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REVIEW ARTICLE

# Multifaceted Housekeeping Functions of Autophagy

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**Abstract** | Autophagy is an evolutionarily conserved intracellular degradation process in which cytoplasmic components are captured in double membrane vesicles called autophagosomes and delivered to lysosomes for degradation. This process has an indispensable role in maintaining cellular homeostasis. The rate at which the dynamic turnover of cellular components takes place via the process of autophagy is called autophagic flux. In this review, we discuss about the orchestrated events in the autophagy process, transcriptional regulation, role of autophagy in some major human diseases like cancer, neurodegeneration (aggrephagy), and pathogenesis (xenophagy). In addition, autophagy has non-canonical roles in protein secretion, thus demonstrating the multifaceted role of autophagy in intracellular processes.

## 1 Introduction

Autophagy, an intracellular evolutionarily conserved process, involves engulfment of unwanted proteins and organelles by double-membrane vesicles, called autophagosomes, which then fuse with the lysosomes/vacuole, and the engulfed cargo is subsequently degraded. It is a cell survival mechanism under stress conditions and it also play important roles in many other intra-cellular processes like protein and organelle turnover and transport of some of the vacuolar enzymes. This process can be divided into various steps, including autophagy induction, nucleation, autophagosome formation, maturation, fusion with the lysosomes/vacuole, degradation of the cargo, and recycling of the precursor molecules, such as amino acids, lipids, and nucleotides, back to the cytoplasm. Autophagy is a tightly regulated cellular mechanism and its flux varies depending on the cell type(s) of an organism. Autophagy is involved in various physiological roles, such as cellular homeostasis, embryonic development, antigen presentation, protein quality control, and maintenance of the amino-acid pool during starvation conditions. It is also implicated in various pathophysiological diseases, such as infection, cancer, diabetes, and neurodegeneration.

Although autophagy is predominantly a cytosolic event, the nucleus exerts a considerable control in the extent of autophagy response, especially during adverse conditions, such as starvation. Depending on the cargo it captures, autophagy is broadly classified as general and selective autophagy. For example, as a response to nutrient deprivation, general autophagy is triggered where it captures random portion of cytosol. In contrast, selective autophagy ensures specific capture of cytosolic cargo, such as damaged or superfluous organelles. When selective autophagy captures and degrades mitochondria, the process is termed as mitophagy. Similarly, autophagic degradation of peroxisomes (pexophagy), Golgi (golgiphagy), ER (ER-phagy), ribosomes (ribophagy), etc., have been documented.[1] The genes comprising the autophagy machinery are named as ATG (AuTophaGy related gene).[1]

## 2 Process of Autophagy

### 2.1 Autophagy Induction

The initial characterization of autophagy flux with respect to involvement of molecular players was carried out in yeast extensively. Although recycling of the cytoplasmic contents happens at

**Autophagosomes**: The "Pac-Man" like double membrane vesicles involved in macroautophagy.

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steady state levels by basal autophagy, autophagy flux increases drastically when it is induced. Autophagy induction happens when the cells are under stress conditions, such as amino acid starvation[1] (Fig. 1). Alternatively, autophagy can also be induced using drugs, such as rapamycin,[2] which targets the TOR (Target of Rapamycin), a major serine-threonine kinase involved in nutrient sensing and cell growth regulation.[3] Both amino-acid starvation and rapamycin inhibit TOR activity and induce autophagy. Under the nutrient rich conditions, TOR is active and it negatively regulates kinase activity of Atg1 by hyper-phosphorylating Atg13 and thus disturbing the Atg1–Atg13 association, required for downstream processes of autophagy.[4] When autophagy is induced either by nutrient limitation or by rapamycin, TOR becomes inactive and does not phosphorylate Atg13 and thus increases affinity of Atg13 towards Atg1, further passing the signal for nucleation of different autophagy proteins (Fig. 1).

**Phagophore Assembly Site (PAS):** The site inside cells that gives birth to autophagosomes.

### 2.2 Nucleation of Autophagy Proteins

When autophagy is induced, nucleation of autophagy proteins takes place at a site called the pre-autophagosomal structure or phagophore assembly site (PAS) which is present near the vacuole. The very first autophagy-related protein (ATG) that is recruited at PAS is Atg17. Atg17 and Atg11 act as scaffold in general autophagy and selective autophagy, respectively.[5] In general autophagy, Atg17 interacts with Atg31 which then interacts with Atg29 and thus forms a ternary complex. Atg17 also interacts with Atg13 and thus links the trimer to Atg1.[6–8] Recent study showed that Atg1 tethers Atg9 vesicles at PAS.[9] Atg9 is a transmembrane protein required for autophagy, and its transport from peripheral sources, such as mitochondria, ER, to PAS is believed to be important for providing a membrane source for the formation of autophagosomes.[10, 11] Atg23 and Atg27 are involved in anterograde transport of Atg9, wherein Atg9 vesicles are brought to PAS.[12] Retrograde transport of Atg9 from PAS to peripheral

