Selective Autophagy of Macromolecular Complexes: What Does It Take to be Taken?

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Abstract

Proteins are known to perform an astonishing array of functions thanks to their ability to cooperate and modulate each other's properties. Inside cells, proteins can assemble into large multi-subunit complexes to carry out complex cellular functions. The correct assembly and maintenance of the functional state of macromolecular protein complexes is crucial for human health. Failure to do so leads to loss of function and potential accumulation of harmful materials, which is associated with a variety of human diseases such as neurodegeneration and cancer. Autophagy engulfs cytosolic material in autophagosomes, and therefore is best suited to eliminate intact macromolecular complexes without disassembling them, which could interfere with *de novo* assembly. In this review, we discuss the role of autophagy in the selective degradation of macromolecular complexes. We highlight the current state of knowledge for different macromolecular complexes and their selective autophagic degradation. We emphasize the gaps in our understanding of what it takes for these large macromolecular complexes to be degraded and point to future work that may shed light on the regulation of the selective degradation of macromolecular complexes by autophagy.

Introduction

Cells and organisms require tight control and maintenance of the correct folding state and abundance of proteins according to cellular needs. Distinct pathways, collectively referred to as the proteostasis network, have evolved to maintain the integrity of the proteome. The fundamental importance of this network is exemplified by the common occurrence of non-native protein aggregates in various tissues during ageing and disease-related decline. Macroautophagy (hereafter referred to as autophagy) is a conserved intracellular pathway to isolate and transport bulky superfluous and defective cytosolic components, such as invading pathogens, protein aggregates and complexes, as well as organelles for their destruction in a degradative compartment. Autophagy plays a role under physiological and pathological conditions or upon exposure to environmental stressors. Thus, autophagy is an essential part of the proteostasis network (its function and machinery have been exentisvely reviewed elsewhere[1–4]). The isolation and transport of cytosolic cargo are achieved by its sequestration into an autophagosome, a double-membrane organelle that is synthesized *de novo* and later fuses with a degradative cellular compartment such as the lysosome or the vacuole. Autophagy can be selectively activated by various stress factors that lead to the accumulation of specific cargo, such as mitochondria.[5] In addition, catabolic breakdown of

macromolecules and recycling of their constituents through autophagy enables cells to cope with nutrient deprivation. These two distinct processes are referred to as selective and bulk autophagy, respectively. Selective autophagy describes the targeting of a specific cargo by selective autophagy receptors (hereafter referred to as receptors), which promote cargo-driven autophagosome formation and its selective sequestration. In contrast, bulk autophagy describes the non-selective uptake and degradation of cytosolic contents for nutrient recycling as a cellular response to metabolic stress, such as starvation.

To promote phagophore initiation during selective autophagy, receptors recruit the autophagy scaffold protein Atg11 or its mammalian counterpart FIP200 through an often phosphoregulated interaction to the cargo surface.[6–9] The scaffold protein interacts with the Atg1 kinase complex, leading to local Atg1 activation and subsequent phagophore nucleation. The relevance of the scaffold proteins has been shown by direct tethering of Atg11 to the cargo, which results in selective cargo degradation even in the absence of receptors.[10] Once initiated, receptors couple the cargo to the expanding phagophore by directly binding to the ubiquitin-like proteins of the Atg8/LC3/GABARAP family.[11] These proteins are covalently anchored to the phagophore and ensure membrane growth specifically around the cargo. The interaction between receptors and Atg8/LC3/GABARAP proteins occurs through a short linear motif, called Atg8 interacting motif (AIM) or LC3 interacting region (LIR). Most AIM/LIRs consist of a short, conserved sequence, whose consensus is Θ-X-X-Γ, where Θ represents an aromatic amino acid (W/F/Y), Γ represents a hydrophobic amino acid (L/I/V), and X can be any amino acid. These motifs are often flanked by at least one proximal acidic residue which regulates their binding to Atg8. Beyond these, non-canonical AIMs that do not contain all of these features have also been described.[12] This diversity of sequence requirements makes receptor protein identification tedious, as most protein sequences contain multiple potential AIMs. Besides the AIM/LIR binding pocket of Atg8, another class of proteins bind to Atg8 via direct interaction of their ubiquitin-interacting motifs with a region of Atg8 located opposite to the location of the classical AIM binding site.[13] This further increases the number and complexity of potential receptors, and it is expected that the number of proteins degraded via selective autophagy is much larger than originally thought. Recent approaches to identify receptors and to map their Atg8 binding sites include the use of AlphaFold multimer to predict sites of Atg8-receptor interactions.[14] Although promising, this approach may be limited by the overall conservation of these binding sites across homologues. Cargo can be recognized by the receptor in two distinct ways: either ubiquitin-dependent or ubiquitin-independent. One of the best-studied examples for a ubiquitin-binding receptor is the mammalian protein p62/SQSTM1, which recognizes polyubiquitylated protein aggregates via its C-terminal ubiquitin associated domain.[15,16]

Notably, the interaction between Atg8/LC3/GABARAP family members and AIMs/LIRs typically displays low affinity, thus raising the question of how cargo recruitment and autophagosome biogenesis are coordinated. The current understanding suggests that multiple avid interactions between the receptor and membrane-bound Atg8 molecules strengthen the interaction and allow membrane expansion templating along the cargo surface. However, this also requires that the cargo forms a distinct entity prior to engulfment. Membrane delimited organelles are a well-described cargo of selective autophagy. All known organelles are subject to selective autophagy under certain conditions, establishing autophagy as a quality control pathway for organelle functionality. Often, parts of the organelle are pinched off from the network, as in the case of the ER or mitochondria, and subsequently engulfed by autophagy (for a comprehensive review see.[17]

On the other hand, , selective degradation of cytosolic macromolecular complexes means that individual protein complexes would need to undergo an enrichment process before being selectively engulfed by the autophagy machinery. This implies that macromolecular complexes should form a larger assembly that allows for low-affinity, high-avidity receptor/cargo interactions on its surface requiered for phagophore growth. This would ensure that other cytosolic constituents are largely excluded when the selective cargo is engulfed by the phagophore membrane during autophagosome biogenesis. Using correlative cryo-electron tomography, we recently visualized cargo directly *in situ* and showed that both selective and non-selective autophagosomes exist under nitrogen starvation in budding yeast, with a much higher frequency of non-selective autophagosomes[18] (Figure 1).

