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Autophagosome Biogenesis Comes Out of the Black Box

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Abstract

Macroautophagic clearance of cytosolic materials entails the initiation, growth, and closure of autophagosomes. Cargo triggers the assembly of a web of cargo receptors and core machinery. ATG9 vesicles seed the growing autophagosomal membrane, which is supplied by de novo phospholipid synthesis, phospholipid transport via ATG2 proteins, and lipid flipping by ATG9. Autophagosomes close via the ESCRT complexes. Here we review recent discoveries that illuminate the molecular mechanisms of autophagosome formation and discuss emerging questions in this rapidly developing field.

Introduction

Macroautophagy (henceforward, “autophagy”) is the means by which eukaryotic cells degrade and recycle large objects such as molecular aggregates, organelles, and intracellular pathogens [1]. Autophagic dysfunction is central to many diseases, including viral and bacterial infections, cancer, and neurodegeneration diseases [2]. Autophagy consists of the initiation and growth of a double membrane phagophore (a.k.a. “isolation membrane”) around the object to be degraded, followed by the sealing of the phagophore into the double membrane vesicle known as the autophagosome, and its fusion with the lysosome (Fig. 1). The parts list of proteins and protein complexes responsible for autophagy has been largely delineated [1], an accomplishment recognized by the Nobel Prize in Physiology or Medicine to Yoshinori Ohsumi. Yet how these complexes and proteins orchestrate membrane lipids through phagophore initiation, growth, and closure has been a mystery, of “origins unknown, biogenesis complex” [3]. Within just the past two years, a great deal of light has now shone into the dark corners of this black box, further illuminating the nature of each of the main steps in phagophore formation (Fig. 1).

Bulk autophagy refers to the starvation-triggered non-specific engulfment and degradation of cytosolic material for the replenishment of biosynthetic precursors. Selective autophagy refers to the same process as targeted to particular substrates. While the fundamental machinery of autophagy was initially uncovered in yeast [4], this review will focus primarily on selective autophagy in mammalian cells. We will make reference to data from bulk autophagy in yeast in some cases, mainly where there are homologies between yeast and

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Conflicts of interest

J.H.H. is a co-founder of Casma Therapeutics.

mammals that address knowledge gaps in the mammalian system. Other excellent reviews have covered some of the recent developments in autophagosome biogenesis [5, 6], yet progress continues at a rapid pace. This review covers the most recent developments from a mechanistic perspective, with an emphasis on discoveries emerging from reconstitution experiments.

The cascade of autophagy initiation in mammalian cells begins with the unc-51-like kinase 1 (ULK1) complex, consisting of its namesake, the ULK1 (or ULK2) protein kinase, the non-catalytic FIP200, ATG13, and ATG101 subunits (Box 1), whose counterpart in yeast is the Atg1 complex [7]. The class III phosphatidylinositol 3-kinase complex I (PI3KC3-C1), consisting of VPS34, VPS15, BECN1, and ATG14, is activated early in autophagy initiation to generate phosphatidylinositol-3-phosphate (PI(3)P). PI(3)P enriched membranes recruit the downstream effector WIPIs (WD-repeat protein interacting with phosphoinositides), which in turn recruit and activate the ATG8/LC3 conjugation machinery [1, 8]. The attachment of the ATG8 proteins of the LC3 and GABARAP subfamilies to the membrane lipid phosphatidylethanolamine (PE), termed LC3 lipidation, is a hallmark of autophagosome biogenesis. LC3 lipidation occurs via a ubiquitin-like conjugation cascade. The ubiquitin E1-like ATG7 and the E2-like ATG3 carry out the cognate reactions in the LC3 pathway. The ATG12–ATG5-ATG16L1 complex scaffolds transfer of LC3 from ATG3 to PE [9, 10]. The role of the ATG12–ATG5-ATG16L1 is analogous to that of a RING domain ubiquitin E3 ligase, although there is no sequence homology between any of the subunits and ubiquitin E3 ligases. ATG2 transfers phospholipids from endoplasmic reticulum (ER) to the growing phagophore [11–13], whereas ATG9 translocates phospholipids from the cytoplasmic to the luminal leaflet, enabling phagophore expansion [14, 15]. These above-mentioned proteins are sometimes referred to as the “core complexes” of autophagy.

In this review, we will focus on a subset of selective autophagy pathways, including aggrephagy, mitophagy, and xenophagy, which are initiated by the ubiquitination of cargoes. These ubiquitinated substrates are recognized by a subset of cargo receptors, including p62 (sequestosome-1), NBR1, optineurin (OPTN), NDP52 and Tax1-binding protein 1 (TAX1BP1). All of these receptors contain a LC3-interaction region (LIR), a ubiquitin binding domain (UBD), and a dimerization/oligomerization domain [16–18]. These cargo receptors connect cargo to the phagophore through their interaction with both clustered ubiquitin chains and membrane-conjugated LC3.

