Physiological Functions of Autophagy

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Contents

Abstract The field of autophagy research has advanced rapidly in recent years, with important discoveries made in relation to both molecular mechanisms and physiological functions. Initially, autophagy was thought to be primarily a response to starvation. Although this might be true in lower eukaryotes, this catabolic process exerts various physiological functions in higher eukaryotes. This review summarizes the physiological roles of autophagy in amino acid pool maintenance, intracellular quality control, development, cell death, tumor suppression and anti-aging.

1 Introduction

Intracellular protein degradation systems can be roughly classified into two groups: selective and nonselective. Selective degradation is primarily carried out by the ubiquitin-proteasome system, whereas most nonselective degradation occurs

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in the lysosome. The degradation of cytoplasmic components in the lysosomes is generically referred to as autophagy (Cuervo 2004; Klionsky 2007; Mizushima 2007; Levine and Kroemer 2008; Mizushima et al. 2008). The three types of autophagy are macroautophagy, microautophagy, and chaperone-mediated autophagy (CMA). In CMA, substrate proteins are specifically recognized by chaperones and directly transported across the lysosomal membrane to the lumen (Cuervo 2004). Microautophagy has been proposed to occur by invagination of the lysosomal membrane into the lumen; however, its molecular mechanisms remain unknown. This chapter focuses on macroautophagy, having been extensively studied and closely related to immunology and microbiology.

Macroautophagy (referred to as autophagy hereafter) is mediated by a unique organelle called the autophagosome (Fig. 1). Upon autophagy induction, a portion of the cytoplasm is enclosed by an autophagosome. Not only cytosolic proteins but also organelles such as mitochondria and endoplasmic reticulum are often sequestered into autophagosomes. The outer membrane of the autophagosome then fuses with the lysosome, allowing lysosomal enzymes to degrade the sequestered cytoplasmic materials. Since bulk cytoplasm is sequestered by autophagosomes, autophagy is usually considered a nonselective random degradation system. However, recent studies have indicated that some proteins and organelles are selectively degraded by autophagy, an issue discussed later in this chapter.

Recently, the molecular mechanisms of autophagy have become increasingly clear. Breakthroughs came from genetic analyses performed in yeast, and at least 18 autophagy-related genes have been identified in this organism so far. Since most of these genes are conserved in higher eukaryotes, studies using reverse genetic approaches have been carried out in various organisms. These studies have demonstrated that, although autophagy is a simple membrane-mediated process, it has a wide variety of physiological and pathophysiological roles.

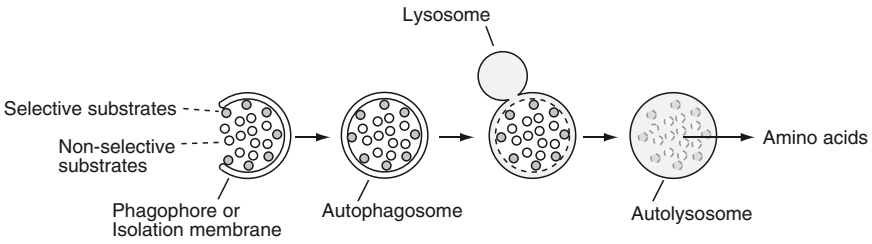

Fig. 1 Schematic model of macroautophagy. A portion of cytoplasm is enclosed by a phagophore or isolation membrane to form an autophagosome. While most substrates are enclosed nonselectively, some proteins such as p62 are selectively recognized by the autophagosome membrane (via LC3 in the case of p62). The outer membrane of the autophagosome subsequently fuses with the lysosome, and the internal materials are degraded. The resulting amino acids are delivered back into the cytosol for reuse or further metabolism

