At the Center of Macroautophagy

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At the Center of Macroautophagy: Autophagosomes

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Abstract

Autophagosomes are double-membrane vesicles that are the hallmark of the intracellular catabolic process called macroautophagy. They are formed by the orchestrated interplay of the AuTophaGy-related (ATG) proteins. The cargo molecules sequestered by autophagosomes include long-lived proteins, protein complexes or aggregates, superfluous or excess organelles, and invading pathogens. Complete autophagosomes fuse with lysosomes delivering the sequestered material in the interior of these organelles where it is degraded by resident hydrolases. Autophagy represents a key survival mechanism because it clears the cytoplasm from unwanted and potentially toxic structures, but it can also represent an intracellular source of metabolites that is induced by cells to generate new macromolecules or energy in times of need.

Nomenclature

AMPK AMP-activated protein kinase
ER Endoplasmic reticulum
ERGIC ER-Golgi intermediate compartments
ERES ER exit sites
HOPS Homotypic fusion and protein sorting
LIR LC3-interacting region
MTOC Microtubule organization center
PAS Phagophore assembly site
PE Phosphatidylethanolamine
PtdIns3KC3 Phosphatidylinositol-3 kinase class 3
PtdIns3P Phosphoinositol-3-phosphate
SARs Soluble autophagy receptors
TORC1 Mammalian target of rapamycin complex 1
Ub Ubiquitin

Glossary

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

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.
Selective Autophagy This term describes forms of macroautophagy in which determined structures (e.g., protein aggregates, organelles or microbes) are exclusively sequestered by autophagosomes.
Autophagy receptors Autophagic receptors are a set of proteins that simultaneously bind the structure targeted by autophagy and the pools of ATG8/LC3 protein family members that localize to the interior of autophagosomes. This property provides the substrate specificity for the selective types of autophagy. Autophagy receptors can be cytoplasmic or anchored in the subcellular structures and organelles.
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 and close it into an autophagosome. In yeast, there is one PAS per cell whereas in mammalian cells there are numerous per cell.
Phagophore The phagophore (also known as isolation membrane) 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 and close it into an autophagosome.

Key Points

- Autophagy is an intracellular lysosomal degradative pathway
- Structures targeted to destruction are either non-selectively or selectively sequestered by double-membrane autophagosomes
- Autophagosomes are generated through the orchestrated action of a specific machinery (ATG proteins) and requires lipid supply from different sources
- Selective sequestration of cargo by autophagosomes is guided by the so-called autophagy receptors

Introduction: Macroautophagy and Autophagosomes

Macroautophagy distinguishes from the two other forms of autophagy, namely chaperon-mediated autophagy and micro-autophagy, because it involves the formation of autophagosomes (Nakatogawa, 2020; Dikic and Elazar, 2018). 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, aberrant protein complexes or dysfunctional 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 or energy (Lahiri *et al.*, 2019) (**Fig. 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 (Galluzzi *et al.*, 2014). 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 post-natal 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 (Choi *et al.*, 2013; Deretic, 2021; Boya *et al.*, 2018).

Together with the proteasome, autophagy is one of the two major protein degradation systems 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 biological components of the cell, including glycans, nucleotides and lipids. While in specific situations autophagosomes randomly sequester cytoplasmic material, often their cargo is a specific structure. Selective types of autophagy include the turnover of organelles (e.g., mitochondria, peroxisomes, endoplasmic reticulum (ER), lysosomes, Golgi, lipid droplets…), protein aggregates, large complexes (ribosomes, mid-bodies, inflammasome…) and invading pathogens (Kirkin and Rogov, 2019) (see Section "Selective Sequestration Into Autophagosomes") (**Fig. 2**). Because of its ability to specifically eliminate unwanted structures, autophagy plays a crucial role in a multitude of physiological processes in eukaryotic organisms. Autophagy is part of our innate immune response through its ability to target invading pathogens for degradation (Deretic, 2021). Moreover the turnover of protein aggregates appears to be important in preventing an early onset of neurodegenerative disorders like Huntington's or Alzheimer's disease (Leidal *et al.*, 2018). Autophagy also plays major roles in stem cell maintenance and development and cell differentiation in multicellular organisms, and contributes to their longevity (Leidal *et al.*, 2018; Cecconi and Levine, 2008). As a result, an impairment or defect in autophagy contributes to a variety of human illnesses, including neurodegenerations, cancer, inflammatory diseases and muscular dystrophies (Mizushima and Levine, 2020; Beek *et al.*, 2018; Levine and Kroemer, 2019).

