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Dynamic regulation of macroautophagy by distinctive, ubiquitin-like proteins

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Abstract

Autophagy complements the ubiquitin-proteasome system in mediating protein turnover. Whereas the proteasome degrades individual proteins modified with ubiquitin chains, autophagy degrades many proteins and organelles en masse. Macromolecules destined for autophagic degradation are “selected” through sequestration within a specialized double-membrane compartment termed the “phagophore”, the precursor to an “autophagosome”, and then hydrolyzed in a lysosome/vacuole-dependent manner. Notably, a pair of distinctive ubiquitin-like proteins (UBLs), Atg8 and Atg12, regulate degradation by autophagy in unique ways, by controlling autophagosome biogenesis and recruitment of specific cargos during selective autophagy. Here we review structural mechanisms underlying functions and conjugation of these UBLs that are specialized to provide interaction platforms linked to phagophore membranes.

Macroautophagy (hereafter autophagy) is a predominant eukaryotic mechanism for mediating bulk degradation of cellular constituents. First, cytoplasmic components destined for degradation by autophagy are encapsulated within a double-membrane sequestering compartment termed the phagophore (**Fig. 1**)^{1,2}. During this process, the phagophore expands to generate an autophagosome, which transports its sequestered cargo to a degradative organelle—either a lysosome in higher eukaryotes or the vacuole in yeast and plants. Ultimately, the autophagosome fuses with the lysosome or vacuole, and the inner vesicle and cargo of the autophagosome are exposed to lytic hydrolases, resulting in degradation. Breakdown is followed by release of the resulting macromolecules into the cytosol for reuse as biosynthetic building blocks or as sources of energy.

Autophagy can be either selective or nonselective. Both selective and nonselective autophagy are carried out via a common overall mechanism directed by the core autophagy machinery made up primarily of autophagy-related (Atg) proteins (**Box 1**). Nonselective autophagy is typically induced by nutrient starvation or certain types of stress. Selective autophagy involves sequestration of specific cargo through recruitment to the phagophore by

receptors and scaffold proteins³ (**Fig. 2**), and has been implicated in a greater range of physiological roles. For example, selective degradation of peroxisomes by autophagy, pexophagy, is used to allow yeast cells to adapt to changing nutrient conditions⁴. The same is true for the selective elimination of mitochondria by autophagy, mitophagy, although this process also occurs when the organelle is damaged^{5,6}. Mitochondria play important roles in energy production, but they also generate reactive oxygen species that can be deleterious to the cell. Accordingly, dysfunctional mitochondria need to be efficiently removed before they cause cell death, and this occurs through mitophagy. Importantly, recruitment of selected cargo to the phagophore and subsequent degradation is not restricted to “self components”, as invasive microbes can also be targeted for sequestration by the phagophore in a process termed xenophagy⁷.

In fungi, 38 Atg proteins have been identified, a remarkable number of which are associated with the two autophagy-specific ubiquitin-like proteins (UBLs). Notably, the autophagy UBLs promote macromolecular degradation in a manner completely distinct from that of ubiquitin in the proteasome pathway⁸⁻¹⁰. One of these UBLs, Atg8 (in yeast), is ligated to a primary amine from the head-group of a lipid, typically phosphatidylethanolamine (PE) or in some cases, phosphatidylserine, which is incorporated into the double-membrane phagophore that is destined to become the autophagosome. This distinctive membrane is decorated on both sides by Atg8-PE, which plays numerous roles in controlling degradation by autophagy. First, Atg8-PE regulates expansion of the membrane compartment itself¹¹; there is a correlation between the amount of Atg8 ligated and the size of the autophagosome¹², although our present knowledge concerning the mechanism by which this occurs remains rudimentary. Although it is possible that Atg8 conjugated to PE (Atg8-PE) acts via a *trans* self-interaction to drive hemifusion of intermediate vesicles¹³, studies with physiological concentrations of PE suggest that Atg8 may not function in this manner, but may instead play a role in tethering vesicles that contribute to phagophore biogenesis¹⁴. Current thinking is that Atg8 ligation to PE, a cone-shaped lipid that destabilizes the bilayer, influences the fluidity of the membrane, enabling its expansion. Furthermore, Atg8-mediated assembly or disassembly¹⁵ of protein complexes at the membrane, and recruitment of specific enzyme activities, also likely influences autophagosome formation, although future studies will be required to elucidate mechanistic details of this process. There are two subfamilies of Atg8 homologs in mammals, MAP1LC3 (hereafter LC3) and GABARAP, composed of at least six members. In general, common overall structural mechanisms seem to underlie conjugation and function of yeast Atg8 and mammalian LC3 and GABARAP family members. Although different functions of the various mammalian Atg8 orthologs are only beginning to emerge, LC3A, LC3B and LC3C have been proposed to function at an early step of phagophore expansion, whereas GABARAP, GABARAPL1 and GABARAPL2/GATE-16 may act at a later step of autophagosome maturation¹¹.

The Atg8 and LC3/GABARAP UBLs play additional key roles in autophagy. Atg8-PE (and LC3/GABARAP-PE) serves as a protein interaction platform that is physically linked to the phagophore membrane¹⁶. Like ubiquitin and other UBLs, Atg8 has two domains. One domain is the C-terminal tail, which is covalently linked to the membrane. The other domain is globular, with a β -grasp fold; this domain mediates a plethora of protein-protein

interactions. Atg8-binding partners include other components of the core autophagy machinery, proteins involved in vesicle regulation, and selective autophagy receptors bound to various cargo. Notably, many of the best-understood functions of Atg8 and LC3/GABARAP surround their roles in initiating cargo recruitment into the forming autophagosome.

The second autophagy-specific UBL, Atg12, comprises a portion of the E3 ligase that links Atg8 and family members to lipids¹⁷. Atg12 is conjugated to Atg5¹⁰, which noncovalently binds Atg16; the latter drives oligomerization of the ternary complex¹⁸. In addition to acting as an E3 ligase, Atg12–Atg5–Atg16 functions in part to determine the subcellular site of Atg8 conjugation to PE¹⁹.

Below we describe recent progress in understanding the biochemical and structural mechanisms by which these UBLs function in autophagy. We first discuss how Atg8 and Atg12 mediate protein-protein interactions that are critical for autophagy. Next, we describe how they become ligated to their targets. Finally, we briefly consider some of the roles of autophagy UBLs in physiology and health.

Atg8-mediated protein interactions in autophagy

A key function of Atg8 is to bind proteins regulating or regulated by autophagy, targeting them to their site of action at the membrane²⁰. In the last few years, our understanding of the role of Atg8/LC3-dependent recognition of partner proteins, and the overall significance of these interactions, has expanded tremendously. Proteins are often recruited to the phagophore by a distinctive “Atg8 interacting motif” (AIM, named for yeast proteins) or “LC3-Interacting Region” (LIR, named for proteins from higher eukaryotes) sequence²¹. The AIM/LIR is generally characterized by sequences resembling WXXI/L, where W is Trp and X is any amino acid. Ile (I) or Leu (L) are typically observed as the 3rd residue downstream of Trp²² (**Fig. 3**).

A key function of AIM or LIR sequences is apparently to recruit components of the autophagy machinery to phagophores. These include the Atg1/ULK1 and Atg13 regulators of autophagy induction, and potentially also the Atg4 and Atg7 regulators of Atg8 conjugation to and deconjugation from PE^{20,23-26}. Another intriguing LC3 partner is FYCO1, which contains both a LIR and a PtdIns3P-binding (FYVE) domain important for autophagosome movement²⁷. Several other LIR-containing proteins identified as being involved in autophagy regulation include TP53INP1, TP53INP2, MAPK15 and TBC1D5²⁸⁻³¹.