Initiation

“On demand” autophagosome biogenesis in selective autophagy—Until early 2019, the leading model in the field was that mammalian selective autophagy was initiated when autophagy receptors bridged cargo to pre-existing ATG8-positive membranes [19]. The ATG8s themselves have been shown, however, to be non-essential for phagophore formation in at least one selective autophagy pathway, mitophagy [20]. ATG8s do contribute to the rate of autophagosome formation and the control of autophagosome size in mitophagy [20], and in bulk autophagy, to the rate of degradation of the inner autophagosomal membrane in the lysosome [21]. This suggested that autophagy receptors must be able to recruit and activate the core autophagy initiation machinery directly, bypassing the need for ATG8 proteins. Indeed,

such a mechanism was identified almost two decades ago in the autophagy-like cytoplasm to vacuole targeting (Cvt) pathway of *S. cerevisiae*. In yeast, the aminopeptidase precursor prApe1 is sorted to the vacuole by the cargo receptor Atg19, which binds to Atg11 and in turn to the Atg1 core complex [22–24].

The mammalian counterpart of the Atg1 complex is the ULK1 complex, and the counterpart of the Atg11 scaffolding subunit in yeast is FIP200 in mammals (Box 1). The C-terminal domain of Atg11 binds to the Atg19 receptor in yeast [25], which suggested that the corresponding C-terminal "Claw" [26] domain of FIP200 might bind to autophagy receptors in mammals. The receptor p62 was found to contain a FIP200 interacting region (FIR) that has weak homology to the Atg11 binding region of Atg19, and overlaps with the p62 LIR [26]. The affinity of the p62 FIR for the FIP200 Claw increases when the FIR is phosphorylated [26]. The p62 phospho-FIR:FIP200 interaction is sufficient to recruit the ULK1 complex to sites of condensed cargo, and so initiate autophagosome biogenesis [26] (Fig. 2a).

FIR motif interactions are not the only mechanism for ULK1 core complex recruitment by cargo receptors. FIP200 binds to another autophagy receptor, NDP52, as revealed by yeast two-hybrid screens [27, 28]. The SKICH domain of NDP52 interacts with the coiled-coil domain of FIP200 [28], in contrast to the FIR-Claw interaction described above for p62. The FIP200-NDP52 interaction is capable of driving both xenophagy [28] and mitophagy [29]. NDP52 engagement induces a conformational change in FIP200 that promotes membrane binding of the ULK1 complex [30]. In xenophagy, TBK1 and the TBK1 adaptor SINTBAD/NAP1 are also required [28]. In mitophagy, TBK1 facilitates FIP200 recruitment by NDP52, but chemically directed recruitment of FIP200 can bypass the need for cargo-NDP52 engagement, demonstrating that ULK1 complex recruitment is sufficient to trigger autophagosome biogenesis [29]. These findings, coupled with the results described above for p62, show that at least two receptors trigger autophagy initiation by recruiting the ULK1 complex to its site of action. This paradigm is not restricted to the ULK1 complex. A third autophagy receptor, OPTN, was proposed to interact directly with ATG9A to initiate mitophagy [31], potentially bypassing FIP200. The targeting of ATG9A and the ULK1 complex by different cargo receptors could explain the long-standing observation that these components could be targeted independently of each other and ATG8s to sites of mitophagy initiation [32]. It will be interesting to see if other core components besides the ULK1 complex and ATG9, such as PI3KC3-C1, the ATG16L1 complex, or WIPIs, can be directly recruited by autophagy receptors. The emerging paradigm is thus that autophagic membranes are made "on demand" when cargo, via the recruitment of core autophagy components by cargo receptors, activates the upstream core complexes (Fig. 2b).

Scaffolding the phagophore in bulk autophagy—In selective autophagy, the cargo itself templates the shape of the phagophore [33]. In bulk autophagy, the mechanism of scaffolding is less clear. First principles of membrane elasticity dictate that in free solution an expanding membrane sheet will fold into the shape of a cup [34], and that crescent-shaped autophagy proteins lower the free energy barrier to promote this process [35]. Whether membrane elasticity alone can drive phagophore shaping in the cytosol without a template is uncertain. p62 was initially identified as the receptor for aggrephagy [36], however, recent studies show that p62 can form phase-separated condensates with polyubiquitin [37, 38]. 15

nm-wide filaments form from purified p62 in vitro [39], and in the presence of model ubiquitinated cargo, condense into 0.5–2.0 μm clusters [37]. These clusters have many of the properties of phase-separated condensates, including high sphericity and a high propensity to fuse [37]. Condensed p62 is capable of recruiting the ULK1 complex [26], explaining how uptake of phase-separated condensates could occur. In cells these condensates are similar in size to other selective autophagy substrates [40]. Another receptor, NBR1, can directly promote the formation of p62 condensates [41]. Thus, at least in cases where there is clear evidence for cargo condensation, bulk autophagy may be actually be "condensate-phagy" or "fluid-phagy" [42]. A membrane elasticity model for the phagophore was recently shown to be compatible with engulfment of fluid p62 condensates, consistent with this idea [43]. This would be just one of the many flavors of selective autophagy, with phagophore growth scaffolded by the size and shape of the condensate.

A multivalent web of low affinity interactions directs initiation—An early and influential model for autophagy initiation based on yeast genetics suggested that the core complexes were recruited in strict order in starvation-induced autophagy, beginning with the FIP200 counterpart Atg17, and culminating with the Atg8 lipidation machinery [44]. Live cell imaging, however, found that ULK1 and the ATG5 component of ATG8 lipidation machinery arrive simultaneously in mammalian starvation-induced autophagy [45]. Evidence is growing that a network of multiple high and low affinity interactions stabilizes the network of autophagy initiation complexes at the nascent phagophore. This contributes to the redundancy and robustness of the process.