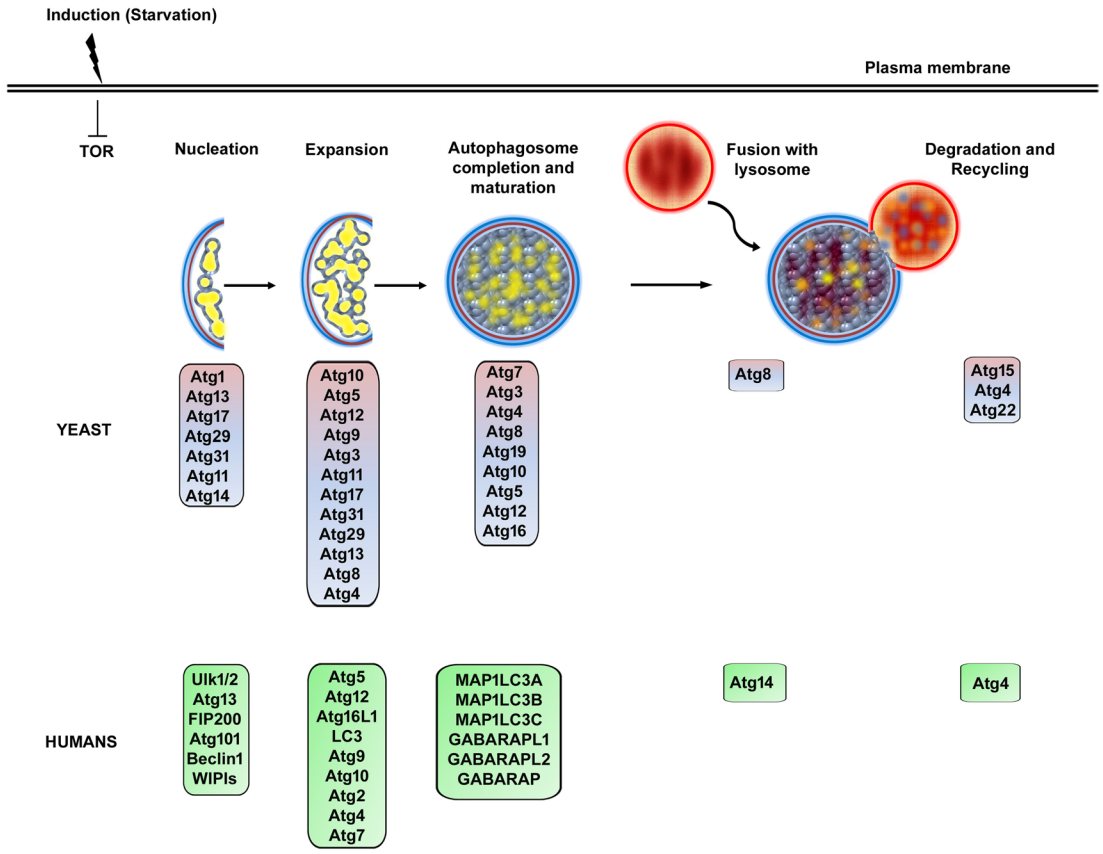


***Figure 1:*** Schematic demonstrating the various steps in the autophagy process. The yeast and human autophagy proteins involved in nucleation, expansion, autophagosome maturation and completion, fusion, and degradation processes are mentioned.

membrane sources require Atg1, Atg2, and Atg18.[13] Another complex important for PAS formation and initiation of autophagosomes is Class III PI3-K complex (VPS34, Atg6/VPS30, VPS15, and Atg14) which forms PI3P (Phosphatidylinositol-3-phosphate) that is present in the autophagosomal membranes.[14] Graef et al. in 2013 also have shown that the PAS containing multiple Atg proteins are tethered to ER exit sites. Localization of all these ATG proteins and the hierarchy of the complexes they form at the PAS have been determined. These orchestrated signaling events lead to a double membrane vesicle formation called an autophagosome [15] (Fig. 1).

### 2.3 Biogenesis, Maturation, and Completion of Autophagosomes

The initiation of the autophagosome biogenesis starts with formation of an isolation membrane at PAS. Atg8 is one of the important proteins that is present on the inner and outer membrane of the autophagosomes and it remains associated with the autophagosomes throughout the process of autophagy right from the formation of isolation membrane to the autophagosome degradation in the vacuole.[16] Atg8 is inserted in the autophagosomal membranes in the form of Atg8-PE (Phosphatidylethanolamine). Two ubiquitin-like conjugation systems help in the formation of Atg8-PE, the first being the Atg7–Atg3–Atg10 conjugation system and the second Atg5–Atg12–Atg16.[17] Atg4 is a cysteine protease that helps in conjugation of Atg8 with PE by cleaving the C-terminal Arg residue and exposes the Gly for conjugation. The recycling Atg8 from the Atg8-PE present at the outer membrane of the autophagosomes also requires Atg4 for the cleavage of PE from Atg8. Thus, Atg4 plays dual role of conjugation and recycling of Atg8.[18] As explained earlier, the membrane source for autophagosome formation is further contributed by transport of Atg9 vesicles along with Atg41.[19] Thus, Atg8, along with Atg4, Atg7–Atg3–Atg10 complex, and Atg5–Atg12–Atg16 help in autophagosome formation and maturation (Fig. 1). An important protein required for autophagosome completion is a PI3P phosphatase, Ymr1 in the absence of which recycling of the Atg proteins from the autophagosomal membrane is blocked and the Atg proteins remain associated with autophagosomes inside the cytoplasm.[20] Once the autophagosomes are completely formed, they are transported to the vacuole and are fused with the vacuole.

### 2.4 Fusion of Autophagosomes

As in the case of any vesicle destined to fuse with a membrane, autophagosomes also involve three major conditions for fusion with the vacuole—(1) interaction of Rab like GTPase, (2) tethering to the vacuole, and (3) SNARE-pair interactions leading to membrane fusion.

**SNAREs**: Proteins involved in fusion of cytoplasmic vesicles.

Ypt7, an yeast Rab GTPase, was shown to be involved in the homotypic vacuolar fusion along with Sec17 and Sec18.[21–23] Tethering of the vesicles is mediated by a complex called as the class C VPS complex or the Homotypic fusion and Vacuolar Protein Sorting complex also known as HOPS. HOPS consists of six subunits Vps18, Vps11, Vps16, Vps33, Vps39, and Vps41.[24–26] HOPS complex functions as an effector for Ypt7.[25]

**Tethering complexes-HOPS**: Tethering complexes-HOPS-Multi subunit protein complex that help anchoring autophagosomes and lysosomes.