At this point, Atg8- and LC3-binding by AIM and LIR motifs is generally best characterized for roles in targeting particular cargos for destruction by selective autophagy. Selective autophagy mediated by Atg8/LC3 serves numerous regulatory functions, including, playing roles in biosynthetic delivery, nutrient- or damage- or pathogen-induced degradation, and regulation of autophagic flux^{20,32,33}. For example, the yeast cytoplasm-to-vacuole targeting (Cvt) pathway is a biosynthetic variant of autophagy in which Atg8 acts to link the cargo with the phagophore via its interaction with a specific receptor (**Fig. 2**)³³. Similarly, during nutrient-dependent or damage-induced turnover of organelles, Atg8 again links the cargo to

the rest of the autophagic machinery through direct binding of multidomain receptors that are part of, or are attached to, the cargo (**Fig. 2**)³⁴⁻³⁶. In addition to an AIM or LIR motif, autophagy receptors typically display at least one other domain that binds cargo directly or indirectly as part of a complex or organelle. Some AIM- or LIR-containing receptors display a ubiquitin-binding motif, mediating recruitment of ubiquitinated proteins to autophagosomes³⁷. Examples of this are seen with SQSTM1/p62³⁸, NBR1³⁹, CALCOCO2/NDP52⁴⁰ and OPTN/optineurin⁴¹. OPTN can also function in a ubiquitin-independent manner⁴². Other autophagy receptors recruit cargo to distinctive protein-interaction domains. For example, STBD1 is a LIR-containing protein that binds both glycogen and GABARAPL1⁴³, whereas the yeast AIM motif protein Atg19 recognizes the prApe1 propeptide, and both Atg19 and Atg32 bind the scaffold Atg11 in addition to Atg8.

Recruitment of a variety of related AIM/LIR sequences to Atg8 and orthologs

Atg8 and its orthologs, although sharing a high degree of similarity, are thought to be able to function in distinct stages of autophagy and recognize particular cargos due to variations in the AIM/LIR binding motifs, which are present in receptor and scaffold proteins. Atg8 family members mediate interactions with the AIM/LIR via their β -grasp fold globular domain. Relative to ubiquitin and canonical UBLs, Atg8 family members also display two additional N-terminal α -helices that pack as part of the globular domain (**Fig. 3a, b**). The molecular basis for canonical AIM/LIR motif binding to Atg8 or LC3B was revealed by several structures including Atg8 complexes with Atg19 (**Fig. 3c**) and Atg32, and a complex between LC3B and SQSTM1/p62 (**Fig. 3d**)^{22,38,41,44}. The AIM/LIR WXXI/L sequence forms an extended structure that becomes a fifth, parallel edge strand in an intermolecular β -sheet. Binding is anchored through interaction with the second β -strand from Atg8/LC3, and through burial of hydrophobic residues from the AIM/LIR motif in two Atg8/LC3 pockets. The N-terminal Trp binds a conserved hydrophobic pocket at the interface between the unique helical extension and β -grasp domain, and the downstream Ile or Leu binds a distinct hydrophobic pocket between the 2nd β -strand and its adjacent helix (**Fig. 3c, d**). Notably, although Atg8 and LC3 interact with a wide range of WXXI/L sequences, intervening and adjacent residues can also make important interactions. For example, in many cases nearby acidic residues bind basic side-chains adjacent to the Atg8/LC3 hydrophobic pockets as seen in the LC3B complex with SQSTM1/p62 (**Fig. 3d**)^{22,38,41,44}.

Several variations on the LIR sequence have been observed. For example, the autophagic receptor NBR1 displays a LIR sequence of YIII. A GABARAPL1-NBR1 LIR complex structure revealed all four hydrophobic residues in the peptide interacting with GABARAPL1 (**Fig. 3e**), with the Tyr docking in the pocket that typically binds a LIR motif Trp⁴⁵. However, a Tyr-to-Trp mutation increases binding by an order of magnitude, raising the possibility that NBR1 interactions with Atg8 family members may be regulated or influenced by other factors.

Insights into Atg8-ortholog specificity and regulation of LIR binding come from a slew of recent studies on selective autophagy restriction of infection by *Salmonella enterica* serovar Typhimurium. When *Salmonella* damage the limiting membrane of their vacuoles and thus

escape their niche inside phagosomes, autophagy destroys the cytoplasmic bacteria⁴⁶. The importance of selective autophagy is reflected by three different receptors recruiting various features of Salmonella or its associated damaged vesicle to Atg8 orthologs. One of these, CALCOCO2/NDP52, is lured to broken Salmonella-containing vacuoles through a domain that binds LGALS8/galectin 8, a lectin that recognizes glycans exposed through vesicle damage⁴⁷ (**Fig. 2**). CALCOCO2/NDP52 is specific for LC3C, displaying a variant “CLIR”, LC3C-specific LIR, of sequence Ile-Leu-Val-Val⁴⁸. The crystal structure of a CLIR-LC3C complex revealed binding via an intermolecular β -sheet between the CLIR and the β 2-strand of LC3C (**Fig. 3f**). However, the CLIR does not occupy the deep hydrophobic pocket that normally binds the Trp in a canonical LIR, and only LC3C apparently can compensate.

A different autophagy receptor recognizing Salmonella requires regulated “phosphor-LIR-motif” recruitment. Cell wall components from cytosolic Salmonella activate the protein kinase TANK-binding kinase 1 (TBK1), which in turn phosphorylates Ser177 upstream of the LIR motif in OPTN⁴⁹. OPTN also displays a ubiquitin binding domain that recognizes Salmonella that have escaped their vacuoles and are ubiquitinated in the cytoplasm (**Fig. 2**). TBK1-mediated phosphorylation promotes OPTN interaction with LC3B, to bridge ubiquitin-coated Salmonella with phagophores for clearance via autophagy. Structures showed that upstream of the LIR, the phosphate is embraced by Arg11 from LC3B's N-terminal helical domain and Lys51 from LC3B's β 2-strand (**Fig. 3g**)⁵⁰. Conservation of these basic residues among Atg8 orthologs raises the possibility that other phospho-dependent LIRs may remain to be discovered.

An interesting twist to potential sequences recognizing Atg8 orthologs came from a phage display approach that revealed GABARAP-binding peptides, including a high-affinity interactor with sequence DATYTW⁶EHL⁹AW¹¹P (superscripts denote residue numbers)⁵¹. A crystal structure showed that the peptide binds in an inverse orientation relative to canonical Atg8/LC3-AIM/LIR sequences, with Leu9 occupying the Ile/Leu pocket, but the more C-terminal Trp11 occupying the canonical Trp-binding pocket (**Fig. 3h**). The upstream Trp6 strengthens the interaction by docking via a conserved mechanism. It will be interesting if other AIM/LIRs will be found to bind in this reversed LXW orientation. Interestingly, ubiquitin and SUMO bind ubiquitin-interacting and SUMO-binding motifs, respectively, from N to C terminus or in reversed orientation, depending on sequence context⁵².

The global importance of LIR-binding was revealed by systems-level in vitro affinity isolation assays in which mutation of LIR docking sites of GST-tagged GABARAP and LC3B reduced interaction with roughly 60% and 38%, respectively, of the 34 interacting proteins tested²³. Interestingly, in co-IPs from cells, only about half of tested interactions depended on the Atg8 ortholog's ability to be conjugated to membranes, with some differential effects for different orthologs. For example, SQSTM1 interacts with LC3 irrespective of its ability to be conjugated, but conjugation of GABARAP is required for the interaction. Thus, there appears to be a complex “code” for how and where particular LIR-motif containing proteins are recognized by different Atg8 orthologs during the orchestration of autophagy (**Fig. 3i**).

