—Review—

Molecular Mechanisms of Macroautophagy, Microautophagy, and Chaperone-Mediated Autophagy

Hayashi Yamamoto and Takahide Matsui

Department of Molecular Oncology, Institute for Advanced Medical Sciences, Nippon Medical School, Tokyo, Japan

Autophagy is a self-digestive process that is conserved in eukaryotic cells and responsible for maintaining cellular homeostasis through proteolysis. By this process, cells break down their own components in lysosomes. Autophagy can be classified into three categories: macroautophagy, microautophagy, and chaperone-mediated autophagy (CMA). Macroautophagy involves membrane elongation and microautophagy involves membrane internalization, and both pathways undergo selective or non-selective processes that transport cytoplasmic components into lysosomes to be degraded. CMA, however, involves selective incorporation of cytosolic materials into lysosomes without membrane deformation. All three categories of autophagy have attracted much attention due to their involvement in various biological phenomena and their relevance to human diseases, such as neurodegenerative diseases and cancer. Clarification of the molecular mechanisms behind these processes is key to understanding autophagy and recent studies have made major progress in this regard, especially for the mechanisms of initiation and membrane elongation in macroautophagy and substrate recognition in microautophagy and CMA. Furthermore, it is becoming evident that the three categories of autophagy are related to each other despite their implementation by different sets of proteins and the involvement of completely different membrane dynamics. In this review, recent progress in macroautophagy, microautophagy, and CMA are summarized. (J Nippon Med Sch 2024; 91: 2–9)

Key words: autophagy, macroautophagy, microautophagy, CMA

Introduction

In the late 1950s, the phenomenon in which cells degrade cytoplasmic materials in lysosomes was discovered by observing animal cells through electron microscopy. In the early 1960s, this process was named "autophagy"[1,2], which is now used as a general term for intracellular processes by which cells degrade their own components in lysosomes in mammals or vacuoles in yeasts and plants.

Autophagy can be classified into three categories according to its cargo delivery pathway and membrane dynamics: macroautophagy, microautophagy, and chaperone-mediated autophagy (CMA) (**Fig. 1**). Macroautophagy is characterized by membrane elongation, which results in the formation of a double-membraned structure called an autophagosome that contains sequestered cytoplasmic material and eventually fuses with lysosomes to have its contents degraded[3] (**Fig. 1**). Macroautophagy is further classified into selective and non-selective types, with the former specifically recognizing cargo proteins via various macroautophagy adaptors/receptors. Microautophagy involves inward membrane deformation of the lysosomal membranes (or endosomal membranes in endosomal microautophagy[4]) to generate intraluminal vesicles containing cytosolic materials that are eventually broken down in the lysosomes[5,6] (**Fig. 1**). In endosomal microautophagy, a portion of endosomal intraluminal vesicles is thought to be released to the extracellular space as exosomes. In contrast to macroautophagy and microautophagy, CMA does not involve membrane deformation[7]. Instead, cytosolic cargo proteins are transported directly into lysosomes with the help of cytosolic molecular chap-