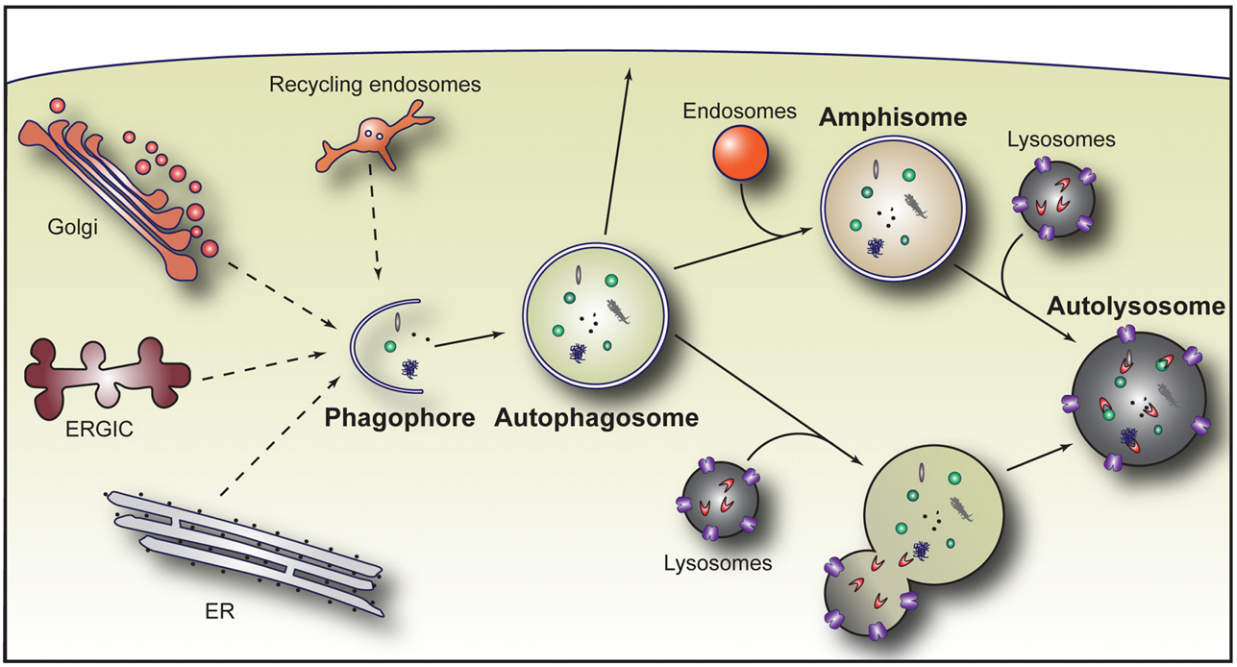

Fig. 1 *The mechanism of autophagy.* Autophagy starts with the formation of a membrane cistern called the phagophore, which nucleates probably through the heterotypic fusion of transport vesicles with different origins, including recycling endosomes, ER, ERGIC and/or Golgi. A subpopulation of these vesicles carries ATG9A. The major source of lipids required to expand the phagophore into an autophagosome is probably the ER, and their transport appears to principally occur at phagophore-ER membrane contact sites. 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 fuses 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 transporters. The autophagosome can also fuse with the plasma membrane and thereby being one of the mechanisms for unconventional secretion.