Atg4 regulates both Atg8 conjugation and deconjugation

Atg8 and its orthologs are synthesized as inactive precursors with a C-terminal extension. Atg4-mediated cleavage of the extension to reveal a C-terminal Phe-Gly (Tyr-Gly in some orthologs) is required for interaction with the conjugation enzymes, starting with Atg7, to ligate the Atg8 C terminus to PE as described below (**Fig. 1**)^{53,54}. The posttranslational modification of LC3 family members is similar to that of Atg8; a proLC3 form is processed at the C terminus by ATG4 to generate LC3-I (the soluble form), followed by activation and conjugation to PE to generate LC3-II (the lipid conjugated form) (**Fig. 1**)⁵⁵. Although there is only one Atg4 family member in budding yeast, higher eukaryotes can have several ATG4 family members, with four in mammals (ATG4A, B, C, and D)⁵⁶⁻⁵⁹. The specificities and functions of the different mammalian Atg4 orthologs remain incompletely understood, but several studies suggest different substrate specificities. In enzyme assays with C-terminally GST-fused Atg8 orthologs as substrates, ATG4B appeared most promiscuous and with high activity toward LC3 family members, ATG4A preferentially cleaves GABARAP family members, particularly GABARAPL2/GATE-16⁵⁴. It seems likely that even more distinctive functions will emerge from studies with more native-like substrates.

Notably, in addition to processing Atg8 and orthologs to promote lipidation, Atg4 also reverses this modification by catalyzing removal of conjugated Atg8 from PE on the surface of the completed autophagosome (**Fig. 1**)⁵³. Although in principle this also serves to liberate Atg8 to enable its re-entry into the conjugation cascade, roles for Atg4 beyond promoting Atg8 ligation came from yeast genetic studies involving expression of Atg8^R, which lacks the extension and exposes the conjugatable Gly C terminus. Although Atg8^R bypasses the need for Atg4 to mediate Atg8 processing, this is not able to fully complement the autophagy defect of the *atg4* mutant, indicating an additional requirement for Atg8 deconjugation, possibly in disassembly/release of other Atg proteins from the forming autophagosome^{15,60}.

Crystal structures of full-length human ATG4B and a C-terminally truncated and catalytically inactive ATG4B in complexes with proLC3B and rat LC3B-I^{25,61,62}, revealed a papain-like protease Cys-His-Asp catalytic triad (Cys74-His280-Asp278 in human ATG4B) (**Fig. 4**). A key Trp side-chain clamps down the LC3B C terminus, which is reminiscent of the active site topology in SUMO deconjugating enzymes (**Fig. 4**). ATG4 family members also contain several fundamentally distinctive features specific for liberating Atg8/LC3 from C-terminal adducts. For example, there are numerous specific contacts with the globular β -grasp domain from LC3B. Also, adjacent to the active site is a pocket whereby the penultimate Phe from the processed/cleaved LC3B docks to position the C-terminal Gly in a narrow channel for bond cleavage. Notably, LC3B binding promotes a conformational change in an ATG4 “regulatory loop”, allowing docking of the penultimate Phe and exposure of the active site for catalysis. Furthermore, the LC3B-bound form of ATG4B reveals displacement of an N-terminal loop, which may enable exit of the short C-terminal peptide product resulting from cleavage of pro-LC3 or of the PE-containing membrane. Thus, it seems that binding of Atg8/LC3 helps initiate the reaction by promoting a conformation of ATG4 that is activated for both cleavage and turnover.

Ligation of Atg8 and Atg12

After Atg4-catalyzed liberation of its C-terminal glycine, Atg8 must be ligated to PE in the phagophore membrane, which promotes phagophore expansion (**Fig. 1**)⁸. Interestingly, a second autophagy-specific UBL, Atg12, which displays a native C-terminal glycine¹⁰, also functions after its own ligation (to Atg5) as part of the Atg8 conjugation machinery (**Fig. 1**)^{17,63,64}. As noted above, Atg12 conjugated to Atg5 acts as an E3 ligase for Atg8. In addition, Atg12 appears to modify other proteins⁶⁵, although the function in this regard has not been determined. The pathways regulating ligation of the autophagy UBLs share core organizational features in common with enzymatic cascades for conjugation of canonical UBLs such as ubiquitin, and include E1 and E2 enzymes reacting with the UBL C-terminal glycine, and in the case of Atg8 a distinctive E3 enzyme.

E1 activation of Atg8 and Atg12, and transfer to E2s

Atg7 is the E1 enzyme for both Atg8 and Atg12, with similar conjugation mechanisms for yeast and human proteins^{9,10,66}. Overall, E1s have two major functions⁶⁷. First, the E1 “activates” a UBL's C terminus, which is otherwise inert. The catalytic mechanism of Atg7-mediated activation of autophagy UBLs has not been probed to the same extent as canonical E1-UBL mechanisms such as for ubiquitin, NEDD8, or SUMO. Nonetheless, the existing data suggest a parallel mechanism whereby Atg7 catalyzes autophagy UBL C-terminal adenylation^{24,68,69}. The resulting covalent acyl-phosphate linkage between the UBL's C terminus and AMP would endow Atg8 and Atg12 with a good leaving group, enabling the subsequent reaction, in which the C terminus of an autophagy UBL becomes covalently-linked to the Atg7 catalytic cysteine, presumably via a thioester bond. The second essential function of an E1 is delivery of a UBL to the active site of an E2. Indeed, Atg8 and Atg12 are transferred from the catalytic cysteine of Atg7 to that of their cognate E2 enzyme, Atg3 or Atg10, respectively^{9,10,68,70,71}.

Structural data provide details into autophagy UBL recognition and activation by Atg7, and transfer to their E2s^{24,68-71}. The E1, Atg7, is organized into three domains^{24,68,69}. The central domain, termed the “adenylation domain”, serves four main functions. The adenylation domain binds a UBL and MgATP, catalyzing UBL activation. Crystal structures of the Atg7 adenylation domain bound to MgATP and Atg8^{24,69} revealed an extensive β -sheet in Atg7 binding to Atg8's β -sheet and C-terminal tail, and directing the C-terminal glycine into the active site. The magnesium ion coordinates a conserved Atg7 aspartate and ATP's three phosphates, with Atg8's C terminus juxtaposed with the ATP α -phosphate for the adenylation reaction (**Fig. 5a**).

A second function of the adenylation domain is to mediate Atg7 homodimerization^{24,68,69,72}, giving rise to a 2:2 Atg7:autophagy UBL stoichiometry. This domain from Atg7 also harbors the catalytic cysteine in a short loop that crosses over the UBL C-terminal tail for adenylation (**Fig. 5a**), but which adopts a range of conformations in different crystal structures that likely enable the catalytic cysteine to drive the third and fourth function of the Atg7 central domain—forming a thioester-linked Atg7~UBL intermediate, and delivery of the UBL's C terminus to its cognate E2.

Atg7's extreme C-terminal domain, not visible in any crystals, is important for binding Atg8 and E1 activity. The current model is that Atg8 is initially recruited via Atg7's extreme C-terminal domain, and then delivered to the extensive noncovalent binding site in Atg7's central domain for adenylation, forming a covalent intermediate with Atg7, and transfer to Atg3²⁴.