We are just beginning to understand how the biophysical properties of cargo influence autophagosome biogenesis and its selective autophagic engulfment . In recent years, liquid–liquid phase separation (LLPS) has emerged as a recurring mechanism for the enrichment of macromolecular complexes. This suggests that autophagy could also play a crucial role in the clearance of membraneless organelles.[19] LLPS describes a physical process by which a homogeneous solution spontaneously separates into two distinct immiscible phases: a dense phase, and a dilute phase. The dense phase is often referred to as a condensate. In many cases, the exact physical state of the condensate is poorly characterized and often ranges from highly mobile liquid-like states to
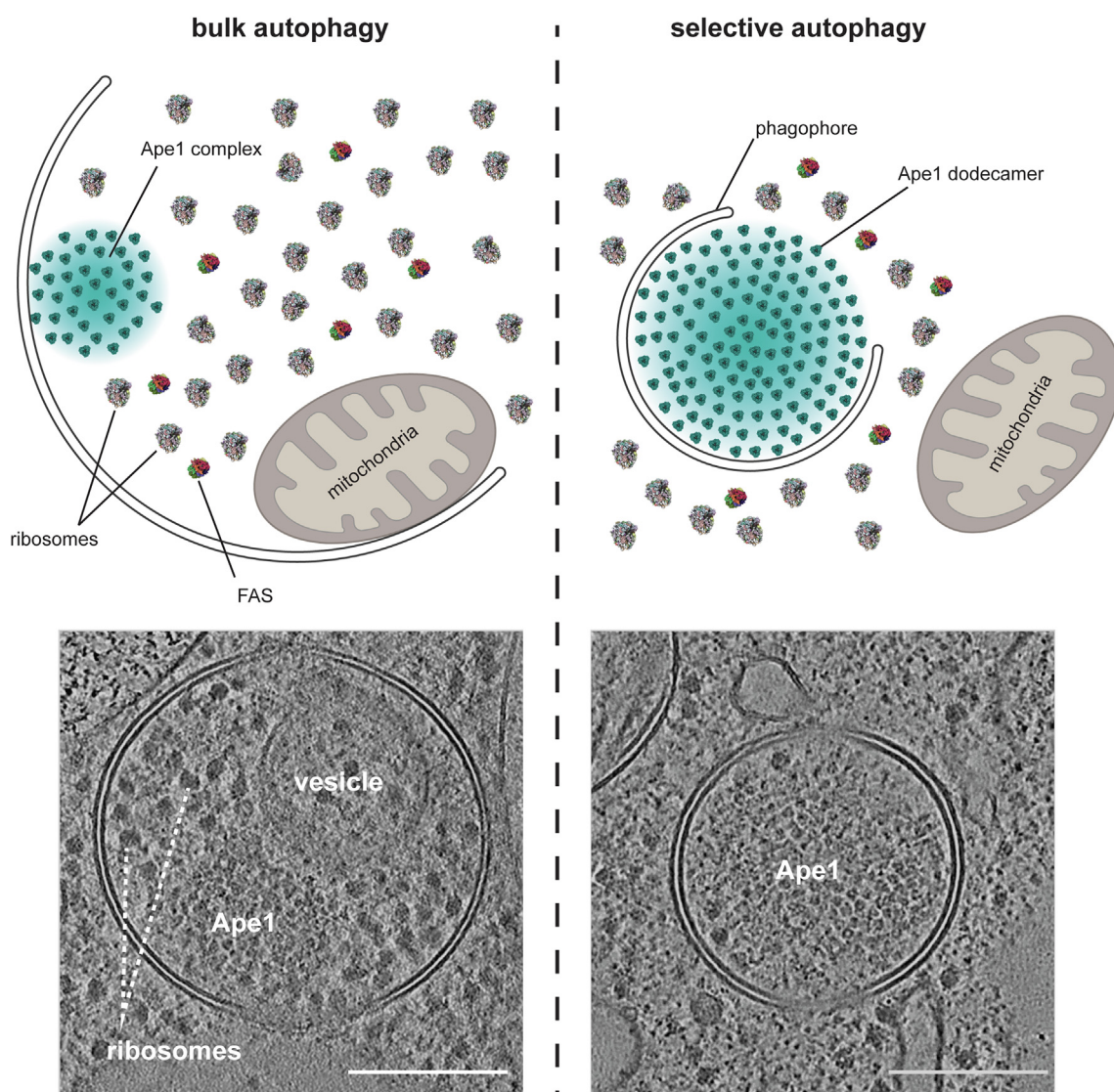
Figure 1. Bulk vs. selective autophagy. In bulk autophagy, cytoplasmic components and organelles are engulfed by autophagosomes for recycling of their cellular components. Here, autophagosomes form without the need for a cargo entity to serve as a template for membrane nucleation and expansion. Even under these conditions, cargo can be attached to the growing phagophore via receptor/Atg8 family member interaction. As a result, selective cargo is engulfed along with other cytosolic components such as ribosomes. In contrast, during selective autophagy, the phagophore specifically grows around a distinct cellular compartment or macromolecular complex (such as the Ape1 complex in *S. cerevisiae*) for its targeted degradation, thereby regulating essential cellular processes. Examples observed using in situ cryo-electron tomography and described in[18] are shown below. Scale bars are 200 nm.

gel-like states in which part of the molecules of a condensate are less mobile or even immobile. For autophagy, phase-separated condensates provide a large and dynamic surface for avid interactions between the receptor and autophagy machinery.

In mammalian autophagy, three major cargo receptors cooperate during selective autophagy of protein condensates and aggregates: p62/SQSTM1, NBR1, and TAX1BP1. These three receptors colocalize into ubiquitin-containing condensates.[20] Phase separation of p62/SQSTM1 into condensates occurs through multivalent inter-

actions established with multiple ubiquitin chains.[21] *In vitro* experiments revealed that NBR1 regulates the condensation of p62 through its ubiquitin associated and PB1 domains, and further recruits the third receptor, TAX1BP1, to the condensates. TAX1BP1 drives the recruitment of the scaffolding protein FIP200 to these condensates, which initiates phagophore biogenesis.[22]

In yeast, the cytoplasm-to-vacuole targeting (Cvt) pathway is the best characterized selective autophagic cargo. It has been shown that the cargo Ape1 forms condensates through phase

separation, and this condensation is essential for autophagic delivery of Ape1 to the vacuolar lumen.[23]

Thus, receptors, such as p62/SQSTM1, NBR1 and TAXBP1, with their distinct properties are crucial factors for the packaging and subsequent disposal of a wide range of cytosolic entities. However, in addition to receptors, cargo enrichment and the dependence on autophagic scaffold proteins such as Atg11 and FIP200 for its degradation should be considered when proposing and studying selective autophagy pathways. In the following sections, we will critically discuss the current understanding of the selective degradation of various macromolecular complexes by autophagy (Figure 2). We will summarize the current state of knowledge, but also highlight what is known about their enrichment mechanisms and dependence on selective autophagy machinery proteins for their targeted degradation (Table 1).

The Ape1 complex

The discovery of the Cytoplasm-to-vacuole Targeting (Cvt) pathway in yeast in the 1990s had a major impact on the field of cell biology. Much of our understanding of selective autophagy comes from studies performed on the Cvt pathway, which is considered a model pathway for selective autophagy.[24] The primary cargoes of the Cvt pathway include the enzymes aminopeptidase I (Ape1) and α-mannosidase (Ams1). In the cytoplasm, Ape1 is synthesized in a precursor form, prApe1. prApe1 contains an N-terminal propeptide that serves as a signal sequence for its targeting and delivery to the vacuole, where it gets processed by vacuolar proteases into its final active form. prApe1 molecules form dodecamers, and these dodecamers assemble into higher oligomeric complexes, referred to as Ape1 complexes, through hydrophobic interactions of propeptides from different dodecamers.