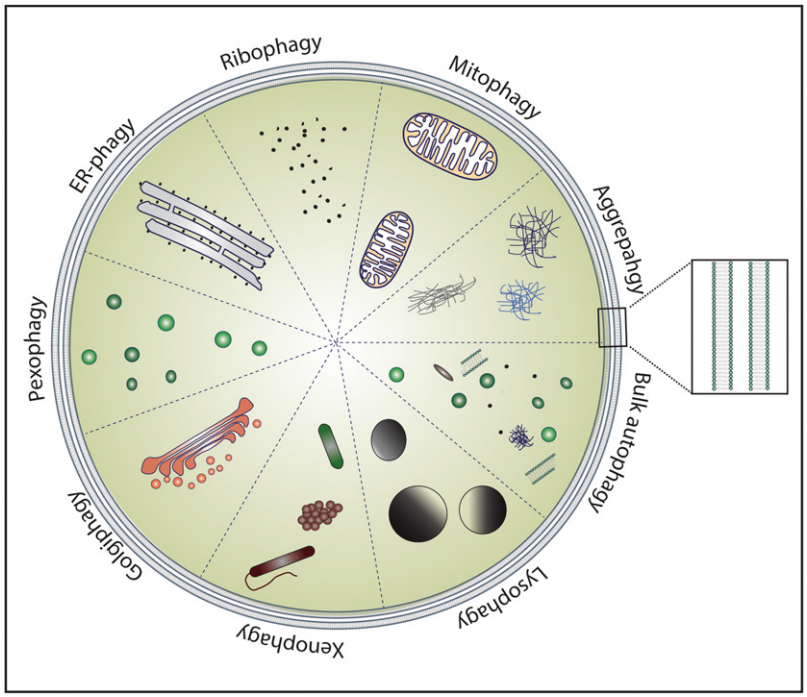

Fig. 2 *The different types of selective autophagy.* In addition to the unspecific sequestration of cytoplasmic material (i.e., bulk degradation), autophagosomes can also specifically target a variety of cellular components. Depending on the conditions, autophagosomes can selectively and exclusively engulf mitochondria (i.e., mitophagy), ribosomes (i.e., ribophagy), peroxisomes (i.e., pexophagy), lysosomes (i.e., lysophagy), parts of the Golgi (golgiphagy), parts of the ER (i.e., ER-phagy), 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.

The 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; Proikas-Cezanne and Robenek, 2011; Cheng *et al.*, 2014). Their size can vary from 500 nm up to 1.5 μm and this flexibility allow them to sequester cargoes of different sizes (Eskelinen *et al.*, 2011). In contrast to other transport vesicles, an autophagosome is both formed *de novo*, i.e., not through budding from a pre-existing organelle, and sequesters cytoplasmic cargo material. 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 (Reggiori and Klionsky, 2013).

The autophagic vesicles are categorized based on their morphology into autophagosomes, amphisomes and autolysosomes (Eskelinen *et al.*, 2011). In autophagosomes, the sequestered cargo is morphologically distinguishable. Amphisomes result from the fusion of autophagosomes and late endosomes, and their content is starting to be degraded, yet some of the morphological features of the cargo are still be recognized. In autolysosomes, which are formed from fusion of amphisomes or even autophagosome directly with lysosomes, the sequestered cargo is already being degraded and is therefore no longer identifiable.

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 most of them are currently called the AuTophaGy-related (ATG) genes (Nakatogawa, 2020). This was a breakthrough discovery in the field of autophagy because the high degree of conservation of numerous *ATG* genes allowed identifying their counterparts in higher eukaryotes. To date 42 ATG genes have been characterized in yeast and many of them have human orthologues (Klionsky *et al.*, 2003, 2021). In higher eukaryotes, there are additional genes essential for autophagy that are not called ATG. It is important to note that beside their role in autophagy, many ATG proteins have additional functions in other cellular pathways and thereby are not exclusive for autophagy (Mauthe and Reggiori, 2016; Galluzzi and Green, 2019).

It is now established that the generation of autophagosomes is regulated by a hierarchical interplay of ATG proteins that is divided in 3 discrete steps: (1) autophagy initiation, (2) phagophore nucleation and (3) phagophore elongation and closure.

Initiation of autophagy is mediated by the ULK/ATG1 kinase complex. The mammalian ULK kinase complex is composed of ULK1 or ULK2 (unc-51-like kinase), FIP200 (focal adhesion kinase family interacting protein of 200 kD), ATG13 and ATG101. The ULK/ATG1 kinase complex is differentially regulated by mammalian target of rapamycin complex 1 (TORC1) and AMP-activated protein kinase (AMPK), and possibly by other signaling cascades. In particular, active TORC1 inhibits the ULK/ATG1 complex whereas active AMPK stimulates the ULK/ATG1 complex (Licheva *et al.*, 2021). TORC1, with its central serine/threonine kinase mTOR, acts as a nutrient sensor in the cell and is activated in presence of nutrients. Under these conditions, TORC1 inhibits both AMPK and the ULK/ATG1 complex through phosphorylation. In nutrient-deprived cells, in contrast, AMPK is activated and phosphorylates TORC1 leading to its inhibition and dissociation from the ULK/ATG1 kinase 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 kinase complex activity and thereby initiating the autophagic process (Licheva *et al.*, 2021).