The ULK1 complex (Atg1 complex in yeast) best illustrates how a multiplicity of interactions can drive autophagy initiation. ULK1, FIP200, and ATG13 subunits of the ULK1 complex all contain larger intrinsically disordered regions (IDRs) [46]. Indeed, the majority of ATG13 consists of a C-terminal IDR. The key contacts between ATG13-FIP200 and ATG13-ULK1 are driven by the ATG13 IDR, which forms the main unifying element of the overall complex [47]. The organization of the complex is therefore structurally loose [48, 49] (Fig. 2c). While the individual subunits are required for canonical autophagy, their assembly is not always strictly required. In starvation induced autophagy, removal of the ATG13 IDR binding sites for FIP200 and ULK1 has moderate to no phenotype [50, 51]. This implies that the ULK1 complex subunits have enough redundant interactions with other components of the autophagy initiation system for them carry out their functions without the strict need to actually assemble into the canonical ULK1 complex.

The Atg1 complex of *S. cerevisiae* is also organized principally by the IDR of its Atg13 subunit, which interacts at multiple sites [52, 53] within the dimeric banana-shaped Atg17 subcomplex [54]. Atg13 can bridge multiple Atg17 molecules [53]. The capacity of Atg13 to bridge multiple Atg17 dimers drives phase separation of Atg1 [42, 53], at least *in vitro*. It takes only ~15–50 copies each of Atg13 and Atg17 to initiate a phagophore [55, 56]. The interpretation of mesoscopic phase separation for biomolecular process involving only a few tens of molecules is an interesting question that is not fully resolved [57]. Many of the physical properties of condensates that are important in biology, such as their ability to sequester their contents and fuse with one another, do not apply on the scale of a handful of molecules [57].

Other aspects, notably assembly through a network of multiple low affinity interactions, are relevant across all scales.

A circuit involving PI3KC3-C1, WIPI2, and the ATG16L1 complex provides another example of cooperativity in autophagy initiation. Upon its recruitment to sites of autophagy initiation, PI3KC3-C1 phosphorylates phosphatidylinositol (PI) to PI(3)P. Membrane bound PI(3)P then recruits WIPI proteins to the membrane (Fig. 2d). At least one of the WIPIs, WIPI2, is key to recruiting the ATG16L1 complex to membranes, and thereby triggering ATG8 lipidation [58]. Reconstitution of the PI3KC3-C1, WIPI2, and ATG16L1 circuit revealed additional weaker interactions between WIPI2 and PI3KC3-C1 that promote positive feedback to drive the net ATG8s lipidation reaction forward [59]. The presence of ubiquitinated cargo, cargo receptors, and the ULK1 complex further enhances the rate of ATG8 lipidation [60].

Growth

Feeding lipids to the autophagosomal membrane—Autophagosomes are remarkable for their ability to grow essentially de novo in a matter of minutes [61]. The nature of the lipid supply for this massive growth, estimated to require the transfer of 4,000 phospholipid molecules per second [62], has been one of the most enduring mysteries in the field [3]. The answer was provided by research into the Atg2 protein, which was identified in 2001 [63, 64] but whose function has been elusive until recently. Human ATG2A is rod-shaped and, in its complex with WIPI4, capable of tethering membranes [65, 66]. ATG2 has sequence homology to the VPS13 proteins, a family of lipid transporters [67]. These observations lead three teams to the seminal discovery that the yeast Atg2 and human ATG2A protein is the lipid transporter linking growing phagophores to membrane sources such as the ER [11–13] (Fig. 3). The lipid transport rates measured *in vitro* are sufficient for a plausible ~10^2 molecules of ATG2 per phagophore to meet the demands of membrane growth [68]. The cryo-EM structure of a thermophilic yeast Vps13 revealed a long and wide hydrophobic tunnel running the length of this rod-like protein [69], explaining how members of the Vps13/Atg2 protein family could serve as a “firehose for phospholipids” [62].

The Atg2 “firehose” model for autophagosome expansion immediately raised several questions. One major question was how to resolve the potential imbalance created by the rapid delivery of phospholipids to the outer leaflet of the phagophore. Atg9 (ATG9A/B in mammals) is the sole integral membrane protein of the core autophagy machinery [70], and its function or functions have a major question in its own right. The cryo-EM structure of ATG9A [71] revealed a trimeric membrane embedded assembly riddled with cavities suggestive of a potential role in lipid transport. Two subsequent cryo-EM structural papers revealed in greater detail the presence of hydrophilic channels on the surface of yeast Atg9 and human ATG9A [14, 15]. The channels run the length of the membrane spanning surface of Atg9, a feature characteristic of the scramblases that equilibrate phospholipids between the two leaflets of the membrane bilayer. These studies and another went on to confirm that Atg9 and ATG9A have phospholipid scramblase activity *in vitro*. Phospholipids in wild-type but not *atg9Δ* yeast are distributed symmetrically, consistent with Atg9 scramblase activity

against multiple phospholipid species [72]. Taken together, the findings for Atg2 and Atg9 explain how phospholipids are directed into the growing phagophore (Fig. 3).

