molecules in the innate immune signaling, thereby negatively regulating the innate immune responses. Therefore, it is of great significance to understand the influence of autophagy on innate immune tolerance for the resolution of infections, tumors, autoimmune diseases, and rejection of organ transplantation that plague human health by relying on autophagy.

#### 28.3.1.2 Autophagy Regulates Innate Immune Tolerance Through Multilayer Mechanisms

Innate immune cells such as natural killer cells, dendritic cells, γδ T, and other cell subgroups and their PRRs form the innate immune regulatory network to control the innate immune responses, and further regulate the adaptive immunity. Therefore, the precise regulation of the signal pathways mediated by innate immune cells and their PRRs is the core of innate immune tolerance. Recently, accumulating reports have revealed the comprehensive function of autophagy in the immunity, the roles of autophagy in the innate immune regulation, and tolerance (Fig. 28.2). First, autophagy regulates the tolerance of innate immune cells. Those innate immune cells are highly heterogeneous, which include mast cells, macrophages, neutrophils, DCs, eosinophils, basophils, natural killer cells, γδ T cells, etc. These cells are not able to divide on their own to proliferate but instead are produced by the differentiation of pluripotent hematopoietic stem cells residing in bone marrow. Autophagy can positively or negatively regulate pluripotent hematopoietic stem cells from the bone

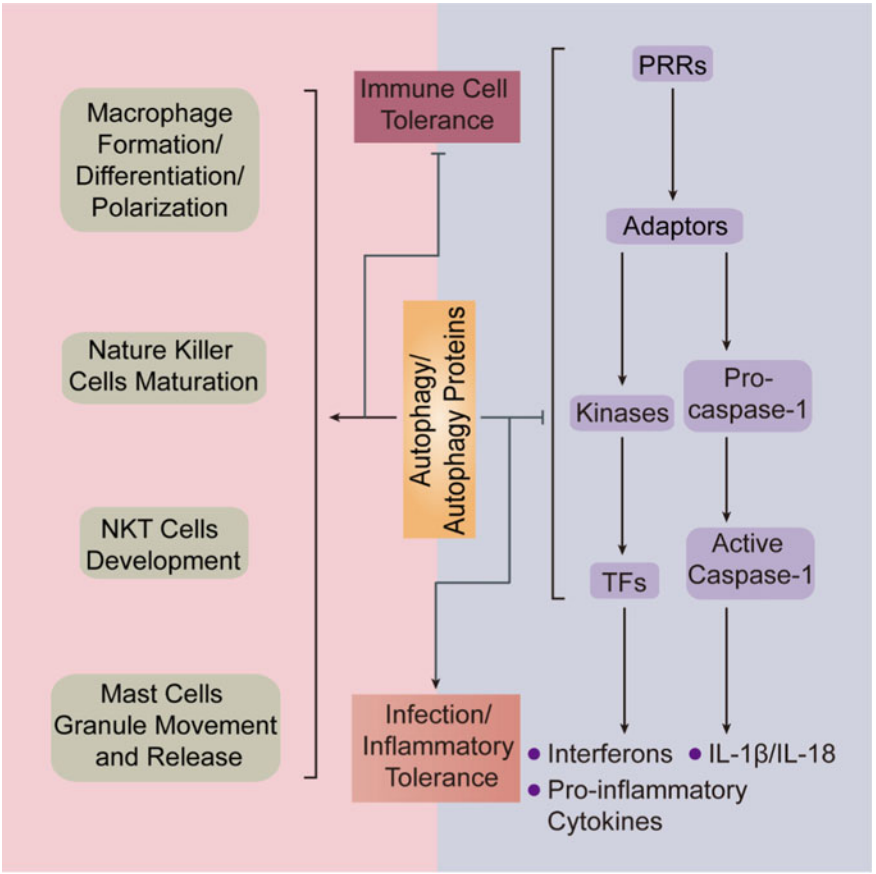


**Fig. 28.2** Autophagy regulates innate immune tolerance. On the one hand, autophagy affects the formation/differentiation/polarization of macrophages, maturation of natural killer cells, development of natural killer T cells, and granule movement and release of mast cells, thus inhibiting immune tolerance at the cellular level. On the other hand, autophagy-related proteins and selective autophagy target innate immune signaling pathways at the levels of pattern recognition receptor, adaptor, kinase/caspase, and transcription factor, respectively, thus negatively regulating antiviral and inflammatory signaling and the activation of inflammasomes and eventually leading to infection tolerance and inflammatory tolerance

marrow to develop into a variety of innate immune cells through complicated mechanisms. Autophagy also inhibits the function of the innate immune cells, leading to tolerance during the process of pathogen elimination in innate immune cells. Second, autophagy negatively regulates innate immune signaling and mediates innate immune tolerance. The innate immune system recognizes PAMPs or DAMPs of pathogens through the pattern recognition receptors, which then transmit signals to adaptor proteins such as TRIF, MyD88, RIG-I, MAVS, and ASC. Subsequently, adaptor proteins transduce signals to the enzymatic proteins, such as IKK complex,

TBK1/IKKε kinase, and caspase-1, which in turn lead to the activation of downstream transcriptional and non-transcriptional cascades. These events include the production of IFNs induced by IRF3/IRF7, the expression of pro-inflammatory cytokines mediated by NF-κB, phagocytosis, autophagy, pyroptosis, cytokines maturation, etc. Autophagy plays a negative regulatory role in multiple levels of innate immune signaling pathways, thereby mediating innate immune tolerance. Autophagy-related proteins can block the cascade reaction of the innate immune signal and execute the innate immune tolerance through negative regulation of the innate immune signaling pathways. In addition, autophagy can selectively degrade the core proteins of the innate immune signaling pathway and restrict the excessive activation of the innate immune signaling pathways, thus contributing to the innate immune tolerance (Deretic et al. 2015).

## *28.3.2 Autophagy Regulation of Immune Tolerance in Innate Immune Cells*

### 28.3.2.1 Macrophages

Macrophages play an important role in the innate immune response with high functional heterogeneity. It is a kind of white blood cells derived from monocytes in tissue, which can phagocyte and digest the debris of dead cells and invading pathogens in the form of immotile cells or free cells, thus activating lymphocytes and other types of immune cells to accelerate their response to invading pathogens. Macrophages will polarize in different microenvironments, producing the classic M1-type macrophages that promote inflammation and the selective M2-type macrophages that inhibit inflammation. Autophagy plays an important regulatory role at different stages of macrophage formation, including maintenance of hematopoietic stem cells, monocyte/macrophage migration, monocytes differentiation into macrophages, and macrophage polarization. Macrophages build a bridge between innate and adaptive immunity. In the senescent immune system, macrophages and other bone marrow cells produce excessive cytokines, which is also known as “inflammatory aging”. The mechanism underpinning how healthy macrophages maintain immune tolerance effectively is still unclear, but autophagy has been reported to regulate the aging process of macrophages. In the absence of the necessary autophagy key protein gene *Atg7*, the number of macrophages in the body increased, and the inflammatory cytokine responses were significantly enhanced, while the phagocytosis, nitrite outbreak, and other key functions were weakened. This phenotype has also been observed in aging macrophages. Moreover, the deficiency of autophagy activity decreased the expression of antigen on cellular surface and causes macrophage metabolism to tilt toward glycolysis. Autophagy flux in the macrophage of elderly

mice was significantly lower than that in younger mice. Therefore, autophagy functions and maintains the homeostasis in macrophages. To prevent excessive inflammation, autophagy induces tolerance during the aging process in macrophages, which in turn affects the immune response and alters the metabolism to reduce the morbidity and mortality associated with "inflammatory aging".

In addition, excessive lipid accumulation will suppress autophagy flux, and impaired autophagy in macrophage may lead to excessive activation of the innate immune response in obese patients. High-fat diet (HFD) results in decreased levels of autophagy in bone marrow derived macrophages (BMDM) and peritoneal macrophages. Through studying the *Lyz2-Cre*-mediated knockout of *Atg5* in macrophages, the researchers have found that feeding HFD and using low dose of LPS treatment caused systemic and liver inflammation in an *Atg5* specific knockout mice model. However, this effect was liver specific, because knockout mice showed no enhancement of inflammation in adipose tissue. Autophagy deficiency promotes inflammation by regulating macrophage polarization. The BMDMs and Kupffer cells of *Atg5* knockout mice showed abnormalities in the polarization process and the number of pro-inflammatory M1-type macrophages increased whereas the number of anti-inflammatory M2-type macrophages decreased. In *Atg5* knockout mice treated with HFD and LPS, the inflammatory response of the liver enhanced and liver damage occurred, but the phenomenon of steatosis was not accompanied. Decreased autophagy of macrophage in obesity could induce hepatitis and liver injury (Stranks et al. 2015). Therefore, autophagy might be the basis for immune tolerance in macrophages.

#### 28.3.2.2 Nature Killer Cells

Natural killer (NK) cells are typical members of the family of innate lymphoid cells (ILCs) recently discovered, also known as granulosa lymphocytes. Their development depends on the bone marrow or thymus microenvironment, and they have the ability to recognize and destroy infected and transformed cells. The potent cytotoxicity of NK cells is controlled by a series of surface receptors that can transmit stimulus or inhibitory signals. Whereas inhibitory NK cell receptors are mainly engaged by MHC class I molecules, the ligands of their stimulatory counterparts encompass plenty of molecules that are specifically exposed on the surface of cells experiencing stressful conditions, including viral infection and malignant transformation. Autophagy contributes to the presentation these molecules. Furthermore, immature NK (iNK) cells from bone marrow of mice have shown some ongoing autophagy, including lipidation of MAP1LC3B/LCB3 and degradation of p62 at the basal level. During homeostatic expansion upon adoptive transferred into $Rag2^{-/-}Il2rg2^{-/-}$ immunodeficiency mice, the researchers found that mature NK (mNK) cells and group 1 ILCs showed enhanced autophagy flux, indicating that autophagy is involved in different steps of NK cell maturation and homeostatic proliferation. The numbers of circulating iNK and mNK cells were significantly reduced in $Ncr1^{cre}Atg5^{flox/flox}$ mice when *Atg5* was specifically knocked out in NK cells, while the numbers of T

cells and B cells in peripheral blood were normal. Additionally, the NKPs isolated from *Atg5* knockout mice could not differentiate iNK or mNK cells after IL-15 treatment, and similar results could be obtained by silencing *Atg3* or *Atg7* in wild-type NKPs. Therefore, autophagy also functions in the tolerance of NK cells, and the detailed mechanism remains to be further explored.

For tumor therapy, solid tumors can establish and maintain an immunosuppressive microenvironment to prevent cytotoxic effector immune cells from infiltrating into tumors. In B16-F10 tumors, BECN1/Beclin-1, a key autophagy protein, inhibits tumor growth by targeting and inducing large amounts of functional NK cells to infiltrate into the tumors. This infiltration is mainly caused by the overexpression and release of cytokine CCL5 in the tumor microenvironment through activating MAPK8/JNK-JUN/c-Jun signaling pathway with *BECN1* deficiency in tumor cells. In human melanoma, there is a positive correlation between NK cell markers and the expression of CCL5. More importantly, the survival rate is significantly improved in melanoma patients with high expression of CCL5. Therefore, targeting autophagy has an important effect on breaking down the barrier of tumor immunosuppressive microenvironment and allowing cytotoxic NK cells to enter the tumor. The key issue in NK cell-based anticancer therapies is the ability of tumor cells to activate resistance mechanisms to avoid NK cell-mediated killing. It has been confirmed that these mechanisms may be formed under hypoxic conditions in tumor microenvironment. Hypoxic-induced autophagy can weaken the susceptibility of breast cancer cells to NK cell-mediated solutes, and such damage is recovered by targeting autophagy. In hypoxic cells, activation of autophagy is involved in the selective degradation of NK-derived serine protease GZMB/granzyme B, thus blocking NK cell-mediated apoptosis of target cells (Lopez-Soto et al. 2017). In conclusion, autophagy plays an important role in the tolerance of NK cells. By targeting autophagy to regulate the activity of NK cells and making full use of the antitumor characteristics of NK cells in the clinical environment, new tumor treatment strategies are expected to be developed.

#### 28.3.2.3 NKT Cells

Natural killer T (NKT) cells are a subset of innate lymphoid cells with T cell receptors that recognize lipid antigens presented by CD1d. CD1d is a class of antigen-presenting molecules that bind to both autogenous and allogeneic lipids and glycolipids. After activation, NKT cells not only have direct effector functions, but also can transform NK cells and mature dendritic cells and activate B cells by secreting a variety of cytokines and interacting with homologous TCR-CD1d. NKT cells are endowed with the ability to coordinate a comprehensive immune response and play a crucial role in mediating the immune response against pathogens and cancer cells. Both tumor cells and tumor-infiltrating immune cells are subject to tumor microenvironmental stress due to the lack of nutrients, such as hypoxia, or the accumulation of toxic metabolites. This change in microenvironment leads to the upregulation of autophagy flux, and tumor cells undergoing autophagy process

will become stronger to persist and metastasize. Before recirculating to the plasma membrane, CD1d molecules bind lipid antigens through endocytosis in MHC class II-containing compartments (MIICs). Autophagosomes are associated with MIICs, and autophagy-related proteins support antigen loading to enhance $CD4^+$ T cell-mediated immune response. CD1d1 restricted glycolipid expression was better presented in mice with DC-specific deletion of the necessary autophagy gene *Atg5*. This effect increased the recognition of antigens with pseudomonas infection, thereby reducing the bacterial load in host cells. Enhanced NKT cell activation was independent of receptor-mediated glycolipid uptake or synergistic stimulus signals. On the contrary, the absence of *Atg5* in DCs impaired the clathrin-dependent internalization of CD1d1 molecules through the transduction of protein complex 2 (AP2), thus increasing the surface expression of the stimulated CD1d1-glycolipid complex. These findings suggest that autophagy assists the recruitment of AP2 to CD1d1, leading to decreased activation of NKT cells, contrary to the supporting function of autophagy in $CD4^+$ T cells stimulation. However, it has also been shown that the absence of *Atg5* is not associated with the expression of CD1d during the development of NKT cells in the thymus (Gapin 2016). The specific regulatory mechanism may be related to cell type and time, and more in-depth studies are needed to determine the role of autophagy in CD1d expression in the future.

#### 28.3.2.4 Mast Cells

Mast cells are innate immune cells with heterogeneous phenotypes, which are regulated by cytokines and other microenvironmental stimuli. They contain heparin, histamine, SRS-A, and ECF-A, which degranulate after being stimulated by allergens. Mast cells play a protective role in parasitic, bacterial, and viral infection and also participate in the pathogenesis of allergy, asthma, and autoimmune disease. In mast cells, LC3-II is continuously converted from LC3-I under sufficient nutritional conditions, and LC3-II is located in the secretory granules of mast cells. Although knockout of *Atg7* in mice did not affect the development of the bone marrow derived mast cells, mast cells lacking *Atg7* displayed the phenomenon of impaired degranulation. In addition, depletion of *Atg7* did not alter the production of cytokines in mast cells. Interestingly, LC3-II, but not LC3-I, colocated with secretory lysosomal marker CD63 in the extracellular released granules of mast cells. And this colocation did not exist in mast cells lacking *Atg7*. In *Atg7* and *Atg12* knockout mast cells, the formation of normal particles was not affected, but deletion of *Atg7* and *Atg12* led to the blockade of IgE-mediated degranulation. It has been confirmed that autophagy plays an important role in the movement and release of granule in mast cells. In addition, mast cells with autophagy disorders also exist in a variety of disease states, such as systemic sclerosis, chronic sinusitis, and asthma (Ushio et al. 2011). Therefore, it is of great significance to study the autophagy process in mast cells and explore the influence of autophagy on the tolerance of mast cells to develop new therapy toward tumor and other diseases.

## *28.3.3 Autophagy Regulation of Infection Tolerance*

### 28.3.3.1 Negative Regulation of Infection Signaling by Autophagy-Related Proteins

Resistance and tolerance are two defense strategies of host against microbial infection. Autophagy-mediated degradation of pathogens is widely recognized as the main resistance mechanism of host to infection. However, autophagy protein plays a major role in the prevalent community associated *Staphylococcus aureus* USA300 infection by mediating tolerance rather than resistance. In *Atg16L1* hypomorphic (HM) allele mutant mice (*Atg16L1*$^{HM}$), autophagy level was significantly reduced, and higher susceptibility and mortality were observed in both sepsis and pneumonia models after USA300 infection. Autophagy provides host protection by limiting the damage caused by α-toxin in endothelial cells. Notably, *Atg16L1*$^{HM}$ showed a greater survival rate rather than susceptibility to α-toxin deficient *staphylococcus aureus* infection. α-toxin binds to toxin receptor A Disintegrin and Metalloprotease 10 (Adam10) on the surface of endothelial cells, epithelial cells, and monocytes, leading to the septicemia and pneumonia of host. Adam10 protein expression was accumulated in autophagy-deficient cells, which can explain why the endothelial cells with *Atg16L1* deletion are more sensitive to α-toxin. Therefore, autophagy plays an important regulatory role in the tolerance of *staphylococcal* infection, and a single virulence factor encoded by the pathogen distinguishes between tolerance and resistance in host cells (Maurer et al. 2015).

Once infected by pathogenic microorganisms, RIG-I-like receptors and cGAS recognize the nucleic acids of RNA viruses and DNA viruses and transduce signals to adaptor proteins MAVS and STING, respectively. Both of them can activate the kinase TBK1 to phosphorylate IRF3, which leads to dimerization and translocation of IRF3 into the nucleus, thus initiating transcriptional activation of type I IFN (Joubert and Albert 2013). The production of type I IFN is a marker of cellular response to cytoplasmic nucleic acid stimulation. Type I IFN itself does not have direct antiviral activity, but it can induce the state of immune activation in virus-infected cells and their adjacent cells, thereby limiting the proliferation of the virus. Meanwhile, it can enhance the antigen-presenting function of dendritic cells and the function of natural killer cells to promote the innate immune responses. And adaptive immune responses can be regulated by type I IFN to promote the maturation and activation of B cells and T cells. Once released into the extracellular environment in an autolytic and paracrine dependent manner, type I IFN stimulates the expression of IFN-stimulated genes (ISGs) by binding to IFN receptors. The proteins encoded by ISGs can not only aim for several essential virus replication processes and perturb the virus amplification, but also positively or negatively regulate the innate immune responses in host cells.

In RIG-I like receptor-mediated antiviral response, autophagy-related proteins are capable of restricting the production of type I IFN to exert the function of infection tolerance. *Atg5* deficiency enhanced the production of type I IFNs induced by RNA virus such as vesicular stomatitis virus (VSV) infection and effectively

inhibits virus replication in host cells. The ATG5-ATG12 complex and its binding protein (elongation factor Tu, mitochondrial TUFM) inhibit dsRNA-induced type I IFN production, thus suppressing the effective process of RIG-I signaling pathway. In addition, induced by IFN, dsRNA-activated protein kinase (EIF2AK2, PKR) can bind Beclin-1 in autophagy VPS34 complex and promote the formation of autophagosome. Autophagy can also be induced by phosphorylation of eukaryotic translation initiation factor 2 subunit 1 (EIF2S1, eIF2α) mediated by PKR (Choi et al. 2018). Deubiquitination enzyme USP19 targets Beclin-1 for stabilization. On one hand, USP19 promotes the formation of autophagosome and the process of autophagy flux. On the other hand, USP19-Beclin-1 complex binds to the CARD domain of MAVS in an autophagy-independent manner and blocks the interaction between RIG-I and MAVS, which in turn negatively regulates antiviral innate immune responses (Jin et al. 2016). Taken together, autophagy provides negative regulation of RLRs-mediated immune responses, and autophagy-related proteins regulate infection tolerance through RLRs.

Cytoplasmic DNA sensor, cGAS recognizes dsDNA of bacteria or DNA viruses and generates a second messenger cGAMP. Subsequently, cGAMP binds and activates STING in the endoplasmic reticulum, thereby inducing type I IFN signaling pathway. In the case of HSV-1 infection, DNA-mediated type I IFN signaling pathway and autophagy also integrate with each other. Beclin-1 interacts with cGAS and inhibits its transferase activity to reduce the production of cGAMP to restrict STING activation. On the contrary, cGAS competes for the combination of Beclin-1 with autophagy inhibitor Rubicon, thus triggering autophagy and causing the degradation of viral DNA in host cells. Furthermore, cGAMP leads to the dissociation of ULK1 from AMPK complex and induces the phosphorylation of STING by ULK1 and subsequent degradation of STING, thereby inhibiting the production of type I IFN and obtaining infection tolerance. The absence of ATG9 can result in STING aggregation on Golgi bodies and enhance STING-dependent type I IFN production. Additionally, cGAS-dependent signal promotes pathogen clearance by autophagy and shuts down the signal in a negative feedback loop. Together, autophagy is also involved in immune tolerance of cytoplasmic DNA sensors.

#### 28.3.3.2 Negative Regulation of Infection Signaling by Selective Autophagy

Although autophagy is a high-throughput degradation system, it could specifically degrade a series of core immune factors to control anti-microbe immunity via a group of cargo receptors (Deretic and Levine 2018; Jin et al. 2017). Selective autophagy can degrade nucleic acid sensors. Upon RNA virus infection, the stability of RIG-I-like receptors is regulated by selective autophagy to generate infection tolerance. Leucine-rich repeat sequence protein 25 (LRRC25) is a member of the leucine-rich repeat sequence protein family, which plays a key role in innate immunity, apoptosis, and nuclear mRNA transport. Recent studies have indicated that LRRC25 is a negative regulator of RIG-I-mediated type I IFN signaling pathway. When the host is

infected by RNA virus, LRRC25 specifically binds to ISG15-associated RIG-I and promotes the interaction between RIG-I and autophagy cargo receptor p62, to mediate the degradation of RIG-I through selective autophagy. The absence of LRRC25 or ISG15 abrogates the interaction of RIG-I-p62 and the autophagic degradation of RIG-I. Thus, LRRC25, as the second receptor that assists RIG-I degradation, plays an indispensable role in p62-dependent selective autophagy in the regulation of immune tolerance. Autophagy also plays an important role in DNA virus infection tolerance. The K48-linked poly-ubiquitin chains at lysine 414 site on cGAS serve as an autophagic degradation signal specifically recognized by p62, and it keeps the protein amount of cGAS at low level. During HSV-1 infection, TRIM14 is upregulated by IFN and then recruits deubiquitinase USP14 to remove the K48-linked poly-ubiquitin chains on cGAS, leading to the stabilization of cGAS as well as the amplification of IFN production. *TRIM14* knockout mice are highly sensitive to HSV-1 infection and death of mice is due to reduced type I IFN production.

Selective autophagy also regulates the degradation of adaptor proteins and mediates infection tolerance. Tetherin/BST2 is an important ISG protein, which can inhibit the release of HIV-1 from the surface of infected cells and has a similar function to a variety of enveloped viruses. Tetherin not only inhibits the release of virus particles from infected cells through "physical interaction", but also regulates the infection tolerance against RNA viruses. Through recruiting E3 ubiquitin ligase MARCH8, Tetherin specifically enhances the K27-linked poly-ubiquitination of mitochondrial antiviral protein MAVS at the lysine 7, thus providing a degradation signal for cargo receptor CALCOCO2/NDP52, leading to the selective autophagic degradation of MAVS to prevent excessive activation of type I IFN signaling. Moreover, when cGAS-STING signaling pathway is activated, STING is ubiquitinated and p62 is phosphorylated by TBK1, so that the ubiquitinated STING directly enters into autophagosomes for degradation. In the absence of p62, STING cannot be delivered into autophagy-related vesicles. Therefore, DNA mediates cGAS-STING pathway to activate TBK1, which further phosphorylates IRF3 and induces type I IFN production. Meanwhile, phosphorylated p62 promotes the selective autophagic degradation of STING and confers infection tolerance. Recently a report showed that in *Salmonella typhimurium* infection, TRIM32-TAXBP1 complex specifically downregulated the innate immune response mediated by TLR3/4 through promoting the autophagic degradation of adaptor TRIF. Taken together, these results demonstrated that the selective autophagy mediated by cargo receptors could specifically degrade PRRs as well as adaptor proteins to generate infection tolerance.