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 PAS structures can be observed upon autophagy induction whereas only one PAS is generated in yeast. The phagophore nucleation requires the local generation of phosphoinositol-3-phosphate (PtdIns3P) by the phosphatidylinositol-3 kinase class 3 (PtdIns3KC3/hVPS34). This protein is in complex with the regulatory subunits BECLIN1/ATG6, VPS15, ATG14L and NBRF2, which together compose the PtdIns3KC3 complex (Simonsen and Tooze, 2009; Nakatogawa, 2020). The activity of this complex is controlled by the differential regulation of its subunits (e.g., BECLIN1 is negatively regulated by BCL-2 protein family members and positively stimulated by the ULK1 kinase (Licheva *et al.*, 2021; Hill *et al.*, 2019)). One of the established functions of PtdIns3P is to mediate the recruitment of PtdIns3P-binding proteins (also called PtdIns3P effector proteins) to the PAS, including DFCP1 and WIPI protein family members, which are necessary for autophagosome biogenesis (Axe *et al.*, 2008; Proikas-Cezanne *et al.*, 2015). As also discussed below (Section "Generation of the Autophagosomes: The Membranes"), ATG9-containing vesicles are a key contributor of autophagy initiation. In addition to act as a membrane seed for the phagophore nucleation (Mari *et al.*, 2010; Yamamoto *et al.*, 2012; Sawa-Makarska *et al.*, 2020), studies in yeast have shown that Atg9 (and PtdIns3P) mediates the formation of membrane contact sites between the phagophore and the ER, through the sequential recruitment of Atg2 and Atg18 (Gomez-Sanchez *et al.*, 2018). Since ATG2 proteins can transport lipids from adjacent membrane and ATG9 proteins scramble lipids in vitro (Maeda *et al.*, 2020; Osawa *et al.*, 2020, 2019; Matoba *et al.*, 2020; Maeda *et al.*, 2019; Valverde *et al.*, 2019), the current working model is that these proteins promote to convey of lipids from the ER into the elongating phagophore, necessary for the biogenesis of an autophagosome.

On a molecular level, the elongation but also the closure of the phagophore is executed by two ubiquitin-like conjugation systems as well, 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 (Mizushima, 2020). The formation of the ATG16L1 complex as well as the LC3 lipidation are critical for the autophagosome biogenesis. The generation 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 resulting ATG12-ATG5 conjugate interacts with ATG16L1 to form a multimeric complex, the ATG16L1 complex, which localizes to the expanding phagophore but dissociates from complete autophagosomes (Mizushima, 2020).

In the second conjugation system, the members of the ATG8/LC3 protein family, i.e., LC3A. LC3B, LC3C, GABARAP, GABARAPL1 and GABARAPL2, becomes covalently linked to phosphatidylethanolamine (PE). Initially a preform of LC3 proteins is post-translationally cleaved at the C-terminus by the ATG4 proteases. The resulting cytoplasmic form of LC3 proteins, also called LC3-I, gets also activated by the E1-like enzyme ATG7 and transferred onto ATG3, an 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 conjugates, also known as LC3-II, are 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). 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, e.g., the autophagosomes, can be an indicator of autophagy induction (Klionsky *et al.*, 2021). Furthermore, it allows assessing autophagy induction and progression by analyzing the LC3-II protein levels by western blot (Klionsky *et al.*, 2021). Of note, there are additional assays that can and should be employed to measure the autophagic flux beside those (Klionsky *et al.*, 2021).

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 a major subject of ongoing research in the field of autophagy. Membranes from multiple sources have been shown to contribute to the nucleation of the phagophore and its expansion into mature autophagosomes. In general, autophagosomes form in close association with the ER, but do not bud or form directly from the ER but rather originate in close proximity of this organelle in structures called omegasomes (Axe *et al.*, 2008). The nucleation of the phagophore requires the ATG9-containing vesicles and probably their heterotypic fusion with transport vesicles originating from recycling endosomes, specific ER subdomains called ER exit sites (ERES) and/or the ER-Golgi intermediate compartments (ERGIC) (Gomez-Sanchez *et al.*, 2021). Subsequent expansion of the phagophore into an autophagosome entails a massive supply of lipids and their major source is very likely the ER, probably through membrane contact sites between this organelle and the phagophores (Graef *et al.*, 2013; Suzuki *et al.*, 2013; Gomez-Sanchez *et al.*, 2018; Yla-Anttila *et al.*, 2009; Hayashi-Nishino *et al.*, 2009; Uemura *et al.*, 2014). The elongation of phagophores occurs adjacent to the ER in regions that are in close contact with mitochondrial membranes, i.e., the so-called mitochondria-associated membranes (Hamasaki *et al.*, 2013). Additional potential membrane sources that may contribute to the autophagosome formation are mitochondria, Golgi, lipid droplets and/or the plasma membrane (Gomez-Sanchez *et al.*, 2021).