Atg9 vesicles are the seeds of autophagosome biogenesis—The functions of ATG9 vesicles have been one of the leading questions in autophagosome biogenesis. As characterized in yeast, trans-Golgi network (TGN)-derived Atg9 vesicles are required for autophagy initiation [73, 74]. The size and number of these vesicles fail to account for the amount of lipid needed to make a phagophore by several orders of magnitude, however. This led to the hypothesis that Atg9 vesicles are not the membrane source, but rather the seeds of phagophore growth. A recent reconstitution of yeast autophagy initiation including the cargo receptor Atg19, the Atg11 scaffold (a FIP200 counterpart in yeast), PI3KC3-C1, the Atg8 lipidation system, Atg2, and Atg9, confirmed that Atg9 vesicles can function as seeds for phagophore growth [75]. In this system, Atg19 and Atg11 linked a model cargo to the rest of the machinery. Recruitment of the lipid transporter Atg2 was sufficient to catalyze lipid transfer from donor vesicles. Newly transferred phosphatidylethanolamine (PE) was capable of serving as an acceptor for Atg8 lipidation by the conjugation machinery. A key point is that lipid transfer can occur upstream of Atg8 lipidation. This is consistent with the findings in mammalian cells that ATG8 conjugation machinery proteins are not essential for phagophore initiation [20, 21, 76], and that ATG8s lipidation can be bypassed by cargo receptor TAX1BP1-driven FIP200 clustering [77]. Phagophore seeding by ATG9 vesicles thus seems likely to be operative in mammals as well.

The autophagosome itself supplies fatty acids for its growth—ATG2 and ATG9 are not ATPases nor are they known to be coupled to other energy inputs, therefore their lipid transfer and scramblase activities must be driven thermodynamically by a concentration gradient. This gradient appears most likely to be established by de novo phospholipid synthesis. In yeast, the acyl CoA-synthetase Faa1 is localized to the phagophore, and its presence is required for phagophore growth [78]. Isotopic labeling was used to track fatty acids activated by phagophore-localized Faa1 and show that these fatty acids are incorporated into phospholipids which can then be returned to the phagophore by Atg2 [78]. Consistent with this idea, mammalian autophagy initiation occurs at ER sites enriched in PI synthase-enriched domains [79], and choline phospholipid synthesis was shown to be required for phagophore growth in cancer cells [80]. Fatty acid activation in the phagophore thus appears to be a key step in powering phagophore growth (Fig. 3).

Closure

As the phagophore grows and engulfs its substrate, eventually the double membrane converges until only a single unclosed membrane neck is left. The open neck allows communication between the cytosol and the interior. Until the neck is closed, is not possible to acidify the interior and so degrade its contents. Membrane necks whose interior is connected to the cytosol, such as the neck of the closing phagophore, are resolved by the ESCRT machinery [81, 82]. Neurodegeneration-linked ESCRT-related phenotypes in autophagy were noted more than a decade ago [83–86]. Only recently has it been possible to create assays that pinpoint the closure step in autophagy [87–89] (Fig. 4a). Human ESCRT-I is required for autophagosome closure, in particular the VPS37A subunit [90]. It is not yet

known how the ESCRTs are recruited to the closing phagophore, although it presumably somehow involves VPS37A. Once recruited to its site of action, ESCRT-I forms a ring-shaped assembly of ~12 copies of the complex that helps recruit downstream ESCRTs and coordinates closure [91] (Fig. 4b). On large cargoes such as bacteria and mitochondria, autophagy may initiate at more than one site, and the ESCRT might also be involved in piecing several membrane fragments into a single phagophore (Fig. 4c), as demonstrated in nuclear envelope reformation [82]. Key questions remain about how closure is correctly synchronized with the termination of growth, how ESCRTs are targeted to the phagophore neck, and how lysosomal fusion is forestalled until closure finishes.

Conclusions and outlook

The findings outlined above highlight what we consider the most important developments in autophagy mechanisms over the past two years. Seven years ago, a comprehensive review concluded that “There remain, however, many unanswered questions about the precise protein and lipid composition of the autophagic membranes, the mechanisms behind their shape, how they detach from the ER and how the autophagosome is finally closed.” Over the just past two years, the field has made major inroads into answering these questions. It is truly time for the textbooks to be rewritten. Of course, these developments raise many new questions. Now that the individual steps of initiation, growth, and closure are becoming much better understood, questions arise as to their coordination with each other and with other cell processes. What are the cues to transition from initiation to growth, and growth to closure? As our understanding becomes more quantitative, we can begin to ask what the rate limiting reactions at each step are. As we understand these parameters, we can begin to rationally modify these pathways, leveraging the basic insights described above to develop potential pro-autophagic therapies.

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Box 1–

Autophagy core complexes and cargo receptors

ULK1c:

Composed of ULK1 (Ser/Thr kinase), FIP200 (scaffold), ATG13, and ATG101. The kinase is activated by mTOR inhibition, and the complex is recruited to its sites of action by a subset of cargo receptors. The ULK2 kinase is partially redundant with ULK1. The orthologous complex in *S. cerevisiae* consists of the ULK1 ortholog Atg1, Atg13, and, unique to budding yeast, Atg17, Atg29, and Atg31.

PI3KC3-C1:

Class III PI 3-kinase complex I, comprised of VPS34 (lipid kinase), VPS15 (scaffold), ATG14 (scaffold, unique to complex I and to autophagy initiation), and BECN1 (regulatory subunit). ATG14 is unique to complex 1, and is replaced in the endosomal and late autophagy complex II by UVRAG. The catalytic subunit VPS34 phosphorylates phosphatidylinositol lipid headgroups to generate PI(3)P. The orthologous complex in *S. cerevisiae* consists of Vps34, Vps15, Atg14, and Vps30.