## *28.3.4 Autophagy Regulation of Inflammatory Tolerance*

### 28.3.4.1 Negative Regulation of Inflammation by Autophagy-Related Proteins

After recognizing the ligands, PRRs recruit and activate a series of downstream signaling molecules such as TRAF6, TAK1, and IKK complex through adaptor proteins such as MyD88 or TIRF, leading to transcription of pro-inflammatory cytokines such as TNF, IL-6, IL-1β, and IL-18 in an NF-κB dependent pathway. Maturation and release of IL-1β and IL-18 also depend on caspase-1 activation in inflammasome-dependent manner. Autophagy balances and regulates immune activation to mediate innate immune tolerance, thus avoiding excessive inflammatory responses. The close association between inflammatory disease and autophagy is originally derived from the genome-wide association study (GWAS), which provides information on genetic polymorphisms associated with susceptibility to a range of human diseases. Most notably, previous researches link Crohn disease (CD) susceptibility with polymorphisms in *ATG16L1* and *IRGM* genes, whose products interact to orchestrate autophagy in cooperation with NOD2, a familial risk factor in CD. Further studies of inflammatory bowel disease, Crohn's disease, and ulcerative colitis have shown that polymorphisms in other autophagy-related genes (which span almost all stages of autophagy process), such as ULK1 proteins and selective autophagy factors, such as SMURF1 and NDP52, are also associated with these inflammatory diseases. Other autophagy genes are also associated with inflammatory disorders and autoimmune disease inheritance, which include *IRGM*, *ATG5*, *PRDM1-ATG5*, and *DRAM1* mutations in systemic lupus erythematosus, *ATG5* mutation in asthma, *ATG5* rs6568431 mutation in rheumatoid arthritis, *EPG5* mutation in Vici syndrome, mutations of *CLEC16A*, *ULK3* and MicroRNA genes targeting autophagy in CD, CLEC16A mutation in multiple sclerosis, disorders of the nervous system, such as amyotrophic lateral sclerosis and frontotemporal dementia, and especially *TBK1* mutations in patients with severe neuroinflammation. CLEC16A is also genetically related to type I diabetes, and it involves immune infiltration and plays an important role in mitophagy and the fusion of autolysosome.

Inflammasomes, which contain several different forms according to various core proteins, are cytoplasmic responders to pathogenic products and sterile endogenous agonists (Zhong et al. 2016). Once activated, Nod-like receptors (including NLRP1, NLRP3, NLRC4, etc.) or AIM2, ASC, and pro-caspase-1 constitute classical inflammasomes that rapidly assemble to exhibit proteolytic activity, and lead to the cleavage and release of IL-1 and IL-18 cytokines. Nonclassical inflammasomes do not require ASC and NLRP proteins but are directly activated by cytoplasmic agonists, such as LPS caspase-4/caspase-11 (mouse) or caspase-4 and caspase-5 (human), which promote the activation of GSDMD (gasdermin D) and cause pyroptosis. Classical and nonclassical inflammasome signaling pathways overlap partially because caspase-1 can also hydrolyze and activate GSDMD (Deretic and Levine

2018). The anti-inflammatory effect of autophagy is usually going through the inhibition of inflammasomes. The initial reports of autophagy gene genetic mutation linked inflammation originated from a mouse model of Crohn's disease in which *ATG16L1* was absent in hematopoietic cells, causing elevated levels of IL-1β and IL-18 that could be canceled by blocking antibody treatment of IL-1β and IL-18 in mice. This is the first time to demonstrate that autophagy controls inflammasome tolerance in vivo. A recent study showed that macrophage-specific autophagy-deficient mice has appeared to lead to inflammasome-dependent IL-1β release and uveitis, an inflammatory eye disease, was often observed in patients with CD. The main reason that autophagy molecules regulate inflammatory tolerance is that autophagy can remove inflammatory agonists in cells. For example, autophagy can eliminate damaged or irreversible depolarized mitochondria, the process of which is often termed as mitophagy. Autophagy can also reduce the release of inflammasome agonist, such as reactive oxygen species (ROS) and mitochondrial DNA, and ultimately restrict the activation of inflammasomes to achieve the effect of inflammatory tolerance. After activation of TLR2 and TLR4 signaling pathways, the expression of type 2 plasminogen activator inhibitor protein (PAI-2) is induced, which stabilizes the protein level of Beclin-1 to promote autophagy and reduces the production of mitochondrial ROS, the quality of NLRP3 protein, and the processing of pro-IL-1β. Overexpression of Beclin-1 in PAI-2 deficient cells inhibited the response of NLRP3 to LPS.

#### 28.3.4.2 Negative Regulation of Inflammation by Selective Autophagy

Selective autophagy inhibits transcriptional activation of pro-inflammatory cytokines, thereby mediating inflammatory tolerance at the transcriptional level. A prominent feature of cellular senescence is the senescence-associated secretory phenotype (SASP), which produces an inflammatory response that promotes tumor and senescence. GATA4 is an important transcription factor that regulates aging and SASP, and it can be degraded by p62-mediated selective autophagy, but such degradation is inhibited during aging. Accumulated GATA4 then activates the transcription factor NF-κB to enhance the transcription of pro-inflammatory cytokines, thereby initiating SASP and mediating inflammation-related aging (Kang et al. 2015). The activation of NF-κB signaling pathway is negatively regulated by F-box protein SKP2 (S-phase kinase-associated protein 2). SKP2 deletion amplifies the activation of NF-κB signaling pathway and increases the production of inflammatory cytokines. Molecular mechanism studies have shown that SKP2 mediates the interaction between IKKβ and p62 and promotes the p62-dependent selective autophagic degradation of IKKβ, thus inhibiting the activation of NF-κB signaling pathway to generate inflammatory tolerance. Moreover, LRRC25 interacts with the Rel homologous domain (RHD) of p65/RelA through its own leucine-rich repeat sequence (LRR). It promotes p65/RelA recognition by p62 and ultimately mediates the selective autophagic degradation of p65/RelA, thereby inhibiting NF-κB signaling pathway.

Selective autophagy also inhibits the activation of inflammasomes, thereby mediating inflammatory tolerance. The macrophage-specific loss of p62 resulted in

the accumulation of damaged mitochondria in the cells, which in turn led to the inflammasome-dependent production of IL-1β, and ultimately caused macrophage death. Similarly, when inflammasome activator was present, lack of Fanconi anemia (FA) gene, *Fancc* (a newly discovered factor mediating selective autophagy) could accumulate the damaged mitochondria and enhanced inflammasome-dependent mitochondrial ROS. In addition to removing damaged mitochondria and inhibiting the activation of inflammasomes, autophagy has been indicated to directly target the components of inflammasomes to regulate inflammatory tolerance. As the direct substrates of autophagic degradation, the degradation of components of inflammasomes represent another way by which autophagy inhibits the activation of inflammasomes. Activation of AIM2 or NLRP3 inflammasomes in macrophages will lead to the activation of G-protein RalB and the formation of autophagosome. The assembled inflammasomes will be ubiquitinated and then recruited to the cargo receptor p62. It has been reported that ASC and p62 have a strong co-aggregation. AIM2 is also removed by autophagy in a process involving p62 recruitment to K63-ubiquitinated ASC. E3 ubiquitin ligase TRIM11 functions as a negative regulator of AIM2 inflammasome upon DNA virus infection. TRIM11 binds to AIM2 through its PS domain and undergoes auto-ubiquitination, which serves as an important binding signal for p62. Functioning as a secondary cargo receptor, TRIM11 delivers AIM2 to autophagosomes for degradation in a p62-dependent manner, which plays a role in inflammatory tolerance. In addition, recent report has indicated other TRIM family proteins which also act as autophagy receptors to specifically degrade key components of inflammasomes. TRIM20 targets pro-caspase-1, NLRP1, and NLRP3. TRIM20 recognizes these substrates through its SPRY domain, and it has three LC3 binding motifs (LIR) that guarantee its interactions with ATG8 family members (LC3 s and GABARAPs). TRIM20 can then be assembled into ULK1, Beclin-1, and ATG16L1 complexes to ensure the effective degradation of substrates. It is worthy to state that TRIM20 mutation is associated with familial Mediterranean fever, and the ability of TRIM20 mutation to bind with autophagy core protein is significantly reduced, suggesting that TRIM20-mediated autophagy of inflammasome components plays a key role in inflammatory tolerance.

In conclusion, autophagy mediates inflammatory tolerance to prevent excessive inflammatory responses mediated by endogenous and infection-derived agonists caused by inner membrane damage and plays an important role in immune balance. Inflammation is involved in many diseases' pathologic processes. Therefore, a better understanding of the relationship between autophagy and inflammatory tolerance is of important guiding significance for drug development and disease treatment in the future.

## 28.4 Conclusion

Autophagy plays a crucial role in both adaptive and innate immune tolerance. It is essential to reveal and understand the mechanisms of autophagy in immune tolerance, so as to manipulate the immune tolerance by orchestrating autophagy, which provides

important theoretical basis for tumor therapy that breaks the immune tolerance and the treatment of autoimmune disease that reconstructs the immune tolerance. Notably, there is a cross talk between autophagy and immune response, so the "one size fits all" approach with interventions of autophagy flux may have some risks in the course of disease treatment. In this case, a better strategy might be to manipulate the selective autophagic degradation of specific substrates to specifically regulate immune tolerance. Therefore, immunotherapies that rely on selective autophagy have broad application prospects in the treatment of a variety of diseases that harm human physical and mental health.

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# Chapter 29
# Autophagy—Cell Survival and Death


**Xiaojie Yan, Ruimin Zhou and Zhenyi Ma**

**Abstract** Autophagy, which is one of the most important ways to maintain cell homeostasis plays an important regulatory role in cell survival and death. Currently, it is agreed that autophagy promotes or inhibits cell death depending on the internal and external environment and cell type. On the one hand, under normal nutritional conditions autophagy regulates cell survival by energy sensing through the main energy sensing cascade kinases. On the other hand, autophagy regulates the process of cell death. mTOR, Beclin 1, caspases, FLIPs, DAPK, and Tp53 play important regulatory roles in autophagy and apoptosis highlighting the crosstalk between the mechanisms underlying the two death modes. However, energy deficiency caused by PARP1 over-activation and DAPK-PKD pathway activation induces necrosis and autophagy, highlighting the interaction between the two pathways. In addition, autophagy regulates cell death through epigenetic regulation such as histone modification. More investigations on the relationship between autophagy and cell death is ongoing. In the future, there will be more challenges in the study of the relationship between autophagy and cell survival and death. As research increasingly focuses on cell death, the relationship between autophagy and existing and newly discovered cell death types is likely to become more complex. The elucidation of the regulatory role of autophagy in cell survival and death requires more research. Some research results are likely to provide hot topics for further investigations on diseases related to cell death disorders and an experimental basis for the targeted regulation of autophagy for specific treatment of diseases.

**Keywords** Autophagy · Survival · Cell death · Crosstalk

X. Yan · R. Zhou · Z. Ma (✉)
Department of Biochemistry and Molecular Biology, School of Basic Medical Sciences, Tianjin Medical University, 22 Qixiangtai Road, Heping District, Tianjin 300070, China
e-mail: zhyma@tmu.edu.cn



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## Abbreviations

| | |
|---|---|
| ACD | Accidental cell death |
| AIF | Apoptosis-inducing factor |
| AMPK | Adenosine 5′-monophosphate (AMP)-activated protein kinase |
| Bruce | BIR-containing ubiquitin-conjugating enzyme |
| CMA | Chaperone-mediated autophagy |
| CSE | Cigarette smoke extract |
| DISC | Fas-dependent death-inducing signaling complex |
| DRAM | Damage-regulated autophagy modulator |
| FADD | FAS-associated death domain |
| FAK | Focal adhesion kinase |
| FLICE | FADD-like interleukin-1 beta-converting enzyme |
| FLIP | Flice inhibitory protein |
| GABARAP-L1 | Γ-aminobutyric acid receptor-associated protein-like 1 |
| HAT | Histone acetyltransferase |
| HDAC | Histone deacetylase |
| HMGB1 | High mobility group B1 |
| IAPs | Inhibitor of apoptosis proteins |
| IFN | Interferon |
| IL3 | Interleukin 3 |
| IMS | Intermembrane space |
| LAMP2A | Lysosome-associated membrane protein type 2A |
| MEF2D | Myocyte enhancer factor 2D |
| MEFs | Mouse embryonic fibroblasts |
| MFN1 | Mitofusin 1 |
| MOMP | Mitochondrial outer membrane permeabilization |
| mTOR | Mammalian target of rapamycin |
| NBR1 | The neighbor of BRCA1 gene 1 |
| NCCD | Nomenclature Committee on Cell Death |
| PARKIN | Parkinson's disease protein |
| PINK1 | PTEN-induced putative kinase 1 |
| PKA | Protein kinase A |
| PKD | Protein kinase D1 |
| PTP | Permeability transition pore |
| RCD | Regulated cell death |
| RIP1 | Kinases receptor-interacting protein 1 |
| ROS | Reactive oxygen species |
| SMAC | Second mitochondria-derived activator of caspase |
| SQSTM1 | Sequestosome 1, p62 |
| TNF | Tumour necrosis factor |
| TNF/NGF | Tumor necrosis factor/nerve growth factor |
| TRAIL | TNF-related apoptosis-inducing ligand |
| VDAC1 | Voltage-dependent anion-selective channel 1 |
| VMP1 | Vacuole membrane protein |

## 29.1 Introduction

Autophagy, which includes macroautophagy, microautophagy, and chaperone-mediated autophagy is an evolutionarily conserved catabolic process used to deliver damaged organelles and misfolded proteins to the lysosome for degradation. Macroautophagy (hereafter referred to as autophagy in this chapter), which is the most prevalent form of autophagy, plays a critical role in cellular homeostasis. Under starvation conditions cells can reuse metabolic precursors through autophagy to survive. Autophagy also promotes cell survival by maintaining the cell energy balance through the degradation of aged organelles and protein aggregates. Although early studies have suggested that autophagy is a mechanism responsible for cell survival, accumulated recent findings have confirmed that autophagy can also regulate the process of cell death including programmed cell death (such as apoptosis, necrosis, etc.), the inflammatory response, and acquired immune processes. Different mediators (such as different proteins in mitochondria, the endoplasmic reticulum, Golgi apparatus, etc.) and different pathways responsible for autophagic degradation regulate the physiological process of cells such as the occurrence and development of diseases. Currently, the causal relationship between autophagy and many regulatory or nonregulatory cell death patterns remains unclear. Autophagy can be associated with necrosis-like cell death induced by caspase inhibition; autophagy and apoptosis can occur simultaneously or inversely depending on the experimental conditions and the signaling pathways shared by both; and autophagy may regulate some other types of cell death such as necrosis. Recent studies have found that autophagy can inhibit the inflammatory response including inflammasome-dependent caspase-1 activation and proinflammatory body cytokine maturation; autophagy still participates in regulating caspase-1-dependent inflammatory cell death (pyroptosis). Due to the intricate relationship between autophagy and cell death, regulating the autophagy process in cell death disorder-related diseases has become a hot research field.

## 29.2 Autophagy and Cell Survival

As the “cell guard”, autophagy can enable cells to survive in harsh environments until the environment improves. From yeast to mammals autophagy has evolved from an ancient self-protection function in response to external stimuli. In addition, autophagy is associated with many diseases such as heart diseases, neurodegenerative diseases, and tumors. These diseases are related to changes in the cellular sensitivity to death caused by autophagy disorders. Although great progress has been made in exploring the mechanism and regulation of autophagy pathways, many problems still need to be elucidated. The relationship between the function of autophagy in metabolism and the process of cell life, the effect of autophagy flow on the metabolic status and cell death sensitivity, the metabolic pathway, metabolic and energy sensing, metabolic substrate supply, and metabolic transformation mechanism all require further investigation. In

this section, we mainly discuss how autophagy regulates cell survival by sensing the energy environmental conditions and providing metabolic substrates, how autophagy regulates cell death through its effects on apoptotic and necrotic pathways, and how autophagic histone codes regulate cell death. By explaining the regulatory role of autophagy in cell survival and death we can understand the role of autophagy in the maintenance of cell homeostasis and regulation of the threshold of cell death and survival.

### *29.2.1 Pro-survival Function of Autophagy Under Stress*

Autophagy plays an important role in the survival of cells, tissues, and whole organisms. When mammals were born the lack of nutrients caused by the sudden interruption of the placental nutrition supply activates autophagy to maintain sufficient amino acid metabolic pools for energy metabolism before the nutrition supply is guaranteed by breastfeeding. Another classic example in which autophagy promotes survival is the metabolic process of the liver when mammals have no food. The rapid activation of autophagy occurs in the liver in fasting rats which can be delayed by treatment with large doses of Cycloheximide (also known as actinomycin, a protein synthesis inhibitor). The number of autophagosomes in the starved liver is very small because the clearance rate of autophagosomes is higher than their formation rate. Studies investigating autophagy have observed the recruitment of LC3-labeled autophagosomes in some organs by transgenic techniques. The results indicate that nutritional deficiency could induce systemic autophagy. This finding demonstrates that tissues other than the liver, such as muscle tissue can provide metabolic substrates for the whole body mainly the brain and red blood cells to maintain life during fasting. However, in humans, starvation for more than three days can lead to a decline in autophagy; simultaneously, ketone replaces glucose as the major energy source to avoid the inadequate synthesis of key proteins in cells (see Chap. 16, Sect. 16.2).

Autophagy can also help maintain redox homeostasis. Under oxidative stress, reactive oxygen species (ROS) are the main intracellular signal transducers of autophagy. Nutrition deficiency could induce the production of reactive oxygen species and an imbalance in the thiol redox state which are important mediators of autophagy. ROS and reactive nitrogen species (RNS) irreversibly oxidize DNA and other cell macromolecules resulting in damage to the biological system and cell death. It has been reported that under oxidative stress autophagy is mainly triggered by the p62/Keap1/Nrf2 pathway which eliminates all irreversible oxidized biological molecules (proteins, DNA and lipids), plays an important role in the antioxidant and DNA damage repair system and promotes cell survival (Ichimura et al. 2013).

Misfolded proteins are toxic to the cells and the accumulation of some toxic proteins leads to neurodegenerative disorders. It is generally believed that the toxicity of misfolded proteins is due to the exposure of their hydrophobic surfaces which leads to their interaction with other normal proteins and interferes with key interactions between these proteins. To minimize the toxicity caused by misfolded

proteins, the protein quantity control system in cells monitors protein folding in real time and removes misfolded components from the cytoplasm in a timely manner. Molecular chaperones can recognize and conceal the hydrophobic surfaces of misfolded monomers and transport these proteins to the ubiquitin-proteasome system or chaperone-mediated autophagy. To remove soluble misfolded proteins the autophagy-lysosome system is used to degrade these proteasome-resistant toxic proteins. In addition, microtubule-dependent transport systems can isolate soluble polymers or aggregates of these misfolded proteins into inclusion bodies. These systems are regulated by stress-induced transcription factors, cochaperone factors, and other cofactors thereby effectively eliminating misfolded proteins. Therefore, autophagy can be used as a supplement for the proteasome degradation system. Specifically, for example, mice with autophagic disorder exhibit neurodegenerative symptoms in the brain. Recently, the following two proteins have been found to associate polyubiquitination with autophagy: SQSTM1 (also known as sequestosome 1, p62) (Zaffagnini et al. 2018) and NBR1 (a neighbor of BRCA1 gene 1, BRCA1 gene 1 adjacent gene 1). These two proteins can serve as a bridge between ubiquitin which is a degradation marker molecule and LC3 which is a key member of autophagy. SQSTM1 and NBR1 can recognize soluble protein aggregates and guide their degradation through the autophagy-lysosome system. Another type of molecular chaperone-mediated autophagy (CMA) can directly lead cytoplasmic proteins to the lysosome for degradation thereby skipping the step of autophagosome entry. In the CMA pathway, the specific motifs (KFERQ and related sequences) of target proteins are first recognized by the molecular chaperone HSC70 to form complexes with HSP40, Hip, and Hop. Thirty percent of cytoplasmic proteins contain KFERQ homologous sequences suggesting that these proteins are likely to be degraded by CMA. Substrate proteins are transported into the lysosome by molecular chaperone complexes with the help of lysosome-associated membrane protein type 2A (LAMP2A). For example, mutations in the encoding gene of α-synuclein, which is associated with Parkinson's disease can block its own degradation process; meanwhile, CMA plays an important role in the degradation of α-synuclein. Recent studies have found that CMA can degrade myocyte enhancer factor 2D (MEF2D), which is a transcription factor necessary for the survival of nerve cells. Although the inhibition of CMA can increase the content of MEF2D in the cytoplasm and whole-cell, the content of MEF2D in the nucleus and its DNA binding ability decrease simultaneously. The overexpression of α-synuclein decreases the transcriptional activity of MEF2D, leading to enhanced cell death. Therefore, CMA plays an important role in the quality control and cell survival of MEF2D and associates the degradation of α-synuclein with the activity of MEF2D, thus playing an important role in the pathological process of Parkinson's disease. Therefore, autophagy plays an important role in the protein quantity control system that protects cells from toxic damage caused by protein misfolding and promotes cell survival (see Chaps. 18 and 20).

### 29.2.2 Autophagy-Related Genes and Cell Survival

Organisms or cells that lack key autophagy genes are more likely to die suggesting that autophagy is essential for cell survival. For example, mice lacking *BECN1* die during the early stage of embryonic development while mice lacking *Atg5* die within one day of birth unless forced breastfeeding is initiated immediately suggesting that autophagy is a key way to overcome hunger. In addition, the deletion of *Atg5* or *Atg7* in mouse nerve cells can lead to age-related neurodegeneration and shortens the life span of mice. A disruption in the Beclin 1-BCL2 interaction could significantly increase the level of autophagy, inhibit premature aging, and significantly improve the life span in mice (Fernandez et al. 2018). Autophagy genes are essential for maintaining cell homeostasis and cell survival when cells are unable to absorb nutrients such as growth factors from the environment. Autophagy also promotes cell survival through other mechanisms such as by removing damaged organelles and degrading pathogens and large protein aggregates that cannot be degraded through the ubiquitin-proteasome system. Therefore, in aging, infection, neurodegenerative diseases, and tumors autophagy is closely related to the maintenance of cell survival (see Chaps. 22 and 24).

### 29.2.3 Autophagy-Mediated Homeostasis for Cell Survival

Autophagy plays an important role in maintaining cell homeostasis. Under a normal nutrient supply autophagy regulates cell survival mainly through energy sensing. Recently, it has been found that the mechanism of the autophagy and metabolism interaction presents a complicated dynamic feedback loop that regulates the energy state of cells to prevent cell death. The process and activity of autophagy can be directly or indirectly affected by the metabolism or energy state inside and outside the cell. Autophagy performs the following three main functions in cell metabolic networks in response to external stimuli: first, autophagy synthetically senses the energy state of cells; second, autophagy generates many metabolic substrates as feedback by adjusting the flow of autophagy; and third, autophagy balances ATP consumption and mitochondrial restoration activity through the autophagy process to ensure cell survival.

The three main energy sensing pathways link the cell energy levels to the autophagy process to guarantee an adequate ATP supply, higher productivity, and cell restoration capacity. Therefore, some downstream amplification effects can regulate the stability of cell metabolism more effectively, which, in turn, could improve cell survival. The main energy sensing cascade kinases in cells include protein kinase A (PKA), AMPK, and mTOR. These three signal transduction pathways are also closely related to the regulation of autophagy.

#### 29.2.3.1 mTOR Complex

The mTOR pathway can regulate protein synthesis and degradation, and mTOR can sense the intracellular ATP concentration. In yeast, the interaction between Atg1 and Atg13 initiated by the hyperphosphorylation of Atg13 is mediated by rapamycin receptor complex 1 (TorC1) and triggers the formation of autophagosomes. The interaction between Atg1 and Atg13 seems to be very stable because it cannot be altered by nutrient conditions or mTOR inhibition. In mammals, mTORC1 phosphorylates ATG1 homologous protein ULK1 which forms a complex with ATG13 and RB1CC1/FIP200/ATG101 to inhibit the initiation of phagophores. TOR/mTOR is a central regulatory pathway that connects the autophagic activity of cells to the energy condition thus enhancing ATP generation efficiency under energy-deficient conditions. For example, ATG13 could be highly phosphorylated by TOR leading to autophagy activation. TOR activity depends on its high sensitivity to available nitrogen sources, such as L-glutamine or L-asparagine. The sensitivity of mTOR to amino acids is based on the outflow of L-glutamine, the inflow of L-leucine and the flow of other essential amino acids in the bidirectional transport system. L-leucine is one of the most effective amino acids activating mTOR signaling. Therefore, mTOR participates in the regulation of the metabolism efficiency of amino acids by a negative feedback loop. During *C. elegans* embryogenesis, mTORC1 can regulate the liquid–liquid phase separation (LLPS) of PGL-1/-3 to help embryos adapt to developmental stresses through autophagic degradation (Zhang et al. 2018). However, the more specific mechanisms by which mTOR regulates autophagy have not been fully elucidated (see Chaps. 3 and 16).