2 Physiological Functions of Autophagy

2.1 Maintenance of the Amino Acid Pool

An evolutionarily conserved role of autophagy is adaptation to starvation through the generation of amino acids inside cells. Under normal conditions, the intracellular amino acid pool can be maintained by the proteasome, which continuously degrades cytoplasmic proteins (Vabulas and Hartl 2005). In contrast, this pool is largely maintained by autophagy during starvation. In yeast, autophagy is suppressed to undetectable levels under growing conditions, but is rapidly upregulated during nitrogen starvation (Takeshige et al. 1992). The levels of amino acids in autophagy-deficient yeast cells are lower than those in wild-type cells during starvation. Likewise, autophagy is immediately activated in cultured mammalian cells following amino acid withdrawal. Accordingly, autophagy has been shown to be crucial for surviving starvation in *Sacharomyces cerevisiae* (Tsukada and Ohsumi 1993), *Dictyostelium discoideum* (Otto et al. 2003), *Drosophila melanogaster* (Scott et al. 2004) and *Caenorhabditis elegans* (Kang et al. 2007). Moreover, autophagy is upregulated in most tissues except the nervous tissues in starved mice (Mizushima et al. 2004). It is also upregulated shortly after birth, when nutrient supply from the placenta is abruptly terminated (Kuma et al. 2004). Mice lacking Atg5, an essential factor in autophagosome formation, die about 12 h after birth. The amino acid levels of these mice are normal at birth but immediately decrease thereafter. These experiments illustrate the important role of autophagy in response to starvation.

The maintenance of the amino acid pool during starvation is important for the production of subsets of proteins needed for adaptation to starvation conditions. Starved yeast cells upregulate the synthesis of chaperones and enzymes for amino acid synthesis, which is severely affected in autophagy-defective mutants (Onodera and Ohsumi 2005). This defect might account for the low survival rate of these mutants under starvation conditions (Tsukada and Ohsumi 1993).

Amino acids produced by autophagy can also be utilized for energy production. Although amino acids are generally considered poor fuel sources, they can be metabolized through the tricarboxylic acid (TCA) cycle to produce energy. This is particularly apparent in muscle (Shimomura et al. 2004), but may also be the case in cultured cells (Lum et al. 2005). Indeed, the autophagy-deficient phenotype of IL-3-dependent cells can be restored by methylpyruvate, a cell-permeable substrate for the TCA, supporting the idea that autophagy produces energy. In addition to direct energy production, amino acids generated by autophagy can be used for gluconeogenesis, which is an important physiological response to starvation. One of the well-known pathways is the glucose-alanine cycle. Under starvation conditions, peripheral tissues such as muscle secrete alanine, which is converted to glucose in the liver. However, the extent that autophagy contributes to this pathway remains unknown.

2.2 Intracellular Quality Control

Although massive induction following nutrient withdrawal is the most prominent feature of autophagy, this process also occurs constitutively at low levels under normal growth conditions. The primary role of this basal autophagy is seemingly not the maintenance of the amino acid pool, because the intracellular amino acid pool is not affected in autophagy-deficient cells and animals as long as sufficient nutrients are available (Kuma et al. 2004; Komatsu et al. 2005; Onodera and Ohsumi 2005). However, recent mouse genetic studies have revealed that basal autophagy is quite important for intracellular quality control through the constitutive turnover of cytoplasmic components. Abnormal ubiquitinated proteins and organelles accumulate in the cytoplasm immediately after the deletion of autophagy genes such as *Atg5* and *Atg7* in the liver (Komatsu et al. 2005; Hara et al. 2006) and nervous system (Hara et al. 2006; Komatsu et al. 2006). These proteins are present in both the diffuse cytosolic and intracellular protein-aggregated forms. Interestingly, ubiquitinated proteins accumulate extensively in the liver, neurons and some endocrine glands, and much more slightly in other tissues such as the skeletal muscle, heart, and kidney (Hara et al. 2006). Since protein turnover in the liver is very high, it is not surprising that basal autophagy is very important in this organ. However, the reason underlying the critical importance of autophagy in the brain remains unclear. Autophagic activity in the brain is very low and is not induced during starvation (Mizushima et al. 2004; Nixon et al. 2005). Quality control may be more important in nondividing, quiescent cells than in rapidly dividing cells. In agreement with this concept, primary mouse embryonic fibroblasts prepared from *Atg5*$^{-/-}$ mice do not show protein aggregates at early phases, but do so in later senescent phases. Intracellular accumulation of abnormal proteins is also observed in *Atg7*-deficient *Drosophila* (Juhasz et al. 2007).