Correspondence to Hayashi Yamamoto, Department of Molecular Oncology, Institute for Advanced Medical Sciences, Nippon Medical School, 1–1–5 Sendagi, Bunkyo-ku, Tokyo 113–8602, Japan
E-mail: hayashi-yamamoto@nms.ac.jp
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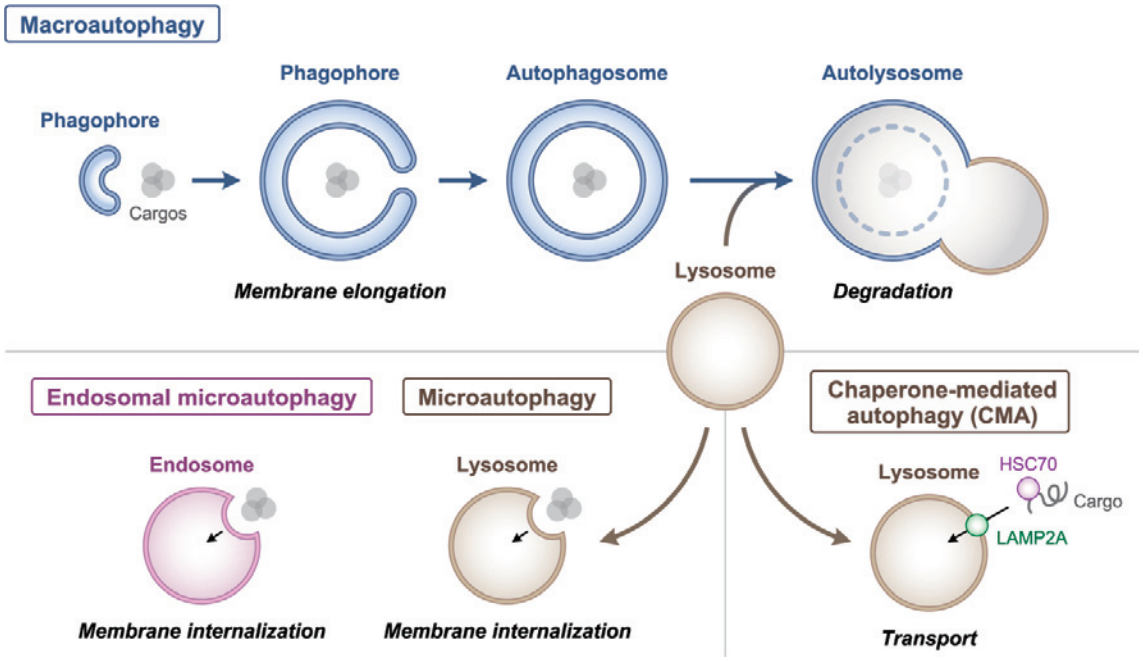

Fig. 1 Membrane dynamics of macroautophagy, microautophagy, and chaperone-mediated autophagy (CMA)
Autophagy is classified into macroautophagy (top), microautophagy (bottom left), and CMA (bottom right). Macroautophagy involves "membrane elongation" of the phagophore membrane to generate the autophagosome sequestering cytoplasmic cargos. The closed autophagosome then fuses with lysosomes to mature into the autolysosome within which materials are degraded. Microautophagy involves "membrane internalization" of the lysosomal membrane (or endosomal membrane in endosomal microautophagy) to generate intraluminal vesicles containing cytosolic cargos. CMA involves "transport" of the cytosolic proteins into lysosomes without membrane deformation, in which proteins containing the KFERQ-like motif are recognized by the cytosolic molecular chaperone HSC70 and recruited to the lysosome.

erone HSC70 (also known as HSPA8) and lysosomal membrane protein LAMP2A (**Fig. 1**). In this review, we describe molecular mechanisms of the three categories of autophagy, with emphasis on mammalian macroautophagy and microautophagy.

Molecular Mechanisms of Macroautophagy

Studies on the molecular mechanisms underlying macroautophagy began following the discovery of macroautophagy in the yeast *Saccharomyces cerevisiae* in 1992[8] and identification of most of the core autophagy-related (*ATG*) genes in 1993[9]. Studies on macroautophagy then expanded from yeast to mammals and plants, leading to discoveries of the significance of macroautophagy in various biological phenomena and human diseases[3,10,11], thereby attracting much attention to the field.

Macroautophagy is strongly induced by starvation or stress. In the initiation stage, the ULK complex composed of ULK1 (or ULK2), FIP200 (also known as RB1CC1), ATG13, and ATG101 assemble to form a punctate structure near the endoplasmic reticulum (ER) membrane to serve as a scaffold for autophagosome formation (**Fig. 2A, 2B**). A recent study revealed that calcium transients on the ER membrane promote the formation of biomolecular condensates of FIP200 (likely when it is a component of the ULK complex) by liquid-liquid phase separation (LLPS)[12] (**Fig. 2B**). Similarly, in yeast, the homologous Atg 1 complex acts as a scaffold known as the pre-autophagosomal structure (PAS), which is also a biomolecular condensate generated by LLPS via the multivalent interactions among its components, Atg1, Atg13, and the Atg17-Atg29-Atg31 subcomplex[13]. Thus, the starvation-induced assembly of the ULK/Atg1 complex driven by LLPS is thought to be a prerequisite for autophagosome formation (**Fig. 2B**).

