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At the Center of Autophagy: Autophagosomes

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Glossary

ATG proteins The AuTophaGy-related (ATG) proteins are the key players regulating and mediating macroautophagy. On a note, there are also genes essential for the progression of autophagy that are not called *ATG*.

Autophagosomes They are vesicles with a double lipid bilayer, which are formed during macroautophagy. Autophagosomes sequester and deliver cytoplasmic material into the lysosomes for degradation.

Autophagy An evolutionary conserved lysosomal degradative pathway. The term encompasses 3 processes: macroautophagy, microautophagy, and chaperone-mediated autophagy. Basal autophagy constantly recycles the cytoplasmic components and thereby maintains the cellular homeostasis. When cells are exposed to stress, the enhancement of autophagy contributes to overcome them.

Autophagy receptors Autophagic receptors are a set of proteins that simultaneously bind specifically a structure targeted by autophagy and ATG proteins found in the interior of autophagosomes, mainly LC3. This property provide the substrate specificity to the selective types of autophagy.

PAS The Phagophore Assembly Site or Pre-Autophagosomal Structure is the nucleation site where the ATG proteins assemble to first generate a phagophore and subsequently expand it into an autophagosome. In yeast there is one PAS per cell whereas in mammalian cells there are several per cell.

Phagophore (also known as isolation membrane) The phagophore is a membrane cistern that is the precursor structure of the autophagosomes. It is formed at the PAS by the orchestrated actions of the ATG proteins, which also elongate it into an autophagosome.

Selective autophagy This term describes forms of macroautophagy in which a specific structure (i.e. an aggregate, organelle, or microbe) is specifically and exclusively sequestered by autophagosomes.

Macroautophagy and Autophagosomes

Macroautophagy can be distinct from the two other forms of autophagy, namely, chaperon-mediated autophagy and microautophagy, because it involved the formation of autophagosomes (Mizushima *et al.*, 2008; Kraft and Martens, 2012). Macroautophagy (hereafter autophagy) is an evolutionary conserved cellular pathway that is active at basal levels in every cell. Thus, cytoplasmic material including unfolded or obsolete proteins, and damaged organelles are continuously degraded into lysosomes even if the flux of autophagy can vary from tissue to tissue. The constant turnover of the cytoplasmic components by autophagy is crucial in maintaining cellular homeostasis and also provides metabolites such as amino acids, sugars, nucleotides, and lipids to generate new macromolecules (Kraft and Martens, 2012; Levine and Kroemer, 2008; **Figure 1**). Autophagy can be further stimulated when a cell is exposed to cellular stresses such as nutrient starvation and helps the cell to overcome them (Shintani and Klionsky, 2004). The physiological relevance of this type of responses was initially revealed by a study showing that mice unable to undergo autophagy are unable to survive the postnatal starvation period and die shortly after birth (Kuma *et al.*, 2004). In addition to stresses, specific developmental programs, immune responses and numerous other signals can also induce autophagy (Levine *et al.*, 2011; Choi *et al.*, 2013; Mizushima and Levine, 2010).

Together with the proteasome, autophagy is one of the two major protein degradation pathways of the cell. However, while the proteasome is mainly devoted to the turnover of short-lived proteins, autophagy degrades long-lived proteins but also numerous other cell components including sugar chains, nucleotides and lipids. While in specific situations autophagosomes randomly sequester cytoplasmic material, often their cargo is a specific structure (Wong and Cuervo, 2010). Selective types of autophagy include the turnover of organelles (e.g., mitochondria, peroxisomes, ER, lysosomes, lipid droplets…), protein aggregates, large complexes (ribosomes, mid-bodies, inflammasome…) and invading pathogens (Choi *et al.*, 2013). These forms of autophagy rely on a series of the so-called autophagy receptors, which recognize the targeted structure and mediate the subsequent recruitment and assembly of the ATG machinery (Boyle and Randow, 2013; Isakson *et al.*, 2013). This results in the formation of the autophagosome around the selected material providing the specificity to this process (Shintani and Klionsky, 2004; **Figure 2**). Because of this ability to specifically eliminate unwanted structures, autophagy plays a crucial function in a multitude of physiological processes in eukaryotic organisms. This pathway is thus part of our innate immune response through its ability to target invading pathogens for degradation (Levine *et al.*, 2011). Moreover, the turnover of protein aggregates prevents an early onset of neurodegenerative disorders like Huntington's or Alzheimer's disease (Choi *et al.*, 2013). Autophagy also plays major roles in development and cell differentiation in multicellular organisms and contributes to their longevity (Yoshimori, 2007; Melendez *et al.*, 2003). As a result, an impairment or defect in autophagy causes a variety of human illnesses, including neurodegenerations, cancer, inflammatory diseases, and muscular dystrophies (Levine and Kroemer, 2008; Choi *et al.*, 2013).