Basal autophagy is apparently crucial for cellular homeostasis. Liver-specific *Atg7*$^{-/-}$ mice develop hepatomegaly and hepatic failure (Komatsu et al. 2005), and neural cell-specific *Atg5* and *Atg7* knockout mice show neurodegeneration accompanied by progressive motor deficits (Hara et al. 2006; Komatsu et al. 2006). These phenotypes are cell autonomous because Purkinje cell-specific *Atg5* and *Atg7* knockout mice show Purkinje cell degeneration (Komatsu et al. 2007a; Nishiyama et al. 2007). Abnormal proteins and organelles are also detected in heart-specific *Atg5*-deficient mice (Nakai et al. 2007). Therefore, autophagy serves as a housekeeper under normal conditions in order to prevent cell degeneration, particularly in the nervous tissue, even if animals do not express disease-associated mutant (aggregate-prone) proteins.

Although the accumulation of protein aggregates and autophagic vacuoles is a hallmark of many neurodegenerative diseases such as Alzheimer's disease (Okamoto et al. 1991; Cataldo et al. 1996), polyglutamine (CAG) repeat diseases (Petersen et al. 2001; Ravikumar et al. 2002), and Parkinson's disease (Anglade et al. 1997), it remains unknown whether autophagy is indeed involved in the pathogenesis of these diseases. In some familial neurodegenerative diseases such as

amyotrophic lateral sclerosis-like motor disease and frontotemporal dementia, the causative mutations in dynein and *CHMP2B* do indeed affect autophagosome–lysosome fusions, which should impair autophagic clearance of abnormal proteins (Ravikumar et al. 2005; Filimonenko et al. 2007; Lee et al. 2007). Irrespective of whether the autophagy defect is the direct cause or not, autophagy could be a good therapeutic target in these neurodegenerative diseases (Rubinsztein 2006). Inhibitors of Tor, a potent endogenous suppressor of autophagy, have been shown to be effective in attenuating symptoms in fly and mouse Huntington disease models (Ravikumar et al. 2004). In addition, other molecules such as lithium and trehalose were shown to modulate autophagy (Sarkar et al. 2005; Sarkar et al. 2007a; Zhang et al. 2007a). Furthermore, small-molecule enhancers of the cytostatic effects of rapamycin (SMERs) enhance autophagy in an mTOR-independent manner, and accelerate the clearance of mutant huntingtin and α-synuclein in a fly Huntington disease model (Sarkar et al. 2007b). Finally, lithium, which induces autophagy by inhibiting inositol monophosphatase independently of mTOR, delays progression of amyotrophic lateral sclerosis in humans (Fornai et al. 2008). To achieve the maximum effect, combination therapy using lithium and mTOR inhibitors has been proposed (Sarkar et al. 2008). Since most neurodegenerative diseases progress slowly, slight modulation of autophagy could produce dramatic effects on disease prognosis.

2.3 Selective Degradation by Autophagy

Whether abnormal proteins and inclusion bodies are selectively degraded by autophagy has been a continuing issue for debate. In the temporary controlled liver-specific *Atg5* knockout model, a loss of autophagy first leads to the accumulation of diffuse ubiquitinated proteins in the cytosol followed by the generation of inclusion bodies (Hara et al. 2006). This suggests that the accumulation of inclusion bodies in autophagy-deficient models is a secondary phenomenon, and large inclusions are not primary substrates. If protein turnover is generally impaired by random autophagy, proteins would have more opportunities to be damaged, misfolded, ubiquitinated and finally aggregated.