In addition to starvation-induced assembly, recent studies also reported cargo-driven assembly of the ULK complex (**Fig. 2B**). The autophagic cargo is frequently marked with ubiquitin, which is then recognized by the ubiquitin-binding macroautophagy adaptor SQSTM1 (also known as p62). SQSTM1 forms a liquid-like biomolecular condensate with the ubiquitinated cargos and recruits the ULK complex through interaction with FIP 200 in the ULK complex[14], thereby promoting selective

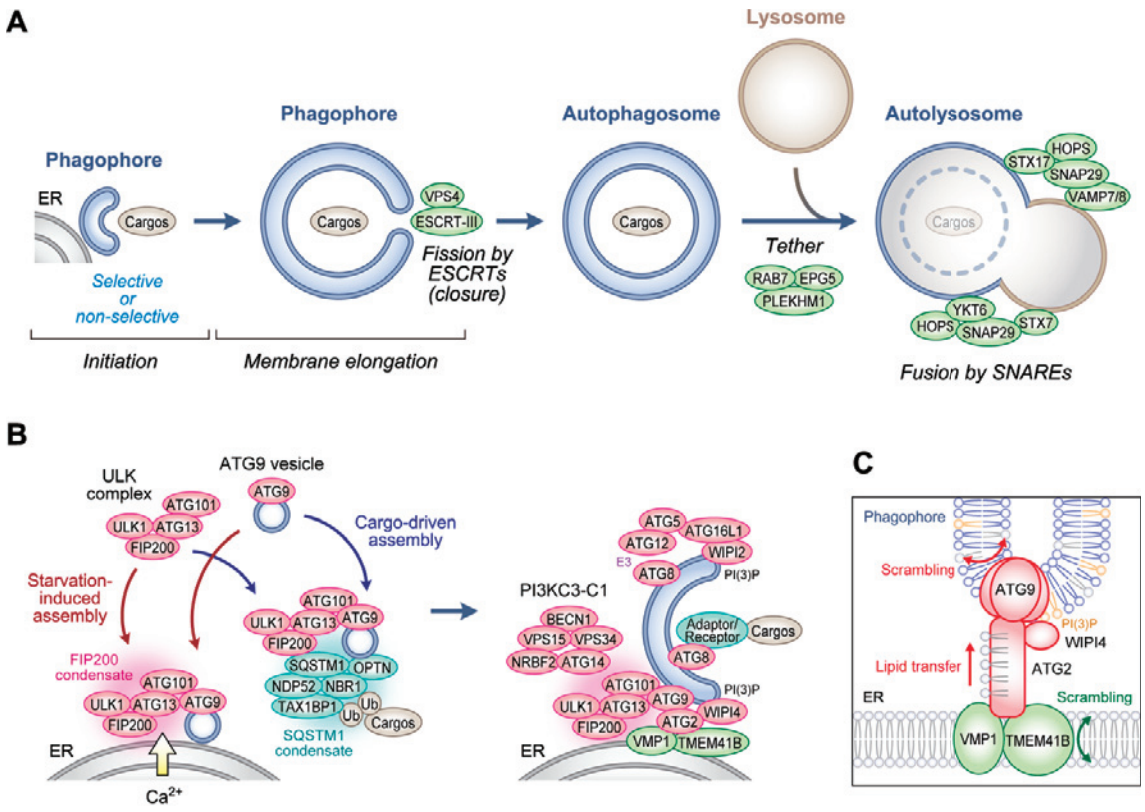

Fig. 2 Molecular mechanisms of macroautophagy

(A) Macroautophagy consists of initiation, membrane elongation, membrane closure, autophagosome formation, membrane tethering, and lysosome fusion.

(B) Molecular details of the initiation and membrane elongation processes. Starvation-induced assembly and cargo-driven assembly of initial ATG proteins are shown in red and blue arrows, respectively.

(C) Molecular mechanism of lipid supply to the phagophore membrane. ATG2 transfers phospholipids from the ER membrane to the phagophore membrane. The ER-derived phospholipids are redistributed to the inner leaflet of the phagophore membrane by the ATG9 scramblase complex.

degradation of the ubiquitinated cargo proteins in the SQSTM1 condensate. Other ubiquitin-binding macroautophagy adaptors NDP52 (also known as CALCOCO2) and TAX1BP1 are also present in the SQSTM1 condensate and serve to further promote recruitment of the ULK complex by binding to FIP200[15–17]. Another ubiquitin-binding macroautophagy adaptor, NBR1, interacts with SQSTM1 to modulate SQSTM1 condensate formation and also recruits the ULK complex through binding to TAX1BP1[17,18].