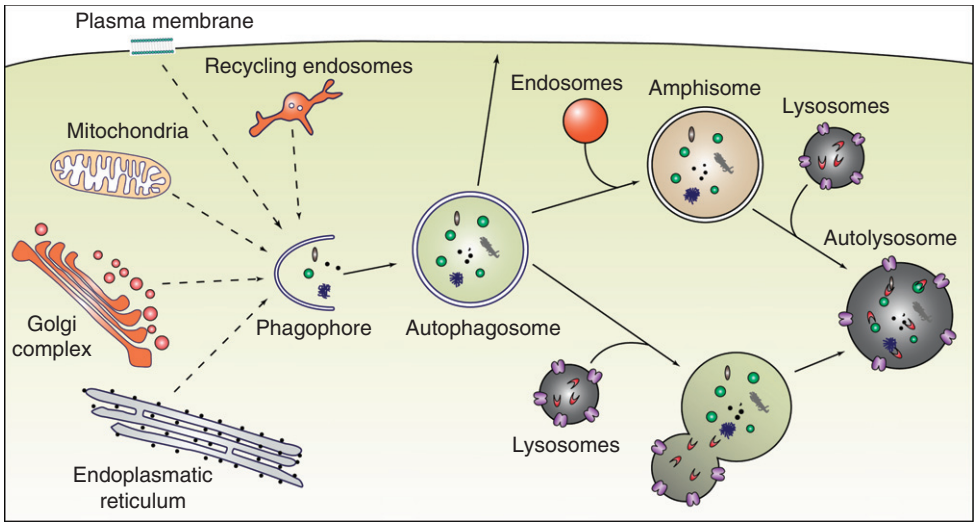

Figure 1 The mechanism of autophagy. Autophagy starts with the formation of a membrane cistern called the phagophore, where all the ATG proteins localize to. Often the initial phagophore is formed in close proximity to the ER. In addition to this organelle, other cellular compartments such as mitochondria, the plasma membrane, the Golgi complex and recycling endosomes have been postulated to contribute membranes to form the phagophore and then expand it into an autophagosome. During the formation of autophagosomes, the cytoplasmic material is sequestered within the forming vesicle. Complete autophagosomes fuse either directly with lysosomes to form autolysosomes or with endosomes to generate an organelle called amphisome. This latter eventually fuse with lysosomes too. In the lysosome lumen, resident hydrolases degrade the autophagosomal cargo and the resulting metabolites are transported back in to the cytoplasm by lysosomal permeases. The autophagosome can also fuse with the plasma membrane and thereby being one of the mechanisms for unconventional secretion.

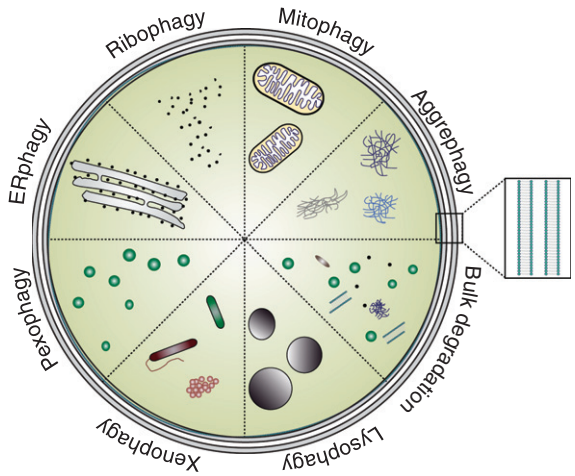

Figure 2 The different types of selective autophagy. In addition to the unspecific sequestration of cytoplasmic material (i.e., bulk degradation), autophagosomes can also specifically target a variety of cellular components. Depending on the conditions, autophagosomes can selectively and exclusively engulf mitochondria (i.e., mitophagy), ribosomes (i.e., ribophagy), peroxisomes (i.e., pexophagy), lysosomes (i.e., lysophagy), parts of the ER (i.e., ERphagy), protein aggregates (i.e., aggrephagy), pathogens (i.e., xenophagy), or lipid droplets (i.e., lipophagy; not shown here), and transport these cell components to the lysosomes for degradation.