ATG12-ATG5-ATG16L1:

The ubiquitin-like ATG12 is covalently linked to ATG5 (by ATG10 and ATG7), which further associates non-covalently with ATG16L1. The coiled-coil protein ATG16L1 homodimerizes, forming a heterohexameric complex composed of two copies each of ATG12, ATG5, and ATG16L1. ATG16L2 is partially redundant with ATG16L1. The complex functions like an E3 ubiquitin ligase with respect to the conjugation of ATG8/LC3 family proteins to membrane phosphatidylethanolamine, although the subunits are not orthologs of ubiquitin E3s. The orthologous complex in *S. cerevisiae* consists of Atg12, Atg5, and Atg16.

ATG2:

ATG2A and ATG2B are high capacity, low specificity lipid transporters whose structure is centered on a long tunnel that carries phospholipids from their source, normally the ER, to the growing phagophore. The sole *S. cerevisiae* ortholog is Atg2.

ATG9:

ATG9A and ATG9B are the sole integral membrane proteins of the core autophagy machinery, and function as trimers. ATG9 vesicles are the seeds for autophagosome growth and the protein has phospholipid scramblase activity that equilibrates the lipid composition of the two leaflets of the bilayer in an ATP-independent manner. The sole *S. cerevisiae* ortholog is Atg9.

WIPIs:

Also known as PROPPINs, these β-propeller proteins bind to the lipids PI(3)P and $PI(3,5)P_2$. In humans, the WIPI family comprises WIPI1, 2, 3, and 4. WIPIs target ATG16L1 and ATG2 to sites of autophagosome biogenesis. The *S. cerevisiae* orthologs are Atg18, Atg21, and Hsv2.

ATG8/LC3 family proteins:

Small ubiquitin-like proteins LC3A, LC3B, LC3C, GABARAP, GABARAPL1, GABARAPL2 which are covalently linked to phosphatidylethanolamine lipid headgroups on the phagophore membrane. Their ortholog is Atg8 in yeast. Most cargo receptors and most of the core autophagy proteins bind LC3 through LC3 interacting region (LIR) motifs.

Cargo receptors:

A group of proteins which selectively bind autophagy cargo (often, although not exclusively, through a ubiquitin-binding domain), which include, among many others, p62, NDP52, OPTN, NBR1, and TAX1BP1.