#### 29.2.3.2 AMPK

AMPK is a sensor of the cell energy level and is necessary for the regulation of autophagy because a mutation in AMPK or the inhibition of AMPK could block autophagy. AMPK can be activated by excessive ADP and AMP or the lack of ATP due to glucose starvation. AMPK can regulate autophagy by affecting the following two key signal transduction complexes: inhibition of mTOR or direct phosphorylation of ULK1. Therefore, AMPK acts as a "fail-safe" mechanism by regulating autophagy activity through two different molecular pathways regulating the induction of autophagy and providing ATP-producing metabolites. Furthermore, AMPK plays a key role in remodeling whole-cell metabolism and improving the metabolic ability of autophagy induction (Mihaylova and Shaw 2011). For example, by closing the ATP-dependent metabolic process, AMPK can promote the retention of ATP. This process may be important when oxygen levels are low and the yields of ATP per mole of oxygen need to be increased. Therefore, AMPK improves ATP levels by the following dual actions: first, AMPK improves the ATP levels available to cells by providing metabolites that can be used as ATP synthetic substrates and second, AMPK reduces ATP consumption and minimizes ATP loss by closing the ATP consumption metabolic process (see Chaps. 4 and 16).

#### 29.2.3.3 PKA

In HeLa cells treated with amino acids and serum starvation PKA could mediate the phosphorylation of DNM1L/DRP1, thus inhibiting cell proliferation, breaking the division/fusion balance, and promoting cell development (Chang and Blackstone 2007). This process results in a larger mitochondrial network containing more elongated mitochondria which leads to higher ATP synthesis efficiency. This higher energy production rate is due to the increased mitochondrial ATP synthesis and higher mitochondrial ridge density and quantity. If a cellular energy state with sufficient residual oxygen and metabolite supply can be maintained the growing energy demand could be met. In this case, ATP is usually retained because of the mitophagy caused by depolarization, and damaged mitochondria are reduced. In addition, the available ATP in the cytoplasm can be more effectively utilized because nonelongated mitochondria consume more ATP in the cytoplasm to maintain their membrane potential leading to the consumption of the ATP storage and accelerating the initiation of cell death.

In addition to the above three forms it has also been mentioned that autophagy could regulate cell survival under pressure by regulating the removal of damaged organelles and misfolded proteins to reduce functional mitochondrial stress and the risk of toxic protein aggregates. In conclusion, these results suggest that changes in the autophagic flow can effectively regulate metabolic efficiency, thus regulating cell survival.

## 29.3 Autophagy and Cell Death

It is generally believed that cell death is an irreversible process leading to the termination of cell life. Recent studies have shown that there are reversible cell death procedures under certain conditions but the regulatory mechanism is still unclear. The current consensus regarding cell death is that cell membrane permeability cannot be reversibly restored and that macromolecules in cells are completely fragmented; it is conceivable that under such conditions cells will surely die. However, cell death often occurs in normal tissues, which is necessary for maintaining the normal function and morphology of life. According to the latest recommendations of the Nomenclature Committee on Cell Death (NCCD) regarding the classification of cell death, cell death can be divided into the following two categories: accidental cell death (ACD) and regulated cell death (RCD). ACD mainly refers to the rapid collapse of the cell structure caused by extreme physical, chemical or mechanical stimulation leading to cell death in an uncontrollable way. RCD can be considered a part of normal physiological development or an adaptive response of cells to intracellular and extracellular environmental changes. According to the specific biochemical mechanisms of cell death these death patterns can be subdivided into different subtypes. From a biochemical perspective apoptosis can be defined as a caspase-dependent form of RCD. According to the origin of the death signal apoptosis can be divided

into internal induced apoptosis, external induced apoptosis, and anoikis (Fig. 29.1). Necroptosis, which is a key subprogram of death programs is considered a caspase-independent form of cell death. In addition, many different types of RCDs can be identified according to the different substances (short peptides, metal ions, etc.) on which cell death depends. Therefore, the classification of cell death is always in the process of continuous improvement. The factors leading to cell death are intricate. There are also intersections among the mechanisms of different types of cell death. Under stress conditions, different types of cells under different experimental conditions may also have various characteristics of cell death (Galluzzi et al. 2018). It is believed that cell death is a type of specific behavior depending on the cell and environment to some extent. Due to the complexity of cell death classification the most classical classification method was chosen in this chapter and cell death was classified into the following three categories: type I cell death (apoptosis), type II cell death (autophagic cell death), and type III cell death (necrosis) (Table 29.1).

In the first section, it was mentioned that autophagy was originally considered a means of maintaining homeostasis and resisting environmental stresses, including nutritional deficiency, energy shortage, endoplasmic reticulum (ER) stress, reactive oxygen species, and hypoxia. Then, the phenotype and biochemical markers of autophagy were frequently found in dead cells and the relationship between autophagy and cell death became a new research hotspot. However, autophagy plays a causal role in a small number of cell deaths. Moreover, many studies claiming that autophagy causes cell death are insufficient. The concept of "autophagic cell death" was proposed in the 1970s as programmed cell death, which was initially described as caspase-dependent necrotizing cell death that is usually accompanied by the accumulation of autophagosomes in cells. This concept sometimes misleads people to believe that autophagy plays a role in the mechanism of cell death. In fact, the function of death mediation is not a part of this definition. Currently, "autophagy-mediated cell death" can be understood according to the following three different meanings: (1) autophagy-associated cell death, which is the original meaning of this concept (2) autophagy-mediated cell death, which suggests that cell death such as apoptosis is triggered by autophagy, and (3) a unique mechanism of cell death independent of apoptosis or necrosis. Of the three explanations the explanatory specificity of (2) is stronger than that of (1), which can be proved by observing whether cell death is blocked when autophagy is inhibited by biological or chemical methods. Explanation (3) is more specific because even if the inhibition of autophagy blocks cell death subsequent evidence is needed to prove that the mechanism of cell death is not apoptosis or necrosis. Therefore, some scholars believe that if the proof requirements of the interpretation of (3) cannot be met, it is better to replace the concept of "autophagy-related cell death" with "autophagy-mediated cell death". According to the above principles it is recommended that autophagy-dependent cell death be used instead of autophagy-mediated cell death because while autophagy has been shown to be a precondition of cell death, it cannot be proven that autophagy mediates the cells to switch to the death state. The term "phagocyte death" is controversial but still widely accepted; thus, this term is still used in the follow-up of this book and most of the literature.

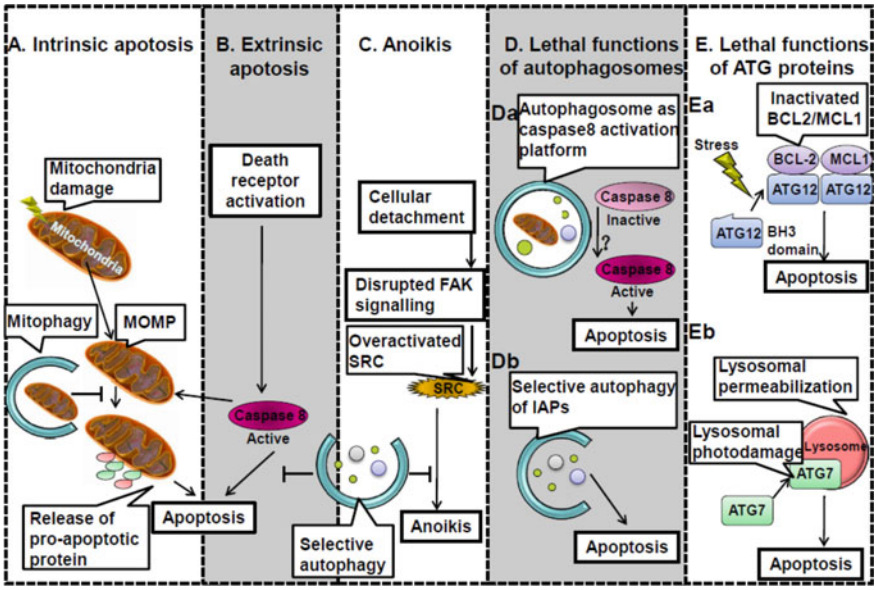


**Fig. 29.1** Crosstalk between autophagy and apoptosis. **A** Intrinsic apoptosis pathway. Many proteins, lipids, and metabolites can induce mitochondrial damage further triggering mitochondrial outer membrane permeabilization (MOMP) which leads to the release of catabolic hydrolases such as AIF (apoptosis-inducing factor) or Endo G (Endonuclease G), and caspase activators (such as cytochrome c and SMAC (second mitochondria-derived activator of caspase)) into the cytosol. These changes usually indicate that the internal trigger of apoptosis is irreversible. Selective autophagy clearance of damaged mitochondria (mitophagy) can limit the release of pro-apoptotic factors thereby delaying the initiation of apoptosis. **B** Extrinsic apoptosis pathway. The extrinsic apoptotic pathway is triggered by the binding of a trimer ligand to a so-called death receptor. A key event in this pathway is the activation of caspase 8. Autophagy selectively removes active caspase 8 and delays the onset of externally triggered apoptosis after death receptor activation. Since caspase 8-induced apoptosis depends on the cleavage and activation of BID (BH3-interacting domain death agonist), BID is a possible MOMP-inducer and mitophagy may inhibit the lethal signaling of death receptor transduction. **C** Anoikis. Autophagy can selectively eliminate overactivated SRC induced by disrupted FAK (focal adhesion kinase) signal transduction thereby delaying anoikis due to cell detachment from the extracellular matrix. **D**, **E** Role of autophagy proteins in apoptosis. Although autophagosomes and ATG proteins usually constitute cytoprotective mechanisms they may also be involved in lethal signal transduction processes under certain special conditions. **D**(a) Autophagosomes can serve as a platform for caspase 8 activation although the detailed mechanism of this process has not been elucidated (marked with a question mark in the figure). **D**(b) In *Drosophila melanogaster*, the clearance of anti-apoptotic proteins, such as Bruce (BIR-containing ubiquitin-conjugating enzyme; an orthologue of the mammalian inhibitor of apoptosis proteins (IAPs)) by selective autophagy can promote the induction of apoptosis. **E**(a) Specific pro-apoptotic stimulation signals such as C6 ceramide or tunicamycin can induce an interaction between ATG12 and the anti-apoptotic BCL-2 or MCL1 (myeloid cell leukemia sequence 1) proteins thereby inhibiting their functions to enhance apoptosis. **E**(b) ATG7 promotes lysosomal membrane permeabilization after lysosomal damage thereby promoting the activation of the apoptotic pathway

**Table 29.1** Main biochemical features of cell death modes

| Cell death mode | Main biochemical features |
|---|---|
| Autophagic cell death | LC3 lipidation, SQSTM1 degradation |
| Apoptosis | Downregulation of EGFR, Inhibition of ERK1 signaling, Lack of β1-integrin engagement, Overexpression of BIM, Caspase-3 (-6,-7) activation (Anoikis), MOMP, Irreversible $\Delta\Psi$m dissipation, Release of IMS proteins, Respiratory chain inhibition (Intrinsic apoptosis), Death receptor signaling, Caspase-8 (-10) activation, BID cleavage and MOMP, Caspase-3 (-6, -7) activation, PP2A activation, DAPK1 activation, Caspase-9 activation (Extrinsic apoptosis) |
| Necroptosis | Death receptor signaling, Caspase inhibition, RIP1 and/or RIP3 activation |

Since the exact relationship between autophagy and cell death has not been confirmed many studies have focused on clarifying the relationship between autophagy and the cell death process. Recent studies have also confirmed that crosslinks exist between signaling pathways regulating autophagy and cell death processes including apoptosis and necrosis. This section focuses on the relationship between autophagy and cell death and the regulation of the death process.

### *29.3.1 Autophagy and Apoptosis*

Apoptosis is a cell-regulated mode of death requiring protease and nuclease that plays a synergistic role in the whole plasma membrane. The morphological characteristics of apoptosis include DNA breakage, vesicles in the plasma membrane, cell condensation, and eventually cell decomposition into apoptotic bodies surrounded by membrane, which are eventually removed by cell phagocytosis. The typical biochemical characteristics of apoptotic cells include mitochondrial dysfunction, respiratory chain inhibition, the loss of mitochondrial internal membrane potential, increased mitochondrial membrane permeability, and exposure of phosphatidylserine on the outer side of apoptotic bodies. Apoptosis plays an important role in maintaining tissue homeostasis and development under physiological conditions. In addition, apoptosis may play an important role in the process of some diseases. Many signaling pathways can activate apoptosis. The "internally initiated" (mitochondrial-dependent) apoptotic pathway is the main cause of apoptotic initiation and is usually triggered by harmful extracellular stimuli. This pathway depends on a series of chain reactions regulated by BCL-2 family proteins. The externally activated apoptotic pathways need to be triggered by receptor–ligand interactions and some downstream signaling pathways are shared with the internally initiated apoptotic pathways (Fig. 29.1).

Autophagy and apoptosis are strictly regulated biological processes in cells that play important roles in development, tissue homeostasis, and diseases. Similar stimulus signals could activate these two pathways and the interaction of some signaling

molecules in these two pathways indicates that crosstalk exists between these two mechanisms. For example, studies have shown that both apoptosis and autophagy can be activated by metabolic stress. The lack of growth factors, nutrition, and energy metabolism could activate the LKB1-AMPK pathway in mammalian cells, improve the stability of the cyclin-dependent kinase inhibitor $p27^{kip1}$, and promote cell survival by inducing autophagy. Conversely, silencing $p27^{kip1}$ under the same conditions activates apoptosis. In addition, autophagy could be used as an adaptive response to ER stress. Notably, a disturbance in the ER calcium balance or function of ER can simultaneously increase autophagy and apoptotic cell death. In different tissues, autophagy plays different roles in resisting ER stress and promoting survival. In colorectal and prostate cancer cells, ER stress-induced autophagy plays an important role in clearing aggregates of ubiquitinated proteins thus protecting cells from death. However, in normal human colon cells and nontransformed mouse embryonic fibroblasts autophagy cannot alleviate endoplasmic reticulum stress but tends to promote endoplasmic reticulum stress-induced apoptosis. Studies have increasingly shown that autophagy and apoptosis could cooccur and antagonize or promote each other resulting in different effects on cell fate. Recent studies have also found that many pathways mediating autophagy can interact with apoptotic complexes laying the foundation for a profound understanding of the regulatory networks of the two pathways.

#### 29.3.1.1 Crosstalk Between Autophagy and Apoptosis

**(1) Autophagy inhibits apoptosis**

Studies using different experimental systems suggest that the role of autophagy in cell death depends on the environment and cell type. Autophagy can delay the initiation of apoptotic processes including starvation, DNA damage, and hemodynamic stress-induced apoptosis. Fasting for one day leads to autophagy in the liver of rats but if starvation lasts for several days liver cells undergo apoptosis. The lack of growth factor IL-3 in hematopoietic cell lines first activates autophagy and eventually leads to apoptosis. This finding indicates that *ATG* genes can promote survival in cells via a complete apoptotic mechanism. SQSTM1-labeled protein aggregates have protective effects on cell death because SQSTM1 mutants lacking the domain of ubiquitin ligase activity inhibit the formation of SQSTM1-positive aggregates and promote cell death.

However, inhibition at an early stage of autophagy enhances the sensitivity of cells to stress-induced apoptosis. In rectal cancer cells, the inhibition of autophagy by 3-MA due to p38 inhibition induces autophagic cell death and apoptosis. These findings suggest that autophagy plays a protective role in promoting survival against harmful stimuli allowing cells to die only after sustained stimulation. In fact, the removal of death signal stimuli can reduce the number and volume of autophagic vesicles in colon cancer cells and the cells could re-enter the cell cycle. Studies using genetic techniques have also shown that autophagic proteins are directly involved in the balance between cell survival and cell death. The inhibition of autophagy induced by nutritional deficiency via the silencing of autophagy genes in HeLa cells leads to

apoptosis which can be delayed by apoptotic inhibitors. When autophagy is inhibited during autophagy nucleation fasting cells accelerate cell death through classical apoptotic processes. Similarly, HeLa cells lacking the ubiquitin-like genes required for autophagosome formation are more sensitive to starvation-induced apoptosis. In vitro experiments have confirmed that ATG gene knockdown induces cell death. *AMBRA1*-deficient embryos exhibit neurodevelopmental deficits associated with autophagy disorders and excessive apoptosis confirming the regulatory role of autophagy-related genes in cell death. In contrast, cell death with apoptotic and autophagic characteristics occurs if autophagy is blocked at the late fusion stage by drugs or *LAMP2* gene silencing. Therefore, the observed accumulation of autophagic vesicles does not necessarily imply an increase in autophagic activity because autophagy is inhibited under these conditions. When autophagy is induced through different pathways and the autophagy process is inhibited during the early or late stages, apoptosis occurs due to the inability to adapt to stress. However, in malignant glioma cells 3-MA can inhibit apoptosis induced by alkylating reagents, while Bafilomycin A1 enhances the sensitivity of cells to apoptosis induced by alkylating reagents indicating that the inhibition of autophagy at different stages can lead to different effects on apoptosis.

Autophagy reportedly inhibits cell apoptosis mainly through the mechanism of mitophagy. Mitochondria constitute the "battlefield" on which survival and death signals struggle to determine whether endogenous apoptotic pathways marked by mitochondrial outer membrane permeability (MOMP) are activated. In fact, hundreds of different factors including proteins, lipids, and metabolites affect the function and physiological integrity of the mitochondrial membrane. During cell death, MOMP causes the release of hydrolytic enzymes (such as apoptosis-inducing factor (AIF) and endonuclease G) in catabolism and caspase activating factors (such as cytochrome c and secondary mitochondria-derived activator of caspase, SMAC, also known as DIABLO) from mitochondria leading to the disappearance of mitochondrial transmembrane potential ($\Delta\Psi_m$) and finally, biological energy could be severely lost. These changes are irreversible in endogenous apoptotic pathways (Fig. 29.1A). Damaged mitochondria are particularly prone to activating apoptotic processes; thus, removing substances released due to mitochondrial damage via autophagy by increasing the threshold of apoptotic induction.

How does autophagy specifically degrade damaged mitochondria? A decrease in $\Delta\Psi_m$ usually indicates the loss of respiratory chain function or an increase in endometrial permeability resulting in the ubiquitination of the Lys63 of the protein anchored on the outer membrane. These outer membrane proteins include voltage-dependent anion-selective channel 1 (VDAC1), mitochondrial fusion protein 1 (mitofusin 1, MFN1), and MFN2 which connect organelles required by mitophagy. This pathway is feasible because the disappearance of $\Delta\Psi_m$ shuts down a protein enzyme that destroys PTEN-induced putative kinase 1 (PINK1) leading to PINK1 aggregation on the surface of damaged mitochondria. Then, PINK1 can recruit and phosphorylate PARKIN (Parkinson's disease protein) which is an E3 ubiquitin ligase that ubiquitizes the substrate of the mitochondrial outer membrane, thus marking out organelles that need to be degraded by autophagy. In addition to the disappearance of $\Delta\Psi_m$, mitochondrial fragmentation is necessary for mitophagy which constitutes

a fail-safe mechanism to prevent the unnecessary degradation of functional mitochondria. Mitochondria in starving cells undergo a loss of $\Delta\Psi_m$ but do not break up suggesting that they do not undergo mitophagy. In contrast, the open mitochondria in permeability transition pores (PTPs), which are pores composed of many proteins and can permeate the inner membrane of mitochondria leading to a decrease in $\Delta\Psi_m$ and the fragmentation of mitochondria; then, these mitochondria could be cleared by autophagy.

Autophagy also plays a role in inhibiting unnecessary cell death in the pancreas. Pancreatic acinar cells contain many inactive enzyme precursors (zymogens) such as trypsinogen (progenitors of trypsin) which exist as isolated particles but may also leak into the cytoplasm and cause cell death. In a cerulein-induced pancreatitis model, the overexpression of vacuole membrane protein 1 (VMP1) by a transgene could promote the clearance of microparticles containing abnormally activated trypsin. The overexpression of VMP1 can alleviate some enzymatic, histopathological and naked eye indicators of pancreatitis, and blocking the autophagic flow by reducing the expression of VMP1 can aggravate pancreatitis induced by ethanol and bacterial lipopolysaccharide. Therefore, VMP1 can inhibit cell death by promoting the removal of microsomes through autophagy.

Autophagy can also alleviate apoptotic cell death by selectively reducing the abundance of pro-apoptotic proteins in the cytoplasm (Fig. 29.1B). In fact, autophagy can specifically select ubiquitinated proteins as target proteins. Ubiquitination modification enables these proteins to interact with autophagic receptors that belong to a series of adaptor proteins including SQSTM1 which can bind both ubiquitinated substrates and LC3. For example, colorectal cancer cells lacking BAX can resist cell death induced by TNF-related apoptosis-inducing ligand (TRAIL) but this resistance disappears when autophagy is suppressed because autophagy can mediate the selective scavenging of active caspase 8. Similarly, the inhibition of autophagy by a specific *Atg7* knockout can enhance the activity of caspase 8 in a TNF-induced mouse hepatocyte apoptosis model which may be due to the inability to effectively eliminate caspase 8 by autophagy. In addition, autophagy can specifically degrade the proto-oncogene tyrosine kinase SRC thus avoiding anoikis induced by blocking the signal transduction of focal adhesion kinase (FAK) (Fig. 29.1C). Under these conditions SRC binds the E3 ubiquitin protein ligase CBL, which contains an LC3 binding region that can be used as a molecular junction to localize SRC on LC3-positive autophagosomes and thus be degraded. In addition, autophagy can inhibit the production of reactive oxygen species (ROS) and cell death induced by SQSTM1 by degrading SQSTM1.

**(2) Autophagy promotes apoptosis**

Some experimental results suggest that the inhibition of autophagy can block the apoptotic process; thus, autophagy can induce apoptosis. When 3-MA was used to inhibit autophagy in the breast, prostate, colorectal cancer cells, and glioma cells apoptosis could not occur in these cells. Resveratrol-induced autophagy can be switched into the apoptotic process which can be blocked by the inhibition of autophagy. Recent studies have confirmed that autophagy plays a role in upstreaming of apoptosis in $CD4^+$ T lymphocytes that are not infected by HIV because apoptosis

can be inhibited by the gene silencing of *BECN1* and *ATG7* indicating that autophagy can induce apoptosis under certain conditions. In *Drosophila* tissues, the overexpression of Atg1 induces autophagy and inhibits Tor signal transduction and cell growth while high levels of autophagy lead to apoptosis.

There are many examples of apoptotic activation via the induction of autophagic cell death. Proteins that play key roles in autophagy may also play roles in apoptotic signal transduction. What is the mechanism by which autophagy promotes the induction of apoptosis? Autophagosome formation rather than degradation is thought to be associated with the activation of caspase 8 after SKI-I (a pan-sphingosine kinase inhibitor) and bortezomib (also known as Velcade, a proteasome signaling pathway inhibitor) treatment (Fig. 29.1D(a)). Under these conditions caspase 8 can form a complex with FADD (FAS-associated death domain) and ATG5, colocalize with ATG5, LC3, and SQSTM1 finally become activated in an ATG5, FADD and, SQSTM1dependent way. In SKI-I- or bortexamib-treated cells, inhibition at the early stage of autophagy (through *ATG3* or *ATG5* knockout) decreases the activation of caspase 8 and caspase 3 effector factors while inhibition at the late stage of autophagy (through bafilomycin A1 treatment) enhances caspase-dependent cell death. These data suggest that the autophagosome formation process rather than the whole autophagy process may provide favorable conditions for the activation of caspase 8. However, caspase 8 is usually not activated when autophagy is induced and the activation of caspase 8 can be confirmed by a TNF test in vivo in mouse models following the liver-specific knockout of *Atg7*, which functions in the early stage of autophagy. The factors determining caspase 8 activations upon autophagosome formation are unclear.

Autophagy may also promote apoptosis by removing endogenous inhibitors of apoptosis. In *Drosophila melanogaster*, autophagy is mainly regulated by the interaction between caspases and inhibitors of apoptotic proteins (IAPs). One IAP called Bruce (BIR containing ubiquitin-conjugating enzyme, ubiquitin-conjugating enzyme containing BIR) can be degraded by autophagy which explains why inhibiting autophagy genetically (by targeting mutations in *Atg1*, *Atg13* or *vps34*) can prevent oocyte developmental apoptosis at later stages of oogenesis (Fig. 29.1D(b)). Recently, autophagy has been reported to promote palmitic acid-induced apoptosis by degrading Caveolin-1, which is a key protein that promotes astrocyte survival (Chen et al. 2018).