General Characteristics of Autophagosomes

Autophagosomes were first described by the Nobel laureate Christian de Duve in 1966 (De Duve and Wattiaux, 1966). One of the peculiarities of these carriers in comparison to other transport vesicles is their double lipid bilayer, which has also a low content in transmembrane proteins. Therefore, autophagosomes appear as very smooth vesicles when analyzed by freeze-fracture electron microscopy (Fengsrud *et al.*, 2000). Their size can vary from 500 nm up to 1.5 μm and this flexibility allow them to sequester cargoes of different sizes (Fengsrud *et al.*, 2000; Klionsky *et al.*, 2012; Mizushima *et al.*, 2002). In contrast to other transport vesicles, an autophagosome is formed *de novo*, that is, not through budding from a pre-existing organelle, and it sequesters cytoplasmic cargo material during its completion. This different solution adopted by the cell to generate a vesicle is probably due to the topology of the cargo, which is cytoplasmic, and therefore cannot be engulfed by a single lipid bilayer with exposed extremities, something thermodynamically highly unfavorable (Reggiori and Klionsky, 2013).

The autophagic vesicles are categorized based on their morphology into early autophagosomes and late autophagosomes or autolysosomes. In early autophagosomes the sequestered cargo is morphological unchanged and whole organelles or part of organelles are found in the autophagosomal lumen. Late autophagosomes or autolysosomes are fused with lysosomes and the sequestered cargo is already being degraded (Yla-Anttila *et al.*, 2009b).

Generation of the Autophagosomes: The Machinery

The initial characterization of the molecular machinery mediating the biogenesis of autophagosomes has mostly been done in the yeast *Saccharomyces cerevisiae*. In particular the genes mediating the process of autophagy were identified through genetic screens in this organism and they are currently

called the AuTophaGy-related (ATG) genes. This discovery was the breakthrough in the autophagy research because the high degree of conservation of numerous ATG genes allowed identifying their counterparts in high eukaryotes. To date 36 ATG genes have been characterized in yeast and many of them have human orthologs (Klionsky *et al.*, 2003; Yang and Klionsky, 2010). In high eukaryotes there are additional genes essential for autophagy that are not called ATG. It is now established that the generation of autophagosomes is regulated by a hierarchical interplay of the ATG proteins and it is divided in 3 discrete steps: (1) initiation, (2) nucleation, and (3) elongation and closure.

Initiation of autophagy is mediated by the ULK/ATG1 complex. The mammalian ULK kinase complex is composed of ULK1/2 (unc-51-like kinase), FIP200 (focal adhesion kinase family interacting protein of 200 kD), ATG13, and ATG101. The ULK/ATG1 complex is differentially regulated by TORC1 (mammalian target of rapamycin complex 1) and AMPK (AMP-activated protein kinase), and possibly by other signaling cascades. In particular, active TORC1 inhibits the ULK/ATG1 complex whereas active AMPK stimulates the ULK/ATG1 complex (He and Klionsky, 2009; Yang and Klionsky, 2010). TORC1, with its central serine/threonine kinase mTOR (mammalian target of rapamycin), acts as a nutrient sensor in the cell and is activated in presence of nutrients. Under these conditions, through phosphorylation, TORC1 inhibits both AMPK and the ULK/ATG1 complex, to which it is bound. In nutrient-deprived cells, in contrast, AMPK is activated and phosphorylates TORC1 leading to its inhibition and dissociation from the ULK/ATG1 complex. In addition, AMPK phosphorylates the ULK/ATG1 complex and thereby activates it. The activation by AMPK and simultaneously release of inhibition by the TORC1 stimulates the ULK/ATG1 complex kinase activity initiating the autophagic process (He and Klionsky, 2009; Yang and Klionsky, 2010).

After the initiation step, the ATG proteins assemble at cellular structures that has been called the phagophore assembly site or pre-autophagosomal structure (PAS). In mammalian cells numerous early autophagosomal structures can be observed upon autophagy induction whereas only one PAS is generated in yeast. The assembly of the ATG proteins, called nucleation, requires the local generation of the phosphoinositol-3-phosphate (PtdIns3P). PdtIns3P is generated by the PtdIns3 kinase class 3 (PtdIns3KC3), also called hVPS34. This protein is in complex with the regulatory factors BECLIN1/ATG6 and p150 (human VPS15), which together compose the PtdIns3KC3 complex (Simonsen and Tooze, 2009). The insertion of BECLIN1 into the PtdIns3KC3 complex is negatively regulated by BCL-2 protein family members and positively stimulated by the ULK complex (Russell *et al.*, 2013; Pattingre *et al.*, 2005). The PtdIns3KC3 complex fulfils several functions in autophagy and in endosomal vesicle transport pathways depending on which proteins are associated with the complex. Binding of ATG14L (or Barkor) and AMBRA1 (activating molecule in BECLIN1-regulated autophagy) to the PtdIns3KC3 complex recruits the complex to the PAS stimulating autophagosome formation (Kang *et al.*, 2011). When this complex is associated with UVRAG (ultraviolet irradiation resistant-associated gene), which is in turn bind to BIF-1 (Bax-interacting factor 1), it is engaged in autophagosome maturation as well as endocytific trafficking (Kang *et al.*, 2011). However, when UVRAG is interacting with RUBICON (RUN domain and cysteine-rich domain containing, BECLIN1-interacting protein), autophagosome maturation is blocked (He and Klionsky, 2009; Kang *et al.*, 2011).

