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REVIEW ARTICLE

Aggrephagy: lessons from *C. elegans*

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Autophagy is a lysosome-mediated degradation process that involves the formation of an enclosed double-membrane autophagosome. Yeast genetic screens have laid the groundwork for a molecular understanding of autophagy. The process, however, exhibits fundamental differences between yeast and higher eukaryotes. Very little is known about essential autophagy components specific to higher eukaryotes. Recent studies have shown that a variety of protein aggregates are selectively removed by autophagy (a process termed aggrephagy) during *Caenorhabditis elegans* embryogenesis, establishing *C. elegans* as a multicellular genetic model to delineate the autophagic machinery. The genetic screens were carried out in *C. elegans* to identify essential autophagy genes. In addition to conserved and divergent homologues of yeast Atg proteins, several autophagy genes conserved in higher eukaryotes, but absent from yeast, were isolated. The genetic hierarchy of autophagy genes in the degradation of protein aggregates in *C. elegans* provides a framework for understanding the concerted action of autophagy genes in the aggrephagy pathway.

Key words: aggrephagy, *atg* gene, autophagosome, autophagy, *Caenorhabditis elegans*, *epg* gene.

INTRODUCTION

Macroautophagy (hereinafter referred to as autophagy) is an evolutionarily conserved catabolic process involving the delivery of a portion of the cytosol to the lysosome for degradation. The autophagy process can be dissected into a series of sequential membrane remodelling events [1]. Upon induction, a cup-shaped membrane sac, known as the isolation membrane (or phagophore), is nucleated in the cytoplasm. The isolation membrane expands further and eventually seals to form a double-membrane compartment called the autophagosome. After fusion with a lysosome to yield an autolysosome, the inner membrane of the autophagosome, together with the sequestrated materials, is degraded by hydrolytic enzymes. The digested material is released back into the cytosol for recycling, and lysosomes are regenerated from autolysosomes for lysosome homoeostasis [2,3]. In response to various stress conditions, autophagy acts as a cell survival mechanism by non-selectively degrading cytosolic components. Under normal physiological conditions, autophagy selectively enwraps damaged or superfluous organelles, protein aggregates or invading pathogens, thus functioning as a quality control system [4,5]. Autophagy plays an important role in many biological processes, such as development, tumour suppression, prevention of neurodegeneration and pathogen resistance [4,6].

YEAST GENETIC SCREENS LAY THE GROUNDWORK FOR A MOLECULAR UNDERSTANDING OF AUTOPHAGY

Autophagy was first described in the early 1960s with the discovery of partially degraded but still recognizable cytoplasmic elements inside membrane-bound bodies in kidney and liver cells under the electron microscope [7]. The molecular understanding of autophagy, however, did not begin until the introduction of the budding yeast *Saccharomyces cerevisiae* as a genetic model to study autophagy in the early 1990s [8,9]. Under nitrogen starvation conditions in proteinase-deficient yeast, autophagy activity is induced and autophagic bodies (autophagosome inner membranes and their enclosed contents) accumulate in the vacuole where they can be observed by light microscopy [10]. Under vegetative growth conditions, cytoplasmically synthesized vacuolar proteases, such as α-mannosidase and the precursor form of aminopeptidase I (prApeI), are transported to the vacuole via the autophagosome-like Cvt (cytoplasm to vacuole targeting) vesicle, a process called the Cvt pathway [2]. Yeast genetic screens have identified approximately 18 Atg (autophagy-related) genes that are essential for formation of autophagosomes and Cvt vesicles. These Atg proteins form distinct complexes that act at discrete steps of autophagosome formation [2,3]. The serine/threonine kinase Atg1 complex and the class III PI3K (phosphoinositide 3-kinase) complex are required for the initiation and nucleation of the isolation membrane. Two ubiquitin-like conjugation systems are involved in the expansion and closure of the isolation membrane. The Atg2–Atg18 complex is involved in cycling of the transmembrane protein Atg9, which supplies membranes for the formation of autophagosomes.

DIFFERENCES BETWEEN THE AUTOPHAGIC PROCESSES IN YEAST AND HIGHER EUKARYOTES

Study of autophagy in higher eukaryotes is greatly facilitated by the functional conservation of yeast Atg proteins in autophagosome formation. However, the autophagy process in higher eukaryotes exhibits several fundamental differences from that in yeast. In yeast, all autophagosomes arise from a

Abbreviations used: Atg, autophagy-related; Cvt, cytoplasm to vacuole targeting; DFCP1, double FYVE-domain-containing protein 1; EI24, etoposide-induced gene 24; EM, electron microscopy; ER, endoplasmic reticulum; FIP200, 200 kDa focal adhesion kinase family-interaction protein; GATE-16, Golgi-associated ATPase enhancer of 16 kDa; LC3, light chain 3; MVB, multivesicular body; PAS, pre-autophagosomal structure; PE, phosphatidylethanolamine; PI3K, phosphoinositide 3-kinase; PIG8, p53-induced gene 8; RER, rough ER; SQST-1, sequestosome 1; TOR, target of rapamycin; mTOR, mammalian TOR; Vps, vacuolar protein sorting; WIPI, WD40 repeat protein interacting with phosphoinositides.