To date, many studies investigating the genetic manipulation of autophagy have indicated that autophagy is closely related to the promotion of apoptosis in selective toxicity models. Recent studies have found that exposure to cigarette smoke extract (CSE) can activate the exogenous apoptotic pathway and cause death in human lung epithelial cells. CSE-induced cell death involves the activation of a Fas-dependent death-inducing signaling complex (DISC) and downstream activation of caspases (8, 9, 3). When exposed to CSE, autophagy formation and transformation from LC3B-I to LC3B-II occur simultaneously in human lung epithelial cells. The silencing of the autophagic protein Beclin 1 or LC3 can inhibit apoptosis induced by CSE exposure in vitro indicating that the increase in the autophagy level is closely related to epithelial cell death. Subsequent studies have found that LC3B may function as

an exogenous apoptotic regulator in this model. LC3B can form a complex with Fas which is the key component of DISC in the form of lipid raft protein caveolin-1-dependent. Exposure to CSE can lead to a rapid dissociation between LC3B and Fas which is related to the activation of apoptotic signal transduction. In conclusion, these results from RNA interference silencing experiments suggest that LC3B can promote apoptosis in a CSE-induced toxicity specific model, but the function of autophagic activity in promoting cell death in this model is still unclear. Notably, CSE-induced autophagy may differ from starvation-induced autophagy because the former requires the formation of complexes by members of different pathways which may alter the function of autophagy. Therefore, the concept of "toxic autophagy" may include this altered function depending not only on whether the degree of the CSE-induced activation of autophagy is at the physiological level or excessive but also on the nature of the exogenous substrates (such as intricate symbiotic organisms in vivo including tar or virus fragments) and their interaction with autophagosomes.

There are also many examples of simultaneous autophagy and apoptosis; for example, Tp53-dependent autophagy requires the upregulation of damage-regulated autophagy modulator (DRAM) which is consistent with the upregulation of apoptosis. TNFα can induce autophagy in embryonic trophoblast cells which leads to the activation of endogenous apoptotic pathways. In this model, the silencing of *Atg5* inhibits TNFα-dependent apoptotic caspase activation. Moreover, *Atg5* knockout protects mouse embryonic fibroblasts from death-promoting stimuli. However, the authors attributed this effect to a chaperone-dependent autophagic compensatory activation rather than autophagy inhibition. These studies also propose the following important conclusion, i.e., under any conditions, genetically silencing certain autophagy-related factors cannot explain whether autophagy has a cytoprotective function because the silencing of any gene expression may affect or trigger other signal transduction pathways unrelated to autophagy or some compensation mechanisms such as other forms of autophagy.

It has also been found that many ATG proteins do not depend on autophagy to participate in lethal signaling. ATG12 is thought to play a role in activating caspases through the mitochondrial pathway because its absence can reduce caspase activation induced by treatment with C6 ceramide, epipodophyllotoxin glucopyranoside, Taxol, and chlamydiamycin (Fig. 29.1E(a)). This ATG12 activity requires the BH3 domain rather than its autophagic activity to mediate its interaction with BCL-2 and MCL1. Similarly, in mouse hepatoma Hepa-1c1c7 cells, Atg7 (not Atg5) promotes autophagy induction after lysosomal injury induced by lysosomal membrane permeation which is similar to MOMP and both can induce apoptosis (Fig. 29.1E(b)). These examples illustrate the mechanism by which a single ATG protein promotes lethal signal transduction.

However, whether autophagy can be used as a mechanism of death when cells have complete apoptotic mechanisms remains unclear. The recovery of beclin-1 expression in breast cancer cells with a single allele knockout of *BECN1* results in enhanced autophagy. In human cervical cancer cells treated with IFNα, ATG5 can promote autophagic cell death by interacting with FADD. Correspondingly, cells may die from apoptosis unless they are stimulated by other sufficiently strong death

mechanisms. Autophagy is more likely to serve as a survival-promoting mechanism than a death-promoting mechanism. At least in cells with complete apoptotic mechanisms the characteristics of autophagy can be used as a resort to maintain survival during the process of death.

#### 29.3.1.2 mTOR

Mammalian target of rapamycin (mTOR) can sense and balance signals from nutrition, growth factors, energy, and stress thus plays an important regulatory role in early development, growth, and adult aging. Low insulin and insulin-like growth factor-1 (IGF1) signaling, nutrient or energy deficiencies, and stress signals can be converted into the downstream regulation of TOR activity. The inhibition of TOR activity can lead to a decrease in the rate of gene translation and thus activate autophagy. Under starvation, TOR is rapidly suppressed to activate autophagy. Interestingly, recent studies have found that mTOR signaling is suppressed at the beginning of autophagy but reactivated when starvation persists. As a result, reactivated mTOR can alleviate the autophagic process by producing prelysosomal tubules and prelysosomal vesicles (also known as autophagic lysosome reformation, ALR) from the autophagic lysosome, which can develop into functional lysosomes thus restoring lysosome balance (Yu et al. 2010). This negative feedback regulation ensures the termination of autophagy under conditions of sufficient nutrients thus avoiding cell death caused by the excessive accumulation of vesicles in the cytoplasm.

mTOR reportedly performs distinct functions in apoptosis depending on the cell environment and different downstream targets such as Tp53, BAD, and BCL-2 proteins. Recently, the following two new mTOR-acting proteins have been identified: the proline-rich AKT substrates (PRAS40) and Q6MZQ0/FLJ14213/CAE45978 proteins; these proteins have been suggested to play a role in apoptotic regulation thereby controlling the balance between cell growth and death. Recent studies have found that MCL1 which is an anti-apoptotic protein BCL-2 homolog can act as a stress sensor to coordinate the control of autophagy and apoptosis. The final result is determined by the interaction between BAX and Beclin 1 activation downstream of MCL1 degradation. Consistent with the possibility of the simultaneous regulation of autophagy and apoptosis by TOR, the inhibition of mTOR leads to inadequate nutritional supply causing the degradation of MCL1. While it is widely accepted that TOR can regulate various anabolic and catabolic processes in a positive or negative way further studies are required to understand the regulation mechanism of TOR in autophagy and apoptosis.

#### 29.3.1.3 Beclin 1

BECN1 is a mammalian ortholog of yeast Atg6. Its expression products form core complexes with VPS34 and VPS15 which can induce autophagy and play a key role in autophagosome formation (Fig. 29.2). Beclin 1 is widely expressed in many tissues

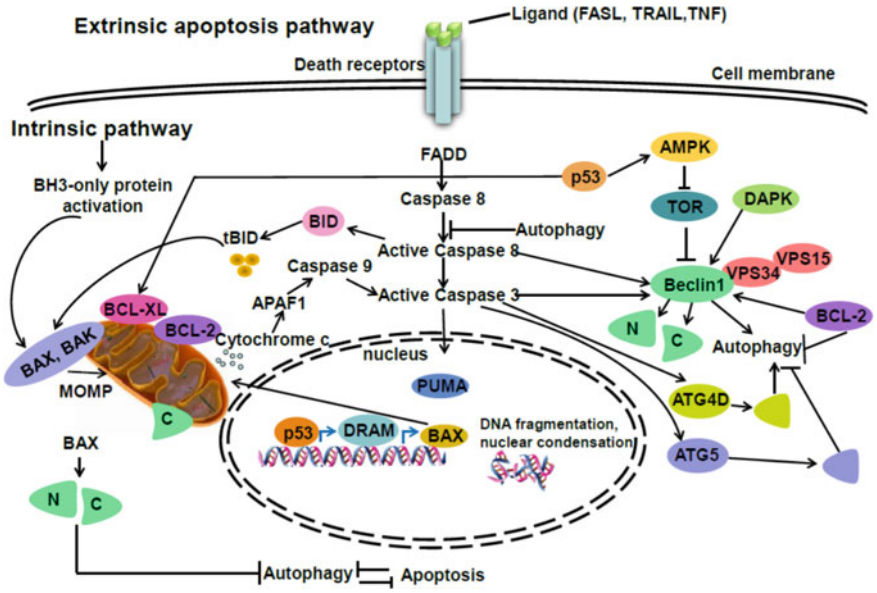


**Fig. 29.2** Mechanism of the complex interplay between apoptosis and autophagy. Apoptosis can be stimulated by external receptor-dependent signaling or internal mitochondria-mediated signal transduction. The extrinsic pathway is initiated by the binding of a death receptor to its cognate ligands such as FASL, TRAIL or TNF. This results in the binding of FADD (FAS-associated death domain protein) to the death receptor and subsequent activation of caspase 8. Activated caspase 8 can directly cleave and activate caspase 7 and caspase 3 to promote apoptosis. The intrinsic pathway is regulated by a BH3 domain-containing protein that is activated by different types of cellular stress, such as DNA damage or endoplasmic reticulum stress which then activates BAX/BAK located in the mitochondrial outer membrane (MOM) and induces mitochondrial outer membrane permeabilization (MOMP). MOMP leads to the release of different apoptosis mediated factors such as cytochrome c, which activate caspase 9. In turn, caspase 9 cleaves and activates caspase 3 and caspase 7 which induce apoptotic cell death. Both pathways converge on the point of caspase 3 activations. Autophagosome formation requires beclin 1 with members of the polyprotein (PI3K) complex such as VPS34, VPS15, etc. The crosstalk between the autophagy and apoptotic pathways is at least partially mediated by the structural and functional interactions between beclin 1 and the anti-apoptotic proteins BCL-2 and BCL-$X_L$. Diverse apoptotic stimuli (internal or external) can result in the caspase-mediated cleavage of beclin 1. Ultimately, beclin 1 loses its ability to induce autophagy. Instead, the C-terminal fragment translocates to the mitochondria, rendering the cell sensitive to apoptotic signals. In contrast to the phenomenon in which beclin 1 and ATG5 lose their ability to induce autophagy after cleavage, caspase cleavage of ATG4D can enhance its ability to promote autophagy. Tp53 plays a key role in both the apoptosis and autophagy pathways. At the transcriptional level Tp53 upregulates BAX, PUMA, and BID or reduces the expression of BCL-2, which inhibits BAX. In addition to apoptosis Tp53 is capable of inducing autophagy through the inhibition of TOR and the transcriptional activation of DRAM. Interestingly, the inhibition of autophagy by cytosol Tp53 suggests that an extremely complex relationship exists between Tp53 and the autophagy/survival pathways. DAPK belongs to a class of $Ca^{2+}$/CaM-regulated Ser/Thr kinases and is closely related to apoptosis and autophagic cell death. See text for details

in humans and mice. Similarly, *Bec-1* is a homologous gene of mammalian *BECN1* which is expressed in all remodeling tissues during dauer larval formation (a stage of developmental arrest). *Bec-1* is critical during embryonic development and is necessary for normal larval development and the whole adult growth process. Moreover, the loss of *BECN1* leads to the death of mouse embryos, and *Becn* $1^{+/-}$ mice are more likely to suffer from tumors. These phenomena are closely related to autophagy deficiency but there is no defect in apoptotic cell death. It has been confirmed that the interaction between autophagy and apoptosis is partially mediated by the functional and structural interaction between Beclin 1 and BCL-2/BCL-$X_L$. The Beclin 1/BCL-2 interaction is very evolutionarily conservative. In *C. elegans*, Bec-1 forms two complexes with the Bcl-2 homologs Ced-9 and Let-512/Vps34 which have different functions. The absence of the lipid product PtdIns 3-phosphate of LET-512/VPS34 in Bec-1-deficient larvae confirms that Bec-1 is essential for let-512/Vps34 which plays a key role in autophagy membrane trafficking, and endocytosis. In addition, the inactivation of *Bec-1* induces apoptosis, which can be confirmed by the increase in the number of apoptotic cells in animal germ cells and body tissues. In conclusion, these studies confirm that *Bec-1* plays a key regulatory role in both autophagy and apoptosis. Recently, the structural basis of the Beclin 1/BCL-2/BCL-$X_L$ interaction has been elucidated (Pattingre et al. 2005). Beclin 1 contains a BCL-2 homology (BH) 3 region which is responsible for the interaction with the BCL-2/BCL-$X_L$ protein. The BH3 domain interacts with the BH3 receptor to inhibit anti-apoptotic proteins, such as BCL-2 and BCL-$X_L$, or activate members of the apoptotic BCL-2 family such as BAX and BAK. Interestingly, mutations in the BH3 domain of Beclin 1 or the BH3 receptor domain of BCL-$X_L$ block the interaction between Beclin 1 and BCL-$X_L$ thus eliminating the inhibition of BCL-$X_L$-mediated autophagy. Notably, BCL-2 is specifically located in the endoplasmic reticulum rather than mitochondria which can effectively inhibit starvation-induced autophagy in yeast, mammalian cells and the heart muscle in transgenic mice coexpressing cardiac BCL-2 and the fluorescent autophagy marker GFP-LC3. The depletion or deletion of BH3-only protein BAD by siRNA has been shown to reduce starvation-induced autophagy, while the overexpression of BAD or the addition of BH3 analogues induce autophagy in human cells. Similarly, a defect of the BH3-only protein Egl-1 in *C. elegans* alleviates starvation-induced autophagy while a gain-of-function mutation in Egl-1 can activate autophagy. In conclusion, these studies suggest that BH3 domain-containing proteins or BH3 analogues can be used not only as inducers of cell death but also as regulators of autophagy. Interestingly, although the binding of BCL-2 to Beclin 1 weakens the ability of Beclin 1 to activate autophagy and Beclin 1 contains only one BH3 motif similar to apoptotic proteins, Beclin 1 cannot regulate the anti-apoptotic ability of BCL-2 to induce apoptosis. The interaction between Beclin 1 and Bcl-2 could not regulate apoptosis in autophagy-deficient mouse embryonic fibroblasts (MEFs), arguing against the protective effect of Beclin 1-mediated autophagy in cells.

#### 29.3.1.4 Caspases

Recent studies have provided new clues further elucidating the molecular mechanism of the crosstalk between autophagy and apoptosis. If the growth factor interleukin 3 (IL3) is depleted from mouse hematopoietic cell lines autophagy is induced as a mechanism of cell survival. If the deprivation is sustained, apoptotic cell death occurs. Apoptosis caused by growth factor deficiency is closely related to caspase-mediated cleavage in Beclin 1 and PI3K. This deficiency impairs the function of Beclin 1 in autophagy. In addition, the degradation of Beclin 1 and PI3K does not depend on the cell type, and the initiation of apoptosis is either endogenous (through the release of mitochondrial death-promoting factors) or exogenous (death receptor-dependent). Importantly, in IL3-dependent mouse pre B cell Ba/F3, the C-terminal fragment of Beclin 1 produced by caspase-mediated degradation is located in mitochondria rendering cells sensitive to apoptosis likely due to the release of pro-apoptotic factors (Fig. 29.2). The apoptotic protein BAX can inhibit autophagy by enhancing the caspase-mediated cleavage of Beclin 1 at D149; Beclin 1 and BCL-$X_L$ which cannot be degraded by caspase and can rescue BAX-induced autophagy (Luo and Rubinsztein 2010). These two phenomena indicate that apoptosis can inhibit autophagy. Further, evidence supporting the association between autophagy and apoptosis is that other autophagic proteins can also be caspase-induced apoptotic degradation substrates. Caspase 3 cleaves human ATG4D producing serine protease which, in turn, degrades the C-terminal of newly synthesized ATG8 (called LC3 in mammals) (Fig. 29.2). Atg4D cleavage by caspase results in increased priming and delipidation activities against yeast Atg8 homologous protein γ–aminobutyric acid receptor-associated protein-like 1 (GABARAP-L1), while silencing the expression of Atg4D inhibits autophagy and renders cells sensitive to starvation and staurosporine-induced cell death. This phenomenon confirms that caspase can activate autophagy-mediated by Atg4D to promote the survival of starved cells. Interestingly, the overexpression of caspase cleaved ATG4D induces apoptosis in human cells which requires the rapid recruitment of ATG4D to mitochondria. ATG5 is essential for the formation of autophagosomes also enhances cell sensitivity to apoptotic stimuli upon cleavage by caspase. Truncated ATG5 is transferred to mitochondria to regulate the mitochondrial apoptotic pathway. Notably, the caspase-mediated cleavage of ATG5 and Beclin 1 can switch autophagy to apoptosis and the truncated products produced by ATG4D cleavage can enhance autophagy activity (Fig. 29.2). These phenomena strongly suggest that autophagy is closely related to apoptosis.

#### 29.3.1.5 FLIPs

Based on the discovery of the crosstalk between the autophagic and apoptotic mechanisms, recent studies have found that the anti-apoptotic protein Flice inhibitory protein (FLIP) can function as a new negative regulator of autophagy. Inhibitors of apoptosis such as the cellular and viral orthologs of the FADD-like interleukin-1 beta-converting enzyme (FLICE)-like inhibitory proteins (c-FLIP and v-FLIP,

respectively), are triggered by known death receptors in the TNF/NGF (tumor necrosis/nerve growth factor) family. Strong evidence suggests that FLIPs compete with LC3 to bind ATG3 which is an E2-like enzyme in the normal environment. As a result, FLIPs inhibited ATG3-mediated autophagosome extension, thus decreasing the autophagy levels. However, under stress conditions FLIPs allow the ATG3-LC3 interaction to induce autophagy (Lee et al. 2009). In conclusion, FLIPs can not only act as an anti-apoptotic factor but also function as an autophagy inhibitor due to inhibitory binding to ATG3.

#### 29.3.1.6 DAPK

DAPK is a serine/threonine kinase regulated by $Ca^{2+}$/CaM (calmodulin) that can regulate cell death induced by various death signals. DAPK inhibits tumors and its expression is inhibited by DNA methylation in most tumors. DAPK is thought to be associated with apoptosis and autophagic cell death. DAPK is activated under endoplasmic reticulum stress, which, in turn, induces apoptotic and autophagic cell death (Gozuacik et al. 2008). In addition, *Dapk* knockout can protect against ER stress in vitro in isolated fibroblasts and in vivo in a mouse kidney toxicity model. Under these two experimental conditions, both apoptosis and autophagy were inhibited confirming the view that DAPK could integrate signals from both apoptosis and autophagy pathways to induce cell death under ER stress. The regulatory function of DAPK on autophagy has been confirmed in mammalian cells and *C. elegans* whereby reducing the expression of the *DAPK* homologous gene *Dapk-1* in the worms by mutation or RNAi silencing reduced the occurrence of starvation-induced autophagy in the pharyngeal muscle cells. However, the mechanism through which DAPK promotes autophagy remains unclear. A recent study investigating this issue found that Beclin 1 can be used as a target for the downstream regulation of DAPK (Fig. 29.2). DAPK mediates Thr119 phosphorylation on the BH3 domain of Beclin 1 and promotes the dissociation of Beclin 1 from its inhibitor BCL-2 family members thereby activating Beclin 1-induced autophagy.

#### 29.3.1.7 Tp53

As mentioned in the previous chapters, Tp53 can be activated by a wide range of stress signals in cells such as DNA damage, hypoxia or abnormal oncogene expression induced cell cycle checkpoints, DNA repair, cell death, and apoptosis. The regulation of apoptosis by Tp53 through endogenous and exogenous pathways has been well studied. Tp53 functions to integrate pressure signals and induce apoptosis by activating the transcription of BAX, PUMA, and BID which are members of the BCL-2 family with multiple domains or reducing the expression of BCL-2 which inhibits BAX (Fig. 29.2). Notably, Tp53-induced apoptosis usually depends on the environment. For example, Tp53 can induce apoptosis in fibroblasts transformed by an oncogene under BAX inactivation but Tp53 has no obvious ability

to mediate apoptosis in normal thymocytes. In addition to controlling the transcription of apoptotic members in the BCL-2 protein family, Tp53 can transactivate the core members of the apoptotic mechanism such as the gene encoding caspase 9 costimulator APAF1. In addition, Tp53 can regulate caspase 6 effector expression through transcription regulation. Recently, it has been reported that Atg7 can interact with Tp53 in *Cnot3*-depleted mouse cardiomyocytes and regulate the activity of Tp53 to adjust the expression of cell death-promoting genes (Yamaguchi et al. 2018). Although Tp53 can also activate gene transcription which plays a role in exogenous apoptotic pathways, it mainly functions in apoptosis through endogenous mechanisms. Surprisingly, Tp53 has the function of not only transcriptional activation but also transcriptional inhibition which may be involved in the regulation of apoptosis. Although most studies have focused on the apoptotic regulatory activity of Tp53 many recent reports have also highlighted the functional relationship between Tp53 and autophagy. Tp53 can inhibit mTOR by activating AMP-dependent protein kinase (AMPK) or trigger autophagy by activating DRAM which is a gene encoding a lysosomal protein with an autophagy induction function (Fig. 29.2). Under genomic instability conditions, Tp53 induces autophagy through DRAM, which will eventually lead to apoptotic cell death. Therefore, DRAM seems to be a key component in the apoptotic and autophagic pathway network regulated by Tp53. Interestingly, new studies have found that Tp53 has a novel function in the cytoplasm in addition to inducing apoptosis and inhibiting autophagy. The knockout or inhibition of Tp53 expression by drugs or interference can induce autophagy in human, mouse, and nematode cells. The enhancement of autophagy can promote survival in Tp53 inactivated cancer cells under hypoxia or nutrition deficient conditions indicating that the inhibition of autophagy induced by Tp53 is a self-protection mechanism in cells. Notably, Tp53 in the cytoplasm but not the nucleus can inhibit the intense autophagy observed in Tp53-inactivated cells suggesting that Tp53 can play a role in the regulation of autophagy by forming complexes. Valid evidence confirms that AMPK is activated in $Tp53^{-/-}$ cells while rapamycin target protein (mTOR) which is a nutrient-sensing kinase is inhibited. Because AMPK and mTOR play key roles in autophagy regulation the inhibition of Tp53 is likely to regulate autophagy through AMPK/mTOR dependent pathways. Overall, these findings suggest that Tp53 depends on different subcellular localization to regulate autophagy. Thus, Tp53 associates autophagy with apoptosis in an intricate and environment-dependent manner and coordinates these processes to restore metabolic balance in cells and organisms (see Chap. 6 for details).

#### 29.3.1.8 Mitophagy

Mitoptosis is a process of mitochondrial suicide that is usually caused by outer membrane permeabilization (MOMP) of mitochondria and subsequent potential loss. Recent studies have shown that after BAX/BAK mediated MOMP an intermembrane space (IMS) protein called DDP/TIMM8a is released into the cytoplasm where it binds the dynamin-like GTP enzyme DRP1. This interaction activates

DRP1-mediated mitochondrial fission and subsequent mitoptosis. Mitochondrial dysfunction and ROS production are the main factors inducing mitoptosis. Interestingly, growing evidence suggests that programmed mitochondrial damage leads to autophagy. In fact, recent studies have suggested that dysfunctional mitochondria can be removed by the formation of autophagosomes during mitophagy or the formation of mitoptotic bodies which are eventually released into the extracellular space through atypical exocytosis processes (Jangamreddy and Los 2012). Recently, it has been reported that the inhibition of proteasome function by the proteasome inhibitor MG132 can activate BAX/BAK-dependent mitoptosis in epithelial cancer cells. However, further clarification of the molecular mechanism of mitoptosis is needed to clarify its significance in pathophysiology (see Chap. 19).

### *29.3.2 Autophagy and Necrosis*

Necrosis is a type of cell death caused by severe, accidental or nonphysiological damage which is usually closely related to cell lysis caused by membrane destruction and subsequently the cell contents leak into the extracellular space which can lead to local inflammation and the destruction of surrounding tissues. In some specific cases, swelling or carcinogenesis of cells may precede necrosis. Although necrosis and apoptosis greatly differ in morphological characteristics the two processes are not mutually exclusive. Apoptosis and necrosis can respond in a dose-dependent manner to injury stimuli caused by various reagent treatments. Many reagents that cause apoptosis at low doses can eventually cause necrosis at relatively high doses. Many intracellular events determine the balance between apoptosis and necrosis. Changes in cell energy such as the ATP level can represent one of the factors influencing cell fate. Because ATP is necessary for some specific steps in caspase activation a rapid reduction in ATP levels in cells usually leads to necrotic cell death.