While the precise role of PtdIns3P in autophagy is unclear, it is known that the phagophore is formed at PtdIns3P rich regions mostly adjutant to the ER. It is also clear that this lipid mediates the recruitment of PtdIns3P-binding proteins (also called PtdIns3P effector proteins) that are necessary for autophagosome biogenesis to the PAS (Mari *et al.*, 2011; Isakson *et al.*, 2013; Codogno *et al.*, 2012). Several of these PdtIns3P effector proteins are known that are involved in autophagosomal formation, including ALFY, DFCP1, and WIPI protein family members (homologs to the yeast ATG18). It was shown in yeast that ATG18 localization to the PAS depends on its interaction with ATG2 and the presence of PtdIns3P (Obara *et al.*, 2008; Rieter *et al.*, 2013). The ATG18-ATG2 complex interacts with ATG9, the only transmembrane ATG protein, at the PAS (Reggiori *et al.*, 2004). ATG9 thereby at least partially contributes membranes to the forming phagophore even if its interaction with the forming autophagosomes is very dynamic in both yeast and mammalian cells (Mari *et al.*, 2011; Isakson *et al.*, 2013; Codogno *et al.*, 2012; Chan and Tang, 2013; Orsi *et al.*, 2012; Yamamoto *et al.*, 2012).

The elongation and closure of the phagophore (also called isolation membrane), the third step in autophagosome biogenesis, is mainly executed by two ubiquitin-like conjugation systems, which have the ultimate result of forming the ATG16L1 complex and of lipidating the members of the ATG8/LC3 (microtubule associated protein 1 light chain 3) protein family (Shpilka *et al.*, 2012; Yang and Klionsky, 2010). The formation of the ATG16L1 complex as well as the LC3 lipidation are essential for the autophagosome biogenesis. The ATG16L1 complex contains the ATG12-ATG5 conjugate. The formation of this complex is mediated by the E1-like enzyme ATG7, which activates ATG12 and transfers it to ATG10, an E2-like enzyme, which finally covalently binds ATG12 to ATG5. The ATG12-ATG5 conjugate interact with ATG16L1 to form a multimeric complex, the ATG16L1 complex, which localizes to the phagophore and early autophagosome intermediates but dissociates from complete autophagosomes (Ohsumi and Mizushima, 2004; Yang and Klionsky, 2010).

In the second conjugation system, LC3 becomes linked to phosphatidylethanolamine (PE). Initially a preform of LC3 is posttranslationally cleaved at the C-terminus by the ATG4 proteases. The resulting cytoplasmic form of LC3, also called LC3-I, gets also activated by the E1-like enzyme ATG7 and transferred onto ATG3, another E2-like enzyme before being conjugated to PE. The ATG16L1 complex promotes this latter step by specifically bringing ATG8-ATG3 in proximity of the PE pool present in the autophagosomal membranes. The LC3-PE conjugate, also known as LC3-II, is present in the inner as well as at the outer surface of the expanding autophagosomal membrane. Prior to the autophagosome fusion with lysosomes, the LC3-II pool on the outer autophagosomal membrane is released from PE by ATG4 for reuse (Kabeya *et al.*, 2000; Yang and Klionsky, 2010; He and Klionsky, 2009). The fact that a subpopulation of LC3 is present in the interior

of autophagosomes makes this protein the ideal marker to monitor the generation of these carriers and their subsequent fusion with lysosomes (Kabeya *et al.*, 2000). In particular LC3 subcellular distribution can be analyzed by fluorescence microscopy and the redistribution of this protein from the cytoplasm into punctuate structures, for example, the autophagosomes, is an indicator of autophagy induction. Furthermore it allows assessing autophagy induction and progression by analyzing the LC3-II protein levels by Western blot (Klionsky *et al.*, 2012; Mizushima, 2004; Rubinsztein *et al.*, 2009).