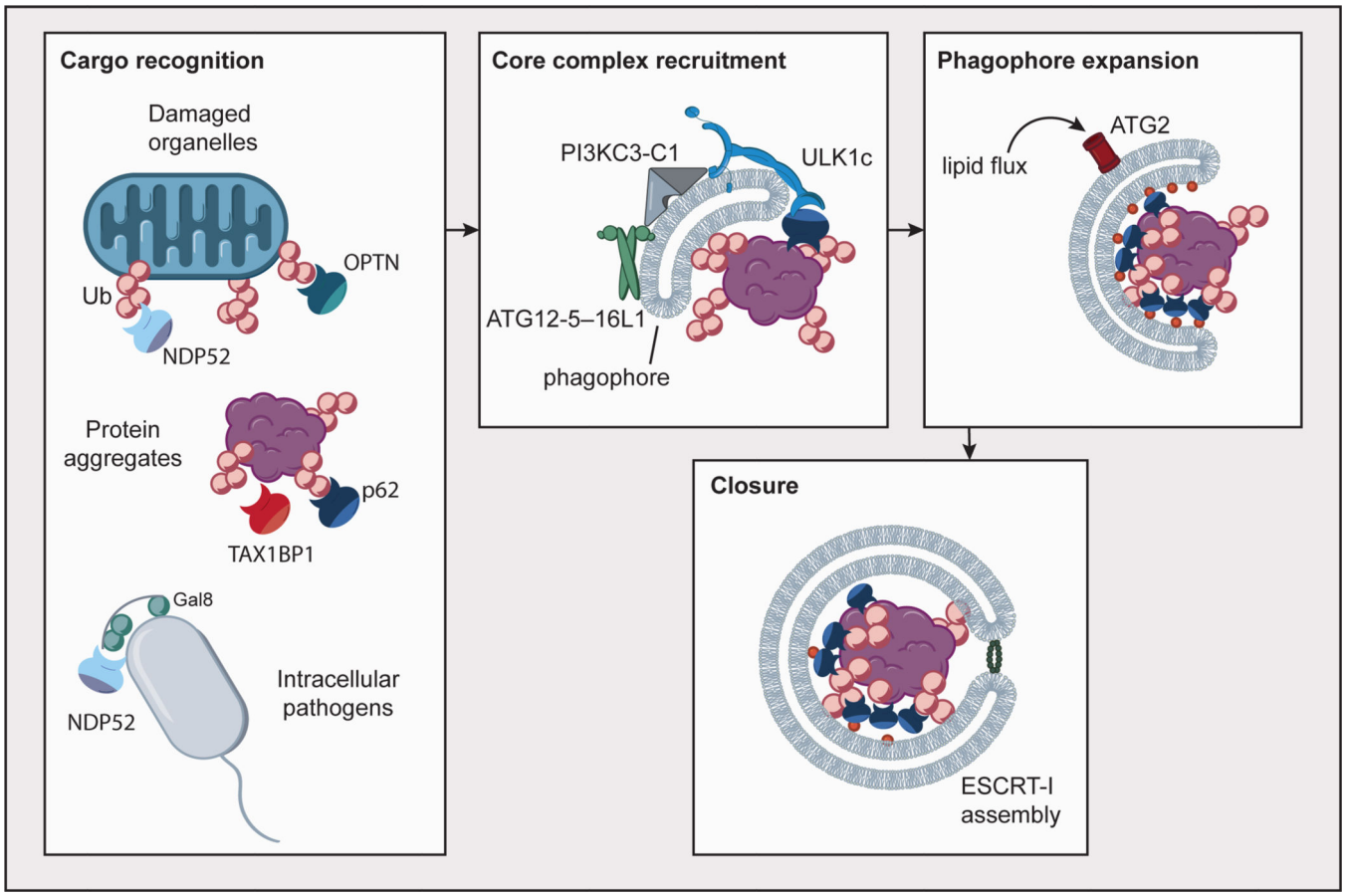

Fig. 1. Overview of autophagosome formation.

Autophagy cargoes such as damaged organelles, protein aggregates and intracellular pathogens are recognized by cargo receptors such as p62, NDP52, TAX1BP1, and OPTN, most commonly through modification with ubiquitin (Ub), as well as the presence of other signals such as Galectin-8. Core autophagy complexes (ULK1c, PI3KC3-C1, ATG12–ATG5–16L1) are recruited to the nascent phagophore through interactions with cargo receptors, the phagophore membrane, and one another in a positive feedback mechanism. Phagophore expansion is driven by lipid flux through ATG2. Autophagosome closure is mediated in part by assembly of ESCRT-I proteins in a ring at the aperture of the closing phagophore.

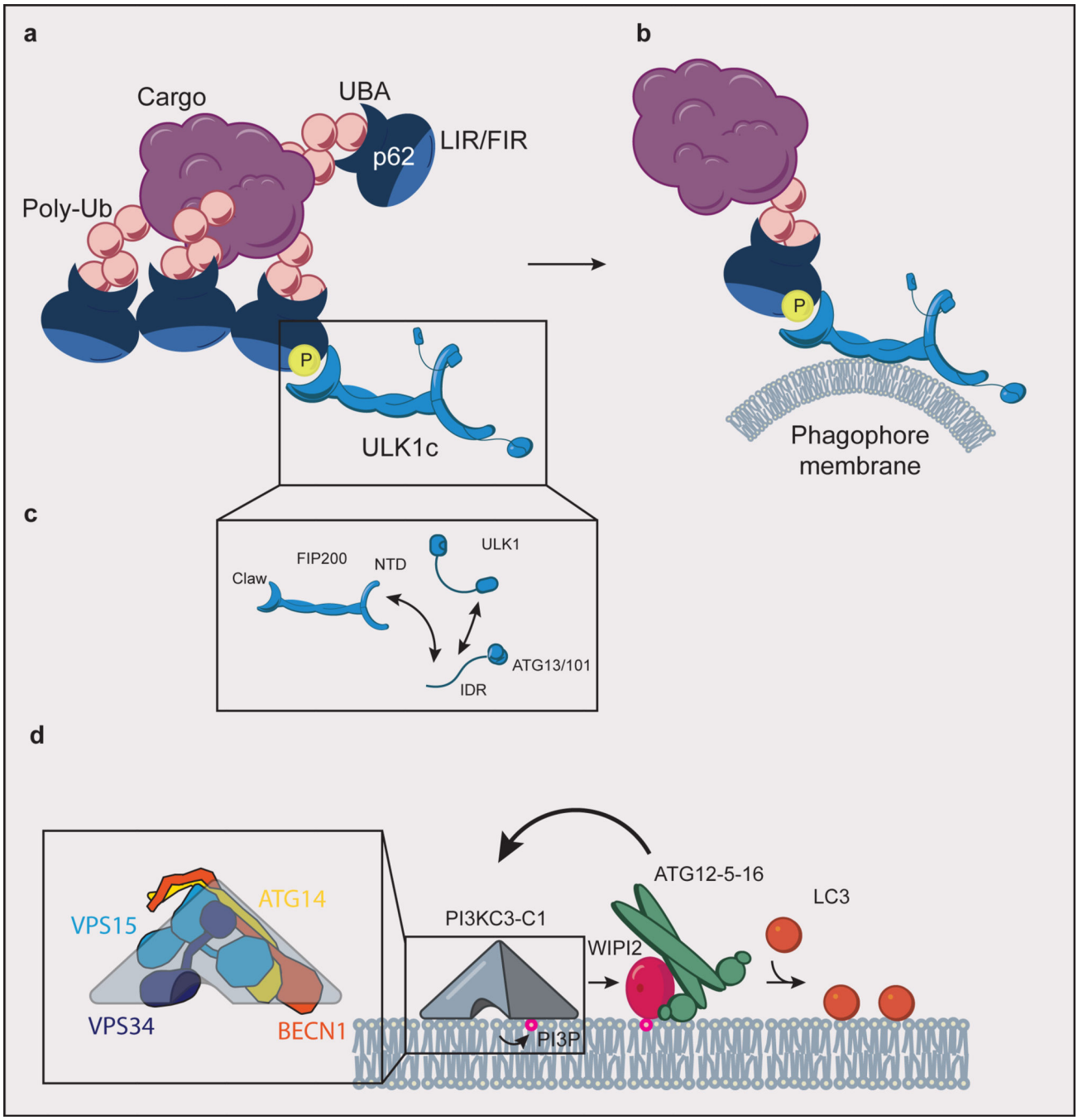

Fig. 2. Autophagy initiation.