The interplay between autophagy and necrosis is more complex. These two processes can be activated simultaneously or sequentially and they can lead to the same or opposite results. The ability of autophagy to inhibit various forms of necrotic cell death which is usually caused by blocking apoptosis or inhibiting necrotic cell death is considered one of the most important pro-survival functions of autophagy.

#### 29.3.2.1 Crosstalk Between Autophagic Death and Necrosis

Some experiments in cancer cells suggest the possibility of crosstalk between the mechanisms of autophagy and necrosis. In the process of the metabolic stress response autophagy can resist tumor necrosis and inflammation thus performing a protective function in cells. Although autophagy buffers against metabolic stress the damage caused by apoptosis and autophagy can promote cell death caused by necrosis both in vivo and in vitro. Although the triggers of necrosis in cancer cells are unclear an inadequate ATP content which may affect the integrity of the plasma

membrane is likely to play a role leading to serious metabolic disorders and cell lysis. It can be inferred that the need for ATP rapidly decreases in the process of cell necrosis. In contrast, autophagy integrates the signals of the metabolic feedback system to help produce enough ATP to maintain cell survival. Natural polyamine spermidine can enhance autophagy and inhibit the destruction of the plasma membrane integrity and can cause the release of high mobility group B1 (HMGB1), which is a biomarker of necrosis.

However, recent studies have confirmed that Necrostatin-1 (Nec-1), which is a specific necroptosis inhibitor cannot only inhibit the process of nerve cell necrosis in nerve cells but also inhibit autophagy. These phenomena suggest that autophagy may be caused by necroptosis and there is a possibility that autophagy can be induced by increasing cell stress during cell death. Necrosis can be triggered when the cell metabolism and integrity are disrupted by nonphysiological factors. The cascade activation of programmed self-destruction includes cathepsin activation and lysosome breakdown. Therefore, autophagy and necrosis are closely interrelated because they share cytotoxic events. Some studies have confirmed the possibility of autophagy transforming into necrosis. For example, it was recently found that when hypoxia-induced autophagy reaches an irreversible point necrotic cell death can be triggered in a BNIP3-dependent manner. In protists of Dictyostelium, the inactivation of Atg1 inhibits vacuolation rather than cell death transforming the cell death caused by developing autophagy into a very similar death mode that is not identical to that caused by mammalian cell necrosis. Interestingly, in L929 mouse cellulosome cell lines TNFα can induce necrosis through the well-known autophagy and apoptosis-inducing factor FADD.

Similar to the relationship between autophagy and apoptosis evidence suggests that autophagy can promote, inhibit, or be unrelated to necrosis. For example, treatment with rapamycin combined with glucocorticoid dexamethasone causes autophagy-dependent cell death with necrotic apoptotic characteristics in acute lymphoblastic leukemia cells suggesting that autophagy can promote necroptosis in this particular system. Recent reports have shown that Atg9a drives necrosis in an autophagy independent manner during developmental bone formation in mice (Imagawa et al. 2016). However, most reports have the following opposite conclusion that autophagy can inhibit necroptosis in many cells such as L929 cells, lymphocytes or human cancer cells treated with TNFα, antigen stimulation or starvation. Currently, additional studies investigating the relationship between autophagy and necroptosis are ongoing. In addition, autophagy can be used as a means of cell survival against PARP-mediated cell necrosis. Many studies have shown that ROS, DNA destructive agents or ionizing radiation can lead to the following events including PARP activation, ATP consumption, AMPK activation, mTOR inhibition, and autophagy induction. More importantly, this induced autophagy can protect cells against cell death caused by PARP activation caused by DNA damage which is a way of cell survival.

#### 29.3.2.2 RIPK1 and RIPK3

Necrosis has been previously described as death caused by extreme physical and chemical stress. However, the widely accepted theory is that specific genes can regulate necrosis which is a process known as necroptosis. Receptor-interacting serine/threonine protein kinase 1 (RIPK1), which is also known as kinase receptor-interacting protein 1 (RIP1), and RIPK3 are key signaling molecules in the process of necroptosis. Some studies suggest that the treatment of L929 cells with the general caspase inhibitor zVAD leads to autophagy and cell death and that this process requires the involvement of RIPK1 suggesting that autophagy is also involved in the process of necroptosis. In many models autophagy has been found to regulate necroptosis. In epithelial cells the inhibition of autophagy can rescue necroptosis induced by palmitic acid. Recently, it has been reported that *Map3k7*-deleted mouse prostate cells are more sensitive to TRAIL (TNF-related apoptotic inducing ligand)induced cell death and that this necroptosis is mainly induced by the assembly of the necrosome in association with the autophagy machinery which is mediated by the p62/SQSTM1 recruitment of RIPK1 (Goodall et al. 2016).

The replication of coxsackievirus in the intestinal epithelium reportedly uses autophagy regulated by RIPK3 to aid in the assembly of its replication mechanism while inhibiting the initiation of necrosis. The knockdown of RipK3 in Atg7-deficient mouse pancreatic cells enhances the process of necroptosis due to defects in autophagy. Photodynamic therapy based on 5-aminolevulinic acid can render the human glioblastoma cell line LN-18 sensitive to RIPK3-dependent cell death, and this process can be reversed by the activation of autophagy. These studies suggest that RIPK1 and RIPK3 associate autophagy and necrosis either synergistically or antagonistically to regulate the process of cell death. However, further research is needed to clarify the complex interactions between the two processes and their molecular mechanism. These experimental phenomena illustrate the functional correlation between autophagy and necroptosis. However, further studies are needed to elucidate the molecular mechanisms underlying the complex interplay between these two processes.

#### 29.3.2.3 PARP

PARP1 belongs to a family of nuclear enzymes that can regulate DNA repair, transcription regulation, chromatin modification, and genome stability through poly-ADP ribosylation. The over-activation of PARP1 leads to an inadequate ATP supply, which can induce necrotic cell death and inhibit energy-dependent cell apoptosis. Interestingly, PARP1 activation may be involved in known signal transduction pathways that promote autophagy. As mentioned above AMPK can be used as a biosensor of intracellular energy and is activated in the absence of ATP. AMPK can promote autophagy through the inhibition of the mTOR signaling pathway (Fig. 29.3) or activation of the ULK1 complex (Egan et al. 2011). The activation of PARP1 in response to DNA damage can lead to ATP deficiency, AMPK activation, mTOR inhibition,

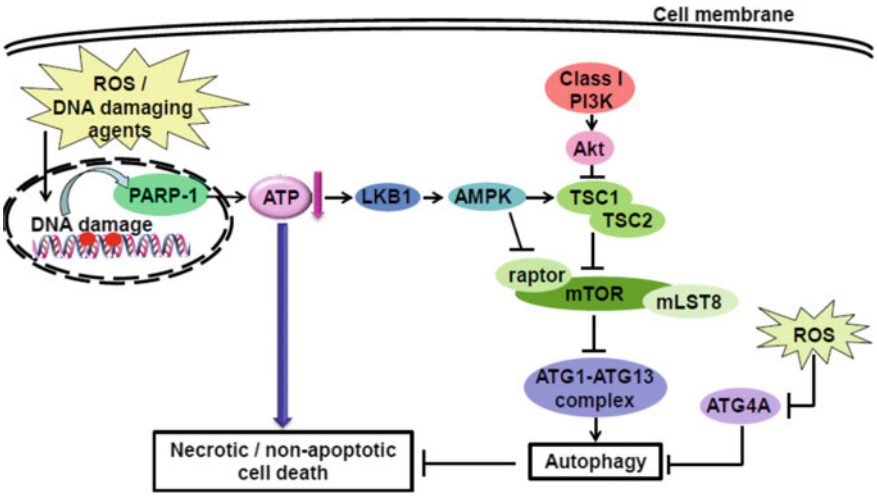


**Fig. 29.3** Modulation of cell death mode by caspase activity. PARP-1 mediates oxidative stress-induced autophagy via the LKB1-AMPK-mTOR signaling pathway and acts as a cellular survival mechanism against ROS-induced necrosis

and autophagy induction. DNA damage-induced autophagy protects cells against necrotic cell death induced by PARP1 activation and may serve as a mechanism for cell survival. These findings suggest that the activation of PARP1 can elicit the following opposing functions: ATP deficiency induces necrosis while autophagy is triggered simultaneously to protect cells from death. Ultimately, whether a cell survives or dies depends on the balance between autophagy and necrosis. In this setting, autophagy induction, which promotes survival, becomes the final means of survival for cells before they die under extreme stress.

#### 29.3.2.4 DAPK-PKD

Protein Kinase D1 (PKD) is a type of serine/threonine protein kinase that involves in many cellular biological processes including cell proliferation, migration, and death and this kinase can be activated by oxidative stress. The accumulation of ROS (reactive oxygen species) eventually leads to cell damage and the activation of various cellular responses including autophagy, apoptosis, and necrosis. The activation of autophagy promotes cell survival by removing damaged proteins and organelles to maintain cell homeostasis. Recently, PKD has been reportedly used as a new regulator of autophagy under oxidative stress. PKD can promote autophagy formation by phosphorylating VPS34. DAPK can also respond to oxidative stress, release Beclin 1 from BCL-$X_L$ through phosphorylation, and activate PKD by phosphorylation. Interestingly, PKD plays a role downstream of DAPK and both are required for the induction of autophagy under oxidative stress (Eisenberg-Lerner and Kimchi

2012). Therefore, DAPK can coordinate the induction of autophagy under stress through the following two different mechanisms: first through the phosphorylation of Beclin 1 and second through the phosphorylation of PKD resulting in activated PKD phosphorylating VPS34 to activate autophagy.

### *29.3.3 Autophagy and Other Forms of Cell Death*

As research progressed increasing forms of cell death have been identified. Autophagy, as a means of maintaining cell homeostasis and fighting against death is bound to interact with these newly discovered forms of death. For example, entosis can be induced by AMPK under glucose starvation. AMPK is also a positive regulator of autophagy. TM9SF4 plays an important role in the phagocytosis of human malignant tumor cells and is also involved in the regulation of autophagy induced by nutritional deficiency (Sun et al. 2018). These phenomena suggest that autophagy and entosis are coregulated. Recent studies have shown that autophagy can help muscle stem cells prevent senescence and thus maintain stemness (Garcia-Prat et al. 2016). In erastin-treated mouse embryonic fibroblasts autophagy can also increase labile iron and ROS by degrading ferritin leading to ferroptosis. In addition, in acrolein-treated human umbilical vein endothelial cells (HUVECs) the inhibition of autophagy by 3-MA aggravates the process of pyroptosis while rapamycin which is an autophagy inducer alleviates pyroptosis. In the non-alcoholic steatohepatitis (NASH) model established by treatment with $As_2O_3$, the inhibition of autophagy alleviates the process of pyroptosis. Based on the above observations the relationship between autophagy and pyroptosis is very complex and more in-depth research is needed to clarify their relationship.

### *29.3.4 Epigenetic Regulation of Autophagy and Cell Death*

The concept of a "histone code" was originally proposed by Allis and Turner who summarized the effect of histone modification on the chromosome structure and the corresponding regulation of nuclear function. According to this hypothesis, histones with specific covalent modifications affect the structure of chromatin and consequently affect the transcription process. This hypothesis was further developed, i.e., the post-translational modification of histones has been proposed to be regulated by specific chromatin-related functions and processes (Tan et al. 2011).

Histone acetylation and deacetylation are known to be in a dynamic balance in the nucleus accurately regulating gene transcription and expression. The process of adding acetyl groups to histone lysine residues under the action of acetyltransferases is a mechanism by which cells control gene expression, protein activity or physiological processes. Histone acetylation occurs mostly in specific lysine residues in the N-terminal alkaline amino acid-rich region of core histone, i.e., the acetyl group of

acetyl coenzyme A is transferred to $-NH_3^+$ of lysine to neutralize a positive charge. Histone acetylation is determined by histone acetyltransferase (HATs) and histone deacetylase (HDACs).

Initially, the post-translational modification of histones during autophagy has not attracted much attention. Until recently, nuclear regulation was not considered to exist in autophagy because it was reported that autophagic vesicles could still accumulate in cells without nuclei under the action of autophagy inducers. However, recently, contrary experimental evidence suggested that the nucleus plays a key role in autophagy. The transcription factors TFEB, ZKSACN3, and TFE3 have also been shown to be closely related to autophagy regulation (Pan et al. 2017). In fact, autophagy seems to involve transcriptional and epigenetic processes, including the epigenetic processes of post-translational modification of histones. The demethylation of H3K9 in histone modification participates in the early stage of autophagy, and the deacetylation of H4K16 participates in the late stage of autophagy, ensuring an adequate level of autophagy required for survival. Notably, this modification of autophagy is also significantly negatively correlated with apoptotic histone markers, suggesting that autophagic histone codes can promote cell survival (Fullgrabe et al. 2014). Moreover, this histone modification may regulate the transcription of autophagy-related genes by regulating the affinity of the autophagy-related transcription factors TFEB, ZKSCAN3, and TFE3 to corresponding DNA sequences thus regulating the autophagy process (see Chap. 11).

Unsurprisingly, the regulation of histone modification is significantly related to apoptosis, autophagy, and cancer progression. Apoptosis, autophagy, and the dysregulation of histone modification are closely related to tumorigenesis. Undoubtedly, the survival related histone code represents only the tip of the iceberg of the regulatory mechanism of autophagic histone modification. Since the concept of survival related histone code has only been born the concept of death-related histone code is still in infancy. Furthermore, more in-depth research is needed to elucidate how these histone post-translational modifications participate in autophagy regulation processes (transcription, silencing, stagnation, flow control of autophagy, etc.).

## 29.4 Autophagic Cell Death and Diseases

Recently, experimental evidence has increasingly confirmed that autophagy plays a key regulatory role in different aspects of human health. These studies have reported both the positive and negative effects of autophagy depending on the type and progression of the disease. Generally, autophagy plays a certain role in bacterial and viral infection diseases caused by protein aggregation, lysosomal storage diseases, tumors, and other diseases autophagic cell death participates in the above processes.

During pathogen infection autophagy locates bacteria in the cytoplasm (*Streptococcus pyogenes*), immature autophagosomes (*Mycobacterium tuberculosis*) or damaged autophagosome-like vesicles (*Salmonella typhi*). During *Salmonella typhimurium* infection of macrophages (rather than epithelial cells), bacteria can

induce autophagy-dependent host cell death rather than lysosome degradation. This process is likely the host's self-protection mechanism thus avoiding bacterial growth and limiting further bacterial infection of nearby cells. In the process of virus infection host cells secrete interferon (IFN) which triggers cellular antiviral mechanisms to restrict virus replication. IFN has been shown to upregulate autophagy. Type I IFN such as IFN-α can induce the activation of PKR. PKR is a type of eIF2α kinase that can inhibit protein synthesis and restrict viral replication. PKR signal transduction can promote autophagy induction, etc. Type II IFN and IFN-γ can also induce autophagy or autophagic cell death and cause the autophagic degradation of intracellular *mycobacterium*.

The correlation between autophagy and cancer has already been reported as early as 20 years ago and some possible molecular mechanisms have recently been widely investigated. Although autophagy has been reported to have both positive and negative effects on tumorigenesis it plays context-dependent roles in cancer. Due to this finding the therapeutic targeting of autophagy in cancer is sometimes considered controversial. In fact, autophagic cell death mainly plays a role when apoptosis is defective which can lead to unregulated cell growth. In this case, cytotoxic stimuli can induce autophagic cell death to kill cancer cells mainly through the inhibition of the mTOR pathway or the activation of the type I PI3K-kinase/Akt pathway or type III PI3K-kinase/beclin 1 pathway. In this case, the self-killing function of autophagy provides a possible way to inhibit the process of cancer. In addition, necrosis can occur if cells can effectively resist apoptosis or nutritional deficiencies are very severe. It has recently been reported that autophagy can promote tumor growth through circulating arginine (Poillet-Perez et al. 2018). Therefore, understanding the relationship among nutritional deficiency, autophagy, and cell death through apoptosis and necrosis is critical for inhibiting the metabolism of cancer cells through new metabolic therapy strategies.

Studies have increasingly confirmed that intricate relationships exist among the three main cell death pathways, i.e., apoptosis, necrosis, and autophagy. Autophagy plays a dual role in cell survival and death. Therefore, autophagy can be used as a mechanism to balance cell survival and death. With the development of cell imaging technology and drug inhibitors with higher specificity the understanding of the process of cell death has become increasingly profound. Numerous cell death subtypes have been identified because of the more detailed definition of different cell types responding to different death stimuli. One such example is the study of the treatment of mouse embryonic stem cell death by etoposide (ETO) which is an inhibitor of DNA topoisomerase II that produces DNA double-strand breaks. This type of cell death is caspase-independent and necrosis independent but is partially dependent on lysosomal protease activity. Moreover, autophagy is not considered to play a role in the process of cell death, suggesting that etoposide induces a new form of programmed cell death in this cell in which Endo G (an endonuclease) participates in DNA breakage. The inhibition of Tp53 transcriptional activity or the role of Tp53 in mitochondria by pifithrinα which is a Tp53 inhibitor, can significantly rescue cell death. The author of this report named this type of cell death in mouse embryonic stem cells after Charon, the ferryman of the dead in Greek mythology, a unique programmed death process called "charontosis".

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# Chapter 30
# Coordination of Autophagy and Other Cellular Activities


**Hui-Ling Zhang, Yong-Ming Zhu and Xian-Yong Zhou**

**Abstract** Conventionally, autophagy (=self-eating) is thought to be a catabolic cellular process that is responsible for regulating cell homeostasis. However, the newly evidence have expanded the range of the impact of autophagy in biology. Autophagy interplays with endocytosis through shared factors such as phosphatidylinositol 3 kinase complex (PI(3)K complex), autophagy associated gene (Atg), and lysosome. Autophagy and phagocytosis orchestrate in maintaining homeostasis, in MHC class II antigen processing, in the removal of pathogens, in cell death, immunity, and inflammation. There are numerous cross talks of autophagy with biosynthetic processes such as conventional and unconventional secretion of biologically active cargo and trafficking of integral membrane proteins, as well as the exosome secretion. There are also links between autophagy and trafficking events from plasma membrane, including lateral plasma membrane proteins connexins, cell connections, and ciliogenesis.

**Keywords** Autophagy · Endocytosis · Phagocytosis · Conventional and unconventional secretion · Trafficking events

## 30.1 Introduction

Autophagy (=self-eating) is a catabolic cellular process that is responsible for regulating cell homeostasis. Autophagy is induced in response to a variety of intrinsic and environmental factors. In the past decades research on autophagy topic has been well developed. Autophagy maintains the cellular homeostasis by digesting macromolecules to nutrients and by removing misfolded or unfolded proteins and damaged cell organelles. Autophagy may participate in the destruction of intracellular bacteria or even the destruction of the whole cell. Autophagy plays crucial roles in multiple physiological processes and pathophysiology conditions including

H.-L. Zhang (✉) · Y.-M. Zhu · X.-Y. Zhou
Department of Pharmacology, Laboratory of Cerebrovascular Pharmacology, Soochow University, 199 Ren-Ai Road, Suzhou, Jiangsu 215123, China
e-mail: zhanghuiling@suda.edu.cn



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aging and longevity, cellular metabolisms, cell development and differentiation, cellular stress, cell survival and death, immune responses and inflammatory response, and degradation of disease-related protein aggregates (Fig. 30.1). These physiological and pathophysiological effects regulated by autophagy have been discussed in other chapters. In this chapter, we focus on the coordination of autophagy and other cellular activities, such as coordination between autophagy and endocytosis between autophagy and secretion and between autophagy and trafficking events from the plasma membrane.

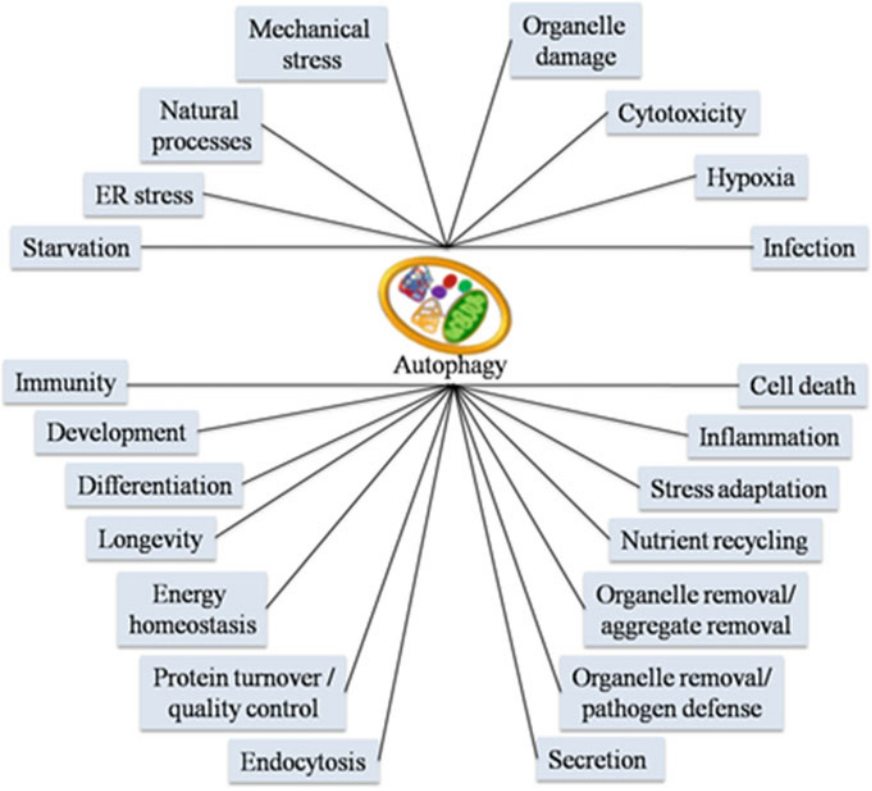



**Fig. 30.1** The role of autophagy in physiological and pathological conditions

## 30.2 Coordination Between Autophagy and Endocytosis

### *30.2.1 The Classification of Endocytosis*

Endocytosis is a cellular process in which substances such as large molecules, parts of cells, and even whole cells are brought into the cell. The target particle to be internalized is surrounded by an area of the plasma membrane of the cell, which then buds off inside the cell to form an intracellular vesicle containing the ingested material. It is a form of active transport. Endocytosis pathways can be subdivided into three categories: namely, receptor-mediated endocytosis (also known as clathrin-mediated endocytosis), pinocytosis (cell drinking), and phagocytosis (cell eating).

#### 30.2.1.1 Phagocytosis

Phagocytosis is the process that cells bind and engulf large particles (larger than around 0.75 μm in diameter), such as small-sized dust particles, cell debris, and apoptotic cells, as well as microorganisms invaded the human body. These processes involve the uptake of larger membrane areas than clathrin-mediated endocytosis and caveolae pathway.

#### 30.2.1.2 Pinocytosis

In human, pinocytosis occurs primarily for the absorption of fat droplets which usually occurs from highly ruffled regions of the plasma membrane. This process is the invagination of the cell membrane around desired extracellular fluid and molecules to form a pocket which then pinches off into the cell to create an internalized vesicle (0.5–5 μm in diameter). Pinocytosis occurs in a nonspecific manner in the substances that it transports. The vesicle then travels into the cytosol and eventually fuses with other vesicles such as endosomes and lysosomes (Nagata et al. 2010).

#### 30.2.1.3 Receptor-Mediated Endocytosis

Receptor-mediated endocytosis (RME) also known as clathrin-mediated endocytosis is a process by which the plasma membrane of cells invaginates and absorbs metabolites, hormones, proteins—and in some cases viruses, by forming a vesicle. This process is strictly induced by receptors on the cell surface. Through this process only the receptor-specific substances can be brought into the cell.

Clathrin-mediated endocytosis remains the best explored although receptors and their ligands can enter the cell through a few mechanisms (e.g., caveolin). The cargo ligands in the luminal compartment of the cell bind to receptors on the cell membrane is the first step for clathrin-mediated endocytosis of many receptor types. Then, the

cargo ligand and receptor recruit adaptor proteins and clathrin triskelions to the binding site of the outside cell membrane. Subsequently, budding of the plasma membrane forms leading to a clathrin-coated pit formed. Other receptors can nucleate a clathrin-coated pit allowing formation around the receptor. A mature pit is cleaved from the plasma membrane by using the membrane binding and fission proteins including dynamin and other BAR domain proteins generating a clathrin-coated vesicle and then this vesicle uncoats and usually fuses to an early endosome. Once fused, the endocytosed cargo (receptor and/or ligand) is destined to lysosomal, recycling, or other transport pathways (Kaksonen and Roux 2018).