After its assembly, the ULK complex recruits ATG9 vesicles and the class III phosphatidylinositol 3-kinase complex I (**Fig. 2B**). The former serve as the basis of autophagosomal membranes in yeast[19,20] and the latter produces PI(3)P at the autophagosome formation site to recruit PI(3)P-binding effectors, such as WIPI1, WIPI2, WIPI3, and WIPI4 in mammals, and eventually the lipid transfer protein ATG2. Generally, ATG9 vesicles localize to the autophagosome formation site by interacting with the ATG13-ATG101 subcomplex[21–23] within the ULK complex (**Fig. 2B**). However, during macromitophagy, they can also be recruited to ubiquitinated mitochondria by the ubiquitin-binding macroautophagy adaptor OPTN[24]. In addition, overexpression of NBR1 leads to accumulation of ATG9 vesicles, implying that NBR1 also recruits ATG9 vesicles[25]. This type of ATG9 recruitment is thus induced by the cargo itself, similar to the aforementioned cargo-driven assembly of the ULK complex. Thus, cargo-driven assembly of the initial ATG factors may be a general mechanism to promote selective macroautophagy (**Fig. 2B**).

Elongation of the phagophore membrane (i.e., the precursor to the autophagosome) then takes place. Although the molecular mechanism underlying this process has been a long-standing mystery, major revelations have been made recently (**Fig. 2C**). ATG9 is a unique ATG protein that forms a homo-trimeric complex embedded in the phagophore membrane. It functions as a scramblase that translocates phospholipids between the outer and in-

ner leaflets of the phagophore membrane[26–28] while interacting with the lipid transfer protein ATG2[29–31], which is recruited to the autophagosome formation site directly by the WIPI3/4 family proteins[32] and indirectly by the WIPI 1/2 family proteins[33,34]. ATG2 also interacts with the ER membrane proteins VMP1 and TMEM41B, which both have scramblase activity[28,35–37]. In the recent model of membrane elongation (**Fig. 2C**), ATG2 bridges the ER membrane to the phagophore membrane and is thought to transfer phospholipids from the outer leaflet of the ER membrane to the outer leaflet of the phagophore membrane. These ER-derived phospholipids are then redistributed to the inner leaflet of the phagophore membrane by the ATG9 scramblase complex, resulting in phagophore membrane elongation.

During membrane elongation, two ubiquitin-like conjugation systems, called the ATG8 and ATG12 conjugation systems, are also involved (**Fig. 2B**). Through the actions of the E1-like enzyme ATG7 and the E2-like enzyme ATG 3, the ubiquitin-like ATG8 family proteins (LC3 and GABARAP in mammals) are conjugated to phosphatidylethanolamine (PE), resulting in ATG8-PE (LC3-PE and GABARAP-PE in mammals). Similarly, with the help of ATG7 and the E2-like enzyme ATG10, ubiquitin-like protein ATG12 is conjugated to its substrate ATG5. The ATG 12-ATG5 conjugate then associates with ATG16L1 to form the ATG12-ATG5-ATG16L1 complex, which has E3-like activity in the LC3/GABARAP conjugation reaction. LC3 and GABARAP, likely in their lipidated forms, localize on the phagophore membrane and are thought to function in the late and early steps of autophagosome formation, respectively.

LC3 and GABARAP are known to interact with the LC 3-interacting region (LIR), GABARAP-interacting motif (GIM), and ATG8-interacting motif (AIM) (collectively referred to as LIR). The consensus tetrapeptide motif, W/F/Y-x-x-L/I/V (where "x" represents any amino acid), is found in some core ATG proteins, such as FIP200, ULK1, ATG13, ATG14, and ATG2, and facilitates interaction with the ATG8 family proteins. Recent studies reported that the PI(3)P effector WIPI2 interacts with ATG16L1 to promote lipidation of ATG8 family proteins at the autophagosome formation site[33], thus leading to LIR-mediated recruitment of ATG2[34] (**Fig. 2B**). These interactions among the ATG8 family proteins and core ATG proteins are thought to be crucial for autophagosome formation.