(a) ULK1c is recruited to p62 condensate cargo through an interaction of the FIP200 claw domain with phospho-p62. p62 binds to ubiquitinated (Ub) cargo via its UBA domain, to LC3 via its LIR motif, and to FIP200 of the ULK1c via its FIR motif. (b) Interaction of ULK1c with p62 condensates stimulates “on demand” phagophore membrane generation. (c) The subunits of ULK1c (FIP200, ULK1, ATG13, ATG13/101) are held together in large part through a network of interacting IDRs. (d) PI3KC3-C1 (inset: comprised of ATG14, BECN1, VPS15, and VPS34) phosphorylates phosphatidylinositol lipid headgroups to form

PI(3)P, which recruits WIPI proteins to the phagophore membrane. WIPI2 subsequently recruits ATG12–5–16 to the phagophore in a positive feedback loop with PI3KC3-C1. ATG12–5–16 covalently links LC3 to phosphatidylethanolamine lipid headgroups (orange circles) in the phagophore membrane.

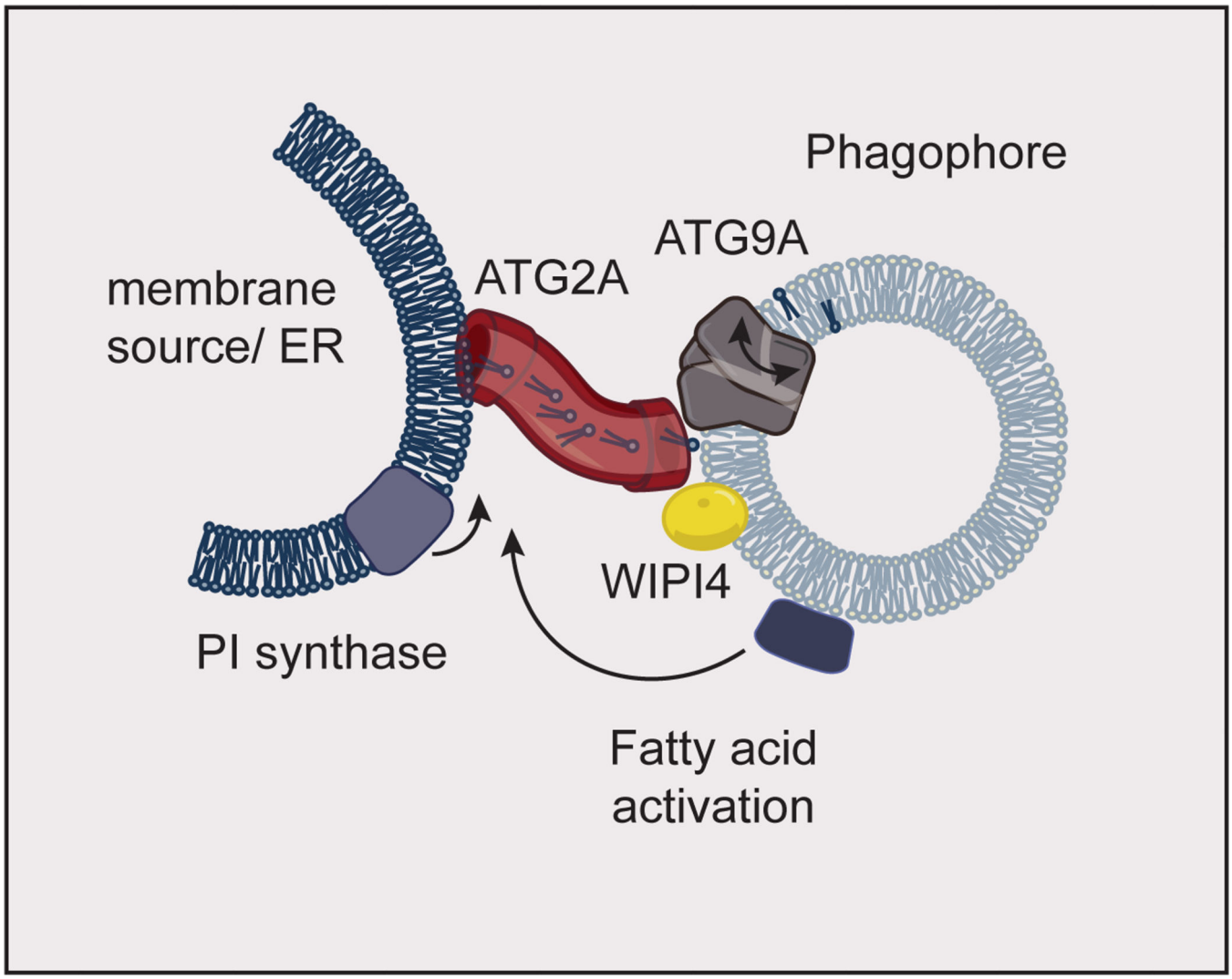

Fig. 3. Autophagosome growth.