Atg19, the receptor for the Cvt pathway, binds to Ape1 and disrupts the propeptide self-interaction, as two molecules of Atg19 bind to one propeptide molecule, forming a receptor layer on the surface of the Ape1 complex and recruiting the autophagy machinery for phagophore initiation.[25] Additionally, Atg34 has been suggested as a receptor for Ams1[26] but its role during cargo recruitment has not yet been defined mechanistically. The autophagosomes resulting from Ape1 complex engulfment are smaller in size (∼150 nm) than autophagosomes formed under bulk autophagy, which span a wide range (300–900 nm).[27] Fluorescence microscopy experiments using a chimeric construct consisting of prApe1 and the C-terminus of Atg19 showed that the interaction between Atg19 and the prApe1 not only regulates the size of the engulfed Ape1 complex, but also its degradation. In spite of the Atg8 and Atg11 binding regions being conserved in this chimeric construct, removal of the propeptide binding region of Atg19 yields Ape1 complexes whose size are too large to be degraded by selective autophagy.[25]

Classical EM analysis has described prApe1 correlated autophagosomes as being filled with a dense homogenous protein content.[28] Recent work on Ape1 has demonstrated its ability to undergo LLPS *in vivo* and *in vitro*.[23] *In vitro* purified Ape1 showed the formation of spherical droplets that coalesced over time into larger droplets. These droplets were 1,6-hexanediol sensitive, a feature characteristic of weak multivalent interactions. Similarly, Ape1 complexes in cells dissolved after 1,6-hexanediol treatment, further supporting the liquid-like nature of the Ape1 complex.[23] Fluorescence recovery after photo bleaching (FRAP) experiments revealed different liquid-like properties across the droplet, with higher liquidity at the periphery of droplets compared to a more rigid core. The saturation concentration required for Ape1 droplet formation *in vitro* was determined to be below the physiological concentration of Ape1 found within cells, supporting the idea that Ape1 can form a semi-liquid droplet *in vivo*.[23] The mutant Ape1 P22L, which enhances propeptide self-interaction, was shown to alter the semi-liquid properties of Ape1 towards a more solid state. High-speed atomic force microscopy revealed the formation of highly dynamic honeycomb-like patterns on the surface of wild type Ape1. Both the organization and dynamics of the Ape1 complex were lost for the P22L mutant. Furthermore, it was shown that the organization of Atg19 around the Ape1 condensate was determined by its N-terminal domain which is unable to penetrate into the condensate and therefore restricts it to the Ape1 complex surface.[23] This "floatability" of Atg19 was shown to be required for the delivery of the Ape1 complex to the vacuole and *in vitro* experiments showed that altering the floatability of Atg19 affects its interaction with Atg8.

Additionally, the liquidity of the Ape1 cargo also determines the selectivity of its engulfment, as the Ape1 P22L mutant which is unable to condensate did not allow efficient membrane growth and selective targeting to the vacuole.[23] However, it should also be noted that, besides disrupting the liquidity, the P22L mutation also affected general parameters such as the size and shape of the Ape1 complex as well as its degradation under rapamycin. Although Ape1 is the major component of the Ape1 complex, the liquidity might additionally be modulated by other components of the Cvt pathway. So far, five cellular components have been described as cargo proteins of the Cvt pathway. These are prApe1, Ape4, Ams1, leucine aminopeptidase III (Lap3) and retrotransposon Ty1.[29]

The lack of high-resolution structural data of the Ape1 complex organization leaves room for speculation about how the fluidity of the Ape1 complex is regulated. It is possible that certain components are transported along with the Ape1
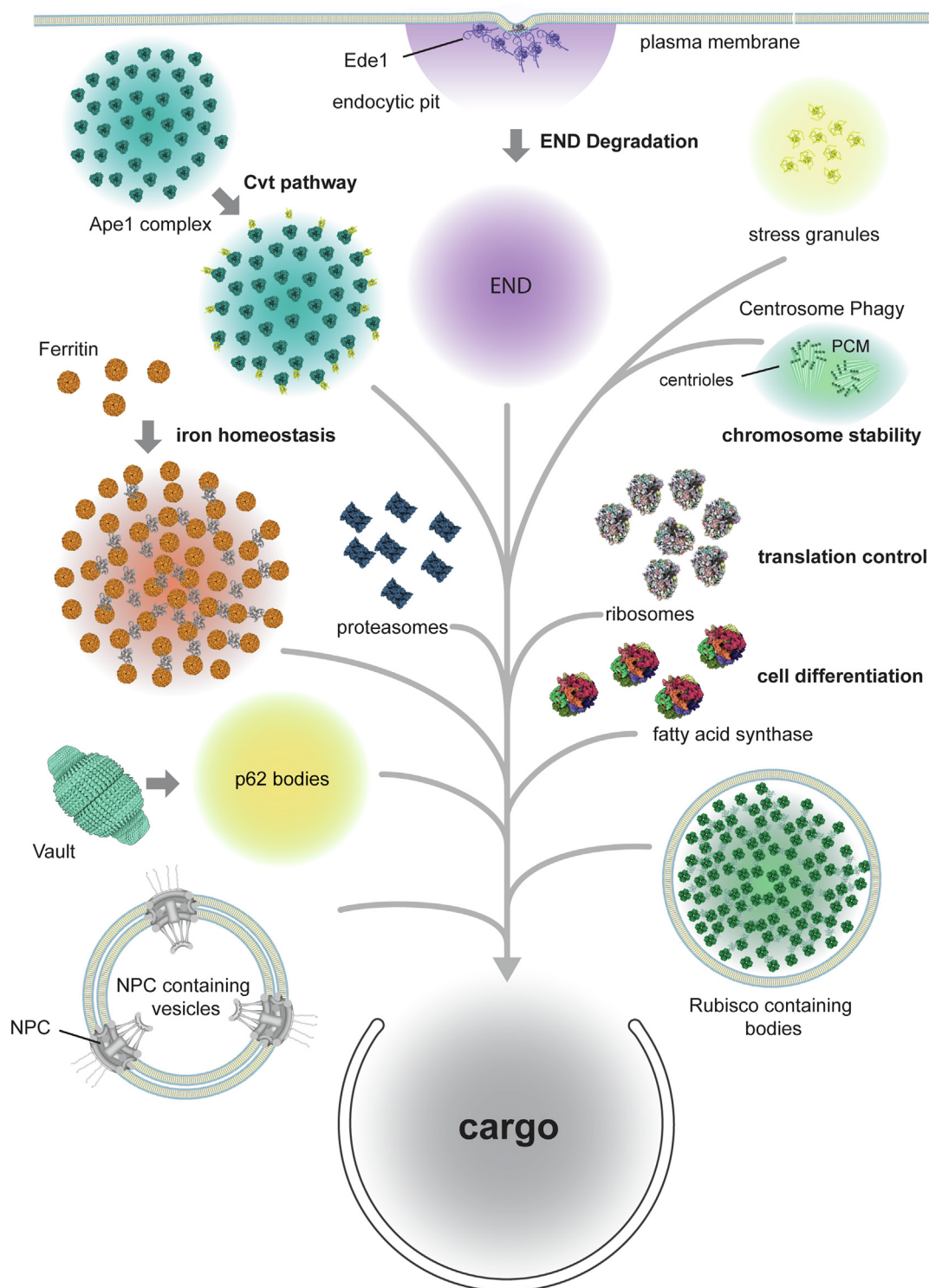
Figure 2. Overview of molecular complexes degraded by autophagy. For selective autophagy, complexes should be enriched into a distinct entity to allow selective growth of the phagophore membrane around them. Cytosolic complexes, such as Ape1, ferritin and END, are found to be enriched by liquid–liquid phase separation. Other complexes, such as Rubisco or the NPC, are engulfed in membranous compartments, such as NPC-containing vesicles or Rubisco-containing bodies. Molecular complexes which undergo LLPS as a cargo enrichment mechanism are shadowed in colors. For some of these complexes, the mechanism of cargo accumulation remains to be elucidated. Molecular complexes are depicted from PDB IDs 7PKZ, 6OLG, 8PRW, 8IOL, 4R8F, 7A6B, 6HWA and AlphaFold IDs AF-B4DZ85-F1, AF-P35193, AF-A0A2K3A85-F1 and AF-P34216.