ATG8 family proteins also play pivotal roles in cargo recognition in selective macroautophagy, which can be divided into ubiquitin-dependent and -independent types. In ubiquitin-dependent selective macroautophagy, the ubiquitin-binding adaptors, such as SQSTM1, NBR1, OPTN, NDP52, TAX1BP1, and TOLLIP, bind to ubiquitinated cargo proteins/organelles and, as they all contain LIR, are recognized by ATG8 family proteins on phagophores, leading to their selective degradation[11] (**Fig. 2B**). Recent studies have reported that the interaction between LC3/GABARAP and the LIR in SQSTM1 leads to selective engulfment of the SQSTM1 condensate by the autophagosomal membrane[38], which is thought to be caused by "membrane wetting"[39], and thus the SQSTM1 condensate can deform the autophagosomal membrane[39] and facilitate membrane elongation along the surface of the SQSTM1 condensate. In addition to the SQSTM1 condensates, ferritin-NCOA4 condensates in mammals[40] and Ape 1 condensates in yeast[41] are other examples of cargo-receptor condensates cleared by this form of macroautophagy, which has been defined as "fluidophagy"[39]. In ubiquitin-independent selective macroautophagy, the cargo-resident macroautophagy receptors—including CCPG1, TEX264, FAM134A/B/C (also known as RETREG2/1/3), SEC62, RTN3L, and ATL3 for macroER-phagy, BNIP3, NIX (also known as BNIP3L), FUNDC1, FKBP8, BCL2L13, and TRIM5 for macromitophagy, CALCOCO1 for macroGolgi-phagy, and TAX1BP1 for macroferritinophagy[11]—possess LIR(s) that are recognized by LC3 for their incorporation into autophagosomes.

After elongation, the phagophore membrane eventually closes to form a double-membraned autophagosome (**Fig. 2A**). During closure, membrane scission of the two membranes is coordinated by the endosomal sorting complex required for transport (ESCRT) complex[42,43]. Mediated by two soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) complexes, the STX 17-SNAP29-VAMP7/8 and YKT6-SNAP29-STX7 complexes[44,45], the closed autophagosome then fuses with lysosomes to mature into the autolysosome within which material is degraded. In addition to the SNARE proteins, membrane tethering factors such as the homotypic fusion and vacuole protein-sorting complex, PLEKHM1, EPG5, and RAB7 are also involved in the process of autophagosome-lysosome fusion[46] (**Fig. 2A**).

Molecular Mechanisms of Microautophagy

Microautophagy, in which cytosolic proteins are incorporated into lysosomes by invagination of the lysosomal membranes, was also discovered by electron microscopic observations of animal cells in the early 1980s[47,48]. Microautophagy is classified into two types: (1) invagina-