### *30.2.2 Interplay Between Autophagy and Endocytosis*

In order to efficiently maintain autophagic flux, a functional endocytic pathway is needed in fusing with the lysosome to transport lysosomal hydrolases such as lipases, proteases, and glycosidases that can acidify the proton vacuolar-ATPase of the vesicle and in delivering the amino acids back to the cytosol after the sequestered proteins are degraded. Generally, the endocytic pathway contains a complicated set of compartments through internalization of the plasma membrane. The occurrence and maturation of the endocytic compartments is triggered by cytosolic proteins and multi-subunit complexes including clathrin coats, Rab proteins, ESCRTs and Retromer and regulated by active recycling, vesicular components from the Golgi complex, and homotypic fusion. A large amount of the internalized membrane and material is delivered back to the plasma membrane via the recycling endosome (RE) whereas the remaining content transfers from the early endosome (EE) to the late endosomes containing the multivesicular bodies (MVBs) (Fig. 30.2). The formation of the intraluminal vesicles (ILVs) is triggered by the ESCRT complexes. The ILVs comprise receptors derived from the EE surface including the EGF receptor and are transported to the lysosome. As the lysosome contains a significant amount of glycosylated proteins on its membrane, such as the LAMPS to protect cell against self-degradation it is a stable organelle in the cell. The lysosome membrane experiences a process of reformation that causes refilling the reformed empty lysosome with newly synthesized lysosomal proteases (Lamb et al. 2013; Munz 2017).

#### 30.2.2.1 Autophagy and Endocytosis Shares Molecular Pathways

Phosphatidylinositol 3 Kinase Complex (PI(3)K Complex)

The Beclin-1 complex is present on early endosome (EE). Rubicon (RUN domain, cysteine rich domain containing Beclin 1 interacting protein) one of the major PI(3)K accessory proteins which affects both endocytosis and autophagy at this stage. Rubicon resides at EE containing Rab5. Rubicon is a negative controller for endocytosis and autophagy through binding and preventing the Rab7 protein activation required

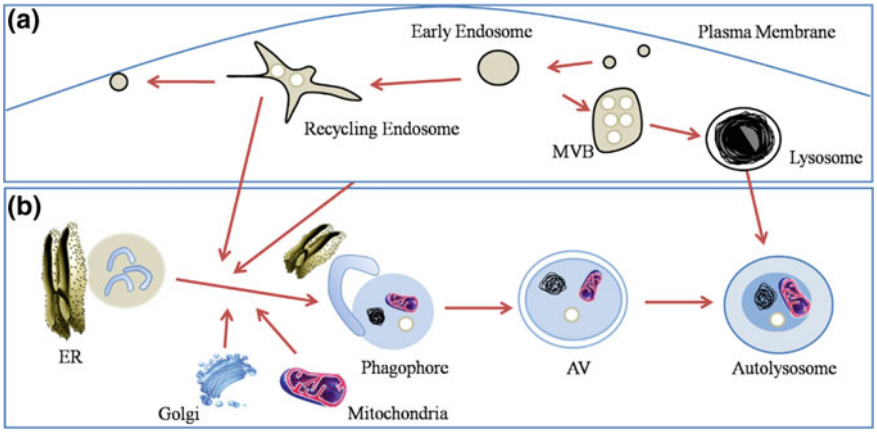



**Fig. 30.2** Membranes and mechanisms that underlie autophagy signaling, and the formation and maturation of autophagosome. **a** Endocytosis in the presence of amino acids and growth factors would occur from the plasma membrane via early endosome (EE). Recycling endosomes transport receptors which are not down-regulated (e.g. transferrin receptor) back to the plasma membrane while receptors targeted for degradation are delivered through the MVB/late endosome to lysosomes. **b** Autophagosomes are formed during amino acid and nutrient starvation on ER-derived sites, with contributions from the Golgi complex and mitochondria. Recycling endosomes and early endosome (EE) also contribute to the forming phagophore. After the closure of the phagophore to form an autophagosome, the autophagosome fuses with endocytic compartments, including EE, multivesicular bodies (MVBs) or late endosomes, and lysosomes to finally form an autolysosome (Lamb et al. 2013)

for the later stages of lysosomal fusion and autophagosome maturation. Rubicon and ATG14L were found in the same study. ATG14L positively regulates autophagy also called Barkor which guides the PI(3)K complex departing from the endosomal system to the sites on the ER where autophagosome is formed through its Barkor/ATG14L autophagosome targeting sequence (BATS) of C-terminal 80 amino acid. Different sub-units of the PI3K complexes also function at later stages of the endocytosis (Fig. 30.3) (Lamb et al. 2013; Matsunaga et al. 2010).

## Autophagy Associated Gene (Atg)

Autophagy is a rapid and a tightly regulated process in live and fixed cells, phagophore membranes are detected within 5 min after amino acid and serum are deprived. Autophagy occurs in mammalian cells through the concerted cascade action of at least 18 autophagy or Atg proteins. Functionally, Atg proteins are classified into several categories: the ULK1/2 kinase complex, the Beclin 1 PI(3)P kinase complex, the ubiquitin-like complexes of Atg12–Atg5–Atg16 and the Atg8/LC3 family conjugated to phosphatidylethanolamine (PE), PI(3)P effectors including the WIPI family, and Atg9 the only integral membrane Atg protein. Of note, the ULK1/2 complex is regulated by mTORC1 while the Beclin 1 complex has several different forms

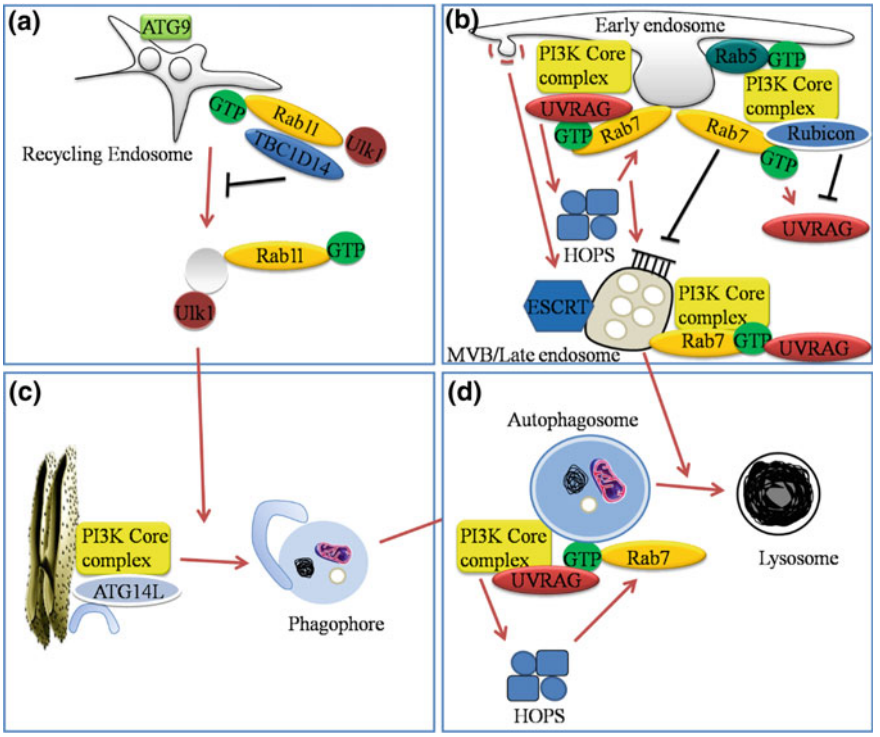



**Fig. 30.3** Endosomal machinery and autophagy. **a** Rab11 positive recycling endosomes contain Ulk1 and Atg9. The RabGAP TBC1D14 binds to active Rab11 and inhibits the transfer of Ulk1 from the circulating endosomal membrane to the growing phagophore. **b** The early endosomal coatomer COPI plays a role in forming proteolytically active late endosomal compartments and is needed for autophagosome maturation. Early endosome (EE) are Rab5 positive and can recruit the Beclin-1 PI(3)K core complex and its effector Rubicon. Rubicon inhibits activation of UVRAG by Rab7-GTP, however, when Rab7-GTP reaches a threshold this inhibition is lifted and UVRAG promotes further Rab7-GTP formation by activating the HOPS complex. This results in endosome maturation and formation of late endosomes/MVBs, in concert with protein complexes such as the ESCRTs. Rab7-GTP facilitates fusion of MVBs with the lysosome. **c** The PI(3)K effector ATG14L/Barkor localizes the Beclin-1 complex to autophagosome formation sites on the ER, generating an ER pool of PI(3)P at the PAS. **d** The UVRAG-HOPS-Rab7 cycle is also important for maturation of autophagosomes, promoting fusion with the lysosome (Lamb et al. 2013)

with the primary purpose of forming PI(3)P on the nascent autophagosome. The ubiquitin-like complex Atg12–Atg5–Atg16 is upstream of the LC3–PE conjugate and downstream of the ULK1/2 complex and the Beclin 1 complex. The Atg proteins are cytosolic proteins responsible for the budding phagophore and autophagosome membranes, except for that Atg9 is present in the trans-Golgi network and endosomal compartments. The Atg8/LC3 family members are modified by covalently binding to PE that provides their stable association with membrane, and the regulatory subunit p150 of the Beclin 1 complex is modified by myristoylation. However, it is unknown

how the remaining Atg proteins are related to membranes. Additionally, the biological activities of the Atg proteins are still not fully elucidated although their activities are starting to be functionally identified (Lamb et al. 2013; Axe et al. 2008).

Lysosome

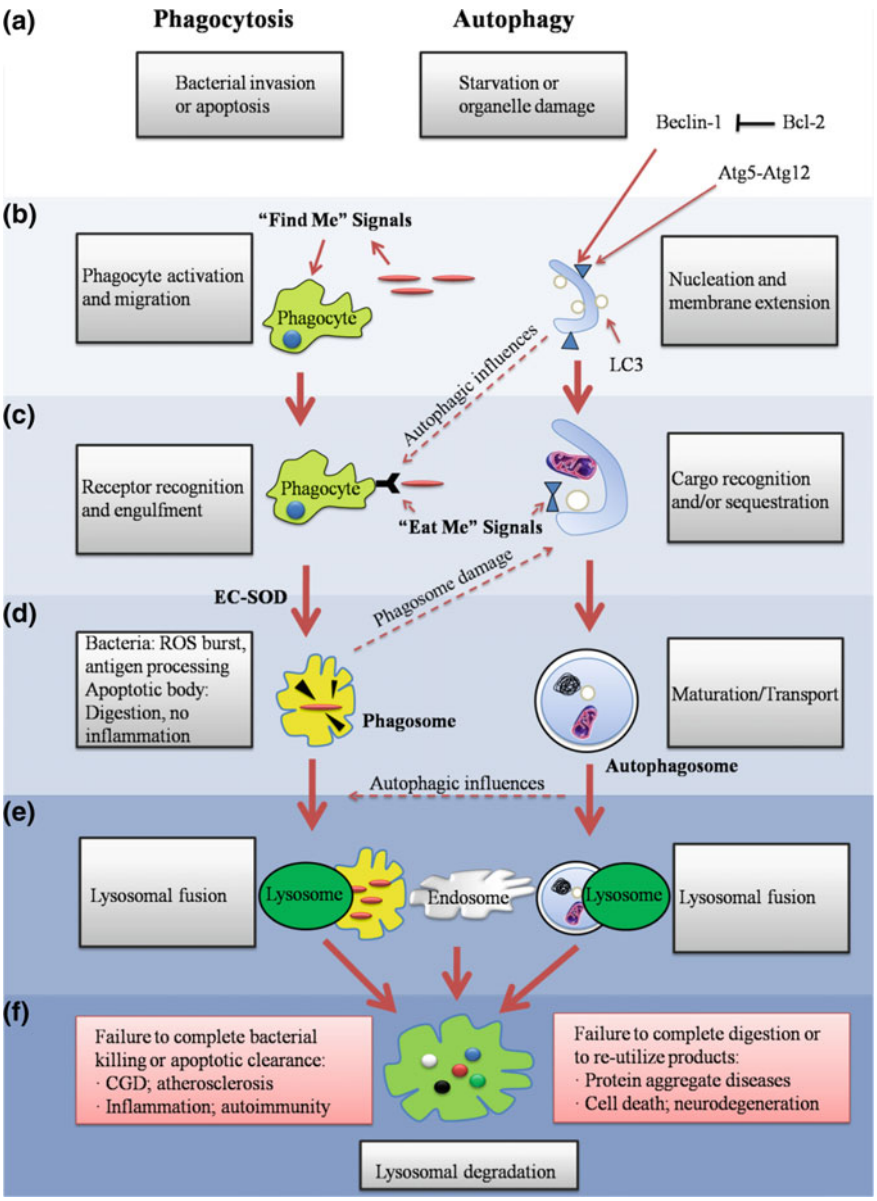
The lysosome acts as a garbage disposal for the substrates of endocytosis and autophagy. However, recent investigations reveal the complicated regulating effects mediated by the lysosome surface proteins. The lysosome serves as a machinery for mTORC1 signaling and regulation and through this process lysosome integrates the early stages of autophagy with the final stages of autophagy in the autolysosome. The transcription factor TFEB coordinated biogenesis of lysosome and autophagy genes underlies the important relation between lysosomes and autophagy.

#### 30.2.2.2 Coordination Between Autophagy and Phagocytosis

Autophagy and phagocytosis possibly each started as a process of facilitating cellular nutrition; however, both methods have evolved to become crucial regulators in cellular homeostasis and defense against pathogens. Therefore, both self-eating and the consumption of other cells whether they are alive or dead play important roles in cell survival and tissue homeostasis. Both autophagy and phagocytosis are usually enhanced simultaneously with apoptosis. Autophagy can act to prevent apoptosis through sequestrating injured mitochondria under some conditions which release mediators for cell death. In addition, autophagy is often related to tumor repressing pathways and autophagy or its major regulatory proteins is possibly needed to facilitate subsequent cell death. Similarly, phagocytosis is employed not only by professional phagocytes to eliminate living (microbial) cells or to clear necrotic tissue but also is involved in a crucial mechanism by which epithelial cells can scavenge the neighboring apoptotic cells. In this way, danger signals that might initiate sustaining inflammation, tissue fibrosis, allergies, or inflammatory carcinogenesis are able to be removed. The final steps for autophagy and phagocytosis converge on similar pathways that are regulated by shared molecules although they are induced by different mechanisms. For instance, phosphatidylinositol-3-phosphate (PI3P) is needed for both phagosome and autophagosome formation and each of them subsequently fuses with the lysosome (Fig. 30.4) (Oczypok et al. 2013).

Coordination Between Autophagy and Phagocytosis in Maintaining Homeostasis

Rapid and efficient eliminating of apoptotic and necrotic cells is a key housekeeping function belonging in the body. There will be no sufficient space for the growth of


(a)
Phagocytosis
Autophagy
Bacterial invasion or apoptosis
Starvation or organelle damage
Beclin-1
Bcl-2
Atg5-Atg12
(b)
"Find Me" Signals
Phagocyte activation and migration
Phagocyte
Nucleation and membrane extension
LC3
Autophagic influences
(c)
Receptor recognition and engulfment
Phagocyte
"Eat Me" Signals
Cargo recognition and/or sequestration
EC-SOD
Phagosome damage
(d)
Bacteria: ROS burst, antigen processing
Apoptotic body: Digestion, no inflammation
Phagosome
Maturation/Transport
Autophagosome
Autophagic influences
(e)
Lysosomal fusion
Lysosome
Endosome
Lysosome
Lysosomal fusion
(f)
Failure to complete bacterial killing or apoptotic clearance:
· CGD; atherosclerosis
· Inflammation; autoimmunity
Failure to complete digestion or to re-utilize products:
· Protein aggregate diseases
· Cell death; neurodegeneration
Lysosomal degradation

◀**Fig. 30.4** Major steps in phagocytosis and autophagy. Both phagocytosis and autophagy process includes the following major steps: the proper induction (**a** and **b**), cargo targeting (**c**), maturation (**d** and **e**), and completion of lysosomal clearance (**f**). For phagocytosis, the recognition of danger signals from dead cells or microbial pathogens induces phagocyte migration. A number of different receptors participate in capturing and internalizing bacterial, apoptotic, or particulate cargoes through a process enhanced by Atg proteins and dependent on a proper local redox environment maintained by EC-SOD. Once the phagocytic cargo is inside the phagosome, concentrated production of ROS or degradative enzymes are stimulated, relying on the ingested contents. Similarly, either general or localized insults within cells serve to trigger the membrane deposition of Atg 5dAtg 12 and of LC3 (through Beclin 1-dependent or -independent mechanisms), which are required for the extension of autophagic membranes. Whereas nonselective sequestration of cytoplasm is mediated by starvation, damaged and potentially harmful cargoes need to be appropriately targeted to LC3-bound membranes. Autophagy is also induced by phagosome-derived signals to sequester pathogens that escape or damage the phagosome membrane. In turn, induction of the autophagic pathway serves to further promote phagosome lysosomal fusion. Thus, these two complementary systems cooperate in eliminating exogenous and endogenous danger signals to prevent proinflammatory, carcinogenic, and pro-death stimuli. For both phagocytic and autophagic pathways, inefficient lysosomal fusion or digestion can facilitate many classes of disease (red-shaded boxes). Accumulation of undigested lysosomal cargo is seen in aging and in chronic granulomatous disease (CGD), neurodegenerative diseases, and many other diseases or disorders. Inefficient utilization of digested products for energy production or in the regeneration of mitochondria and other essential cellular structures may also promote cell death and neurodegeneration (Oczypok et al. 2013)

new cells if the dead cells are not cleared and the dead cells can release inflammatory danger signals that are deleterious to host tissue. Dead cells that keep in tissue increase the risk for autoimmune diseases including systemic lupus erythematosus and other morbidities. Autophagy and phagocytosis cooperate to decrease the number of dying cells blocking the secretion of detrimental inflammatory cytokines. Cells that have experienced apoptosis exhibit phosphatidylserine on their surface labeling them for macrophage ingestion. Macrophages release the MFG-E 8 (milk fat globule epidermal growth factor 8) protein which binds to phosphatidylserine and facilitates phagocytosis of apoptotic cells. This is much like the opsonins in bacterial infections first found in 1903 by Wright and Douglas. Recently, Qu et al. pointed to that the autophagy genes ATG5 or Beclin1 is needed for properly displaying the eat-me signals of the phosphatidylserine on apoptotic cells. They illustrated that phagocytes could not efficiently engulf dead cells in embryoid bodies in the absence of proper autophagic function resulting in developmental abnormalities. Likewise, autophagy regulated by the Toll-like receptor 4 may exert protective effect in a model of pulmonary fibrosis by promoting the clearance of damaged or dead cells, thereby speeding up the resolution of inflammation (Oczypok et al. 2013; Qu et al. 2007).

## Endocytosis Regulation by Autophagy Proteins in MHC Restricted Antigen Presentation

### *LC3 Associated Phagocytosis in MHC Class II Antigen Processing*

After phagocytosis of pathogens or cellular debris MHC class II molecules can present antigens to helper $CD4^+$ T cells. Uptake of pathogens associated molecular patterns (PAMPs) that stimulate PAMP recognition receptors (PRRs) for myeloid cell activation has an effect on the maturation of phagosomes and the processing of antigens in the same phagosome as the PAMPs for improved MHC class II presentation. On stimulation, the PRR toll-like receptor 2 (TLR2) can recruit LC3B to the phagosomal membrane and regulate phagosome maturation. This pathway is termed as LC3 associated phagocytosis (LAP). During this process, LC3B is conjugated to the cytosolic side of the single membrane surrounding phagosomes. Besides TLRs the C-type lectin receptor Dectin-1 and Fc receptors and receptor TIM4 for apoptotic cell debris have abilities to activate LAP. The accumulation of LC3B to the plasma membrane of phagosome needn't the ULK1/Atg1 complex but the Beclin-1/Atg6 complex that puts PI3P at the phagosomal membrane and recruits NADPH oxidase 2 (NOX2). The activity of NOX2 is necessary for LC3B conjugation at phagosomes. The recruitment of Atg5-Atg12/Atg16L1 complex to the phagosomal membrane is required for LC3B lipidation by the WD40 domain of Atg16L1, however, it is still unknown what the WD40 domain identifies. The effect of the conjugation of LC3B to phagosomal membranes might vary from cell population to population. It functions likely to promote fusion with lysosomes in mouse macrophages by accelerating its trafficking along microtubules and its fusion with lysosomes. LC3B can redirect phagosomes to endosomes with TLR for more effectively sensing pathogen and producing type I interferon in plasmacytoid dendritic cells. The of conjugation LC3B possibly postpones the fusion of phagosome with lysosomes for persistently processing antigen towards MHC class II presentation in human dendritic cells and macrophages. In spite of these different kinetic, LC3B can be disassociated from the phagosomal membrane before acidification and fusion with lysosome. Furthermore, LAP promotes antigen processing for MHC class II presentation in both mouse and human macrophages. Therefore, in mice with Atg5 absence in dendritic cells extracellular antigen processing onto MHC class II molecules is also inhibited. Furthermore, during experimental autoimmune encephalomyelitis (EAE) the restimulation of autoimmune $CD4^+$ T cells through LAP to process autoantigen owning cell debris is destroyed in dendritic cells of mice with Atg5 deficient. This contributes to protecting the mouse against central nervous system (CNS) autoimmunity after the primed autoimmune $CD4^+$ T cells are adoptively transmitted to mice. In addition, regulatory $CD4^+$ T cells induced by commensal are damaged in dendritic cells of the gut without Atg16L1. Finally, macrophages with LAP deficiency inefficiently eliminating apoptotic cells may lead systemically to lupus-like autoimmunity with autoimmune antibody production. This lupus-like autoimmunity depends on Atg5, Atg7, Beclin-1, and NOX2 but not components of the ULK1 complex. Hence, LAP assists antigen processing outside the cell for MHC class II presentation by using

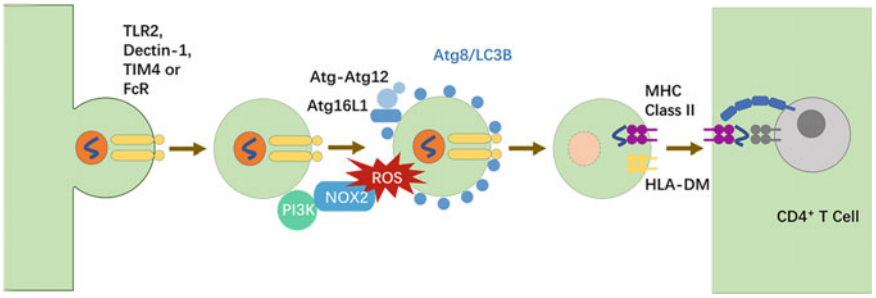



**Fig. 30.5** LC3 associated phagocytosis (LAP) for MHC class II-restricted antigen presentation. Phagocytic cargo that engages TLR2, Dectin-1, TIM4 or Fc receptors results in the recruitment of NADPH oxidase 2 (NOX2) to the phagosomal membrane via PI3 phosphorylation by phosphoinositide 3-kinases (PI3K). NOX2-dependent reactive oxygen species (ROS) production is needed for LC3B conjugation at the phagosomal membrane. The WD40 domain of Atg16L1 is required for the transfer of the Atg5-Atg12/Atg16L1 complex to the phagosomal membrane for LC3B lipidation at this membrane. Lipidated LC3B regulates phagosome fusion with lysosomes, thereby promoting MHC class II presentation of the phagocytosed cargo to helper $CD4^+$ T cells (Keller et al. 2018) accumulation

some of the molecular constituents of the macroautophagy machinery (Fig. 30.5) (Keller et al. 2018; Romao et al. 2013).