Atg7's unique N-terminal domain recruits the E2s, Atg3 or Atg10, in a 2-part manner^{68,70,71}. The E2s, Atg3 and Atg10 display an overall fold of a truncated E2, albeit with distinctive loop insertions for interactions including with Atg7. Despite different sequences and structures, the Atg3 and Atg10 insertions both bind to a common groove in Atg7's N-terminal domain. The Atg7 N-terminal domain also binds the E2 core domains from Atg3 and Atg10, via surfaces distal from the E1 and E2 active sites. Crystal structures of nearly full-length Atg7-Atg3 and Atg7-Atg10 complexes, revealed additional interactions between surfaces surrounding the E1 and E2 catalytic centers⁷⁰

A model of an Atg7-Atg8-Atg3 complex can be generated by superimposing the structure of an Atg7-Atg8 complex onto the corresponding region of the Atg7-Atg3 complex (**Fig. 5a**), and the Atg7-Atg12-Atg10 intermediate likely shares similar overall features⁷⁰. Taken together, the structures, biochemical data, and modeling suggest that recruitment of the E2s to the Atg7 N-terminal domain results in presentation of the Atg3 and Atg10 active sites to the Atg7~Atg8 or Atg7~Atg12 thioester linkage from the opposing monomer in the Atg7 homodimer^{24,68}. Although a trans UBL transfer mechanism explains a requirement for Atg7 homodimerization, at this point it remains unknown whether there is crosstalk between the two active sites, or between the two UBL cascades. It is conceivable that future studies may reveal that one Atg7 homodimer could simultaneously transfer one Atg8 to one Atg3 and one Atg12 to Atg10, in a manner that coordinates the two UBL cascades in autophagy.

The E2 active site regions display a range of conformations or are not visible in some crystals, raising the possibility of active site malleability being important for different reactions, such as receiving the cognate UBL from Atg7 and subsequent ligation^{70,71,73-75}. Comparison of free and Atg7-bound structures reveals dramatic reorientation of the E2 active sites. Atg7-bound Atg3 and Atg10 both display a common active site architecture, with the catalytic center flanked on one side by a conserved Tyr and on the other by a conserved His or Asn (**Fig. 5b**). Notably, an Atg3 Tyr to Ala mutation impairs formation of the Atg3~Atg8 intermediate, whereas the His/Asn is required for the downstream ligation reactions for both E2s.

E3-independent ligation of Atg12

Atg12 is ligated to a single conserved target lysine on Atg5 in an E3-independent manner. Instead, Atg10 interacts directly with Atg5, albeit with low affinity⁷⁵. Surfaces required for Atg12 ligation have been mapped onto the structure of Atg5⁷⁵, which is comprised of a helical region and two ubiquitin-fold domains. Interestingly, residues from all three Atg5 subdomains contributing to Atg12 ligation map to the interface with Atg12 in a structure containing an Atg12-Atg5 conjugate. Thus, it seems likely that both Atg10 and Atg12 within the thioester-linked Atg10~Atg12 intermediate contribute to Atg12 ligation to its target lysine on Atg5.

Atg8 transfer from Atg3 to PE

Atg8 is completely unique among UBLs in that its target is not a protein, but a lipid (PE). Atg8 can be transferred to the primary amino group of PE without an E3, indicating inherent ability of Atg3~Atg8 to bind PE and mediate catalysis. This reaction is enhanced *in vitro* by the Atg12–Atg5 conjugate, which facilitates Atg8–PE formation *in vivo*^{17,76}. Atg5 is typically found in a noncovalent complex with Atg16, which also contains a coiled-coil domain that mediates oligomerization and formation of a large dimeric Atg12–Atg5–Atg16 complex¹⁸. ATG16L1 promotes localization of the ATG12–ATG5 conjugate to membranes associated with autophagosome formation *in vivo*¹⁹, and Atg12–Atg5 association with Atg16 promotes Atg8 ligation to PE as well as tethering of giant unilamellar vesicles *in vitro*⁷⁷. Structures of Atg12–Atg5–Atg16 complexes from yeast and human superimpose well with each other, but display no similarity to any other E3s^{64,78}. The structures revealed a compact arrangement, with Atg12 and Atg5 packing together (**Fig. 5c**). This interface is not only important for Atg12 ligation to Atg5^{56,75,70}, as described above, but also for Atg12–Atg5 to act as an E3 promoting Atg8/LC3 lipidation *in vitro*. Although the role of the Atg12–Atg5 interface in E3 activity remains to be fully characterized, this potentially stabilizes the structure of Atg12 (which is generally insoluble and aggregates on its own), or rearranges to bind other components of the lipidation reaction.

For the human proteins, ATG12 provides the bulk of the high-affinity interaction with ATG3^{64,78}, through binding part of ATG3's loop insertion adjacent to an E1 binding site (**Fig. 5c**)⁷⁹. Intriguingly, ATG12 uses parallel features to recruit ATG3 as Atg8 and LC3 use to recruit their partners⁷⁹.

In vitro biochemical data suggest that binding to Atg12–Atg5 also allosterically opens the structure surrounding the Atg3 active site to expose the catalytic cysteine⁶³. Although the structural basis for Atg12–Atg5 activation of the Atg3 catalytic domain remains to be determined, this may parallel structural opening of the Atg3 active site also observed upon binding to the E1, Atg7. Atg12–Atg5 may also promote recruitment of the Atg3~Atg8 intermediate to membranes, as Atg5 has been reported to bind vesicles in a manner hindered by glutamate substitutions in place of two surface lysines⁷⁷.

Atg UBL proteins in physiology and disease

Autophagy has attracted increasing attention over the past decade in part because defects in this process are associated with numerous pathophysiologies. In addition, there is tremendous interest in manipulating autophagy for therapeutic purposes, and knowing the structures and mechanism of action of the UBL systems provides an avenue for further research with the goal of identifying or designing small molecule inhibitors of the the UBL conjugation or deconjugation enzymes. In particular, rational drug design, coupled with many years of research on ubiquitination, make proteins such as ATG4 and ATG7 potential targets for pharmacological intervention⁸⁰.

As with most of the yeast Atg proteins, deletion of any of the genes encoding the conjugation machinery results in an essentially complete block in autophagy activity at the stage of autophagosome formation. Although basically normal during vegetative growth,

these yeast mutants rapidly lose viability upon starvation, and diploids are defective in sporulation. At the cellular level, the defects associated with mutations or knockdown of the UBLs in mammals is similar to that seen in yeast. The greatest difference is seen with the evolutionary divergence of Atg8 into the separate LC3 and GABARAP subfamilies, which act at different stages of autophagosome formation¹¹, and with ATG16L1, which is much larger than the yeast ortholog; the WD-repeat domains of ATG16L1 interact with proteins such as NOD2 and TMEM59 in response to bacterial invasion^{81,82}. Similarly, the absence of ATG5 results in increased susceptibility to bacterial infection⁸³, and the ATG12–ATG5–ATG16L1 complex is needed for IFNG-mediated antiviral function⁸⁴.

A wide range of phenotypes accompanies autophagy defects in higher eukaryotes. With regard to the UBL systems for example, mice with conditional deletion of *Atg5* or *Atg7* display similar Paneth cell defects⁸⁵. Changes in the expression or properties of LC3 and GABARAP are found in Lewy body disease⁸⁶, altered expression of ATG5 and ATG7 may contribute to sporadic Parkinson disease^{87,88}, and an ATG7 mutation has been associated with earlier onset of Huntington disease⁸⁹. Furthermore, ATG7 deficiency leads to liver disease^{90,91}, and deletion or knockdown of *Atg5* or *Atg7* results in spontaneous tumor formation⁹², neurodegeneration^{93,94}, and muscle myopathies^{95,96}. Notably, ATG16L1 regulates the production of inflammatory cytokines⁹⁷ and a mutation of ATG16L1 is associated with Crohn disease⁹⁸⁻¹⁰⁰.

Autophagy also plays a role in cellular homeostasis and development. For example, selective autophagy mediates removal of some organelles during cellular differentiation, such as is seen with mitophagy during the maturation of red blood cells^{101,102}. The absence of ATG5 and/or ATG7 results in decreased viability of mouse neonates^{90,103}, defects in post-fertilization preimplantation development¹⁰⁴, and defects in the maintenance of hematopoietic stem cells¹⁰⁵.