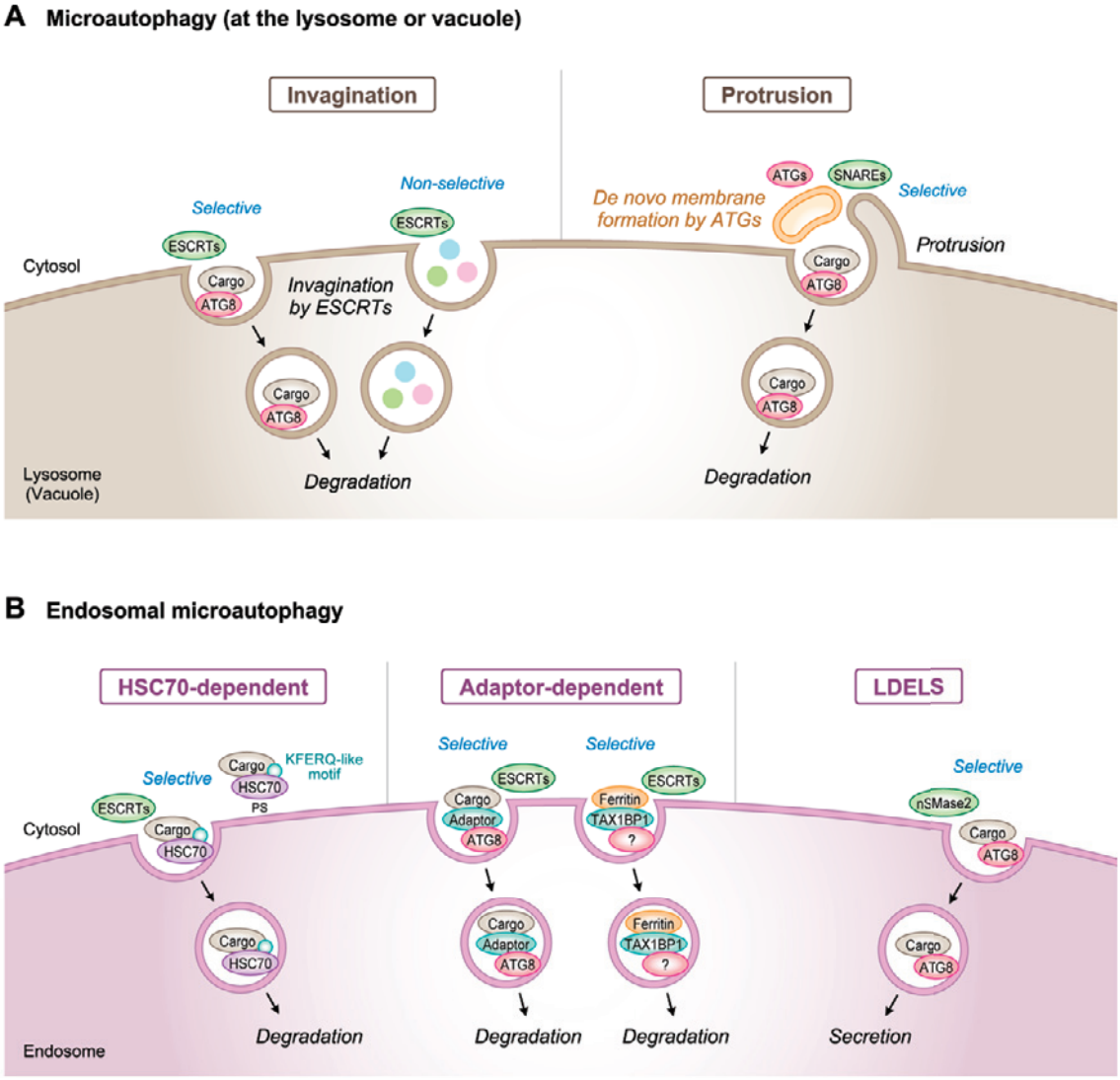

Fig. 3 Molecular mechanisms of microautophagy

(A) Microautophagy involves invagination (left) or protrusion (right) of the lysosomal/vacuolar membrane. The intraluminal vesicles containing cytosolic cargos are degraded in the lysosome/vacuole.

(B) Several types of endosomal microautophagy exist in mammals: HSC70-dependent microautophagy (left), adaptor-dependent microautophagy (middle), and LDELS (right).

tion of the lysosomal membranes (or endosomal membranes in endosomal microautophagy) and (2) protrusion of the lysosomal membranes[6] (**Fig. 3A**). The former type is thought to require the ESCRT complex, which mediates inward deformation and subsequent fission of the lysosomal or endosomal membranes to form intraluminal vesicles, but not the core ATG proteins, except for the ATG8/ATG12 conjugation systems, which are involved in cargo recognition. By contrast, the protrusion type requires core ATG proteins and SNARE proteins (**Fig. 3A**). The best characterized protrusion-type microautophagy pathway is micropexophagy in the yeast *Komagataella phaffii*, which is known to involve membrane deformation dependent on the ATG8/ATG12 conjugation systems and *de novo* membrane formation covering the membrane closure site, likely mediated by macroautophagy machinery such as core ATG proteins and SNARE proteins.

Several types of selective microautophagy have been reported. A key example is the incorporation of portions of the ER directly into lysosomes along with the macroER-phagy receptor SEC62 and the ATG8/ATG12 conjugation systems[49]. Another example is the degradation of procollagen-containing subdomains of the ER by microER-phagy in a manner dependent on the ubiquitin-binding macroautophagy adaptor SQSTM1, the ATG8/ATG12 conjugation systems, and the ESCRT complex. Thus, cargo recognition mechanisms are at least partially

shared between macroautophagy and microautophagy. Microautophagy is also known to selectively degrade subdomains of the nucleus (micronucleophagy), lipid droplets (microlipophagy), lysosomal membranes (microlysophagy)[50,51], and photodamaged chloroplasts (microchlorophagy)[52].