A number of SNARE proteins also mediate the process of membrane fusion. Vam3 is a v-SNARE (also a syntaxin homologue) that localizes to the vacuolar membrane and has been shown to be important for both cytoplasm to vacuole delivery of Ape1 and for the fusion of autophagosomes to the vacuole.[27] Vam7 was later shown to be functioning together with Vam3 in vacuolar fusion.[28] Another v-SNARE Vti1 was reported to interact with Vam3 in both alkaline phosphatase pathway (Golgi-vacuole) and CVT pathway (one of the selective autophagy pathways). Along with these two other proteins which form a complex and function in the fusion step are Ccz1 and Mon1 which were identified in a screen of mutants defective in autophagy and CVT pathways.[29]

**Phagophore/isolation membrane**: The beginning structure that grows into an autophagosome.

**CVT pathway**: Cytoplasm-to-Vacuole pathway that delivers proteins from cytoplasm to the vacuole.

The fusion of outer membrane of the autophagosomes leads to the delivery of single membrane autophagic bodies into the vacuolar lumen which is then degraded.

**Autophagic bodies:** Single membrane vesicles inside yeast vacuoles as a result of autophagosome vacuole fusion.

### 2.5 Degradation of Autophagosomes and Its Contents

Takeshige et al. reported that yeast strain which was defective in vacuolar proteinases showed accumulation of autophagic bodies inside the vacuole.[2] Pep4 and Prb1 were the two mutants that accumulated autophagic bodies post starvation. Aut5/Cvt17 was identified to be an important component of the degradation machinery owing to its lipase activity.[30] Cvt17 was shown to be the lipase which degrades the membrane of the autophagic body in the vacuole.[31] Moreover, acidification of the yeast vacuoles was shown to be important for the degradation per se.[32]

### 2.6 Recycling of Degradation Products

One of the major roles of autophagy is to provide nutrients to the cell during nutrient limiting conditions. This requires not only degradation of part of cytoplasm but also effective recycling of the breakdown products to the cytoplasm. Aut4 which was later named as Atg22 was first identified to be involved in the degradation step as the mutants of Aut4 accumulated autophagic bodies in the vacuole.[33]

## 3 Autophagy in Higher Eukaryotes

The highly conserved nature of autophagy assisted in the identification of orthologs of yeast autophagy genes in mammals. As in yeast, autophagy in mammals is responsible for cellular homeostasis and quality control. Basal levels of autophagy in the cell remove misfolded proteins and damaged organelles. Induced autophagy, on the other hand, combats nutrient starvation, intracellular bacterial infection, oxidative stress, genomic damage, or accumulation of toxic protein aggregates (Fig. 2). The process of autophagy begins with the assimilation of tetrameric ULK1 complex comprising of ULK1, FIP200, Atg101, and Atg13 at the membrane nucleation site or 'Phagophore assembly site' (PAS). The ULK1 kinase activity is necessary for recruiting the Class III PI3-K complex I kinase, Vps34 along with regulatory subunits Beclin1, p150, Atg14L, and AMBRA1 at the PAS. The PI3P produced by Vps34 activity brings FVYE domain containing proteins, such as WIPI2 and DFCP1, to the nucleation site.[34, 35] Expansion of the phagophore is facilitated by Atg9 which brings membrane from various cellular organelles as well as the two conjugation systems; Atg5–Atg12–Atg16L and LC3.[36, 37] Ubiquitin like protein Atg12 is activated by E1 ligase Atg7, transferred to E2 ligase Atg10 and eventually conjugates with Atg5. The Atg5–Atg12 non-covalently binds to Atg16L and forms an Atg5–Atg12/Atg16L complex which is targeted to the PAS. The second conjugation system involves LC3, an ubiquitin like protein, which is generally present in the cytoplasm. It is cleaved by protease Atg4 to expose a C-terminal glycine which gets conjugated to phosphatidylethanolamine (PE) with the help of Atg7 and Atg3 which are E1 and E2 ligases, respectively. The PE conjugated LC3 binds to the inner and outer membranes of the expanding autophagosome.[38–40] The autophagosome cargo recognition and capture are facilitated by ubiquitin-binding adaptor proteins like p62/SQSTM1 which bind to polyubiquitinated cargo on one end and LC3 through the LC3 interacting region (LIR) on the other end.[41] Isolation membrane nucleation and elongation, cargo recognition and capture, and eventual closure result in the completion of double-membrane autophagosomes. Once completed, autophagosomes move along microtubules assisted by cytoskeletal motor proteins dynein and dynactin to fuse with lysosomes. The fusion of autophagosomes with lysosomes is mediated by small GTPases Rab7, autophagosomal SNARE Syntaxin17 (Stx17), lysosomal SNARE VAMP8, and tethering proteins of HOPS complex. Proper lysosomal function is important for autophagosome-lysosome fusion as autophagy inhibitors BafilomycinA1 and Chloroquine (CQ) inhibit fusion by affecting lysosomal pH. The end function of autophagic process is the degradation of cargo inside lysosomes by hydrolases like CathepsinB/D and recycling of biomolecules.[37, 42, 43]

**mTOR:** A protein that negatively controls autophagy.

## 4 Signaling Regulation of Autophagy

The highly conserved serine/threonine kinase mTOR (mammalian Target Of Rapamycin) senses nutrient signals in a cell and regulates its growth and division. Two complexes of mTOR, mTORC1, and mTORC2 are localized to different subcellular compartments. In the presence of amino acids and growth factors like Insulin-like growth factor (IGF), protein kinase B (PKB/Akt) is activated by phosphoinositide-dependent kinase-1 (PDK1). Akt phosphorylates TSC1 which blocks its interaction with TSC2, and hence, TSC1/2 complex is not formed which allows small GTPase Rheb to remain active. The mTORC1 complex is targeted to the lysosome by Ragulator-Rag complex where it is activated by Rheb and the active mTORC1, in turn, negatively regulates autophagy by inhibitory phosphorylation of ULK1 hence preventing ULK1 complex formation. During nutrient and metabolic stresses, the low levels of ATP in cells are sensed by AMPK which phosphorylates and activates TSC1/2 complex thereby inactivating Rheb and further mTORC1, hence allowing autophagy upregulation. AMPK also directly regulates autophagy independent of mTOR by phosphorylating and activating ULK1 independent of mTOR.[44, 45]

## 5 Transcriptional Regulation of Autophagy

Understanding the process of autophagy in an unabridged manner requires study of nuclear events that control autophagy along