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Table 1 Summary of macromolecular machines and their features to be degraded by selective autophagy

Cargo	Receptor	Cargo enrichment mechanism?	Requirement of Atg11/FIP200?	Stimulus	Described in	References
Ape1	Atg19	LLPS	Yes	Starvation, mutagenesis Ape1 P22L	Budding yeast	21–25
Centrosome	p62	LLPS?	Unknown	Inhibition of autophagy (Atg5 deletion)	Mammalian cells	76–78
END	Ede1	LLPS	Yes	Nutrient rich conditions	Budding yeast	41
FAS	N/A, direct interaction with Atg8 non-canonical	unknown	No	Cell differentiation compounds such as ATRA	Budding yeast and mammalian cells	58
Ferritin	NCOA4	LLPS	Yes	Iron deficient conditions	Mammalian cells and zebra fish	62–64
NPC	Nup159	unknown	Yes	mTORC inhibition or prolonged starvation	Budding yeast	81,82
Proteasome	RPN10 in plants and Cue5 in yeast	Unknown	Unknown	Proteasome inhibition, MG132	Plants and mammalian cells	48,49
Ribosome	NUFIP1, p62 in mammals?	Unknown	Unknown	Amino acid starvation	Budding yeast and mammalian cells	31,43,45
Rubisco	N/A, direct interaction with Atg8	Unknown, degraded in Rubisco Containing Bodies	Unknown	Leaf senescence	Plants	93–95
Stress granules	p62	LLPS	Yes	Heat-shock, Arsenite-induced stress	Mammalian cells	98,99
Vault	p62	Incorporation into p62 bodies in a NBR-1 dependent manner	Unknown	Bafilomycin A	Mammalian cells	88

complex by interacting with Atg19 at the Ape1 surface or they can be part of the complex in a yet undefined organization.

The ribosome

Ribosomes are large macromolecular machines, which synthesize proteins by translating genetic information from messenger RNA (mRNA) into peptides or proteins. The 80S eukaryotic cytosolic ribosome is composed of a small (40S) and a large (60S) subunit, which together consist of approximately 80 ribosomal proteins and four ribosomal RNAs (rRNA) with a total size of about 3.5 MDa. In dividing cells, ribosomes are highly abundant cytosolic constituents and have been observed inside autophagosomes by electron microscopy.[30,31]

The first evidence for selective ribosome degradation was described in *S. cerevisiae*, where the turnover of the 40S and 60S ribosomal subunits was shown to be an autophagy-dependent process during prolonged nitrogen starvation.[32] The degradation of ribosomes displayed faster degradation kinetics than other cytosolic proteins, and therefore this process was termed ribophagy. Whether or how ribosomes are packaged into a distinct entity during selective autophagy is unknown. Structural analysis of phagophores and autophagosomes under early bulk autophagy conditions using *in situ* cryo-electron tomography has revealed no significant difference in the spacing of individual ribosomes with respect to each other inside and outside of autophagic structures,[18] arguing for a bulk-mediated uptake of cytosolic material including ribosomes. Along these lines, autophagic flux measurements in mammalian HEK293 or HCT116 cells showed a rather bulk flux of ribosome degradation,[33] whereas SILAC-based protein turnover mass spectrometry experiments in human MCF7 breast cancer cells suggest a selective degradation of proteins important for protein translation,[34] arguing for potential differences in ribophagy between cell types.

How are ribosomes selectively targeted for autophagic degradation? A deletion screen of starvation sensitive genes , performed in the study by Kraft et al., identified the deubiquitinase Ubp3 and its cofactor Bre5 as specific ribophagy factors. Deletion of *UBP3* selectively impaired ribosome degradation, while other autophagy cargoes, such as the Cvt pathway cargo Ape1 or Atg8 itself, were not affected, suggesting that ubiquitylation of the ribosome protects it from autophagic degradation.[32] Notably, the deletion of *UBP3* selectively impaired the degradation of the 60S ribosomal particle while degradation of the 40S particle was unaffected,[32] suggesting specific targeting of newly synthesized 60S subunits or partially disassembled 80S ribosomes. In agreement with the differential degradation observed, Rpl25, a ribosomal component of the 60S subunit, was later found to be ubiq-

uitylated by the ubiquitin ligase Ltn1. Ltn1 is a key player in the ribosome-associated protein quality control (RQC) pathway, which in an Ubp3-antagonistic manner protects the 60S from autophagic turnover.[35] Proteins in the RQC pathway facilitate the removal of ribosomes on stalled mRNAs by first splitting the 80S ribosome releasing and degrading the mRNA. The unfinished polypeptide chain in the 60S is then polyubiquitinated by Ltn1 in concert with other RQC components and extracted by the AAA ATPase Cdc48 before being degraded by the proteasome.[36–40] Under nitrogen starvation, the block in translation leads to rapid depletion of the Ltn1 pool, establishing a direct link between the protective role of Ltn1 and ribophagy.[35] Whether 60S ribophagy is directly linked to the RQC pathway is still unclear, as later studies saw no direct relation between ribosomal quality control and 60S ribophagy.[33] However, both share at least one common factor: the ubiquitin-dependent AAA ATPase Cdc48. Cdc48 and its ubiquitin-binding cofactor Ufd3 physically interact with Ubp3, and impairment of the Cdc48/Ufd3 function, similar to that of Ubp3, protects ribosomes from ribophagy.[41] In addition, proteomic experiments revealed that ribosome degradation is strongly induced by arsenite, a proteotoxic stressor, leading to the stalling of translating ribosomes on mRNAs, providing a potential link to the RQC pathway.[42,43] Similar to yeast, treatment with arsenite as well as treatment with reversine, a drug that induces chromosome mis-segregation, showed increased ribophagic flux, which in both cases was strictly dependent on the core-autophagy machinery protein Atg5.[33,44]

In yeast, the receptor that mediates the selective autophagic degradation of ribosomes remains to be elucidated. Analysis of pulldown experiments using Atg8 as a bait have revealed a strong interaction with ribosomal proteins,[45] suggesting that a ribosomal protein or an associated factor may function as an autophagy receptor. In mammals the protein NUFIP1 has been proposed as a receptor for ribophagy. NUFIP1 is a component of the nucleoplasmic R2TP pathway, which is involved in the assembly of 60S ribosomal subunits.[46] In the nucleus NUFIP1 forms a heterodimeric complex with ZNHIT3 or ZNHIT6, and this heterodimeric complex interacts with the R2TP complex. Amino acid starvation in HEK293 cells led to the cytosolic shuttling of NUFIP1-ZNHIT3 from the nucleus, associating with ribosomes in a nutrient-dependent manner.[47] Additionally, NUFIP1 contains a LIR at the N-terminus that mediates its interaction with LC3B, thus exhibiting all the features of an autophagy receptor.[47] The presence of NUFIP or its LC3B-binding competent form was essential for cellular survival under starvation conditions, suggesting an essential role of ribophagy in amino acid recycling.[47] However, in using Ribo-Keima reporters or unbiased proteomic analysis of autophagic turnover, it was shown that in mammals amino acid

starvation or mTOR inhibition promotes ribosome degradation in a mechanism independent of the Atg8 conjugation machinery.[33,48] Later proteomics experiments further showed no evidence of NUFIP1 relocalization from the nucleus to the cytosol. *NUFIP1*^{-/-} HEK293T cells showed no significant difference in the turnover rate of ribosomes measured by proteomics, nor in the processing of Keima tagged RPS3, suggesting no functional role of NUFIP1 in autophagy.[48] A recent study investigated ribosomal turnover during oncogene-induced senescence, a persistent antiproliferative response.[49] The deubiquitinase USP10 was found to dissociate from ribosomes, leading to ubiquitylation of the small ribosomal subunit in particular. Increased ribosomal ubiquitylation is in turn recognized by p62, resulting in autophagic degradation.[49]

It remains unclear whether ribosomes are selectively degraded under conditions such as starvation or arsenite treatment. The enrichment of ribosomes to form a confined entity for autophagic uptake and the receptor mediating its degradation have not been clearly identified in many cases. Additionally, it is uncertain whether selective autophagic scaffold proteins, such as Atg11 or FIP200, are involved in ribosome turnover. Future work addressing these issues will clarify whether ribosome degradation is part of the selective autophagy collection.