However, these studies do not rule out the possibility that oligomerized misfolded proteins or ubiquitinated proteins might be selectively incorporated into autophagosomes. Recently, it has been proposed that p62/SQSTM1 may serve as an adaptor protein for mediating the binding of ubiquitinated proteins by autophagosomes (Bjørkøy et al. 2005; Pankiv et al. 2007). Apart from the known functions of p62 in various signaling pathways (Wooten et al. 2006; Moscat et al. 2007), it can also bind both ubiquitin and LC3. Therefore, the LC3-p62 complex on the inner membrane of an autophagosome may recruit ubiquitinated proteins into autophagosomes (Fig. 1). However, the extent to which this pathway contributes to the degradation of ubiquitinated proteins under normal conditions remains unclear. A recent study showed that K63-ubiquitinated proteins accumulate in *p62*$^{-/-}$ mouse brain, though this may be due to the reduced activity of a K63-deubiquitinating enzyme

called cylindromatosis tumor suppressor (CYLD), which seems to be independent of autophagy (Wooten et al. 2008).

p62 is mainly degraded by autophagy together with LC3, but accumulates excessively in autophagy-deficient cells (Wang et al. 2006; Komatsu et al. 2007b; Nakai et al. 2007). Interestingly, the maintenance of p62 expression at certain levels by autophagy is critically important for cellular homeostasis. The liver enlargement and dysfunction found in liver-specific *Atg7* knockout mice are significantly rescued by simultaneous ablation of p62 (Komatsu et al. 2007b). Ubiquitin-positive inclusion bodies are not generated in $Atg7^{-/-}p62^{-/-}$ mouse liver, suggesting that the excess amount of p62 accounts for the generation of the inclusions and hepatocyte damage. However, deletion of p62 does not alter the clinical course of neural cell-specific *Atg7* knockout mice (Komatsu et al. 2007b). Therefore, basal autophagy is important for the degradation of not only p62 and its interacting proteins but also other proteins.

Selective degradation by autophagy has been also demonstrated for yeast Ald6 (Onodera and Ohsumi 2004), peroxisomes (Luiken et al. 1992; Iwata et al. 2006), mitochondria (Kim et al. 2007), ribosomes (Kraft et al. 2008) and invading bacteria (Levine and Deretic 2007; Schmid and Münz 2007).

2.4 Development and Cell Death

Autophagy has been reported to be important for the development of various organisms. For example, yeast autophagy mutants are defective in spore formation during starvation (Tsukada and Ohsumi 1993), and autophagy mutants of *D. discoideum* are defective in multicellular development (Otto et al. 2003). Dauer formation is also affected in *Caenorhabditis elegans* autophagy mutants (Melendez et al. 2003). These findings might suggest that autophagy is important for nutrient mobilization during these remodeling processes because these developmental events occur under starvation conditions. However, this idea may be reconsidered based on a recent *Drosophila* study (Juhasz et al. 2007). Previous studies of *Drosophila* revealed that several autophagy mutants show premature death from the third larval to the pupal stages (Juhasz et al. 2003; Scott et al. 2004). It could thus be interpreted that larval tissues are degraded to produce nutrients for generating adult tissues in a pupa. Indeed, in dying larval tissues such as the salivary glands, massive autophagy is observed (Baehrecke 2003). However, recently generated *Atg7*-deficient *Drosophila* have been shown to be viable, although autophagy is virtually suppressed and adult flies are sensitive to nutrient and oxidative stresses (Juhasz et al. 2007). The pupal period is extended but the larval-adult midgut transition proceeds normally. The previously reported *Drosophila* mutants such as the *Atg1* mutant may have defects beyond autophagy. Therefore, some other pathways may compensate for the defective protein breakdown in the autophagy-defective mutant during metamorphosis.