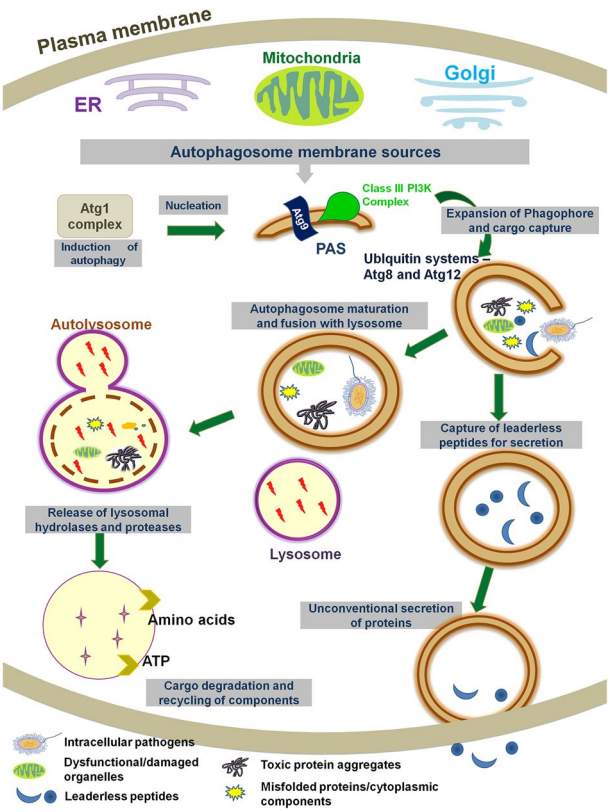


***Figure 2:*** Canonical and non-canonical autophagy flux: under basal levels, autophagy helps in maintaining the cellular homeostasis by getting rid of cellular waste and superfluous components. Stimulation through several factors, such as starvation, stress, or chemicals, leads to induction of autophagy. The initiation complex comprising of Atg1 complex and Class III PI3K complex along with several accessory proteins helps in nucleation at the site of autophagosome biogenesis also referred to as Pre-autophagosomal structure (PAS). Addition of membrane from several different sources leads to the expansion of autophagosomal membrane (phagophore). Atg9 along with accessory proteins is known to provide membrane to the developing phagophore from different sources, such as plasma membrane, endoplasmic reticulum, mitochondria, and Golgi. A ubiquitin ligase like system delivers Atg8 to the developing membrane and leads to the autophagosome expansion around the cargo and finally captures of the cargo. The cargo could be: (1) destined for degradation inside the lysosome through the canonical form of autophagy or; (2) could be secreted out of the cell through non-canonical function of autophagy referred to as unconventional protein secretion. (1) The cargo destined for degradation could comprise of cytoplasmic components like misfolded proteins, dysfunctional or damaged organelles or superfluous components under the basal levels of autophagy. However, autophagy also serves a cytoprotective role by getting rid of any intracellular pathogen or protein aggregates. The mature autophagosome along with its constituents fuses with the lysosome. Lysosomal enzymes act upon the cargo and degrade it into simpler building blocks like amino acids and ATP that are eventually pumped back into the cytosol to be reused by the cell. (2) Many newly synthesized or processed peptides could also be taken up by the autophagy machinery and delivered to the plasma membrane for secretion out of the cell. Such phenomenon of unconventional protein secretion through autophagy has been observed for several peptides that lack any conventional leader sequences for secretion.

with cytoplasmic process that unfold during autophagy. Nuclear regulation of autophagy is mediated by transcription factors, miRNAs, epigenetic marks, and histone modifications. These factors regulate both rapid and long-term responses to autophagy. More than about 20 transcription factors are now known to regulate autophagy.[46] Transcriptional regulation of autophagy can be via both mTOR-dependent and independent mechanisms. The first clue to the transcriptional regulation of autophagy came when in the yeast cells; Atg8 was found to be transcriptionally up-regulated via inactivation of the TOR signaling cascade.[16]

Studies by Settembre et al. gave new impetus to transcriptional regulation of autophagy. They

identified TFEB as the master positive regulator of autophagy. The two extensively studied major regulators of autophagy are TFEB and ZKSCAN3.[47, 48] TFEB is a basic-helix-loop-helix-leucine zipper transcription factor which is a master positive regulator of autophagy. It controls expression from nexus of genes involved in lysosome biogenesis (and function) and autophagy. It regulates the expression of genes that contain Coordinated Lysosomal Expression and Regulation (CLEAR) DNA sequences.[47] ZKSCAN3 is a zinc finger family protein that contains KRAB (KRuppel-Associated Box) and SCAN domains. Silencing of ZKSCAN3 shows induction in autophagy and lysosome biogenesis, while their presence down-regulates the expression of large array of genes involved in autophagy and lysosome biogenesis.[47, 48] TFEB and ZKSCAN3 play antagonistic role to each other in regulating expression of autophagy genes. Under nutrient rich conditions, mTORC1 in its active state phosphorylates TFEB on the lysosome membrane preventing it from entering the nucleus. This, in turn, prevents the activation of the genes harboring CLEAR DNA sequences. On the contrary, ZKSCAN3 has an antagonistic role. It is present in the nucleus where it down-regulates the expression of multitude of genes involved in autophagy and lysosome biogenesis. During starvation conditions, calcineurin dephosphorylates TFEB allowing it to enter the nucleus and positively regulate the expression of genes involved in autophagy and lysosome biogenesis. Concomitant to TFEB translocation to the nucleus, ZKSCAN3 is relocated to the cytoplasm releasing the negative control on the expression genes of autophagy and lysosome biogenesis.[49] Core autophagy genes transcriptionally regulated by TFEB are ATG4, ATG9, BCL2, LC3, SQSTM1, UVRAG, WIPI, and by ZKSCAN3 are ULK1 and WIPI, respectively.

Similarly there are other TFs, such as hypoxia inducing factor (HIF-1),[50] FOXO,[51] p53,[52] NF-κB,[53] and many others, that play a direct or indirect role in autophagy under different environmental stress conditions.