The proteasome

The 26S proteasome is a large protein complex responsible for degradation of intracellular proteins. It is made up of two subcomplexes a catalytic core particle (20S) and a regulatory particle (19S) with a total size of about 2.5 MDa. Proteasomes are found both in the nucleus and the cytosol and, alongside autophagy, proteasomal degradation is an additional branch of the proteostasis network.

Different studies mapping the autophagy network or autophagosomal content by mass spectrometry have identified proteasomes as a potential target during autophagy.[50,51] Selective degradation of proteasomes by autophagy was first described in *Arabidopsis thaliana*, where an increase of proteasomal subunits was observed in mutants compromising autophagy.[52] Fluorescence microscopy experiments under nitrogen starvation showed elevated degradation of fully assembled proteasomes in an autophagy-dependent manner, named proteaphagy. Examination of other stress conditions showed that proteaphagy was specifically induced by pharmacological inhibition of proteasomes. Proteasome inhibition, however, did not increase general autophagic flux, arguing for specific induction of proteophagy. Proteasomes and their targets were highly ubiquitylated under these conditions.[53] RPN10, a subunit of the proteasome, which also exists as a non-proteasome bound form,[53] strongly associated with these ubiquitylated forms.

RPN10 was proposed to be a receptor as it binds Atg8 via its ubiquitin-interacting motif 2 and deletion of RPN10 blocks proteaphagy. The selective degradation of proteasomes has subsequently been confirmed in yeast and mammals. In yeast, the ubiquitin-receptor Cue5 was first identified as a receptor recognizing polyubiquitylated aggregated proteins[54] and later has been suggested to degrade in addition ubiquitylated proteasomes upon inhibition.[55] In addition, Hsp42 an oligomeric chaperone important for sequestering misfolded proteins into aggregates has an essential function in proteaphagy. Fluorescence monitoring of proteasomal subunits in Hsp42 deletion cells after proteasome inhibition showed a diffuse cytosolic localization in contrast to aggregated proteasomal subunits in wildtype cells, suggesting that Hsp42 acts in the accumulation of proteasomes for autophagic uptake. Both RPN10 in *Arabidopsis thaliana* and Cue5 in budding yeast are only mediating selective autophagic degradation of proteasomes under proteasome inhibiting conditions.[52,54]

During nitrogen starvation both RPN10 and Cue5 are dispensable for proteaphagy, arguing for a more bulk-dependent process of proteasome degradation by autophagy under these conditions. It has been suggested that mainly soluble proteasomes are degraded under these conditions, which is in line with a role of bulk autophagy.[56] However, as similarly observed in ribophagy, Ubp3 specifically mediates the degradation of the 20S core particle but not the 19S regulatory particle under nitrogen starvation.[57] Only a double deletion of both the bulk and selective autophagy scaffold proteins (Atg17 and Atg11) led to a complete block in proteasome degradation.[58] Mass spectrometry of lysosome-enriched fractions from mammalian cells after proteasome inhibition showed an increase of proteasomal subunits.[59] Native gel analysis showed that proteasomes reach the lysosome intact resembling a similar pathway as previously shown in plants and yeast. However, the receptor targeting proteasomes for degradation under these conditions is unknown. Moreover, next to macroautophagy, microautophagy pathways contribute to the lysosomal targeting of proteasomes under these conditions as Atg5 and Atg7 deficient cells showed only a partial block of degradation. Proteasomes have been shown to accumulate under various stress-related conditions in liquid-like droplets both in the nucleus and the cytosol.[60,61] Moreover, they have been found in other cellular compartments such as the JUxta Nuclear Quality control compartment (JUNQ), a deposit for protein aggregates.[62] Whether any of these compartments is associated with proteaphagy still has to be addressed in the future.

The fatty acid synthase

Fatty Acid Synthase (FAS) is a 2.6 MDa complex that is essential for the *de novo* synthesis of fatty

acids. It has been reported as one of the overexpressed genes in a number of cancers, including leukemia. FAS is composed of two subunits, Fas1 and Fas2, which in yeast assemble into a homo-dodecamer with an equimolar number of subunits.

It has been proposed that upon nitrogen starvation in yeast, all subunits of FAS are degraded by selective autophagy in a process that requires direct interaction with Atg8.[63] In contrast to a classical receptor, the interaction between FAS and Atg8 is not mediated through the canonical binding pockets of Atg8, but through its N-terminus,[63] and so far, no AIM has been described in the sequence of FAS. Similar to yeast, FAS is also frequently found as part of the common autophagic cargoes in mammalian cells.[50] Experiments on differentiation of acute promyelocytic cells have shown that mammalian FAS is at least partially degraded by selective autophagy. Upon treatment with ATRA, an inducer of cell differentiation, and blockage of lysosomal acidification by bafilomycin A, NB4 cells showed accumulation of FAS along with p62 and lipidated LC3B, suggesting the degradation of FAS by autophagy during granulocytic differentiation.[64] High levels of FAS enhance mTOR activity, thus inhibiting autophagy and preventing cellular differentiation. Importantly, inhibition of the FAS catalytic activity had no effect on cellular differentiation, indicating it is not the activity but the enzyme levels which prevent cellular differentiation. The clearance of FAS by autophagy resulted in the activation of lysosome biogenesis genes improving differentiation of granulocytic cells and validating the potential use of these compounds for differentiation therapy.[64]

FAS is often observed as an abundant autophagy cargo, however the role of selective autophagy in the turnover of FAS has not yet been studied in depth and the mechanism of FAS enrichment as well as the factors involved are unknown.

Ferritin

Iron is an essential element for life as it plays a central role in many essential enzymatic reactions, such as the generation of energy by oxidative phosphorylation. To respond to changing iron levels, cells can sequester and release iron upon different signals. The 480 kDa complex ferritin, formed by 24-subunits comprising equimolar amounts of heavy and light chains (FTH1 and FTL, respectively), can store up to 4500 iron atoms.