Conclusions

Despite the recent explosion in our understanding structural mechanisms of UBL conjugation and protein interactions in autophagy, many questions remain. For example, what regulates the conjugation of Atg8/LC3? In general, the C-terminal extension is removed constitutively and in some protist orthologs the nascent protein has an exposed Gly residue¹⁰⁶, making the function of the additional residues unclear. In addition, several high-confidence candidate interaction proteins that bound human Atg8 orthologs also bind versions lacking the C-terminal Gly, suggesting a network of interactions prior to, or independent of, lipidation²³. Another issue concerns the timing of deconjugation; since Atg4 can remove Atg8 from PE, this step must be temporally regulated. One suggestion is that ATG4 activity is regulated by redox¹⁰⁷, but it is not known how this would operate within the cellular milieu. It is also possible that other Atg proteins sterically protect Atg8–PE at the phagophore assembly site¹⁰⁸. In contrast to Atg8, the conjugation of Atg12 to Atg5 appears to be irreversible, but we do not know if it is otherwise regulated. In some plant and mammalian cells the unconjugated forms of these proteins are not detected and the total amounts do not change significantly^{109,110}, suggesting that regulation might involve other components or be mediated through Atg16.

As mentioned above, it remains unclear for now as to how Atg8/LC3 influences phagophore elongation and autophagosome size. Similarly, beyond recruitment of the Atg3 E2⁷⁹, we do not know how the Atg12–Atg5–Atg16 complex functions as an E3. *Plasmodium falciparum* ATG12 lacks a C-terminal Gly residue that is needed for conjugation to ATG5, suggesting that it might either form a noncovalent complex with ATG5, or act as an E3 on its own¹⁰⁶ in contrast to the yeast protein¹⁷.

Specificity of protein-protein interactions is another issue. Considering the large number of potential Atg8/LC3-interacting proteins²³, how is specificity—including of timing and location of interactions—achieved? Also, the AIM/LIR motif is found in some of the conjugation enzymes including ATG4B and Atg3, and it is important in binding the substrate^{25,111,112}. This motif is a low complexity sequence and additional variations may yet be discovered. Many of the Atg proteins contribute to the conjugation of Atg8, but it is not clear where this process occurs, and whether it marks the donor membrane for phagophore expansion. Also, Atg8 is initially present on both sides of the phagophore, and one role of the population lining the concave surface is cargo recognition—are there differences in Atg8 function, perhaps mediated through interacting partners, on the two sides of the membrane? Although we know that the Atg8 orthologs in higher eukaryotes act at different steps of autophagy, why are there so many isoforms in each subfamily? Considering the potential number of interacting proteins²³, and the variations in LIR recognition we anticipate the identification of additional components that regulate these proteins and explain why they are distinct among UBLs by being anchored to a membrane.

Finally, are there connections between the conventional roles of the conjugation proteins described above and new functions that are beginning to be discovered? For example, LC3-associated phagocytosis¹¹³, ATG12 conjugation to ATG3⁶⁵, ATG16L1-dependent hormone secretion¹¹⁴, the role of ATG12–ATG5–ATG16L1 in IFNG-mediated antiviral activity⁸⁴, and the contribution of cleaved ATG5 to apoptosis¹¹⁵ all provide hints of contributions of the conjugation machinery to processes that are distinct from traditional mechanisms in macroautophagy.

We have only scratched the surface in terms of understanding how the UBL conjugation process is regulated, and how the Atg UBL proteins function. Further studies will provide insight into this unique mechanism of cytoplasmic sequestration and recycling, and may permit the identification of targets for modulating the process therapeutically.

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BOX 1

Currently, more than 40 proteins have been identified that function primarily in macroautophagy. These proteins can be divided into several groups based on the stage at which they function.

Induction. The Atg1/ULK1 kinase complex acts at multiple stages of macroautophagy, but first participates in induction. Macroautophagy occurs constitutively at a basal level, and is induced in response to stress. Atg1 is a Ser/Thr protein kinase, although its key physiological target(s) is not known. Kinase activity depends on interaction with Atg13/ATG13, a phosphoprotein. Regulatory kinases including protein kinase A and TOR inhibit Atg1 and Atg13 during normal growth. Maximal kinase Atg1 kinase activity requires interaction with a stable ternary complex composed of Atg17/RB1CC1-Atg31-Atg29.

Nucleation and expansion. The Vps34/PIK3C3 lipid kinase is part of multiple complexes that act at the nucleation stage. In yeast, Vps34-Vps15-Vps30/Atg6-Atg14 [comprising the phosphatidylinositol (PtdIns) 3-kinase complex I] localize to the phagophore assembly site (PAS), the presumed nucleation site for phagophore biogenesis. The generation of PtdIns3P is needed for the recruitment of certain Atg proteins. There are two ubiquitin-like (Ubl) protein conjugation systems that act at this stage, composed of the following components: Atg12 (Ubl), Atg5 (covalently modified target), Atg16 (noncovalent binding partner), Atg7 (E1-like activating enzyme), Atg10 (E2 conjugating enzyme) and Atg8 (Ubl), Atg4 (processing protease that removes the C-terminal Arg of Atg8 to expose a Gly), Atg7, Atg3 (E2-like). Atg8/LC3 is particularly interesting in that approximately one-sixth of all known Atg proteins are involved in the conjugation of Atg8 to PE, suggesting that it plays a critical role in macroautophagy. The amount of Atg8 determines the size of the autophagosome, and this protein also participates in cargo recruitment. Atg9 is a transmembrane protein that cycles between peripheral sites and the PAS. Anterograde movement to the PAS depends on Atg11 (a scaffold), Atg23 and Atg27, whereas retrograde trafficking requires Atg1-Atg13, and Atg2-Atg18. Atg9 levels control the number of autophagosomes, suggesting that it participates in, or regulates, membrane delivery from donor sources to the expanding phagophore. In selective types of autophagy cargo recognition occurs during the expansion step. Specificity factors include the receptors Atg19 (cytoplasm-to-vacuole targeting pathway), Atg32 (mitophagy) and Atg36 (pexophagy).

Fusion. All intracellular pathways that terminate at the vacuole rely on the same set of tethering and fusion components including Rab proteins and SNAREs. Thus, these proteins are not specific to macroautophagy.

Degradation and efflux. In most cases, the sequestration step of macroautophagy is not sufficient to relieve the inducing stress. Rather, the process must go to completion, which includes breakdown of the cargo and efflux. Lysis of the autophagic body in yeast involves the action of Atg15, a putative lipase. Various hydrolases can then degrade the

cargo. Efflux of the resulting macromolecules back into the cytosol occurs at least in part through membrane permeases including Atg22.