Transcriptional regulation of autophagy has also been addressed in the yeast model. Here, Ume6, Pho23, and Rph1/KDM4 are the three master transcriptional repressors of autophagy related genes in yeast.[54–56] Ume6 is associated with histone deacetylase complex which includes Sin3 and Rpd3, and negatively regulates the transcription of Atg8. Under nutrient replete conditions, the absence of any of these three components leads to an increase in Atg8, and consequently, autophagic activity is augmented. During autophagy, a protein kinase named Rim15 is responsible for phosphorylating Ume6, thereby dissociating it from Sin3 and Rpd3. The absence of Rim15 from cells leads to reduction in the synthesis of Atg8 at basal level. The authors have demonstrated Rim15 as a positive regulator of autophagy that acts upstream of Ume6 to regulate Atg8 synthesis.[54] Pho23 is another transcriptional repressor of autophagy that negatively regulates ATG9 and thus controls the frequency of autophagosome formation. It also down-regulates the expression of other autophagy-related genes, such as ATG7, ATG14, and ATG29. Studies show that deletion of PHO23 in yeast cells leads to an increase in the autophagosome formation and the number of autophagic bodies. This increase is possibly due to an increase in the levels of Atg9.[55] Rph1/KDM4 is a histone demethylase that negatively regulates the expression of ATG7, ATG8, ATG9, ATG14, and ATG29. It regulates autophagy in histone demethylase independent manner. In nutrient rich conditions, Rph1 keeps autophagy induction under check. However, under starvation, Rph1 phosphorylation by Rim15 causes partial degradation of this protein, thereby leading to induction of autophagy.[56] Thus, as in mammalian cells, yeast too has transcriptional machinery devoted to control expression of autophagy genes.

In many genetic and neurodegenerative diseases, autophagy becomes dysfunctional. Mechanisms that promote autophagy and mediate cellular clearance of toxic protein aggregates are being identified that serve as the novel therapeutic targets. For example, over expression of TFEB rescues cytoxicity of α-synuclein in rat model of Parkinson's disease[57] and also clears the polyQ Huntingtin protein.[58] Recently, HEP14 and HEP15 (small molecules) have been shown to increase biogenesis of lysosomes by activating TFEB. This increases the clearance of the cytotoxic aggregates from the cell and also increases the degradation of lipid droplets.[49] Thus, modulating the expression of TFs can help enhance autophagy which may be beneficial in alleviating disease conditions.

## 6 Autophagy in Disease

Dysfunctional autophagy is implicated in various diseases and disorders, such as cancer, intracellular infections, and neurodegeneration.

## 7 Cancer

The role of autophagy in maintaining cellular homeostasis is undeniably important and any perturbations in this can accumulate damaged organelles, oxidative stress, and misfolded proteins in a cell leading to genomic damage and even tumorigenesis. This concept was very elegantly proven in experiments with mice having deletion of essential autophagy genes like BECLIN, *ATG5*, and *ATG7* which made them prone to spontaneous tumors.[59] Beclin1 deletions were also identified in human breast, prostate, and ovarian cancer samples.[60] However, understanding the role of autophagy in cancer is not as simple as that. Autophagy can also provide survival advantage to tumor cells in a solid tumor which are facing nutrient limitation and hypoxia. Cancers, such as pancreatic and lung cancer, have been shown to have high basal levels of autophagy. On gene deletion of essential autophagy genes, tumor regression occurred in these cells. Hence, the role of autophagy in cancer is complex and requires an understanding of the stage and type of cancer. It definitely prevents the onset of tumorigenesis by limiting genomic damage but may be pro-cancer in established tumors.[61–63]

## 8 Xenophagy

Autophagy, apart from serving as a metabolic pathway providing building blocks like amino acids during conditions of nutritional stress, is also involved in degrading intracellular pathogens. The process of capturing and eliminating intracellular pathogens by autophagy is called as xenophagy. The process of xenophagy provides a broad spectrum of defense mechanism to capture bacterial, viral, and protozoan pathogens. Plethora of studies in recent times has shown that xenophagy acts as a part of innate immune system against huge number of intracellular pathogens in both phagocytic and non-phagocytic cells.

Although the conventional autophagy was discovered in 1963 by de Duve,[64] xenophagy remained unknown until electron micrographs of guinea pig polymorphonuclear leukocytes (PMNs) infected with *Rickettsiae* (Gram-negative pleomorphic bacteria) showed autophagosome like structures containing bacteria.[65] Following this, notable discoveries on xenophagy in Group A *Streptococcus*,[66] *Mycobacterium*,[67] *Salmonella*,[68] *Shigella*,[69] HIV,[70] Sindibis virus,[71] *Toxoplasma*[72] showed that xenophagy is a conventional defense mechanism of host against various pathogen types.

### 8.1 Pathogen Capture by Xenophagy

Post entry, some pathogens escape into cytosol to prevent fusion with lysosomes. This also provides them with sufficient nutrition from the cytosol to replicate efficiently.[73] These cytosolic pathogens are targeted by xenophagy machinery that captures them in double membrane vesicles (xenophagosomes) and delivers them to the lysosomes.[74]

Recognition of cargo for xenophagic capture occurs via ubiquitination of the pathogens which, in turn, is recognized by autophagy adaptor proteins like p62, NDP52, Optineurin, and NBR1. These adaptors bridge interactions with the ubiquitin and the autophagy machinery by interacting with LC3. This enables autophagosome formation around the pathogen.[75] Pathogen-specific adaptor proteins like septins (in case of *Shigella* and *Listeria*) and Tecpr1 (in case of *Shigella*) are also shown to recruit autophagy machinery to the pathogens.[76, 77]