Ferritin is degraded in response to low iron levels in the cell in order to release these atoms required for essential cellular functions. A proteomics study identified NCOA4 as the receptor for selective autophagy of ferritin, termed ferritino-phagy.[65] Although some reports suggested that ferritin is degraded by the proteasome,[66] autophagy of ferritin was further confirmed by colocalization of ferritin with NCOA4 and LC3B positive puncta, and accord-

ingly, depletion of NCOA4 led to a decrease in targeting of ferritin to lysosomes.[65] The interaction between NCOA4 and ferritin takes place via the heavy chain of ferritin (FTH1). Particularly, NCOA4 residues I489 and W497 and FTH1 R23 were key for this interaction *in vitro*, and for productive ferritino-phagy *in vivo*.[67] The levels of NCOA4 show an inverse correlation with iron levels in order to regulate autophagy: as iron levels decrease, NCOA4 levels increase to promote ferritino-phagy resulting in iron release. NCO4A levels are regulated by the HECT E3 ligase HERC2 via the ubiquitin-proteasome system and by basal autophagy.[67] The interaction between HERC2 and NCOA4 takes place in the same region as FTH1, but is mediated by different residues. A mechanism has been proposed in which iron binding to NCOA4 enhances its binding to HERC2, thus downregulating ferritino-phagy and iron release.[67] On an organismal level, knockdown of NCOA4 in zebrafish exhibited reduced hemin-induced erythroid differentiation, highlighting the role of ferritino-phagy for heme synthesis and erithroid cell development.[67]

In HeLa cells deficient for the selective autophagy scaffold FIP200, ferritin can form membraneless clusters, and a series of experiments showed their liquid-like properties.[68] While the ferritin light chain seems dispensable for the formation of these condensates, co-expression of human FTH1 and NCOA4 in yeast cells confirmed that NCOA4 is required for their formation. The multivalent interactions of NCOA4 (with itself and with FTH1) are essential for the formation of this liquid-like compartment.[68] Fluorescence microscopy and 3D-CLEM experiments showed that ferritin condensates are degraded by autophagy in a piecemeal fashion, engulfing 500 nm sized portion of the ferritin condensate in autophagosomes.[68] TAX1PB1 was found to be dispensable for ferritin-NCOA4 condensate formation but required for autophagy recognition. Of note, macroautophagy-independent degradation of ferritin by endosomal microautophagy was also observed, where a fraction of the condensate is directly internalized into the endosomal system in a process still depending on TAX1BP1.[68] *In vitro*, ferritin condensates become more solid upon increased iron concentrations, suggesting that increased iron availability turns ferritin condensates into a more aggregate-like/insoluble state. Remarkably, less mobile condensates were found to be degraded by non-canonical autophagy in an Atg7-independent manner.[69] These results infer that the physical state of the autophagic cargo might determine its degradation pathway by either macro- or microautophagy.

Clathrin-mediated endocytosis machinery

Clathrin-mediated endocytosis (CME) is an essential cellular process that enables the transport of a wide range of cargo molecules from

the cell surface to the interior and relies on the concerted assembly and disassembly of a complex protein machinery. More than 60 different proteins assemble at the plasma membrane in a highly interconnected network that enables the production of clathrin-coated vesicles.[70,71]

The key initiator protein of CME in *S. cerevisiae* is Ede1, a homologue of mammalian Eps15, which acts as a scaffold for early CME assembly. During initiation, Ede1 accumulates at the plasma membrane through phase separation and recruits downstream machinery to these sites.[45,72,73] Its ability to phase separate is conserved across budding yeast, plants and humans, and is essential for the efficient production of clathrin-coated vesicles at the plasma membrane.[74,75] Increasing the Ede1 concentration or genetic manipulation of the downstream machinery leads to accumulation of Ede1 at the plasma membrane in stable condensates, termed Ede1-dependent protein deposits (END).[45] ENDs have been observed in both yeast and plant cells and triggers recruitment of the autophagy machinery.[45,76] In turn, this leads to the degradation of ENDs by selective autophagy.

It has been demonstrated that Ede1, in addition to its role as an initiator and phase separation driver during CME, also functions as a receptor for the removal of aberrant CME assemblies.[45] Ede1 contains a C-terminal AIM-rich region that is essential for interaction with Atg8. In addition, pull-down experiments showed that Ede1 interacts with the fourth coiled-coil domain of the autophagy scaffold Atg11, a domain required for binding other known receptors.[45] Deletion of Atg11 blocks END degradation under nutrient-rich conditions, further confirming the selective nature of the pathway. Live cell microscopy and correlated cryo-electron tomography revealed that END condensates are degraded in a piecemeal fashion similar to ferritin-NCOA4 condensates. Interestingly, correlated cryo-electron tomography further revealed that ENDs can be degraded in an exclusive manner during selective autophagy under rich conditions or as a product next to other cytosolic constituents during bulk autophagy. Notably, both modes of degradation require the interaction of Ede1 and Atg8.[45]

The centrosome

Centrosomes are essential cellular organelles that play a crucial role in the organization of the cytoskeleton during cell division. In animal cells, centrosomes consist of a pair of centrioles surrounded by an amorphous zone of pericentriolar material from which small membrane-less granules known as centriolar satellites extend.[77,78]

Autophagy contributes to the regulation of the centrosome number by centrosome-phagy. Healthy cells normally contain 1 or 2 centrosomes. Centrosome abnormalities have often been observed in several types of cancer, and misregulation of the number of centrosomes is known to lead to defects in chromosome segregation and genomic instability.[74,79] Deletion of the core autophagy machinery protein Atg5 in mouse embryonic fibroblasts showed an increase in centrosome number.[80] The centrosomal protein of 63 kDa (Cep63), which is involved in the initial steps of centriole duplication, has been subsequently shown to interact directly with the receptor p62, removing premature centrosomes.[80] Accordingly, deletion of p62 or Atg7 in mice increased the number of centrosomes observed in erithroid cells,[80] placing autophagy as a regulator of centrosome number.

Another type of autophagy involving centrosomes is doryphagy, which refers to selective turnover of centriolar satellites.[81] Misregulation of centriolar satellites results in defects in DNA segregation and postmitotic cell death. Doryphagy is mediated through a direct interaction between a C-terminal LIR motif of the satellite organizer PMC1 and the GABARAP proteins that regulates their selective degradation. Interestingly, the degradation of centriolar satellites is GABARAP specific and does not depend on LC3 proteins.[81] In contrast, Cep131, another resident protein of centriolar satellites, interacts with LC3, indicating that more than one centriolar satellite protein could regulate their degradation.[82]

Autophagy is also involved in the regulation of centrosome stability of mid-body derivatives after cytokinesis, with the centrosomal protein Cep55 binding to the receptor NBR1 and targeting selective degradation of these compartments in order to promote cell differentiation.[83] Highlighting the role of autophagy in the maintenance of cellular homeostasis, inhibition of autophagy of centriolar satellites or mid-body derivates prevents cell differentiation and enhances tumorgenicity of cancer cells.[84] Although still a matter of debate, a common feature of centrosomes with other macromolecular complexes described throughout this review is that they are also suspected to undergo LLPS.[85] This behavior has been suggested particularly for proteins of the pericentriolar material. Whether the liquid (e.g. mobile) phase properties of these regions surrounding the centrioles can regulate their degradation by autophagy, similar to what is known for other macromolecular complexes such as ferritin, remains to be addressed experimentally.