Autophagy has also been thought to be a type of cell death-inducing process, especially during development. It is sometimes referred to as “type 2 cell death” or “autophagic cell death.” During development, autophagy occurs in dying cells in various

embryonic tissues (Baehrecke 2005; Debnath et al. 2005; Levine and Yuan 2005). However, the role of autophagy in cell death execution has been an issue of great controversy, while that of autophagy in cell survival has been well documented. In apoptosis-deficient cells, autophagy contributes to cell death induced by apoptogenic stimuli such as genotoxic stress and staurosporine, and a caspase inhibitor (z-VAD) (Shimizu et al. 2004; Yu et al. 2004). There have been no lines of evidence that autophagy induces cell death during physiological development in mammals because *Atg5*$^{-/-}$ and *Atg7*$^{-/-}$ mice are born grossly normal at birth. However, a recent *Drosophila* study revealed that autophagy is indeed required for the complete degradation of a dying salivary gland in a pupa (Berry and Baehrecke 2007). Cells in the salivary glands rapidly die and whole salivary glands are degraded after pupa formation. This tissue destruction is at least partially mediated by autophagy, because the suppression of several *ATG* genes leads to incomplete degradation (Berry and Baehrecke 2007). Since caspase inhibition also partially suppresses cell death, both apoptosis and autophagic degradation may function in the rapid destruction of the salivary gland. Thus, the physiological role of autophagy in cell death is rather complicated and depends on the presenting situation. The term "autophagic cell death" may not be appropriate in certain cases, even if autophagy is detected in dying cells.

The role of autophagy in mammalian development has not been well understood because mice deficient for *Atg5* or *Atg7* can survive embryogenesis (Kuma et al. 2004; Komatsu et al. 2005). However, these studies overlook the requirement of autophagy during early developmental stages, when maternally inherited proteins remain in the cytoplasm of knockout oocytes. Indeed, autophagy is activated shortly after fertilization, which is essential for preimplantation development (Tsukamoto et al. 2008).

Autophagy is also involved in another step of cell death. Cells undergoing apoptosis expose phosphatidylserine (PS) at the cell surface, which is recognized by phagocytes. However, autophagy-defective cells cannot expose PS efficiently due to low levels of cellular ATP, resulting in the failure of dead cell clearance (Qu et al. 2007).

2.5 Tumor Suppression

The role of autophagy in tumorigenesis and cancer progression has been discussed for a long time. It may be considered that autophagy is also important for the survival of tumor cells, just like normal cells. However, many studies have suggested that autophagy instead acts as a tumor suppressor (Hippert et al. 2006; Jin and White 2007; Levine 2007; Mathew et al. 2007a).

The first genetic linkage between autophagy and cancer was indicated by a study on Beclin 1. Beclin 1 is a mammalian counterpart of yeast Atg6/Vps30, which is a component of the autophagy-related PI3–kinase complex (Liang et al. 1999; Kihara et al. 2001b; Kihara et al. 2001a). Beclin 1 was originally identified as an interacting partner of an antiapoptotic protein, Bcl-2 (Liang et al. 1998). Accordingly, Bcl-2 negatively regulates autophagy by binding with Beclin 1 (Pattingre et al. 2005). Importantly, Beclin 1 is monoallelically deleted in 40–75% of sporadic human breast

and ovarian cancers (Liang et al. 1999). In addition, *beclin 1*$^{+/-}$ mice develop spontaneous tumors such as lung cancer, hepatocellular carcinoma and lymphoma (Qu et al. 2003; Yue et al. 2003). These studies revealed that Beclin 1 is a novel tumor suppressor. Recently, UVRAG (a Beclin 1-interacting protein; Ionov et al. 2004; Liang et al. 2006) and Atg4C were also shown to have tumor-suppressive roles (Mariño et al. 2007). Therefore, autophagy likely has a protective role against tumorigenesis.