*Salmonella enterica* serovar Typhimurium is a well-studied pathogen that gets restricted by xenophagy. Inside the host cells, *Salmonella* can reside either inside membrane bound endosomes or enter into cytosol by rupturing the endosomes. There are temporal changes in the intracellular *Salmonella* replicating niche in terms of morphology and recruitment of host factors. At later time points (6–8 h p.i), membrane bound endosomes develop into replicative vesicles for salmonella called as Salmonella Containing Vacuole (SCVs) which is characterized by its tubular structure. Adaptors like p62, NDP52, and optineurin recognize ubiquitin positive *Salmonella,* and NDP52 also recognizes galectin that are bound to damaged *Salmonella* containing endosomes. In a ubiquitin independent pathway, *Salmonella* gets captured to autophagosomes through diacylglycerol present on SCVs. Almost 25–30% of intracellular bacteria are shown to be captured by autophagosomes at early time points like 1 h post infection and the recruitment drastically falls at later points.[68] One of the speculated reasons for surpassing xenophagy is translocation of *Salmonella* virulence effectors, especially sseL which has deubiquitinase activity that could essentially prevent the ubiquitination of the pathogen. Another reason being repression of autophagy by *Salmonella* at later time points through mTOR activation.[78, 79]

The mechanism of subversion differs between pathogens. Another example is in the case of *Shigella flexneri* which causes shigellosis can escape from the phagosome/endosome and move within the host cells by directing actin polymerization

using its *vir*G gene. VirG is an outer membrane protein that accumulates on one end of the bacterium and mediates bacteria's polar movement. It is also known to be the target of autophagy machinery via interaction with Atg5. Recent studies have shown that an effector protein of *Shigella*, IcsB, acts as anti-Atg5-binding protein, by having a strong affinity for the same binding region on VirG as that of Atg5. Hence, mutants of icsB are captured by autophagosomes more rapidly.[69] Thus, although xenophagy exists, it is suppressed/subverted by most pathogens to evade detection and capture.

Impairment of xenophagy is also known to play role in the chronic infection of Crohn's disease. Genome Wide Association Studies (GWAS) have provided evidence for the contribution of two autophagy genes, ATG16L1,[80] and immunity-related GTPase M (IRGM) in the disease pathogenesis.[81] Subsequent studies show that single-nucleotide polymorphism occurring at ATG16L1 (T300A) does not impair the general autophagy process but show deficits in intracellular bacterial clearance.[82]

### 8.2 Signaling Pathways of Xenophagy

Recent studies have shed light on signaling pathways that lead to xenophagy activation even prior to ubiquitination of pathogens. Pattern recognition receptors are host proteins of immune system that recognize pathogen products initiating anti-microbial signals. These receptors could be either membrane bound (e.g., Toll-like receptors) or cytoplasmic (e.g., NOD-like receptors). Both are shown to play role in inducing xenophagy.[83, 84] IRGM is human gene shown to interact with NOD2 during infection, and together, they recruit Ulk1 and Beclin1 to initiate autophagy.[85] Similarly, membrane bound TLR4 has been shown to be involved in LPS-induced xenophagy. This activation also facilitates incorporation of VPS34 to autophagy vesicle formation.

Among other genetic factors that regulate xenophagy, TFEB, a mammalian transcription factor whose role is well studied in lysosomal biogenesis gets activated during *Staphylococcus aureus* infection in a pathogen-specific manner, while a similar effect is not seen in *E.coli* infection. In addition to lysosomes biogenesis, HLH30 (*Caenorhabditis elegans* homolog of TFEB) is also shown to induce number of autophagy genes, such as Atg2, Atg16, ULK1, among others. TFEB activation also seems to increase the tolerance to bacterial infection by prolonging the life span of infected *C.elegans* in comparison to autophagy mutants.[86]

In addition to the immediate innate response that xenophagy elicits, considerable research has been done to find its contribution to adaptive immunity in macrophages and antigen presenting cells. Atg5-deficient dentritic cells show reduced MHC class II representation of antimicrobial peptides and this, in turn, also affects the T-cell priming.[87] These cells also show reduced IL2 and interferon gamma production in response to viral infections.

These studies suggest that xenophagy is a conserved innate immunity pathway that pathogens evade to establish infection. Thus, enhancing xenophagy that rescind the block imposed by the pathogens would enhance the host immunity to fight against infectious agents. In this direction, screening for compounds that could enhance clearance of intracellular pathogens by xenophagy has been done for pathogens like *Toxoplasma* and *Mycobacterium*.[88, 89]

## 9 Aggrephagy

One of the hallmarks of life threatening neurodegenerative diseases is neuronal death caused by accumulation of misfolded toxic protein aggregates, such as α-synuclein, β-amyloid, huntingtin polyQ repeats, FUS, and TDP43. Cellular proteostasis involving the clearance of superfluous cellular organelles and other cargos, including toxic proteins, is maintained through the chaperones, the Ubiquitin–Proteasome System (UPS), and the autophagy pathways.[90] Chaperone and UPS functions are choked by the misfolded protein aggregates. Misfolded proteins are substrates for autophagy.[91] A selective autophagy pathway, aggrephagy, is a cellular degradation mechanism to clear the toxic, misfolded proteins. Recent studies highlight the importance of autophagy in maintaining organismal homeostasis. Brain-specific autophagy knockout mice (Atg5) accumulate p62 protein aggregates in neurons, and subsequently manifest neurodegenerative phenotypes, illustrating the vital role of basal autophagy for aggregate clearance.[92]

Autophagy is dysfunctional in neurodegenerative disease pathologies.[91] Thus, restoring autophagy through pharmacological approaches using small molecules has been reported to have beneficial neuroprotective effects.[93–95]

## 10 Non-canonical Roles of Autophagy

Besides the canonical role of cellular homeostasis and degradation, autophagy process also has some moonlighting functions which are underexplored. Involvement of autophagy machinery is seen in several contexts which do not involve capture and delivery of the cargo to the lysosome for degradation via a double membraned autophagosome. Such non-canonical autophagy processes include LC3-Associated Phagocytosis (LAP) and autophagy mediated unconventional protein secretion are two such examples. These non-canonical functions were explicitly put forth in a recent review by the pioneers in the field.[96] Some of the pleiotropic functions of autophagy include their role in cell survival and apoptosis, cellular transport, secretion, signaling, transcriptional and translational responses, membrane organization, and microbial pathogenesis.