*Atg Assists Endocytosis of MHC Class I Molecules*

This support of the macroautophagy machinery for endocytosis extends to the internalization of surface molecules. For example, Alzheimer's disease-related protein APP (amyloid precursor protein) can be internalized through a LC3B lipidation-dependent manner. LC3B interaction with the adaptor protein 2 (AP2) complex in one of whose subunits (AP2A1) a LIR motif was identified benefits to clathrin-mediated endocytosis for APP degradation. In addition, inhibition of mTOR or starvation promotes APP internalization depending on Atg5. Furthermore, the direct association between Beclin-1/Atg6 and APP for the recruitment of the respective PI3 kinase complex further facilitates this internalization for APP degradation. Linking the macroautophagy machinery even more with clathrin-mediated endocytosis, clathrin itself contains a LIR motif. Therefore, LC3B likely promotes MHC molecules in a clathrin-mediated endocytosis dependent manner. As different pools of MHC molecules experience different internalization routes, the issue becomes more complicated. MHC class II components rely on clathrin-mediated endocytosis only if these molecules are linked to their chaperone chain or ubiquitinated through membrane-associated RING-CH (MARCH) E3 ubiquitin ligases. But, the loss of the LC3B lipidation machinery didn't cause the changes in MHC class II surface levels. Classical and nonclassical MHC class I molecules are stable on the mouse dendritic cell surface with Atg5 or Atg7 absence. Reduction in classical MHC class I molecules internalization results in $CD8^+$ T cell stimulation enhancement. This

leads to the enhancement of $CD8^+$ T cell responses following influenza A or lymphocytic choriomeningitis virus infection. In macrophage with specific deletion of Atg5 and Atg7 this enhanced $CD8^+$ T cell priming correlates with decreased influenza A virus titers and improved adaptive immune control of influenza A virus infection. However, there are also at least two internalization pathways for MHC class I. Clathrin-independent MHC class I can internalize into recycling endosomes in cell lines with limited phagocytic potential. In contrast, MHC class I internalization into late endosomal compartments destined to be degraded can be enhanced by MARCH E3 ubiquitin ligases presumably utilizing clathrin-mediated endocytosis. In fact, LC3B lipidation-dependent MHC class I endocytosis leads to enhanced MHC class I degradation. Furthermore, LC3B interacts with adaptor associated kinase 1 (AAK1) which causes phosphorylation of AP2M1 a subunit of AP2 leading to more efficient clathrin-mediated endocytosis. Absence of LC3B lipidation destroys AAK1 recruitment to MHC class I molecules and silencing AAK1 causes stabilization of MHC class I surface levels. Thus, the macroautophagy machinery can promote internalized molecules recruitment to MHC class I complexes and then lead to suppression of $CD8^+$ T cell stimulation. In addition to $CD8^+$ T cell responses natural killer T (NKT) cell responses are also dampened by LC3B lipidation which are restricted by CD1d as a nonclassical MHC class I molecule. Loss of Atg5 in dendritic cells can stabilize CD1d molecules on the surface of cell resulting in enhancement in recognizing NKT cell and improvement in early innate immune control of *Pseudomonas paucimobilis*. Therefore, both nonclassical and classical MHC class I molecules are internalized by LC3B-assisted endocytosis possibly benefitting from the internalized components recruitment to MHC class I and CD1d molecules via LC3B lipidation (Fig. 30.6) (Keller et al. 2018; Loi et al. 2016).

## Coordination Between Autophagy and Phagocytosis and Its Mechanisms in the Removal of Pathogens

Autophagy will serve as a backup system to fight against infection when phagocytosis can't kill a pathogen. When phagocytosed, certain bacteria can reside inside the phagosome or can be released into the intracellular space by destroying the phagosome. Modifying or damaging to the phagosome can induce the autophagic pathway. For instance, Listeria monocytogenes bacteria release listeriolysin-O, which lyses the phagosome and then releases phagocytosed bacteria, concurrently, induce autophagy. Salmonella enterica, serovar Typhimurium, stays in the phagosome for protecting; but, it releases proteins as part of its type III secretion system, to activate the entire autophagy vesicle containing salmonella. In addition, Mycobacterium tuberculosis suppresses the maturation of phagosome, however, triggering autophagy is adequate to overturn these effects leading to declined phagosome pH and restoration of bacterial elimination. Upon Toll-like receptor signaling activation on macrophages during phagocytosis the recruitment of LC3 to phagosomes facilitates phagosome fusion with lysosome to assist in killing bacteria. In spite of the dual roles of phagocytosis

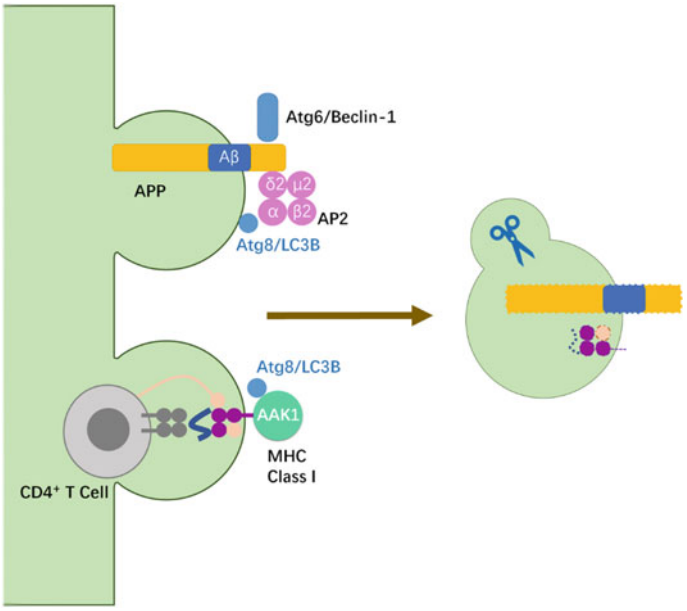



**Fig. 30.6** LC3B-assisted internalization of surface receptors, including MHC class I molecules. Lipidated LC3B recruits components of the internalization machinery to surface receptors. These include adaptor associated kinase 1 (AAK1), which can promote AP2-dependent internalization via phosphorylating the μ subunit of this adaptor complex. The subunit of AP2 also directly binds to lipidated LC3B and might thereby enhance clathrin-mediated endocytosis. Finally, Atg6/Beclin-1 can directly bind to amyloid precursor protein (APP) of Alzheimer's disease for efficient internalization. All these LC3B-assisted internalization events have been suggested to result in the degradation of the respective surface receptors in lysosomes (Keller et al. 2018)

and autophagy in immune surveillance pathogens like hepatitis C viruses have developed mechanisms for preventing being cleared through interfering with autophagosome lysosomal fusion (Oczypok et al. 2013).

## Coordination Between Autophagy and Phagocytosis in Cell Death, Immunity and Inflammation

It is easy to understand that there is a dynamic relationship between autophagy and phagocytosis particularly, under the cell death conditions considering the nature of both processes and the respective roles they take in maintaining the homeostatic balance at the level of both the cell and the organism. For example, molecules originally considered to be specific to one pathway, such as LC3 in the autophagy process, are in fact playing similar roles in the other. In process that macrophages phagocytize pathogens or cellular debris from necrotic, apoptotic, or necroptotic cells that exhibit Phosphatidylserine recruitment of LC3 to phagosomes rapidly occurs which is lack

of the typical double-membrane coat unlike classical autophagosomes in spite of demanding PI3KC3, Atg5, Atg7, and Beclin 1 but not unc-51-like kinase 1 (ULK1). This process is called LC3-associated phagocytosis or LAP. TLRs, TIM4, FccR, and NADPH oxidase-mediated ROS signal as upstream signals are needed for LAP. LAP is essential for the efficient elimination of cell corpses as well as for phagosomal maturation. Abnormalities in cell death process cause a series of diseases such as neurodegenerative disorders, cancer, and autoimmunity. A growing number of literature now investigate the crosstalk between apoptosis and autophagy. Indeed, apoptotic proteins usually directly suppressed autophagic proteins. Moreover, data that apoptotic caspases function as autophagic substrates indicates another aspect of direct interaction between autophagy and apoptosis. This has impelled a lot in these fields to destine for autophagy opposite to apoptosis and this process has been termed as a form of "programmed cell survival". In addition, autophagy itself can act as an alternative type of cell death, although this has not been identified in a consistent fashion. Similar to phagocytosis, during embryogenesis autophagy assists to remove apoptotic cells too. Autophagy is also associated with nutrient acquisition and energy production which is essential for cell survival upon cytotoxic injury. The major function of most phagocytes seems to damage pathogens. Phagocytosis contributes to innate immune sensing and degradation of products of internalized pathogens that may traffic and be modified as nutrients. Moreover, phagocytosis is needed for antigen presentation and adaptive immunity. Autophagy plays a key role in adaptive and innate immunity, such as clearing pathogen, preventing or promoting virus replication, B and T cell homeostasis, and antigen processing and presentation. All such processes collectively for autophagy have been termed as "immunophagy". The apoptosis of inflammatory cells and their subsequent elimination by phagocytosis (also called efferocytosis) is crucial to coordinating between successfully resolving inflammation and inhibiting autoimmune responses. This is partly acquired by the suppression of pro-inflammatory cytokines including TNFa and IL-1β and the release of anti-inflammatory cytokines such as IL-10, TGFb, platelet activating factor (PAF), and prostaglandin E2 (PGE2). In the deficience of LAP, the dead cells engulfment process leads to reduced anti-inflammatory cytokines production and enhanced pro-inflammatory cytokines generation (e.g., IL-1β). The formation of the "inflammasome," is one of the crucial mechanisms of inflammation, which results in the maturation and secretion of IL-1 family mediators and other inflammatory cytokines as well as caspase-1 activation. Previously, autophagy was thought to be an anti-inflammatory mechanism when increased IL-1β secretion was seen during sepsis in Atg16L1-null mice. But recently, it has been revealed that autophagy activation by starvation facilitates IL-1β release in an inflammasome-dependent manner. These facts indicate that autophagy plays dual roles in controlling inflammation relying on the timing and form of autophagic induction (Vernon and Tang 2013; Martinez et al. 2011; Stuart and Ezekowitz 2008).

### *30.2.3 Outlook*

In summary, there are shared regulation factors for the autophagic and endocytic routes. The function of both processes ultimately relies on lysosomal degradation. Since the lysosome serves as a machinery for the energy status sensing of the cell, the perspective that there is interaction between and coordination of the two pathways is interesting. With the improvement of approaches to screen factors regulating for autophagy among known molecules of membrane transporting we can look forward further insights into the common regulation of the two crucial degradative pathways within the cell.

## 30.3 Coordination Between Autophagy and Secretion

Recent studies in mammalian systems have shown that autophagy plays a broad biological role in protein trafficking and secretion. Autophagy supports trafficking pathways for moving integral membrane proteins to the plasma membrane and affects cell secretion including the conventional secretion and unconventional secretion pathways. It is a special way of membrane transportation. The autophagy pathways and the Atg molecular machinery are involved in the regulated secretion of the particulate contents or secondary lysosomes in specialized secretory cells that contain proteins and other effector cargos. These secreted biologically active proteins or other products are engaged in tissue remodeling, inflammatory responses, and development of specialized tissues and organs (Deretic et al. 2012).

### *30.3.1 Secretion*

#### 30.3.1.1 Conventional Secretion(ER-to-Golgi Secretory Pathway)

Definition of Conventional Secretion

Secretion is the cellular physiological process present in every organism that delivers soluble proteins and cargoes to the extracellular matrix of the cell. Although these secreted products can be a variety of nonprotein substances such as steroids, they are often functional proteins. The process of secretion is in contrast to excretion by which waste substances are removed.

The best characterized mechanism of trafficking in eukaryotes is the conventional protein secretion (CPS). In eukaryotes, conventional protein secretion is the trafficking route by which secretory proteins are delivered from the endoplasmic reticulum (ER) to the Golgi apparatus (GA), subsequently to the trans-Golgi network (TGN), and finally to the plasma membrane (PM). The trans-Golgi network

is the organelle where proteins that are destined to be released are segregated from lysosomal/vacuolar enzymes and sorted in budding secretory granules or secretory vesicles (Viotti 2016).

The Role of Golgi in Conventional Secretion

The tasks of the Golgi apparatus in secretory activities are multiple and varied. The specific roles carried out are the following aspects: (1) Receiving substances synthesized from other parts of the cell prior to their packaging for exporting, mainly from the rough endoplasmic reticulum; (2) Concentrating secretory products. After concentration, the secretory material becomes observably more dense and closely packed; (3) Chemical modification (processing) of the materials to be secreted. One example would be adding or removing sugars from glycoproteins; (4) Synthesis of secretory materials; (5) Sorting secreted materials some of which may be routed to lysosomes or other types of vacuoles while other materials may be directly exported to the cell surface. Golgi sorts proteins based on signal peptides or "signal spots" on proteins. (6) Forming secretory vesicles and transporting proteins to the appropriate destination by reading the "signal" on the protein. If there is a "secretion" signal on the protein, the Golgi forms a small vesicle that wraps the protein that needs to be secreted to the outside of the cell. If there is a signal on the protein that the protein should remain in the endoplasmic reticulum, the Golgi will form another small vesicle and the protein will be returned to the ER.

Classification of Conventional Secretion

The secretory pathway in eukaryotic cells is used to send proteins and lipids to the plasma membrane and certain membrane-bound organelles and to release material outside the cell. Conventional secretion is divided into the constitutive secretion and regulated secretion. If the newly synthesized protein is secreted after synthesis, it is called constitutive secretion. If the newly synthesized protein is stored at a high concentration in the secretory vesicle such as secretory lysosomes particles or other parts of organelle until a signal triggers fusion with the plasma membrane and secretion it is called regulated secretion. Secretory lysosomes, granules, and other organelles are derived from post-Golgi vesicles or are affected by post-Golgi vesicles.

#### 30.3.1.2 Unconventional Secretion

Unconventional secretion is known as endoplasmic reticulum (ER)/Golgi-independent protein secretion or nonclassical protein export. It refers to a term for a variety of processes that transport different proteins localized in the cytosol to the outside of the cell or support in delivering integral membrane proteins to the plasma membrane not to necessarily pass through the ER-to-Golgi biosynthetic pathway

to their final destination. Therefore, not all unconventionally secreted proteins are secreted by the same mechanism. Two types of unconventional protein secretion are these: signal peptid-containing proteins and cytoplasmatic and nuclear proteins that are lacking an ER-signal peptide.

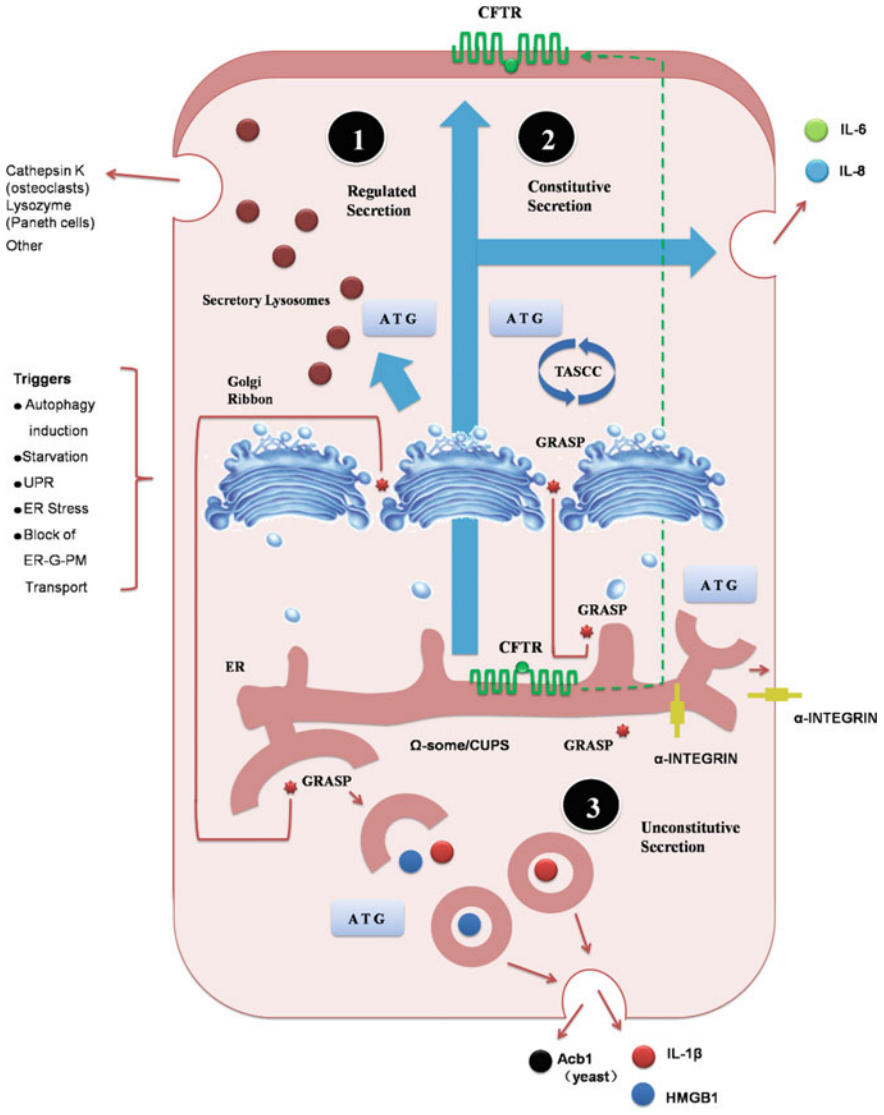
## *30.3.2 Autophagy and Conventional Secretion*

### 30.3.2.1 Autophagy and Constitutive Secretion

Recent evidence suggests the intersection between autophagy and the constitutive secretory pathway (Fig. 30.7). In this case, there is a specialized compartment named TOR-autophagy spatial coupling compartment (TASCC), interfacing with autophagic degradation and indirectly favoring production of a subset of secretory proteins that are transported to the outside of the cell via conventional (ER-to-Golgi-to-plasma membrane) secretion. In senescent cells, TASCC is a group cluster of lysosome-like organelles that are physically discernible in a perinuclear region juxtaposed to the Golgi. For example, during mutant Ras-induced cell senescence TASCC is formed and observed. The location of TASCC is interfered by brefeldin A but is insensitive to nocodazole. Thus, it relies on the maintenance of the conventional secretory organelles. TASCC chiefly consists of degradative organelles that display features of late/mature phagosomal compartments containing LAMP, p62, variably LC3, and mTOR. This compartment seems to play a role in providing building blocks for biogenesis and in favoring secretion of conventional secretory products. For example, it assists a few key proteins secretion, concurrently, degrading bulk proteins through autophagy. In this case, autophagosomes wrap and deliver cellular proteins destined to be modified in TASCC where proteins are degraded while enabling mTOR recruitment to TASCC that is activated by released amino acids from autolysosomes, selectively promoting translation and secretion for a limited number of targeted proteins. This process was seen under the condition of increased IL-6 secretion in senescent cells. But, in differentiated cells this process (even if morphologically not as recognizable) may be in utilizing in tissues to streamline or tighten their repertoire of secreted proteins (Deretic et al. 2012; Narita et al. 2011).

### 30.3.2.2 Autophagy and Regulated Secretion

Autophagy also affects regulated secretion. Several studies have shown that defects in autophagy or Atg genes alter the regulated secretion in delivering the contents stored in secretory granules or lysosomes. The findings of the role of autophagy in the above events include: (1) the enhanced Atg-dependent fusion between phagosomes and lysosomes in the process of microbial ingestion by macrophages (Fig. 30.7, pathway 1); (2) secretion of lysozyme at the plasma membrane from Paneth cells implicated in Crohn's disease; (3) secretion of ATP under certain conditions by


CFTR
1
Regulated Secretion
2
Constitutive Secretion
IL-6
IL-8
Cathepsin K (osteoclasts)
Lysozyme (Paneth cells)
Other
Secretory Lysosomes
ATG
ATG
TASCC
Golgi Ribbon
Triggers
Autophagy induction
Starvation
UPR
ER Stress
Block of ER-G-PM Transport
GRASP
ATG
GRASP
CFTR
ER
α-INTEGRIN
Ω-some/CUPS
GRASP
α-INTEGRIN
GRASP
3
Unconstitutive Secretion
ATG
Acb1 (yeast)
IL-1β
HMGB1

◀**Fig. 30.7** Non-degradative roles of autophagy in conventional (constitutive and regulated secretion), and unconventional secretion. (1) Regulated secretion: secretory lysosomes, granules, and other organelles, partially derived from or affected by post-Golgi vesicles. ATG: symbolizes that Atg factors affect regulated secretion, delivering various biologically active cargos such as that indicated. Other: includes non-proteinaceous cargo (e.g. ATP secreted from drug-treated cancer cells), provided they are competent to undergo autophagy, with inflammatory consequences and clearance of transplanted tumors. (2) Autophagy affects constitutive secretion (e.g. IL-6, IL-8) via a compartment intermixed with autophagic organelles, called the TOR-autophagy spatial coupling compartment (TASCC). (3) A subset of unconventional secretion processes relies on autophagy (autophagy-based unconventional secretion; 'auto-secretion') for secretion of proinflammatory mediators such as IL-1β and HMGB1 in mammalian cells and Acb1 in yeast. GRASP (note in Fig. 30.1 that GRASP is normally localized to the Golgi and affects early stages of autophagy) is needed for autophagy-based unconventional secretion (auto-secretion). The CUPS, a yeast structure implicated in autophagy-based unconventional secretion, may be equivalent to the V-some in mammalian cells. In addition, autophagy plays a role in unconventional transport of the ER form of the cystic fibrosis transmembrane conductance regulator (CFTR) to the apical aspect of the plasma membrane, bypassing the Golgi and rescuing function of the mutant CFTR responsible for cystic fibrosis. GRASP plays a role in autophagy-dependent unconventional delivering of CFTR and in unconventional trafficking of α-integrin to the basolateral plasma membrane in Drosophila (a role for autophagy has not yet been established for α-integrin trafficking). Triggers: conditions (the list is not comprehensive) that contribute to the induction of unconventional secretion or trafficking (Deretic et al. 2012)

autophagy-competent cancer cells, acting as an extracellular signaling molecule in its immunological role as an alarmin; (4) osteoclasts secreting cathepsin K during bone resorption; and (5) LC3-contained secretory granules degranulation in mast cells resulting in anaphylaxis. Autophagy is also required for vestibular epithelial cells to secrete glycoproteins otoconins necessary for normal development of the organic calcium carbonate crystals (otoconia) which are needed for middle ear equilibrioception. Additionally, autophagy contributes to melanosomes maturation. Melanosomes contain specialized lysosome-related organelles with constituents from the Golgi that experience complicated transporting through plasma membrane via sorting endosomes. PMEL17-positive pre-melanosomes colocalizes with LC3 and Atg5, and PMEL17 is the amyloid-like fibrils offering structural foundation to the pre-melanosomes. Pre-melanosomes are loaded with the pigments eumelanin or pheomelanin during maturation finally are transported from melanocytes to recipient cells such as keratinocytes.

In pancreatic β-cell of the islets of Langerhans, autophagy directly or indirectly affects secretory processes. In the islets, autophagy possibly indirectly impacts the status of intracellular organelles including mitochondria and ER which are necessary for the secretion of insulin-induced by glucose through cytosolic $Ca^{2+}$ transients and for the increase of β-cell mass. This process is a part of normal glucose tolerance adaptations and under some conditions such as free fatty acids promoting peripheral insulin resistance and diabetes.

It is still unclear how Atg molecules or autophagy influence regulated secretion. It may involve in common steps of the secretory route, including post-Golgi vesicles and fusion properties of the plasma membrane domains, but could also affect unique

and highly specialized aspects in the maturation of secretory organelles and maintenance of their integrity and quality through autophagy. Moreover, it may also include the roles of autophagy in the expression and stability of clathrin-associated adaptor AP3 complex in vestibular cells and the organization of polarized plasma membrane domains in osteoclasts. In the latter case, the specialized fusion acceptor sites can offer homing addresses for regulated secretory lysosomes and their substances. In this case, lipidated LC3-II or other Atg8 paralogs is possibly needed for marking the acceptor membranes to be fused with lysosomes. This phenomenon is applicable to other cellular membrane domains. For instance, in professional phagocytic cells or in epithelial cells during entosis these relationships are similar to fusion of lysosome with intracellular compartments derived from plasma membrane including phagosomes harboring pathogen. Entosis is the invasion of detached epithelial cell into the cytoplasm of the neighboring cell were it either survives in entotic vacuoles or dies through fusion with lysosomes in an ATG-dependent manner. The above unexpected function of autophagic machinery and LC3 indeed belongs to the function of LC3/Atg8 as a tethering/fusion device and recapitulates the successive steps of the classical autodigestive autophagy pathway because the conventional LC3-positive autophagosomes complete their function by being targeted for fusion with lysosomal organelles (Deretic et al. 2012; Sanjuan et al. 2007).

## *30.3.3 Autophagy and Unconventional Secretion*

### 30.3.3.1 Autophagy-Based Unconventional Secretion (Auto-secretion)

Unconventionally secreted cytosolic proteins lack leader peptides and thus cannot be exported by the conventional ER-to-Golgi secretory pathway. One breakthrough in the role of autophagy in protein secretion and trafficking was the realization that a subset of unconventionally secreted cytosolic proteins depends on the autophagic machinery for extracellular secretion. In this context, autophagy-based unconventional secretion is termed as ‘autosecretion’ to distinguish it from other (Fig. 30.7) (Deretic et al. 2012; Bruns et al. 2011; Manjithaya et al. 2010).