There are two (not mutually exclusive) hypotheses as to why defective autophagy causes tumors. The first is that tumorigenesis is induced by an inflammatory response. As discussed above, autophagy is basically a protective mechanism and its deficiency causes necrotic cell death if the apoptotic pathway is also compromised. This phenomenon is particularly important under metabolic stress (ischemia and nutrient starvation) conditions, which is often observed at the center of solid tumors (Degenhardt et al. 2006). The resulting necrotic cell death induces an inflammatory response, which in turn promotes secondary tumorigenesis (Degenhardt et al. 2006). The second hypothesis is that autophagy can protect cells from genomic damage in a cell-autonomous manner (Karantza-Wadsworth et al. 2007; Mathew et al. 2007b). A high rate of genomic damage and instability were observed in *beclin 1*$^{+/-}$ and *Atg5*$^{-/-}$ kidney (Mathew et al. 2007b) and mammary epithelial cells during metabolic stress (Karantza-Wadsworth et al. 2007). Why autophagy is beneficial for genome protection is not fully understood. One possible explanation is that deficiency in autophagy, which is important for mitochondrial quality control, causes the accumulation of damaged mitochondria (Komatsu et al. 2006; Kim et al. 2007; Zhang et al. 2007b; Twig et al. 2008). In yeast, reactive oxygen species (ROS) tend to accumulate in autophagy-defective cells (Xiong et al. 2007). Also in mouse liver, an oxidative transcription factor, Nrf2, is activated if autophagy is impaired (Komatsu et al. 2007b). Such oxidative stress may promote DNA damage and ultimately tumorigenesis.

2.6 Anti-Aging

As discussed above, autophagy is involved in both nutrient regulation and intracellular quality control. Therefore, it can be assumed that an excess of nutrients suppresses intracellular clearance, while mild starvation promotes such clearance. Indeed, an inverse relationship between autophagy and aging has frequently been postulated (Bergamini et al. 2004; Dröge 2004; Levine and Klionsky 2004). Caloric restriction is the most effective method for extending the life spans of various species from yeast to mammals. The precise mechanisms underlying such extension are not completely understood, but autophagy could be one of the effectors. Autophagy or general protein degradation is upregulated during calorie restriction in rodents and worms (Ward 1988; Donati et al. 2001; Bergamini et al. 2004; Mörck and Pilon 2006). Furthermore, genetic studies have also suggested that autophagy is indeed important for extending life span. A *C. elegans* mutant lacking the insulin signaling gene *daf-2* shows a life-extension phenotype, which is suppressed if autophagy-related genes are simultaneously

silenced (Melendez et al. 2003; Hars et al. 2007; Hansen et al. 2008). Similarly, the life-extending effect of calorie restriction is also impaired in *atg* gene-knockdown worms (Jia and Levine 2007; Hansen et al. 2008). Although both dietary restriction and *daf-2* mutation upregulate autophagy, their underlying mechanism appears to be different. Dietary restriction-induced autophagy requires the transcription factor Pha-4/FoxA, whereas *daf-2* mutation-induced autophagy requires neither Pha-4 nor Daf-16/FoxO (Hansen et al. 2008). Thus, autophagy induction may not be sufficient to extend life span, and a parallel pathway through Daf-16/FoxO is also required. The NAD-dependent deacetylase Sirt1, which is an evolutionally conserved regulator of life span, was also shown to play an important role in autophagy induction both in vitro and in vivo through the deacetylation of several Atg proteins by Sirt1 (Lee et al. 2008). Therefore, there appear to be complicated interrelations between autophagy and life span determination.

In aged cells, various damaged proteins are accumulated, which could be (at least partially) due to the seemingly reduced activity of both macroautophagy and CMA with age (Bergamini et al. 2004; Terman, 2006; Martinez-Vicente and Cuervo 2007). Therefore, autophagic activity may be a critical determinant of life span, and its modulation will be a potential strategy against aging.

3 Conclusion

Autophagy has a wide variety of physiological roles due to its several different modes (induced vs. constitutive) (Mizushima 2005) and steps (sequestration vs. degradation) (Mizushima 2007). In addition to the topics described in this review, recent studies have also demonstrated that autophagy is important for protection against heart failure and liver and muscle diseases. Furthermore, as this book highlights, autophagy is important for the degradation of not only self-proteins but also various microbes. Paradoxically, “degradation” is not the main function of autophagy in some circumstances. The autophagosome can also be used as a special site for the survival and replication of subsets of bacteria and viruses. Thus, as described in this book, autophagy has various roles in infection and immunology.

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