The non-canonical roles can be looked upon from two diverse perspectives:

1. As macroautophagy involves formation of vesicles and membranous structures, these could be harnessed by other cellular and non-cellular processes.
2. Moonlighting functions of Atg proteins.

### 10.1 Harnessing Autophagy Machinery for Other Cellular Processes

The prime role of autophagy is turnover and is accompanied by the process of dynamic membrane biogenesis.[97, 98] The double layered autophagosome membrane formation to entrap cargoes is an orchestrated, dynamic process with the involvement of several Atg proteins and requires PI3-K activity. This property has been elegantly exploited by the pathogens that infect mammalian cells. Virus and bacteria have evolved mechanisms not only to evade the degradative action of autophagy but also to hijack the host autophagy machinery for their multiplication. In this section, we will focus only on the non-canonical role of autophagy proteins in microbial pathogenesis. LC3 in mammals mediates the recruitment of the substrates onto the autophagosomes via their LC3-interacting regions (LIR). Some of the examples that utilize the Atg proteins besides their degradative functions are discussed below:

1. Influenza A virus redirects LC3-conjugated membranes meant for autophagy to the cell surface for budding of stable viruses.[99] The ion-channel matrix protein of the virus (M2) recruits the central player of autophagosomal membrane or the landing pad of cargo receptor, LC3, inhibiting the fusion to lysosomes, thereby aiding in the transport of virions to the plasma membrane.[100]
2. In *Mycobacterium tuberculosis* infection, Atg5 is found to play a unique role of protection by preventing PMN-mediated immunopathology. Knockout studies support an additional, ATG16L1 independent role of ATG5 in protecting the mice from *M. tuberculosis* infection.[101]
3. Another study from an unbiased siRNA screen has indicated the involvement of ATG13 and FIP200 in the picornavirus replication that is independent of their canonical autophagy functions.[102] The host and the viruses exploit the autophagy machinery along with the autophagy-related membranous structures to either restrict or enhance viral replication that is non-canonical of the autophagy functions. Autophagy proteins, including Beclin1, LC3, Atg4B, Atg5, Atg7, and Atg12, positively regulate the Hepatitis C viral replication,[103] whereas in murine norovirus, some of the autophagy proteins are required by the IFN-γ activated macrophages to inhibit viral replication complex.[104] Non-involvement of ULK complex distinguishes the non-canonical from canonical autophagy.[105] There is a general notion that a single ATG gene deletion leads to specific block in the autophagy process, but the above-mentioned examples provide evidence that the Atg proteins also exhibit many of the non-canonical roles during viral infection.[106]
4. In Mouse Hepatitis Virus (MHV) infection, as unlipidated LC3 (LC3-I) promotes viral replication in Double-Membrane Vesicles (DMVs) without utilizing ATG5[107] and LC3-II,[108] it suggests that the canonical autophagy is not involved. Detailed analysis of the vesicles indicates that the DMVs are another LC3-presenting membrane that is distinct from the canonical double membrane autophagosomes.
5. Zikavirus, a member of the Flaviviridae family, causes microcephaly affecting the central nervous system.[109] This virus produces a variety of intracytoplasmic inclusions termed as "virus factories" in the infected cells. The zika virus infected skin fibroblasts demonstrate that the virus not only blocks the autophagic flux but also hijacks the

**Virions:** Virus particles.

**Non-canonical autophagy:** Moonlighting functions of autophagy such as those involved in protein secretion.

**Macroautophagy:** An intracellular mechanism to capture, degrade and recycle unwanted, damaged or surplus cytoplasmic materials. Commonly referred as autophagy.

autophagic machinery for its own replication.[110, 111]

In all the above examples, we see that the ability to form membrane structures of the autophagy proteins is being exploited by the virions to promote their viral budding and replication, thereby aiding in their survival and infection.

### 10.2 Moonlighting Functions of Atg proteins

(i) Role in Unconventional Protein Secretion

Beyond its role of cellular self-eating and homeostasis, autophagy proteins also play an important role in unconventional protein secretion whose mechanism is not well elucidated.

The conventional secretory proteins enter endoplasmic reticulum via signal peptides, whereas the unconventional secretory proteins destined for secretion follow an alternate trafficking route. The process by which proteins that are devoid of canonical leader sequence still get secreted is termed as unconventional protein secretion.

Extensive studies of two main cargoes studied till this date have provided us clues on autophagy-mediated unconventional protein secretion.

1. First, the secretion of mature cytokine, IL1-β, is found to be controlled by the process of autophagy.[112] Its secretion is presumed to involve Rab proteins and MVBs.[113] The matured form of the IL1-β is released outside the cell after cleavage from its precursor form. Although Caspase-1 mediated IL1-β release is reported, elegant studies by Zhang et al, 2015 have demonstrated that the translocation of the unconventional secretory protein, IL1-β into a secretory vesicle, is mediated by autophagy, multivesicular bodies (MVBs), and Golgi-associated proteins (Golgi Reassembly Stacking Protein-GRASPs).
2. The second cargo is the Acyl-CoA-binding protein (Acb1) that gets secreted outside the cell by unconventional protein secretion upon starvation in yeast. Genetic studies in yeast[114] have demonstrated that Acb1 is unconventionally secreted via vesicles and are captured in a new compartment called CUPS (Compartment for Unconventional Protein Secretion).[115] These studies in yeast have revealed that the core autophagy machinery is a necessary requisite for autophagosome construction, suggesting that secretory autophagosomes must be formed. This secretion is found to be GRASP-dependent and autophagy-mediated, and plays an important role in peroxisome biogenesis providing some clues on membrane source for autophagosome biogenesis.[116]

Multiple lines of evidence demonstrate the interplay of autophagy and unconventional protein secretion in the clinical and pathophysiological context.