The nuclear pore complex

The nuclear pore complex (NPC) is a gigantic proteinaceous pore that spans across the inner and outer nuclear membrane (60–110 MDa from yeast to human). It consists of ~550 to 1000 protein subunits and allows bidirectional transport of large macromolecules in and out of the nucleus. In *S. cerevisiae*, NPC degradation has been linked to autophagy in addition to the proteasome in a process called NPC-phagy.[82,87] In this process,

NPC-containing vesicles originating from the nuclear envelope are pinched off from the nucleus into the cytosol and subsequently engulfed by autophagy. NPC-phagy is induced by pharmacological TORC1 inhibition or prolonged nitrogen starvation. The proposed receptor during NPC-phagy is Nup159[86], the largest cytosolic filament protein of the NPC, aspulldown experiments using Atg8 as bait revealed Nup159-dependent binding of NPC components.[86] Nup159 contains an AIM that mediates its interaction with Atg8 and leads to degradation of certain nucleoporins by selective autophagy.[82,87] While some NPC components appear to be dependent on Nup159, others have been found to be independent of the Nup159-Atg8 interaction.[87] This has led to the idea that Nup159 is mainly responsible for the degradation of soluble NPC components in a process called nucleoporinophagy. NPC-phagy was shown to be at least partially dependent on the autophagy scaffold protein Atg11, and deletion of the receptor binding site in Atg11 also impaired Nup159 binding in coimmunoprecipitation experiments. It is possible that there is more than one receptor that determines the degradation of NPCs by selective autophagy. Enrichment of NPCs at the nuclear envelope by NPC clustering appears to be a prerequisite for NPC-phagy. This was demonstrated in strains deleted for the y-complex (outer ring) nucleoporins Nup120 or Nup133, which display a constitutive NPC clustering phenotype[84–90] and show increased turnover of NPCs by selective autophagy.[86] Interestingly, similar to ENDs, the receptor is part of the complex and each NPC has Nup159 incorporated, raising the question of how selective turnover of certain NPCs is activated and regulated to avoid constant degradation of this molecular machine. Future studies will need to address which factors and signals induce NPC turnover.

The vault complex

Vaults are large ribonucleoprotein particles (13 MDa) found in the cytoplasm of eukaryotic cells. Vaults have a distinct barrel-like shape, with the major vault protein forming the cylindrical framework. The barrel encloses a central space and is capped at one end by proteins like telomerase associated protein (TEP1) and vault-poly (ADP-ribose) polymerase (VPARP). The central region of the barrel remains largely hollow, creating an internal space where other molecules, such as small RNAs, may be enclosed. Despite being discovered in the 1980s, the exact biological role of vaults remains a subject of ongoing research. Functionally, vault proteins have been related to cancer resistance as they show overexpression in multi-drug resistance tumors, as well as to intracellular transport related to the NPC.

By providing nutrients under stress conditions such as hypoxia or starvation, autophagy may be beneficial for the survival of cancer cells. Vault

RNA101, an RNA found inside the Vault complex, was shown to directly bind p62 preventing its oligomerization and therefore disrupting p62-dependent autophagy.[87,92] Interestingly, the major vault protein was found by mass spectrometry as a component of p62 bodies during fluorescence-activated particle sorting purification of p62 bodies from human cell lysates.[93] p62 bodies describe a phase-separated compartment of ubiquitylated proteins with p62 as the major constituent. Quantitative proteomics of mouse tissues defective in selective autophagy showed that vault can be a selective autophagy cargo. The targeting of vault to p62 bodies requires the interaction of the major vault protein with the receptor NBR1. The interaction is mediated between the loop region in the shoulder domain of the major vault protein and the ubiquitin-associated domain of NBR1, recruiting vault for p62-dependent degradation via selective autophagy. This enrichment of vault correlated with accumulation of p62 and NBR1 in Mallory-Denk bodies in a distinct form of hepatocellular carcinoma.[93]

Rubisco

Autophagy seems also to play a role in the degradation of protein complexes in plants. Rubisco (Ribulose-1,5-bisphosphate carboxylase/oxygenase) is an essential enzyme responsible for photosynthetic carbon assimilation. This 540 kDa octameric enzyme complex alone accounts for a significant percentage of the total leaf protein mass. During leaf senescence and unfavorable environmental conditions, Rubisco gets degraded in the vacuole releasing nutrients that are recycled towards other organs of the plant or stored inside seeds.

In line with other molecular complexes, Rubisco has been shown to form condensates in pyrenoid organelles in the model alga *Chlamydomonas reinhardtii*[90,95] and in carboxysomes in the cyanobacterium *Syneschococcus elongatus* PCC 7942.[96] In plants, the degradation of Rubisco happens after it has been released from the chloroplast in what is known as Rubisco-containing bodies.[97] Rubisco-containing bodies pinch off from the chloroplast as a membrane enclosed vesicle containing only a fraction of the chloroplast stroma. Rubisco-containing bodies seem to be degraded by autophagy since Atg5 deletion mutants did not show accumulation of such bodies in the vacuole. Reinforcing this, it was shown that Atg8 and a stroma marker accumulated in autophagic bodies in the vacuole.[97] The formation of Rubisco-containing bodies seems to be highly correlated to the availability of carbon in the leaf, as sugars and the presence of starch inhibited their formation.[98] The process of Rubisco-containing body degradation, which takes place in the early stages of leaf senescence,[99] is different from chlorophagy: autophagy of whole chloroplasts. As opposed to the degradation of Rubisco-containing bodies, chloro-

phagy does not seem to be affected by the lack of nutrients but is rather a mechanism for the degradation of whole chloroplasts damaged by photooxidative stress.[100]

Further research is needed to understand the molecular mechanism underlying the selective degradation of Rubisco. The key difference from other autophagic degradation pathways described here is that Rubisco is concentrated in a membrane-enclosed vesicle. How these Rubisco-containing bodies are recognized and signaled for autophagic degradation is still unknown. It may be that, as is the case for membranous organelles such as mitochondria, the receptor is a constitutive or recruited component of the outer membrane subdomains of chloroplasts that is recognized by Atg8 and the scaffold proteins for specific targeting of Rubisco for degradation.

Stress granules

Stress granules are dynamic membraneless organelles formed in the cytoplasm of eukaryotic cells under stress.[101] They are composed of mRNAs associated with translation initiation factors and other RNA binding proteins. Stress granules regulate mRNA expression levels by sequestrating non-translating RNAs, restricting their availability and lifetime in the cell. Like other protein complexes covered in this review, stress granules are formed by LLPS, which is largely mediated by the stress granule assembly factors G3BP1 and G3BP2.[101]

In a microscopy-based deletion screen of non-essential yeast genes, loss of core autophagy proteins led to increased accumulation of cytoplasmatic stress granules.[102] Furthermore, triggering the accumulation of mRNAs by decreased enzymatic decapping activity led to enhanced degradation of stress granules as seen by their accumulation in *ATG15* deletion mutants, which cannot breakdown the autophagic body membrane in the vacuolar lumen. A similar pathway has been observed in mammalian cells, where autophagy appears to play a role in the degradation of stress granules.[102] Moreover, selective autophagy of stress granules is dependent on the AAA ATPase Cdc48 in yeast or its homologue VCP in mammalian cells.[102] A colocalization screen of E3 ubiquitin ligases present in stress granules identified TRIM21, and found that ubiquitination of G3BP1 inhibits its phase separation behavior *in vitro*,[103] implicating ubiquitin as a negative regulator of stress granule phase separation. This is interesting in the context of selective autophagy, as it requires enrichment of the cargo prior to successful engulfment. Thus, ubiquitylation of G3BP1 could interfere with the selective autophagy pathway by dissolving stress granules. However, the same study identified the proteins CALCOCO2 and p62 in the periphery of stress granules as receptors mediating their degradation under arsenite-induced stress.[103] This places ubiquitin at different key regulatory steps.

Recent work has shown how the fate of stress granules depends on their longevity in the cell and on the stimulus that induced their formation.[104] Under heat shock stress conditions, the ubiquitylation of G3BP1 also induces stress granule disassembly by lowering the local concentration below the percolation threshold. Ubiquitylated G3BP1 bound the ER-associated adaptor for the segregase p97/VCP FAF2, which was essential for efficient stress granule clearance.[104] Furthermore, short heat shock treatments resulted in autophagy-independent clearance of stress granules. Only after prolonged heat shock (>90 min) clearance was autophagy dependent, highlighting a role for selective autophagy in the removal of persistent protein condensates.[104] How ubiquitination mechanistically regulates disassembly and degradation of stress granules remains to be elucidated, as stress granules are likely to be degraded in a piecemeal fashion during selective autophagy, similar to other condensates.