Maturation and Consumption of Autophagosomes

Once complete, autophagosomes fuses either directly with lysosomes or with endosomes to forms amphisomes, which ultimately also fuse with lysosomes (Reggiori and Ungermann, 2017; Zhao and Zhang, 2019). 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 (Eskelinen *et al.*, 2011) (**Fig. 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, the homotypic fusion and protein sorting (HOPS) and the MON1-CCZ1 complexes have been shown to be involved in fusion (Reggiori and Ungermann, 2017; Zhao and Zhang, 2019). 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 exported by lysosomal membrane transporters out of the autolysosomal lumens into the cytoplasm, where it can be metabolized by the cell (Lahiri *et al.*, 2019) (**Fig. 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). Specific adaptor proteins (i.e., FYCO1, RILP and JIP1) are responsible to recruit autophagosomes to motor proteins associated to microtubules and mediate their transport (Johansen and Lamark, 2020). On the other hand, lysosomal positioning within the cell is regulated in a nutrient-dependent manner by TORC1. 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 (Johansen and Lamark, 2020).

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 non-degradative function of autophagy plays a role in the unconventional secretion (i.e., secretion that does not involve transport through the Golgi) of a subset of signal molecules (Deretic *et al.*, 2012). Diverting of autophagosomes from their conventional trajectory to the endolysosomal 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).

Selective Sequestration Into Autophagosomes

These forms of autophagy rely on a series of so-called autophagy receptors, which recognize the targeted structure and mediate the subsequent recruitment and assembly of the ATG machinery (Kirkin and Rogov, 2019). Depending on the type of organelles that are targeted for sequestration distinctive terms have been used to describe these selective forms of autophagy, including mitophagy (mitochondria), lipophagy (lipid droplets), lysophagy (lysosomes), pexophagy (peroxisomes), nucleophagy (nucleus), golgiphagy (Golgi) and ER-phagy/reticulophagy (ER) (Kirkin and Rogov, 2019). Additionally, substrates may also include large protein complexes, such as ribosomes and mid bodies, or even single proteins, such as ferritin (Mancias *et al.*, 2014; Kraft *et al.*, 2008; Pohl and Jentsch, 2009; Wyant *et al.*, 2018). Autophagy receptors can be categorized into ubiquitin (Ub)-dependent and Ub-independent, based on the mechanism how they bind to their targets (Kirkin and Rogov, 2019). Autophagy receptors simultaneously bind the cargo and the members of the ATG8/LC3 protein family via a so-called LC3-interacting region (LIR) motif, promoting the specific sequestration of the cargo into the nascent autophagosomes. The principal Ub-dependent autophagy receptors are p62/SQSTM1, NBR1, TAX1BP1, CALCOCO1, NDP52 and optineurin/OPTN (Kirkin and Rogov, 2019). These autophagy receptors are also known as soluble autophagy receptors (SARs) since they are not anchored to their target but rather recruited to specific structures when those are ubiquitinated. In contrast, Ub-independent autophagy receptors are often located on the cargo themselves. Those include the ones mediating mitophagy (e.g., BNIP3, BNIP3L, FUNDC1, BCL2L13, FKBP8, PHB2, NIPSNAP1 and NIPSNAP2) or ER-phagy (e.g., FAM134B, SEC62, RTN3, CCPG1, ATL3 and TEX264) (Kirkin and Rogov, 2019). A prerequisite for the initiation of autophagosomal sequestration of the cargo is the clustering of autophagy receptors for Ub-dependent receptors or the activation of a cargo-embedded Ub-independent receptors by a posttranslational modification (Farre and Subramani, 2016). These events lead to the subsequent recruitment and activation of the ULK kinase complex and the subsequent autophagosome formation initiation (Turco *et al.*, 2019; Ravenhill *et al.*, 2019; Vargas *et al.*, 2019).

Conclusion

The field of autophagy has experienced tremendous advances in our knowledge about the regulation, mechanism and function of autophagy over the past two decades, which have completely changed the initial view of a process exclusively devoted to the bulk turnover of intracellular components. These advances have also been key in uncovering the role of autophagy in different pathologies and valuing the therapeutic potentials of this pathway. Although therapeutic interventions specifically targeting autophagy are not available yet, it is predictable the fast advances in this field and the relevance of this process in many pathologies will lead to the development of medical treatments. What we have seen so far appears to be just the foretaste of what we will witness during the next years.

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Further Reading

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