1. The GRASP-dependent unconventional secretion of CFTR, the Cystic Fibrosis Transmembrane conductance Regulator, demonstrates a physiological relevance of unconventional protein secretion in the cystic fibrosis disease. Autophagy-mediated trafficking of CFTR leads to proper insertion of the protein to the plasma membrane, whereas the transgenic overexpression of GRASP rescued the phenotype of the ΔF508-CFTR mice.[117]
2. Autophagy plays a significant role in polarized secretion of lysosomal contents in osteoclastic bone resorption.[118]
3. Impairment of autophagosome–lysosome fusion promotes tubulin polymerization-promoting protein (TPPP/p25α) to secrete α-synuclein, the hallmark protein of Parkinson's disease, in an unconventional manner.[119]
4. Another unconventionally secreted protein, Insulin Degrading Enzyme (IDE), was found to be mediated through autophagy-based unconventional secretion upon statin induction[120] and also has disease relevance in Alzheimer's disease.[121]
5. Secretion of β-amyloid aggregates formed in the Alzheimer's disease is also mediated by autophagy. Knockout studies in mice neuronal Atg7 was found to influence the β-amyloid secretion thereby affecting the plaque formation, a pathological hallmark of AD.[122]
6. Atg16L1 not only regulates cellular autophagy but also acts as Rab33A effector by secreting the hormone from the dense core vesicles of the neuroendocrine PC12 cells.[123] Another example of the combined role of Atg5, Atg7, Atg4B, and LC3 is observed in the polarized secretion of lysosomal contents (cathepsin) in the osteo-

clasts.[118] Defects in Atg4B and Atg5 in mice are found to manifest balance related disorders due to deficient secretion of otoconins by vestibular sensory cells in the inner ear.[124, 125]

(ii) Role in cell division:

The non-canonical role of autophagy proteins has gained significance, especially in microbial pathogenesis. The functional importance of localization of *Pf*Atg8 to apicoplast, a four membrane-bound non-photosynthetic plastid, provides clue for non-canonical function of autophagy in *Plasmodium falciparum.*[126] In the apicomplexan parasite *Toxoplasma gondii*, *Tg*ATG8 is vital for normal replication of the parasite inside the host cell. Recent studies have demonstrated that another key role of apicoplasts bound *Tg*ATG8 is involved in centrosome-driven inheritance of the organelle during cell division.[127]

In the Zika virus infected patients, microcephaly is brought about by the abnormal function of centrosomes affecting neural brain development.[128, 129] As this process is coupled with hijacked autophagy machinery, it is presumed that autophagy proteins are probably involved in cell division too.

(iii) Role in inflammatory disease control:

The LC3-Associated Phagocytosis (LAP) is one of the prime non-canonical functions of autophagy that is required for effective clearance of apoptotic cells.[130] In canonical autophagy, LC3 conjugates to the autophagosomal membranes facilitating maturation upon fusion with lysosomes. Rubicon, a Beclin-1-binding protein, is found to be required for LAP but not for canonical autophagy.[131] In Systemic Lupus Erythematosus (SLE), the pathogenesis is brought about by the defects in clearance of dying cells. LAP is found to inhibit autoinflammatory responses caused by dying cells implicating its link in inflammatory disease control of SLE.[105] Even in viral RNA-mediated infection, the immunostimulatory RNA (isRNA)-mediated type I interferon production is negatively regulated by the Atg12–Atg5 conjugate[132, 133] demonstrating its suppressor activity in the innate antiviral immune signaling aiding cell survival.

Studies reveal interplay between inflammasomes (multiprotein complex that activates caspase-1) and autophagy. While autophagy negatively regulates inflammasome activation, autophagy induction is dependent on the presence of specific inflammasome sensors. Autophagosomes degrade inflammasomes via the selective autophagic receptor p62 and autophagy plays a role in the biogenesis and secretion of the proinflammatory cytokine IL-1β.[134–138]

The involvement of the adaptor protein, ATG16L1, in the inflammatory bowel disease (Crohn's disease) is characterized by dramatic increase in commensal bacteria.[139] Deletion studies in ATG16L1-WD repeat domain and T300A mutant of mouse embryonic fibroblasts did not affect xenophagy or the normal autophagic function indicating its differential role in Crohn's disease.[140]

(iv) Role in lipidogenesis and development:

Lipid droplet formation in mammalian white adipocytes involves massive cytoplasmic remodeling within the cells. Besides the conventional roles in autophagy, several autophagy genes have been implicated to have "non-autophagy roles". For example, Atg2 and LC3 are also involved in lipid droplet biogenesis in mouse hepatocytes and cardiac myocytes,[141, 142] while knockout studies in mice for Atg5 and Atg7 have revealed their additional roles in adipogenesis.[143, 144] The mice fed with high fat diet in the Atg12 lacking pro-opiomelanocortin expressing neurons exhibited aggravated obesity which demonstrates an auxiliary function of Atg12 in diet-induced obesity.[145] In addition, Atg5-independent non-canonical autophagy generates autophagosomes in a Rab9-dependent manner. Such Atg5-independent autophagy is found to be required for iPSC reprogramming that mediates mitochondrial clearance.[146]

The versatility of the autophagy proteins in all the cellular processes opens new avenues to explore its moonlighting functions. It is imperative to understand the discrete functions of the autophagy proteins besides their central role in degradation and cellular homeostasis.

### *10.3 Open Questions in Autophagy*

Although the field has garnered much interest now with the award of the Nobel Prize to Prof. Yoshinori Ohsumi for his contributions to understanding the mechanism of autophagy, several autophagy-related frontiers remain unchallenged. Questions pertaining to understanding basal autophagy and the mechanisms that regulate it are still open. How various intracellular membrane sources contribute to autophagosome biogenesis and the factors that

govern autophagosome size and number is still an active area of research. In spite of identification of a conserved set of core autophagy proteins, their actual roles in autophagosome construction and mechanisms regulating autophagosome-lysosome fusion are not clear. The contribution of autophagy in cell death is controversial and the case of "cell death by over eating oneself" is highly debatable.[147, 148] Finally, restoration of impaired autophagy in several disease states via small molecule autophagy modulators has been shown to be promising in many cases, but bonafide and exclusive modulators are still elusive. Discovery of such small molecules will not only further our understanding of autophagy flux but will also fuel the tremendous therapeutic potential autophagy holds.

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