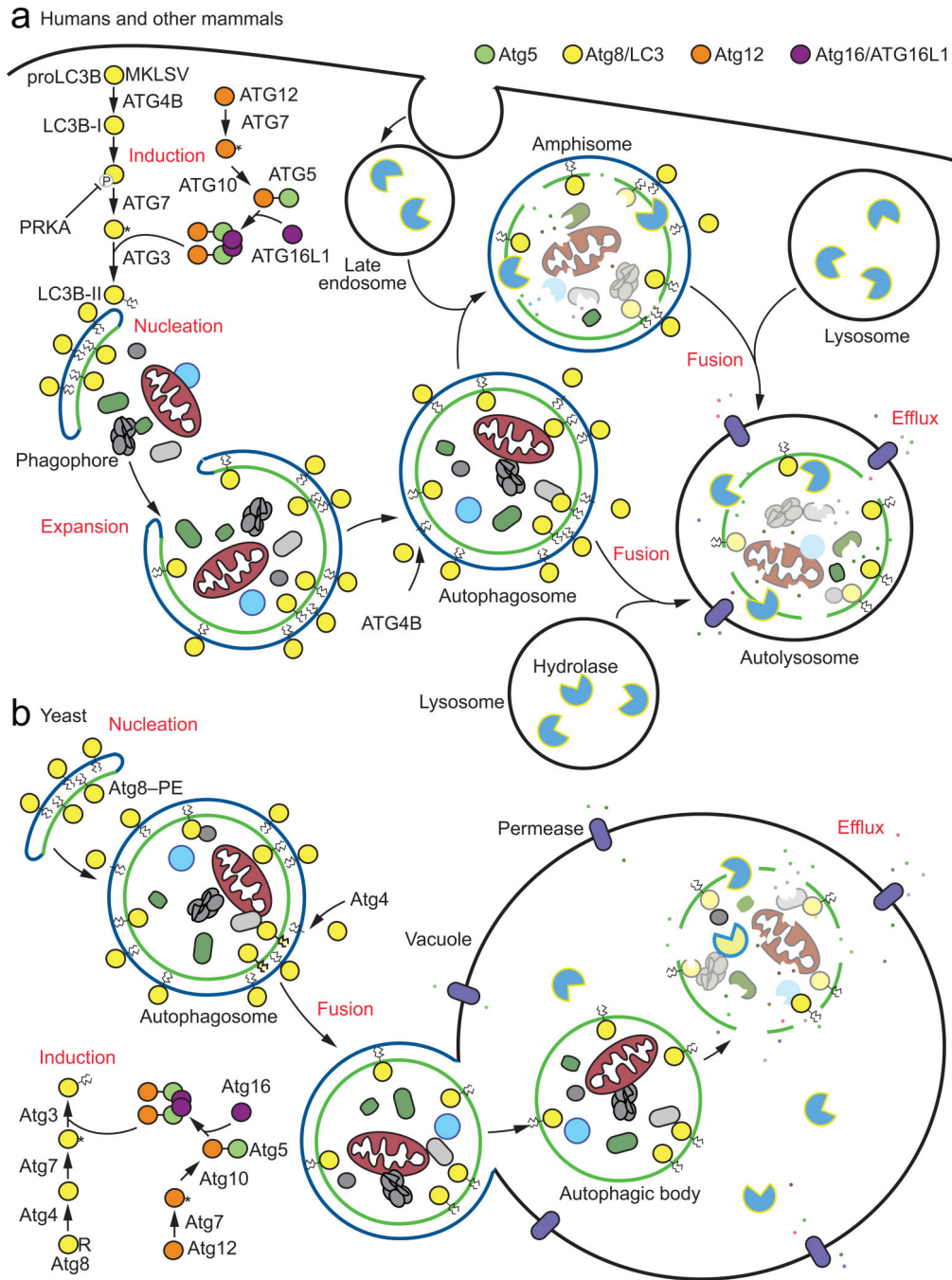
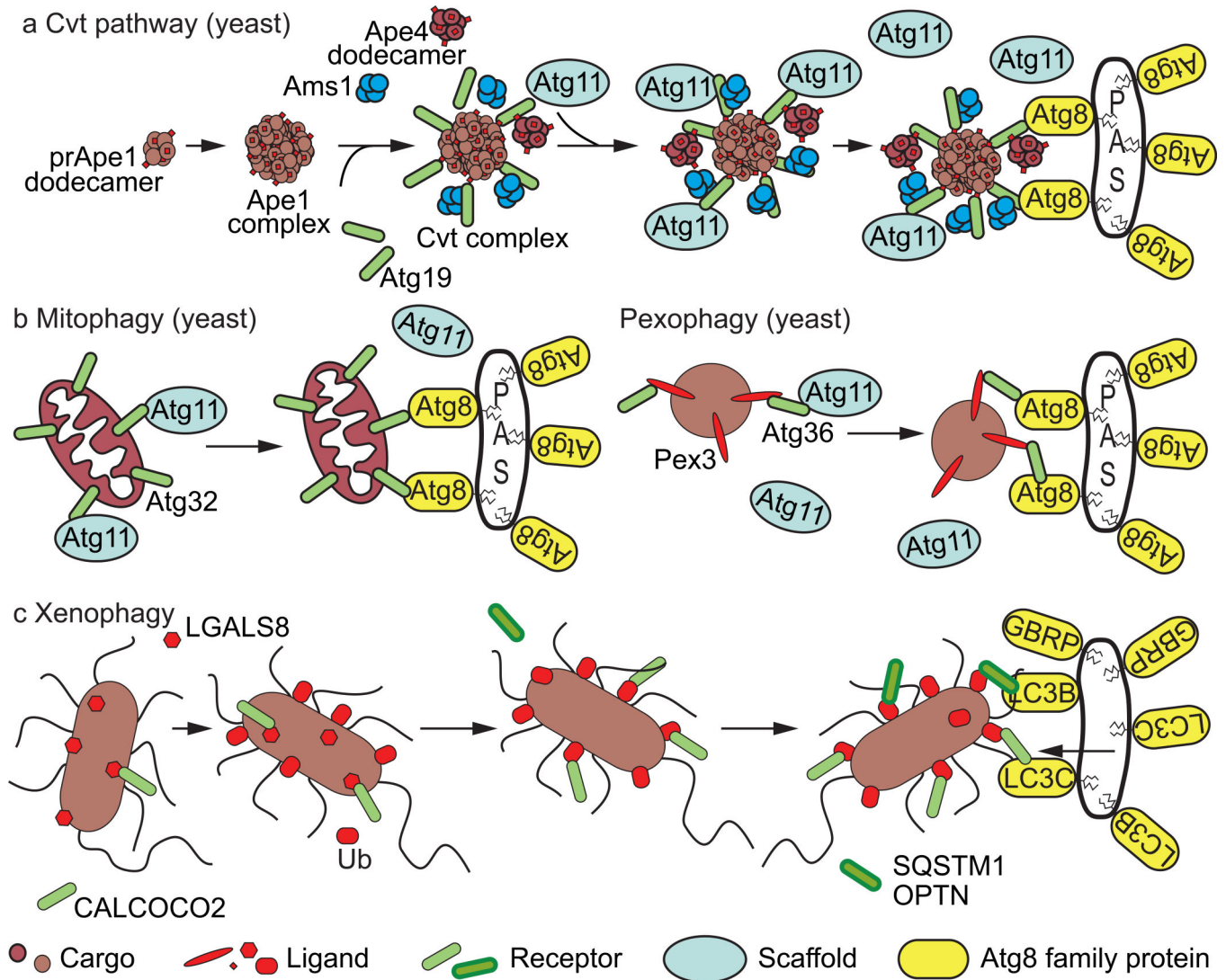


Figure 1. Morphology of macroautophagy and location/functions of UBL Atg proteins. The key morphological intermediates of macroautophagy are shown for mammals (a) and yeast (b). In either case, the process begins with the nucleation of the phagophore, the initial sequestering compartment (for simplicity, an omegasome is not depicted). This step and the subsequent expansion of the phagophore require the function of Atg8/LC3. Upon completion the phagophore becomes a double-membrane autophagosome. In mammals the autophagosome may fuse with a late endosome to form an amphisome, or may fuse directly

with the lysosome to generate an autolysosome. In yeast, the autophagosome fuses with the vacuole, releasing the inner vesicle that is termed an autophagic body. The inner vesicle of the autophagosome is lysed and the cargoes are degraded, with the breakdown products being released into the cytosol for reuse. Atg8/LC3-II is present on both sides of the phagophore, and on the early autophagosome, but is released from the outer membrane by Atg4/ATG4B-dependent deconjugation during autophagosome maturation. Regulatory kinases including protein kinase A and TOR inhibit Atg1/ULK1 and Atg1/ATG13 during normal growth. See the text for details on the functions of the proteins involved in the UBL-protein conjugation systems.