Membrane invagination that occurs at the endosomal membranes is defined as endosomal microautophagy[4,6,53] (**Fig. 3B**). It is a process thought to be nearly identical to ESCRT-dependent multivesicular body formation. Endosomal microautophagy can selectively degrade cytosolic cargo proteins, which contain the KFERQ-like pentapeptide motif recognized by cytosolic molecular chaperone HSC70[53] (**Fig. 3B**). Then, HSC70 recruits cargo to the surface of the lysosomal membrane by interacting with phosphatidylserine and inducing internalization into lysosomes. Additionally, starvation-induced endosomal microautophagy selectively degrades macroautophagy adaptors/receptors and cargos such as SQSTM1, NBR1, NDP52, TAX1BP1, NCOA4, and ferritin, a process which is partly dependent on the ATG8/ATG12 conjugation systems[4], and thus ATG8 family proteins are also degraded by endosomal microautophagy (**Fig. 3B**). The ESCRT complex-dependent phenomenon in the yeast *Schizosaccharomyces pombe*, called the Nbr1-mediated vacuolar targeting pathway, which is independent of ATG proteins, may be similar to endosomal microautophagy in mammals[54].

In addition to ESCRT-dependent endosomal microautophagy, LC3-dependent extracellular vesicle loading and secretion (LDELS) has been reported[55] (**Fig. 3B**). This process is dependent on nSMase2 (also known as SMPD 3) but not the ESCRT complex. In LDELS, RNA-binding proteins such as HNRNPK and SAFB are recognized by LC3 through the LIR, selectively incorporated into the endosomes, and eventually secreted from the cell as exosomes.

A recent study revealed that macroautophagy and microautophagy are closely related during ferritin degradation (**Fig. 3B**). Ferritin forms biomolecular condensates through LLPS driven by NCOA4[40]. Ferritin-NCOA4 condensates are then targeted by macroautophagy (macroferritinophagy) and endosomal microautophagy (microferritinophagy), which both require TAX1BP1 as an adaptor. In contrast to its complete degradation when taken up by macroferritinophagy, a portion of ferritin taken up by microferritinophagy might be destined for secretion, as ferritin is known to be secreted via exosomes.

Molecular Mechanisms of CMA

CMA was first described in the late 1980s with the discovery that the KFERQ-like motif is recognized by molecular chaperone HSC70 and is sufficient for selective incorporation into lysosomes[7]. In contrast to HSC70-dependent endosomal microautophagy, CMA does not involve membrane deformation (**Fig. 1**). Instead, KFERQ-containing proteins are directly transported into lysosomes through the lysosomal membrane protein LAMP 2A. During CMA, HSC70 binds to the KFERQ-like motif with its cofactors, HSP40 (also known as DNAJB1) and CHIP. The HSC70-substrate complex then localizes to the LAMP2A homo-trimeric complex assembled in the lysosomal membrane and enters the lysosomal lumen through the LAMP2A complex. CMA activity is at least partly regulated by the amount of LAMP2A. It has been reported that CMA activity is also correlated with macroautophagy activity, which reflects crosstalk between CMA and macroautophagy.

The direct uptake of cytosolic RNA and DNA into lysosomes has also been reported. Known as RNautophagy and DNautophagy (collectively referred to as RN/DNautophagy), this process is ATP-dependent and involves the direct recognition of RNA and DNA by the cytosolic arginine-rich regions of LAMP2C and SIDT2[6,56,57]. Details of its molecular mechanism are still unknown and RN/DNautophagy is sometimes classified as selective microautophagy.

Conclusions

Several breakthroughs have been made with respect to the molecular mechanisms of autophagy. For macroautophagy, it is now known that biomolecular condensate formation of the ULK complex is driven by LLPS at the initial stage and membrane elongation is achieved by lipid transfer by ATG2 and lipid scrambling by ATG9. Macroautophagy, which is associated with several human diseases, has been found to be highly diverse in its selective cargo, which indicates that the selective cargo can drive assembly of the ULK complex in addition to being recognized by adaptors/receptors and ATG8 family proteins. Microautophagy is also highly diverse in terms of molecular mechanisms and membrane dynamics. The identification of common substrates between macroautophagy and microautophagy (e.g., the SQSTM1 condensates and ferritin-NCOA4 condensates) as well as correlated activity between macroautophagy and CMA point toward crosstalk between macroautophagy (macrofluidophagy), microautophagy (microfluidophagy), and CMA.

Future work clarifying the relationships between the different types of autophagy and how they are related in the context of diseases will deepen our understanding of autophagy and perhaps bring us closer to modulating this cellular process for health benefits.

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