A subset of unconventional secretory pathway use, at least under some circumstances, autophagic organelles or their intermediates as vehicles for extracellular export of proinflammatory factors interleukin (IL)-1β, IL-18 as well as high mobility group protein B1 (HMGB1) in mammalian cells and Acb1 in yeast (Fig. 30.7, pathway 3). The extracellular export of these leaderless secreted proteins relies on Atg molecules and is distinguished by the involvement of the GRASP (Golgi reassembly and stacking protein). GRASP termed as GRASP55 (GORASP2) and GRASP65 (GORASP1) in mammalian cells, GrpA in Dictyostelium, Grh1 in yeast, and dGRASP in Drosophila, is normally localized to the Golgi and was described first as a protein that helps organize Golgi ministacks into Golgi ribbons. GRASP

can affect early stages of autophagy and is required for autophagy-based unconventional secretion (auto-secretion), but appeared not to play a crucial role in conventional secretion through the Golgi apparatus. GRASP plays a role as a specific factor specializing in unconventional secretion and trafficking; examples include AcbA/Acb1 in Dictyostelium and yeast, IL-1β and IL-18 in mammalian cells, α-integrin in Drosophila and CFTR in human epithelial cells.

#### 30.3.3.2 Autophagy-Based Unconventional Secretion, Omegasome/CUPS (Compartment for Unconventional Protein Secretion)

The question is how autophagy promotes unconventional secretion. The omegasome acts as a cradle for forming nascent autophagosomes, or a potentially related structure in yeast termed the compartment for unconventional protein secretion (CUPS), and it may represent the source of organelles or trafficking intermediates for autophagy-based unconventional secretion.

The conventional secretory pathway, auto-secretion, and autophagy converge on interacted domains on the ER, the secretory aspect of ER-localized toward the Golgi named vesicular–tubular clusters or the ER-Golgi-intermediate compartment. This process in autophagy-based unconventional secretion initiates with the omegasome-like structure CUPS, which was formed in the vicinity of the ER exit sites with Sec13 positive but not fully colocalizing with Sec13.

Sec12 and Sec16, as organizers of ER exit sites and Sec23 and Sec24 of COPII components, but not Sec13 and Sec31 of COPII, play key roles in the formation of the autophagosome. The regulators of the early secretory pathway also participate in autophagy in mammalian cells; for example, a GTPase Sar1 interferes with the Sec12 GEF and other regulators of the early ER-to-Golgi secretory pathway in the formation of autophagosome and omegasome. It seems possibly that omegasomes in mammalian cells and the CUPS in yeast are related but this still needs to be determined. Contrastingly, there are some inconsistences regarding which of the molecules play a role in CUPS versus PAS formation in yeast; for instance, COPII component Sec23 is needed for autophagy but its mutation does not affect on Acb1 secretion via unconventional secretory pathway, whereas Sec12 is required for autophagy but seems to be not essential for the formation of CUPS. Whether these discrepancies suggest subcompartmentalization of omegasome domains transported to degradative autophagy versus auto-secretion still needs to be established.

Starvation stimulates the formation of CUPS and omegasome. As noted above CUPS in yeast is much like by morphology and localization the omegasome structures in mammals related to the formation of autophagosome although there are likely minor discrepancies in molecular and physiological layers. The remarkable features of omegasome and CUPS are that both of them are mediated by starvation, are marked by PI3P and are related to Atg molecules. The CUPS is different from the Cvt structure that is Ape1-positive and is not mediated by rapamycin. However, the omegasome and its contribution to autophagy as well as its PI3P-positive feature originally was solely

described in starvation-induced autophagy but were not reported in use of rapamycin. The omegasome is an early structure prior to the typical autophagosomes marked by LC3/Atg8, like the omegasome the formation of CUPS does not depend on Atg8 and Atg9. Upon starvation in yeast cells monitored with Grh1-GFP, one to three CUPS structures per cell form in the vicinity of transitional ER marked by Sec13, identified as Grh1-positive sites. Much like the structures of PASs, another salient feature of the CUPS is that it is marked by PI3P, Atg8, and Atg9. The CUPS also comprises Vps23, named as TSG101 in mammalian systems. Vps23 is one of the components of the ESCRT (endosomal sorting complex needed for transport)-I complex and is often related to multivesicular endosomal sorting and also participates in other cellular processes. Considering that other ESCRT proteins were not seen in the CUPS the role of Vps23 in CUPS is not clear. Loss of Vps23 did not interrupt the formation of CUPS marked by Grh although it is necessary for unconventional secretion of Acb1 in yeast suggesting that Vps23 likely plays a role in the downstream of the CUPS sorting effector functions. Vps27 of ESCRT mutant in yeast, named as Hrs in mammalian systems, along with mutant of some other components of ESCRT complexes 0, I, II, and III produced subtle kinetic effects on the CUPS and thus their roles are not equal to that of Vps23. Further, significance is likely that VPS23/TSG101 in its sorting function can bind to ubiquitinated cargo. This has yet to be investigated in CUPS function and in terms of putative adaptors selecting and transporting cargo destined to be exported through autophagy-based unconventional secretion. Unexpectedly, loss of yeast Atg1 did not block its GRASP (Grh1) translocation to the CUPS, thus separating Atg1 effect from the action of localization on Grh1. Similarly, this absence of effects were seen when Atg8 or Atg18 mutant was examined. Therefore, it remains to be determined following starvation which molecular factors controlled by underlying upstream events result in the yeast GRASP redistribution ortholog to the CUPS. However, in mammalian cells GRASP participates in the formation of autophagosome. Upon starvation in yeast, in addition to Vps23 localization to the CUPS, the GRASP equivalent plays a crucial role in the relocalization of Atg9. The effect of the yeast GRASP ortholog Grh1 on the Atg9 is consistent with the known role of the mammalian GRASPs (GRAPS55) in regulating initiation of autophagy (Deretic et al. 2012; Bruns et al. 2011; Manjithaya et al. 2010).

#### 30.3.3.3 Dual Role of Autophagy in Unconventional Secretion and Control of Inflammasome Activation

At least according to some predictions IL-1β a leaderless cytosolic secretory protein exported from the cell via membranous organelles would be a substrate for autophagy-based unconventional secretion. However, this issue is far more complicated. From the immunological point of view, numerous findings regarding the roles of autophagy in IL-1β activation have revealed that autophagy negatively regulates IL-1β activation. This is in accordance with the first description that Atg16L1 absence enhances mice IL-1β production, and is opposite to the cell biological predictions that autophagy positively regulates the secretion of IL-1β. Autophagy indirectly

suppresses IL-1β activation by decreasing the endogenous factors of inflammasome activation. It may also act directly via autophagic degradation of inflammasome components that has yet to be fully demonstrated. The inflammasome is a protein complex containing at least three proteins, including a Nod-like receptor (NLR) protein-like NLRP3, adaptor protein ASC as well as caspase-1. When inflammasome is activated by activators it processes pro-IL-1β (as well as other pro-proteins) into active IL-1β ready for secretion. Autophagy inhibits the activation of inflammasome via eliminating depolarized or damaged mitochondria that release endogenous inflammasome activators. When dysfunctional mitochondria increase in cells of autophagy defect they provide endogenous inflammasome agonists such as mitochondrial DNA and ROS. This is in keeping with the original function of autophagy in maintaining the cellular interior clean via removing potentially detrimental macromolecular aggregates and dysfunctional organelles. Therefore, in the deficiency of basal autophagy endogenous irritants result in the activation of inflammasome and enhancement of IL-1β process and become factors of sterile inflammation.

Autophagy indeed functions in IL-1β secretion via an unconventional route. However, this is not easy to distinguish from the roles of basal autophagy in maintaining the cellular interior clean and basal levels activation of inflammasome down. On starvation, autophagy is induced. At early time points, autophagy activation leads to enhanced secretion of IL-1β provided that the inflammasome is activated with conventional inflammasome agonists such as silica, nigericin, β-amyloid fibrils, and alum. This process wanes with time and at later time points, negative control of the inflammasome by autophagy becomes dominant. In conclusion, autophagy negatively regulates inflammasome activation and positively regulates IL-1β secretion with the net effect being the product of these two opposing actions. Autophagy induction promotes IL-1β secretion and the secretion of other cytosolic inflammasome substrates such as IL-18 and HMGB1. These acute results can be best detected early after stimulation (Deretic et al. 2012; Manjithaya et al. 2010).

#### 30.3.3.4 Autophagy and GRASP in Unconventional Trafficking of Proteins to Plasma Membrane

The role of GRASP and autophagy also includes transporting integral membrane proteins to polarized domains of the plasma membrane via unconventional secretory pathway, without passing through the ER–Golgi–plasma membrane conventional secretion pathway. DF508 CFTR, which is the most common type of mutant CFTR protein leading to cystic fibrosis, is delivered to the plasma membrane via the unconventional pathway depending on ATG5, ATG7, along with GRASP55. Inhibiting the conventional ER-to-Golgi secretory pathway (using dominant negative expression constructs of Sar1, Arf1, and Syntaxin 5) unexpectedly enhanced plasma membrane expression of the ER form of both DF508 and wild type CFTR (core-glycosylated). Examination of the role of GRASPs (predominantly GRASP55) in the unconventional trafficking of CFTR to the plasma membrane indicates that myristoylation of GRASP is required what's more that GRASP55 phosphorylation at Ser-441 and

the C-terminal signal of CFTR associating with the first PSD95, Dlg1, and zonula occludens-1 anchoring domain (PDZ1) of GRASP55 are needed for this protein transport pathway.

Biologically, the unconventional delivery of CFTR to reach the plasma membrane is functional and CFTR is in the correct apical domain of the plasma membrane. In the DF508 CFTR transgenic homozygous mice CFTR functioned as channel activity and blocked the typical malnourished and growth retardation phenotype. An integrin in Drosophila is also transported to the basolateral plasma membrane domain via the GRASP-dependent unconventional delivery where this integrin completes its physiological function. Therefore, the unconventional delivering pathway to the plasma membrane keeps polarization specificity and this process is not random but is a potentially high fidelity route that has evolved as a default or as a salvage trafficking pathway under certain conditions.

The unconventional CFTR transport can be activated by ER stress signaling and the UPR (unfolded protein response). Depleting $Ca^{2+}$ stores in ER with thapsigargin led to activated unconventional delivery of the core-glycosylated CFTR in a GRASP dependent (either GRASP55 or GRASP65) manner. The signal transducer resulting in these events is specifically associated with the Ser/Thr kinase IRE1 but not with PERK and ATF6, all three proteins as the crucial signal molecules of UPR. The function of IRE1 is outside of its most commonly considered endonuclease role, activating XBP1 (X-box binding protein 1 transcriptional factor) during ER stress because TRAF2 (TNF-receptor associated factor 2)-dependent JNK (c-Jun N-terminal kinase/stress-activated protein kinase) activation seems to play a role. In addition, following ER stress or the ER-to-Golgi transport blocked, GRASP55 is phosphorylated and Ser-441 of GRASP55 is needed for activating the unconventional transport route for trafficking DF508 CFTR to the plasma membrane. The precise connections between IRE1 signaling and GRASP55 phosphorylation remain to be explored; for example, the identity of the kinase phosphorylating Ser-441 of IRE1on GRASP55, however, overexpression of the wild type, phosphorylatable GRASP55 needed IRE1 for its ability to induce unconventional transport of DF508 CFTR to the plasma membrane. Thus, UPR, IRE1, and GRASP55 are sequentially linked in this pathway, which also depends on autophagy factors (Deretic et al. 2012; Gee et al. 2011).

#### 30.3.3.5 Coordination Between Exosome Secretion and Autophagy

Exosomes are small vesicles that are produced in the endosomal compartment of most eukaryotic cells and are released to the extracellular space. Contrary to other forms of extracellular vesicles, exosomes are originated from endocytic source and are formed as ILVs (intraluminal vesicles) though inward budding of the limiting membrane of late endosomes or MVBs (multivesicular bodies). The occurrence of release exosome is a constitutive fashion although cellular stress or irritant signals modulate their secretion. Exosomes transport-specific repertoires of proteins and

nucleic acids in the form of mRNAs and small noncoding RNAs, such as microRNAs, and are regarded as an unconventional secretory pathway. Exosomes deliver their cargo to neighboring cells and modulate at a distance the properties of receptor cells. Thus, exosomes also participate in communication between cells in several physiological actions and contributes to organism development, tissue repair, neuronal communication, and immune responses. However, exosomes might play a role in some diseases, such as benefitting tumor progression or virus spreading. Moreover, given that exosomes transport destroyed cellular products targeted for clearance, they promote toxic types of aggregated proteins spreading including β-amyloid, α-synuclein, and prion proteins and consequently facilitate neurodegenerative diseases progression.

There is a close relationship between the different autophagy processes and the biogenesis and secretion of exosomes. The mechanisms that direct the selective incorporation of proteins during the biogenesis of exosome and the occurrence of membrane invagination during the formation of ILV and the maturation of MVB have been thought to be a form of endosomal microautophagy. The endosomal microautophagy delivers cytosolic proteins into ILVs depending on the ESCRT structure and the chaperone hsc70. This pathway does not need substrate unfolding or the necessary molecule of CMA in lysosomes such as LAMP-2A but it depends on electrostatic binding of hsc70 to endosomal acidic phospholipids.

Autophagosomes may either fuse with lysosomes or with MVBs during macroautophagy. Findings on ESCRT mutants have pointed to a close relationship between the autophagy pathway and the biogenesis of MVB. ESCRT mutants fail to finish autophagic maturation due to the absence of fusion of the autophagosome with the endolysosomal machinery and cause up-regulated number of autophagosomes. ESCRT mutants in different *C. elegans* lead to increased autophagic activity, which assists cells to process the detrimental accumulation of abnormal endosomes, resulting from the deficiency of ESCRT system. In this condition autophagy induction promotes increasing cell survival and organismal lifespan possibly via the selective clearance of late endosomes through the autophagy pathway. Regulators for autophagy remarkably modulate the formation of MVB and the secretion of exosome. Under conditions of autophagy induction such as rapamycin treatment, starvation or LC3 overexpression, autophagy activation suppresses exosome secretion, indicating that in this context, MVBs are guided to the autophagic pathway, consequently preventing exosome secretion. Therefore, the balance between the activation of autophagy and the release of exosome may be controlled by the cellular metabolic status. The current challenge is to identify how metabolic sensors regulate the fate of certain molecules toward the degradation of autophagy or the secretion of exosomes and how this modulation affects the autonomous and non-cell autonomous homeostasis (Baixauli et al. 2014).

### *30.3.4 Multitasking Protein in Autophagy and Secretion*

#### 30.3.4.1 Role of COPII Components in the Secretion-Autophagy Crosstalk

ERES may be a preferred site for autophagosome biogenesis because the curved membranes are available at ERES. Alternatively, it is possible that autophagy and the secretory pathway share common machinery that localizes in ERES. The first evidence that core components of the ER export machinery regulate autophagy came from the observation that yeast strains with genetic deficiencies in certain COPII components or their regulators are unable to form autophagosomes. More recently, several proteins such as Sar1, Ypt1/Rab1, and the CK1 kinase Hrr25 which phosphorylates the COPII coat have been implicated in autophagy. Suppression of the GTPase Sar1, which interacts with its membrane-bound exchange factor Sec12 and triggers COPII vesicle trafficking, impairs autophagy in yeast and mammalian cells, suggesting an evolutionarily conserved dependence of autophagy on ER export and/or COPII components. In support of the former, treating mammalian cells with the ER export inhibitor FLI-06 attenuates the number of autophagosomes under starvation conditions. Moreover, a recent study in yeast demonstrated that ER export of the ER-localized Qa/t-SNARE Ufe1 is induced in response to starvation and that Ufe1 is targeted to sites containing Atg8 and Atg9, where it promotes autophagosome formation.

The COPII machinery may play a role beyond regulating the delivering of autophagy regulators and instead may be redirected to autophagy in response to stress. For example, a recent study suggested that phosphorylation of three amino acids on the membrane-distal surface of the COPII cargo adapter Sec24 facilitates the interaction of Sec24 with Atg9 at the PAS. Although phosphorylation of the distal sites on Sec24 is needed to increase autophagosome number, which facilitates cellular homeostasis during starvation, it is not required for ER-to-Golgi transport. The Sec24 phosphorylation sites are conserved in mammalian SEC24A, and the highly conserved kinase Hrr25 is one of the kinases that phosphorylate this site. The redirection of COPII vesicle machinery for autophagy has also been identified in mammals. Studies demonstrate that the induction of autophagy leads to class III PI3 kinase-dependent relocation of ERES components to the ERGIC (Farhan et al. 2017).

#### 30.3.4.2 TECPR2 as a Bridge Between the Autophagy and Secretory Machinery

Another example of a multitasking regulator is tectonin β-propeller-containing protein 2 (TECPR2), a protein implicated in neurodegenerative diseases. TECPR2 binds to ATG8 (LC3 in mammals) and positively regulates autophagy. ATG8 is a small,

ubiquitin-like molecule that is conjugated to phosphatidylethanolamine in the growing autophagosome membrane through a cascade involving other autophagy proteins. Recently TECPR2 was found to interact with the COPII component SEC24D and to regulate the number of ERES in an LC3C-dependent manner. The mechanism by which TECPR2 switches from being an ERES regulator to an autophagy regulator remains to be elucidated (Farhan et al. 2017).

#### 30.3.4.3 Rab1 and Its Dual Role in Autophagy and Secretion

Rab GTPases regulate all membrane-bound intracellular transport pathways. Therefore, it is reasonable that certain Rabs (Ypt in yeast) regulate both autophagy and secretion. For example, Ypt1 activation on COPII vesicles results in the recruitment of Uso1, which tethers the vesicles to the Golgi. Active Ypt1 is also needed for starvation-mediated autophagy and provides a means of targeting autophagy (i.e., ULK/Atg1) components to the PAS. Given the degree of evolutionary conservation in this class of proteins, it is perhaps not surprising that mammalian Ypt1 (Rab1) and Trs85, a component of the multimeric guanine nucleotide exchange factor (TRAPPIII) that activates Ypt1/Rab1, have been implicated in the regulation of autophagy (Farhan et al. 2017).

#### 30.3.4.4 ULK1/2 Are Multitasking Kinases Regulating Both Autophagy and Secretion

ULK1/2, known for their role in the induction and regulation of autophagy was recently found to play a role in ER–Golgi traffic during normal growth. ULK1/2 localize to ERES and phosphorylate SEC16A a scaffold protein that promotes the biogenesis and maintenance of ERES. Interesting, the phosphorylation of SEC16A by ULK1/2 is necessary for the transport of specific cargo from the ER to the Golgi. Because SEC16A is a target of several kinases its posttranslational modification may provide a means of fine-tuning the regulation of COPII trafficking with the availability of growth factors and nutrients. Although inhibition of COPII trafficking impairs starvation-induced autophagy, additional studies are required to determine whether SEC16A phosphorylation by ULK1/2 is needed for autophagy and to precisely illustrate how the multitasking function of SEC16A is orchestrated in the context of secretion and autophagy (Farhan et al. 2017).

#### 30.3.4.5 UVRAG and Its Dual Role in Autophagy and Retrograde COPI Trafficking

In the absence of cellular stress, the PI3P-binding protein UVRAG localizes to the ER where it assembles into a RINT-1 containing tethering complex that regulates the arrival of COPI vesicles involved in Golgi-to-ER retrograde transport. On the

induction of autophagy, UVRAG dissociates from the RINT-1 complex and associates with the class III PI3K complex to induce the mobilization of ATG9 from the Golgi. UVRAG also regulates autophagosome maturation by binding to the class C Vps complex. Thus, UVRAG is a multitasking protein whose function is altered in response to cellular stress (Farhan et al. 2017).

### *30.3.5 Conclusion*

There are close cross talks between autophagy and biosynthetic pathways including conventional and unconventional biologically active cargo secretory processes and delivering of integral membrane proteins. This expands the range of physiological actions of autophagy. Autophagy affects the biogenesis of several specialized tissues and organs including secretion of inflammatory cytokines melanosome maturation in skin melanocytes and retinal pigment epithelial cells and bone remodeling by osteoclasts. With the developments of these roles of autophagy the area of autophagy impact expands to the extracellular space both in complicated metazoan tissues and in the outside environment of unicellular eukaryotes including yeast. Complicated syndromes including cystic fibrosis and Crohn's disease which are often companied by inflammatory mediators seem to be affected by this novel unexpected role of autophagy. In a word, the breadth and depth of the impact of autophagy in biology seem endless.

## 30.4 Coordination Between Autophagy and Trafficking Events from Plasma Membrane

### *30.4.1 Autophagy and Connexins*

Connexins are one of the groups of lateral plasma membrane proteins which are degraded via autophagy. Currently, it is still unknown whether connexins are degraded through an autophagy receptor-like mechanism such as focal adhesions or directly incorporated into autophagic precursor structures such as Notch1. Surprisingly, there is an intermodulation between autophagy and connexins. Connexins at the plasma membrane suppress autophagy by associating with different proteins that modulate the biogenesis of autophagosome including VPS34, VPS15, ATG16L1, and Beclin-1. When autophagy is activated by starvation, this effect is reversed by the reaching of ATG14 to the ATG-Connexin complex which allows its internalization with ATG9A. The degradation of the connexions by autophagy leads to reduced levels of these inhibitory factors and thus allowing more long-lasting activation of autophagy (Pavel and Rubinsztein 2017).

### 30.4.2 Autophagy and Cell Connections

The degradation of the constituents interacted with the plasma membrane can be induced through autophagic receptors, which act as a connection between the related substrate protein and the constituents of the autophagosome, such as LC3. This mechanism is crucial for degradation of cell-matrix focal adhesions (FAs) when they are disassociated with the basal cell membrane. This process is mainly induced by the autophagy cargo receptor neighbor of BRCA1 (NBR1). In addition, when Src is activated by autophagy the disassociation and turnover of FAs rely on the direct association between LC3 and the conserved LIR motif as an early precursor and important constituent of FAs. These findings may explain why autophagy is needed for cell migration, invasion, and metastasis.

As components of the lateral plasma membrane the functions of adherens junctions (AJ) and tight junctions (TJ) are regulating cell–cell adhesion and paracellular barrier functions both needed for retaining cell polarity. But upon EMT (epithelial–mesenchymal transition) and tumor invasiveness this phenomenon is lost. Autophagy actually enhances the degradation of E-cadherin which is the main transmembrane molecule of adherens junctions and consequently promotes the dissolution adherens junctions during epithelial–mesenchymal transition. Moreover, the levels of E-cadherin can be transcriptionally suppressed in cells of absence of autophagy where up-regulated p62 levels stabilize the oncogenic transcription factor Twist1 which represses the synthesis of E-cadherin and enhances epithelial–mesenchymal transition. However, the molecular mechanisms for the process of autophagy-dependent disassembly of adherens junctions are not fully clear and may involve other events. For instance, autophagy degrades the tight junctions proteins claudin-2 leading to decreased epithelial tight junctions permeability with enhanced paracellular barrier properties. This phenomenon is related to inflammatory bowel disease (Pavel and Rubinsztein 2017).

### 30.4.3 Autophagy and Ciliogenesis

There is also another reciprocal regulation of autophagy by ciliogenesis. Upon starvation, autophagy is activated by relocalizing several autophagic proteins including LC3, ATG16L1, GABARAPL1 along with AMBRA1 to cilia and consequently promoting the enhanced cilia-plasma membrane turnover needed for effective ciliogenesis. Ciliogenesis also increases autophagy, potentially allowing a self-sustaining feedforward mechanism: ciliogenesis–autophagy–ciliogenesis. Additionally, mechanical stimuli including fluid flow initiate and maintain this feedforward loop because autophagy plays a key role in decreasing cell size/shape that further potentiates ciliogenesis. Autophagy might respond to fluid flow in the kidney to modulate epithelial cell size in this way (Pavel and Rubinsztein 2017).

### *30.4.4 Conclusion*

Recent studies indicate multiple layers of regulation between the plasma membrane and its associated proteins and autophagy. Autophagy can modulate cell fate by affecting the degradation of differentiation-related proteins or proteins that influence migration. Connexins localized to the plasma membrane as inhibitory proteins of autophagy can themselves be degraded by autophagy and thus developing autoregulatory positive feedback that potentiates many aspects of autophagy.

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