**Figure 2.**

Cargo recognition and packaging in selective macroautophagy. Different types of selective autophagy are illustrated. The yeast cytoplasm-to-vacuole targeting (Cvt) pathway is a biosynthetic process that delivers certain resident hydrolases to the vacuole. The propeptide of precursor aminopeptidase I (prApe1) is the key ligand that binds the Atg19 receptor. The latter interacts with the scaffold Atg11, and also contains an AIM motif for binding Atg8, connecting the cargo with the phagophore. A similar process occurs during mitophagy and pexophagy, where Atg32 and Atg36 act as receptors that bind both Atg11 and Atg8. During xenophagy in mammalian cells invasive bacteria may first be recognized by LGALS8 and subsequently by ubiquitin, which act as ligands. Receptors, including CALCOCO2/NDP52, SQSTM1 and OPTN bind the ligands and one or more of the Atg8 orthologs. Ams1, α -mannosidase; GBRP, GABARAP; PAS, phagophore assembly site.

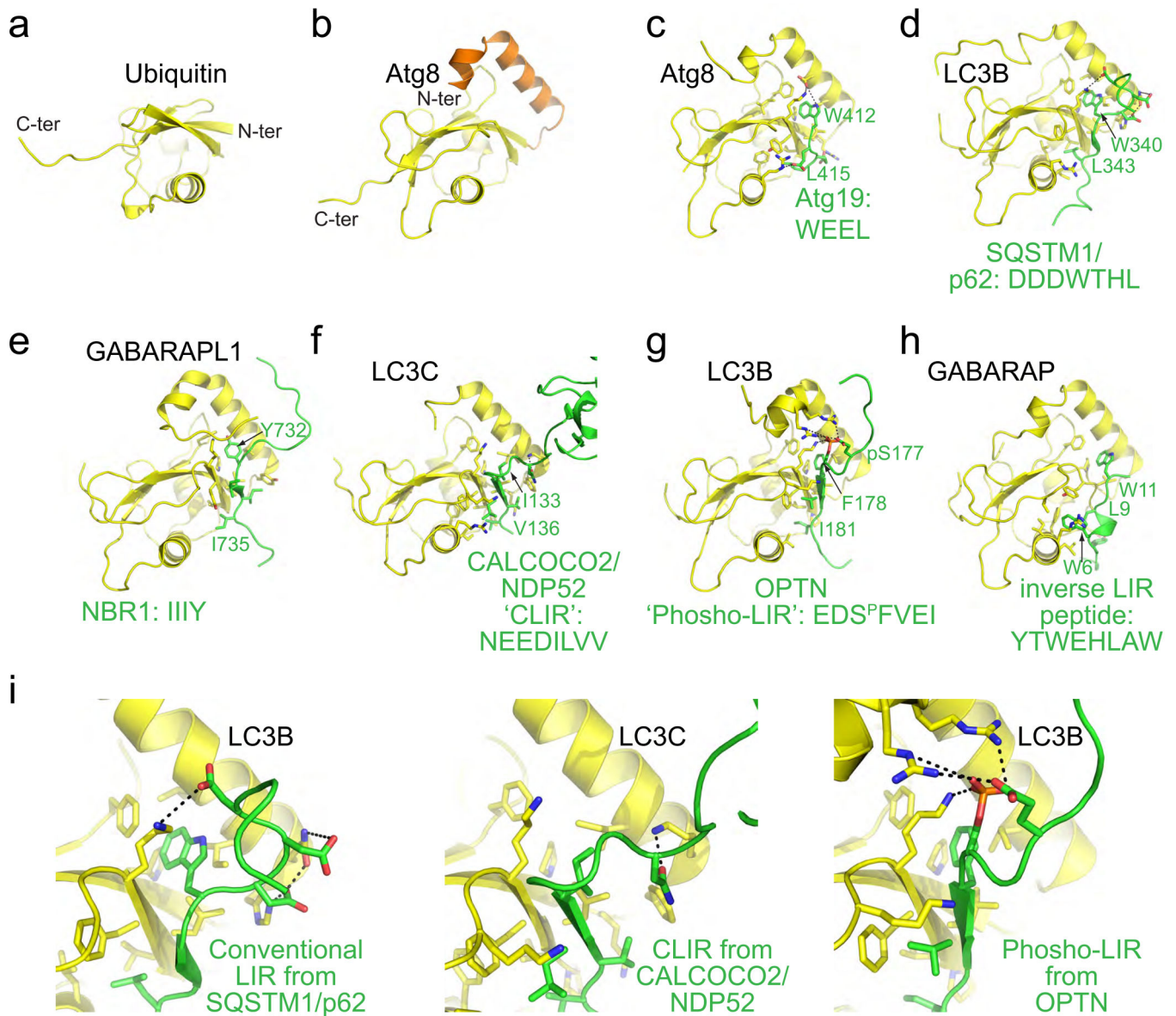


Figure 3.

Structural basis for Atg8 interacting motif (AIM)/LC3 interacting region (LIR) binding to ubiquitin-like protein Atg8 and orthologs. UBLs are shown in yellow, AIM or LIR peptides or proteins in green, and key interacting residues are shown in sticks. (a) Ubiquitin from a complex with E1¹¹⁶. (b) Atg8 from a complex with E1, with distinctive N-terminal helices colored in orange^{24,69}. (c) Overall similar modes of interactions with Atg8, LC3, and GABARAP as in an Atg8 complex with the AIM peptide from Atg19²². (d) LC3B complex with the LIR peptide from SQSTM1/p62^{22,44}. (e) GABARAPL1 complex with the LIR peptide from NBR1⁴⁵. (f) LC3C complex with the CLIR from CALCOCO2/NDP52⁴⁸. (g) LC3B complex with the phospho-LIR from OPTN⁵⁰. (h) GABARAP complex with a synthetic high-affinity inverse LIR peptide⁵¹. (i) Close-up views highlighting selected variations in LIR binding to LC3 and GABARAP family members from structures of LC3B

bound to a conventional LIR (left), LC3C bound to the CLIR (middle), and LC3B bound to phosphor-LIR (right).

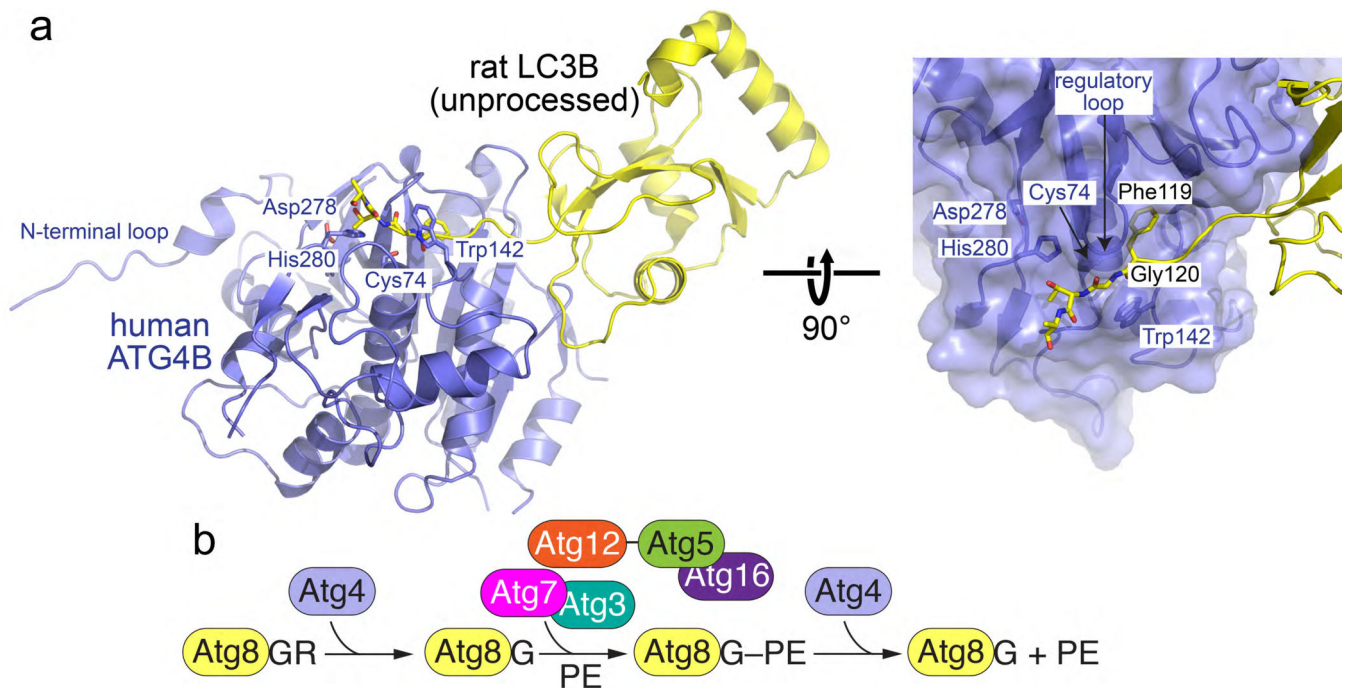


Figure 4.