Fatty acid activation at the phagophore feeds phospholipid synthesis by phosphatidylinositol (PI) synthase at the membrane source for the autophagosome. This phospholipid synthesis drives lipid flux through ATG2A (which binds WIPI4 on the phagophore) into the growing phagophore membrane. ATG9A aids in phagophore growth by scrambling lipids between the inner and outer leaflets of the phagophore. The model shown here is inspired by data obtained in yeast. The proteins implicated in autophagosome growth are conserved between yeast and humans, and human proteins are shown here. ER: Endoplasmic Reticulum.

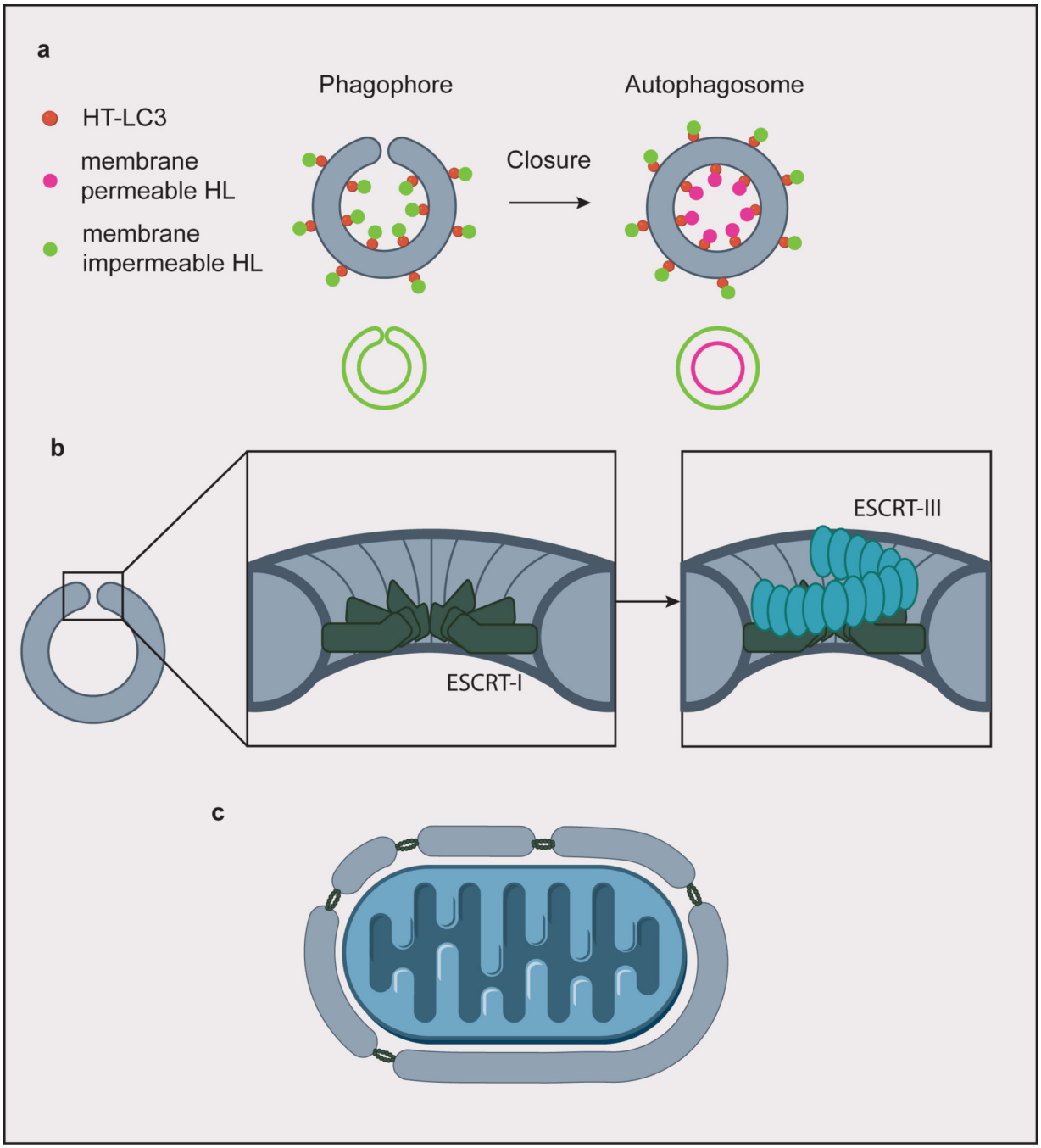

Fig. 4. Autophagosome closure.

(a) A screen for factors involved in autophagosome closure identified ESCRT-I protein VPS37A by using membrane permeable/impermeable Halo ligand (HL) dyes to selectively label Halo tagged (HT) LC3. Hits were identified from cells whose phagophores only stained with the impermeable dye, indicating failure to close the autophagosomes. Closed autophagosomes stain with both the membrane permeable and impermeable dyes. (b) ESCRT-I forms a dodecameric ring assembly at the rim of the closing autophagosome. In canonical ESCRT-mediated membrane scission, subsequent helical assembly of ESCRT-III

constricts the membrane rim. (c) For larger cargoes, ESCRTs could be involved in piecing together membranes to form a single, large phagophore.