Generation of the Autophagosomes: The Membranes

While enormous progresses have been done regarding the characterization of the ATG machinery, the origin of the autophagosomal membranes is still not clear. Membranes from multiple sources have been shown to contribute to the formation and maturation of autophagosomes; therefore, this topic is the subject of intense debate and research. Amongst these there are several organelles, including the plasma membrane (Ravikumar *et al.*, 2010), early endosomes (Razi *et al.*, 2009), the mitochondria (Hailey *et al.*, 2010), recycling endosomes (Longatti and Tooze, 2012; Puri *et al.*, 2013), the Golgi complex (Mari *et al.*, 2011; Orsi *et al.*, 2010) and the ER (Axe *et al.*, 2008; Hayashi-Nishino *et al.*, 2009; Yla-Anttila *et al.*, 2009a; **Figure 1**). In fact, it was shown that most of the autophagosomes form in close association with the ER, in a structure called the omegasome (Axe *et al.*, 2008; Hayashi-Nishino *et al.*, 2009; Yla-Anttila *et al.*, 2009a). The omegasome is a PtdIns3P rich subdomain of the ER that is formed upon starvation. It is a dynamic structure where ATG proteins required for the nucleation step assemble and which gives rise to phagophores and then autophagosomes (Axe *et al.*, 2008; Hayashi-Nishino *et al.*, 2009; Yla-Anttila *et al.*, 2009a). The autophagosomes, however, do not bud or form directly from the ER but rather originate in close proximity of this organelle, possibly in regions that most likely are in close contact with mitochondrial membranes, that is, the so-called mitochondria-associated membranes (Hamasaki *et al.*, 2013).

Maturation and Consumption of Autophagosomes

Once complete, autophagosomes fuse either directly with lysosomes or with endosomes to form an intermediate organelle called amphisome. Amphisomes ultimately fuse with lysosomes too. These events lead to progressive maturation of autophagosomes into a degradative compartment, the autolysosome, through the acquisition of low pH and lysosomal hydrolases, which access the autophagosomal cargo and mediate its turnover (He and Klionsky, 2009; **Figure 1**). The precise mechanism of how the autophagosome fuses with the lysosomes has not yet been fully resolved. However, members of both the Rab GTPase and the SNARE (soluble N-ethylmaleimide-sensitive-factor attachment protein receptor) protein families as well as a low intralysosomal pH have been shown to be involved in fusion (Bento *et al.*, 2013; Moreau *et al.*, 2013; Yamamoto *et al.*, 1998). During the autophagosome fusion events, only the outer lipid bilayer of these double-membrane carriers fuses with the lysosomal limiting membrane and as a result the inner autophagosomal vesicle is also degraded within the autolysosome. The resulting material is finally transported by lysosomal membrane permeases out of the autolysosome lumens into the cytoplasm, where it can be metabolized by the cell (He and Klionsky, 2009; **Figure 1**).

For more efficient fusion between them, autophagosomes and lysosomes have to come into close proximity. This is accomplished on the one hand through the active transport of the autophagosomes via microtubules from the cell periphery to the microtubule organization center (MTOC) (Bento *et al.*, 2013). On the other hand, lysosomal positioning within the cell is regulated in a nutrient-dependent manner by mTOR. That is, under growing conditions lysosomes bind to a kinesin complex that mediated their positioning at the cell periphery. Under starvation, an increase in the intracellular pH provokes an inhibition of the recruitment of the kinesin complex to the lysosomal membrane and thereby allowing lysosomes to move to the MTOC (Bento *et al.*, 2013).

Autophagosomes not only fuse with compartments of the endo-lysosomal system. Recent findings support the notion that these carriers could also fuse with the plasma membrane (Deretic *et al.*, 2012). This nondegradative function of autophagy plays a role in the unconventional secretion (i.e., secretion that does not involve transport through Golgi apparatus) of a subset of signal molecules (Deretic *et al.*, 2012). Diverting of autophagosomes from their conventional trajectory to the endo-lysosomal system is also something implemented by certain pathogens, mainly bacteria, to supply their intracellular inclusions with nutrients and thus fuel their replication and differentiation (Amer, 2013).

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See also: Cell Division/Death: Apoptosis: Autophagy. Cell Division/Death: Regulation of Cell Growth: mTORC1: Upstream and Downstream. Organelles: Structure and Function: Conventional and Secretory Lysosomes; Early Endosomal Compartments; Golgi and TGN; The Late Endosome

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