Structural basis for Atg8/LC3 processing and deconjugation. Top: Crystal structure of unprocessed rat LC3B (yellow) in a complex with human ATG4B (blue) harboring a Ser substitution in place of the catalytic Cys74 (here noted as Cys). The left view shows LC3B in the same orientation as in Figure 3, and the right shows ATG4B with a transparent surface, rotated 90° about the x-axis to provide a close-up view. The C-terminal LC3B residues including the penultimate Phe119, neo C-terminal Gly120, and pro sequence are shown in sticks, as are ATG4B's Cys-Asp-His of the catalytic triad (here the Cys is substituted with Ser) and Trp clamping down the LC3 C terminus. Bottom: schematic view of Atg4 functions, as illustrated with the *S. cerevisiae* Atg8 C-terminal sequence. Atg4 orthologs from other organisms display overall similar functions, including with Atg8 orthologs displaying different C-terminal sequences. The Atg12– Atg5-Atg16 conjugate is shown in its role as an E3 ligase for Atg8.

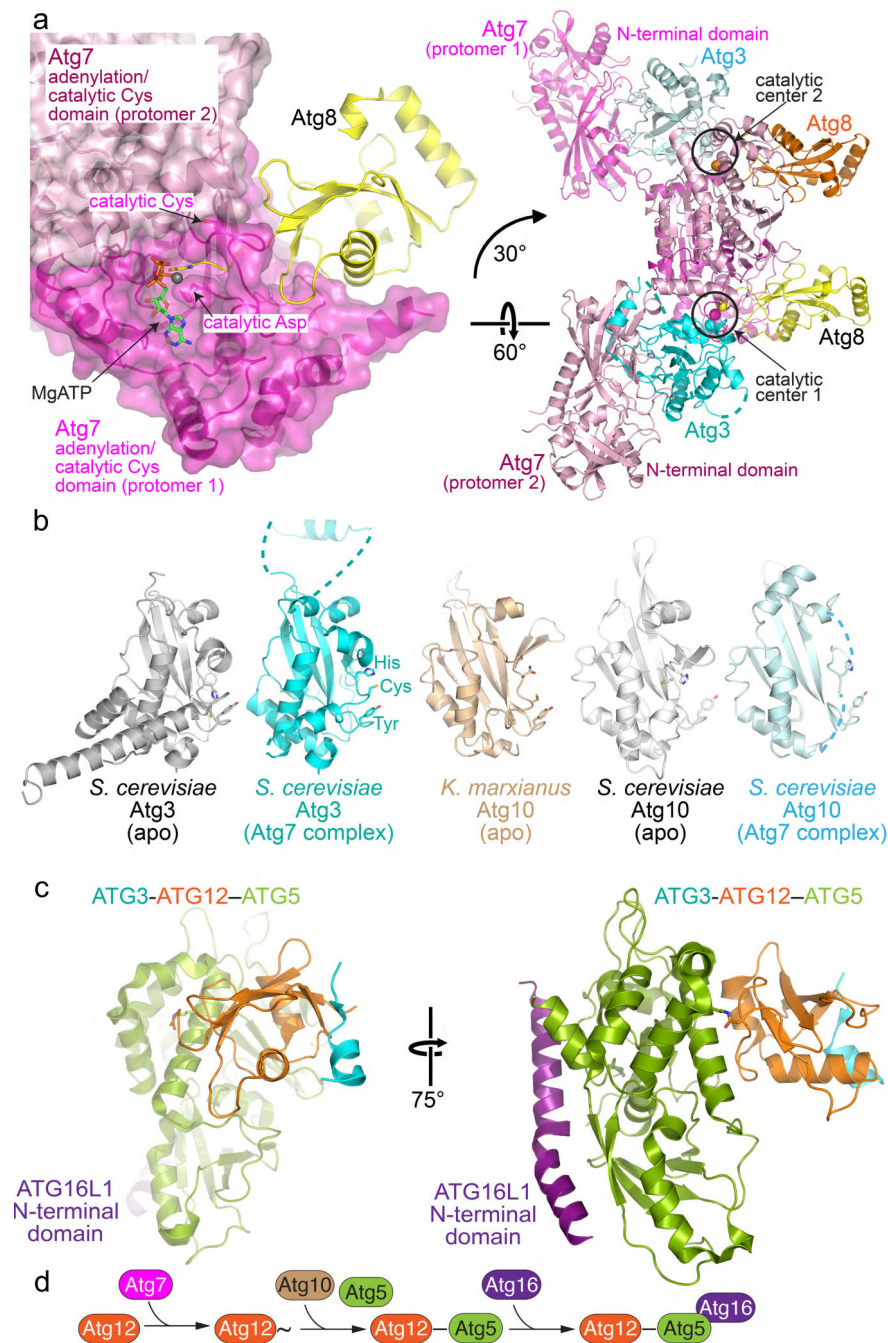


Figure 5. Selected structures of autophagy UBL ligation enzymes. **(a)** Structural insights into autophagy UBL activation by the E1, Atg7. Left, Close-up view showing one of two Atg8 molecules (yellow) in a complex with the homodimeric adenylation/catalytic cysteine domain of yeast Atg7 with the two subunits shown in magenta and pink^{24,69}. The catalytic Cys and Asp residues are shown in sticks. The magnesium ion is shown as a gray sphere, with ATP in sticks. The Atg8 molecule bound to protomer 2 (pink) is not visible in this view. Right, Model of an (Atg7-Atg8-Atg3)₂ intermediate, generated by superimposing the

structure on the left and the structure of an Atg7-Atg3 complex⁷⁰. A key feature is that an autophagy UBL (Atg8 shown here) is transferred in trans from the catalytic Cys of one Atg7 protomer to an E2 (Atg3 shown here) recruited to the amino terminal domain of the opposite monomer of the Atg7 homodimer. **(b)** Conformational variability of the active site region of autophagy E2 enzymes. For Atg3 and Atg10 from *S. cerevisiae*, the active site and other structural regions are dramatically reoriented upon binding to Atg7, although the crystal structure of isolated Atg10 from *K. marxianus* appears closer to the active conformation^{70,71,73-75}. **(c)** Crystal structure of human ATG3 (cyan)-ATG12 (orange)-ATG5 (green)-ATG16L1 N-terminal domain (purple)¹⁷⁹. Note that the overall structures of the Atg12-Atg5-Atg16 subcomplexes are similar between human and *S. cerevisiae*^{64,78}. The isopeptide linkage between the ATG12 C terminus and ATG5 is shown in sticks, and the ATG12 region implicated in binding to the E2, ATG3, is indicated⁷⁸. **(d)** Schematic illustration of Atg12-Atg5-Atg16 conjugate formation.