Finally, studies in *C. elegans* have shown that P granules, specialized ribonucleoprotein compartments in the oocyte, are also degraded by autophagy during embryonic development.[105] Disruption of autophagy induces the formation of intracellular aggregates, known as PGL granules, containing the protein PGL-1/-3. PGL granules are LLPS condensates whose liquidity is regulated by the scaffold protein EGPG2 and by the receptor protein SEPA-1.[106] The timely expression of these proteins during embryonic development allows the degradation of PGL granules.[105] At a higher regulatory level, mTORC also regulates the liquidity of PGL granules, conferring them resistance against heat stress under which they cannot be degraded by autophagy.[106]

Conclusion

Selective autophagy is an important part of the proteostasis network by targeting the degradation of specific cellular components upon need. This selectivity enables the degradation of almost any damaged or unwanted structure in the cell. During the last years we have started to understand better the factors that determine the degradability of autophagic cargo. However, the mechanisms of cargo enrichment and the properties of these cargoes are often understudied.

The selective degradation of organelles is determined by their membrane boundary, which provides at least a partially deformable surface on which phagophore templating can take place. Factors on the membrane surface can laterally diffuse, which is an essential feature for avid interactions between receptors and the autophagy machinery.

For some of the cytoplasmic macromolecular complexes described in this review, concentration by LLPS is key to forming an entity that can be
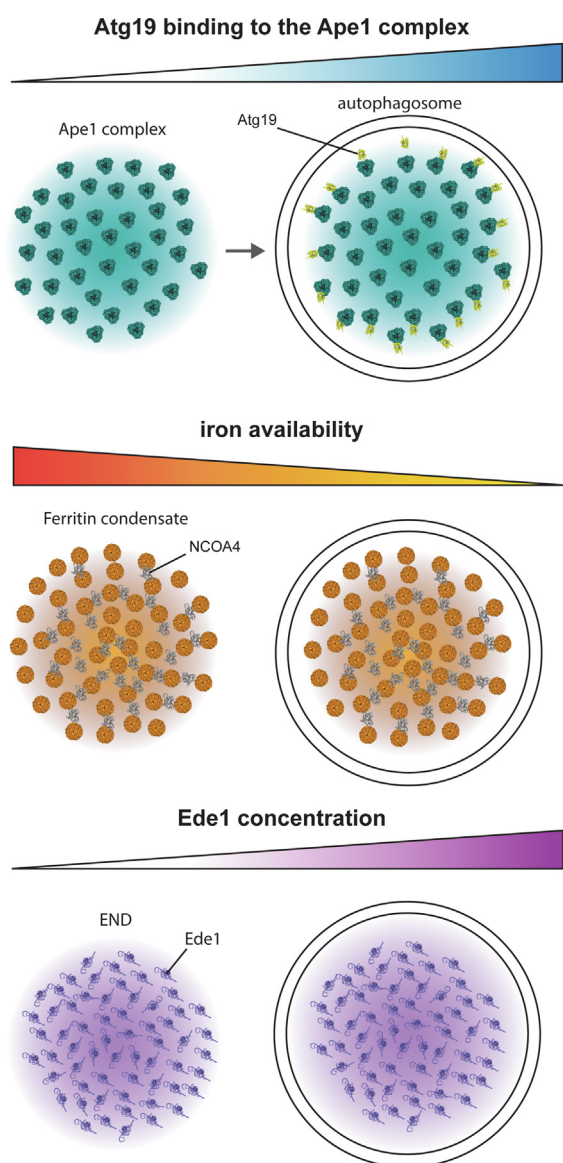
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Figure 3. Mechanism of cargo concentration of different macromolecular complexes. Top: Ape1 dodecamers form a large super assembly referred to as the Ape1 complex which gets targeted by the receptor Atg19. The Ape1 complex forms a liquid-like droplet close to the vacuole with similarities to LLPS. The receptor Atg19 interacts with Ape1 dodecamers at the surface and by this regulates its selective degradation by autophagy. Middle: Ferritin also forms large liquid-like compartments. The fluidity of these compartments is regulated by the availability of iron. Low iron recruits NCOA4 the receptor for selective ferritino-phagy to ferritin. Bottom: Ede1 accumulation during non-productive clathrin-mediated endocytosis serves as a signal for selective autophagy. Ede1 acts as an intrinsic receptor for recruiting the autophagy machinery to aberrant endocytic sites referred to as END, thus removing aberrant endocytic sites from the plasma membrane by selective autophagy.

engulfed by selective autophagy. Such phase transitions have been experimentally demonstrated for ferritin, stress granules, END and Ape1 (Figure 3). Others are still under discussion, such as for centrosomes, or are still being elucidated, such as for ribosomes, proteasomes or FAS. The low affinity interactions of factors within a condensate provides a dynamic and deformable surface on which a growing phagophore can establish the necessary avidity between the cargo and the autophagy machinery. In addition, phase transitions can represent a regulatory step in the process, e.g. triggered by an increase in cargo concentration, as in the case of END. Extracellular stresses are also known to induce phase separation, as in the case of stress granule formation. These LLPS condensates normally dissolve rapidly after stress relief, but persistent stress often triggers their solidification, which could be counteracted by prior removal through selective autophagy. In the future, defining the properties of autophagic cargo by various biophysical methods will be needed to clarify its enrichment mechanism and the factors that modulate it. Moreover, the new developments in structural biology will allow us to monitor and visualize autophagic cargo and its factors directly in the native environment of the cell. Understanding the properties of selective autophagy cargo and its removal will also allow to develop strategies to selectively remove factors during human diseases and open up new therapeutic treatments based on cargo enrichment via modulation of its biochemical properties.

CRediT authorship contribution statement

Javier Lizarrondo: Writing – review & editing, Writing – original draft. **Florian Wilfling:** Writing – review & editing, Writing – original draft, Funding acquisition, Conceptualization.

DECLARATION OF COMPETING INTEREST

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Abbreviations:
AIM, Atg8 interacting motif; **AAA ATPase**, ATPases Associated with Diverse Cellular Activities; **ADP**, Adenosine diphosphate; **ATG**, Autophagy-related; **Cvt**, Cytoplasm to vacuole targeting; **END**, Ede1-dependent protein deposits; **ER**, Endoplasmic reticulum; **FAS**, Fatty acid synthase; **FRAP**, Fluorescence recovery after photobleaching; **HEK**, Human Embryonic Kidney; **HECT**, Homologous to the E6-AP Carboxyl terminus; **HCT116**, human colon cancer cell line 116; **JUNQ**, JUxta Nuclear Quality control compartment; **kDa**, kilodalton; **LIR**, LC3 Interacting Region; **LLPS**, Liquid-liquid phase separation; **MDa**, MegaDalton; **mRNA**, Messenger ribonucleic acid; **NPC**, Nucleapore complex; **PGL**, P granule abnormality protein; **RCBs**, Rubisco Containing Bodies; **RQC**, Ribosome-associated protein quality control; **rRNAs**, ribosomal ribonucleic acids; **SEPA**, Suppressor of ectopic P granules in autophagy; **SILAC**, Stable Isotope Labeling by/with Amino acids in Cell culture; **TORC1**, Target of Rapamycin Complex 1

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