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single perivacuolar site, known as the PAS (pre-autophagosomal structure), whereas, in mammalian cells, there is no evidence for a PAS and isolation membranes can be generated simultaneously at multiple sites [3,11,12].

In yeast, autophagosomes fuse directly with the single large acidic vacuole. In higher eukaryotes, the autophagosome and lysosome are comparable in size. Nascent autophagosomes undergo a series of maturation processes by fusing with components of the endocytic pathway, including early and late endosomes and MVBs (multivesicular bodies), to generate a hybrid organelle, called the amphisome, which is more acidic than the autophagosome. The amphisome then fuses with lysosomes to form degradative autolysosomes [11,12]. Components of the endocytic pathway have been demonstrated to play an essential role in autophagosome maturation, including the small GTP-binding protein Rab7, which regulates the maturation of early endosomes to late endosomes, COPI (coatomer protein 1), which is required for early endosome maturation, and the ESCRT (endosomal sorting complex required for transport) proteins, which participate in sorting ubiquitinated membrane proteins into the MVBs for subsequent lysosomal degradation [13–16]. Furthermore, various signalling pathways need be integrated into the autophagic machinery during development in higher eukaryotes [17]. Thus it is conceivable that, in addition to highly conserved Atg proteins, the more elaborate autophagic machinery in higher eukaryotes has acquired components that are absent from yeast. Extensive biochemical purification approaches have been carried out to identify components that interact with Atg proteins. Many of the interacting factors identified, however, are involved in regulating autophagy activity under starvation conditions. Only very few metazoan-specific autophagy genes have been identified that are essential for autophagosome formation and maturation under normal physiological conditions. In the present review, we mainly discuss the establishment of *Caenorhabditis elegans* as a model system that is suitable for identifying essential components of the autophagic machinery by unbiased genetic screens.

AUTOPHAGIC DEGRADATION OF A VARIETY OF PROTEIN AGGREGATES DURING *C. ELEGANS* EMBRYOGENESIS

Degradation of germline P granule components in somatic cells

During *C. elegans* embryogenesis, germ blastomeres are generated through four sequential asymmetric cell divisions, which give rise to P1, P2, P3 and, finally, P4. P4 divides equally at the ~100-cell embryonic stage, producing two germ precursor cells, Z2 and Z3, which remain quiescent during embryogenesis and proliferate throughout larval development to generate germ cells [18]. P granules, a specialized type of protein–RNA aggregate, are dispersed throughout the cytoplasm of newly fertilized embryos, but become localized exclusively in the germline blastomeres during early embryonic divisions [18] (Figures 1A and 1B). P granules are expressed in all descendants of Z2 and Z3 with the exception of mature sperm. P granules contain both transient and constitutive components [18]. Transient components associate with P granules only during early embryogenesis, whereas constitutive components interact with P granules during all developmental stages [18]. During the unequal divisions that generate germline blastomeres, transient and constitutive P granule components are also segregated into sister somatic blastomeres, but they are quickly removed [19–21]. The transient components, including the CCCH-type zinc-finger proteins PIE-1, MEX-1 and POS-1, are removed by the cullin-dependent degradation system [22]. The constitutive P granule components, including the RGG box-containing RNA-binding proteins PGL-1 and PGL-3, are selectively degraded by autophagy [21]. In the somatic cells of autophagy mutants, PGL-1 and PGL-3 co-localize and accumulate into aggregates, which are named PGL granules to differentiate them from germline P granules [21] (Figures 1C–1F). Compared with P granules, PGL granules lack some constitutive components, such as the RNA helicases GLH-1 and GLH-4, and also transient components, such as POS-1, MEG-1 and PIE-1 [21].

Genetic screens revealed that formation and degradation of PGL granules requires the activity of *sepa-1*, which encodes a novel coiled-coil domain-containing protein. In *sepa-1* mutants and in autophagy mutants with simultaneous depletion of SEPA-1, PGL-1 and PGL-3 fail to be removed and are diffusely localized in the cytoplasm of somatic cells [21]. SEPA-1 is zygotically synthesized and thus is absent from germ P granules. SEPA-1 forms aggregates via its self-oligomerization domain and is also selectively removed by autophagy during embryogenesis [21]. In wild-type embryos, SEPA-1 aggregates are present in early stage embryos, but are absent from the comma stage onwards. In autophagy mutants, SEPA-1 aggregates persist in late-stage embryos and also in early larvae [21]. Degradation of SEPA-1 is independent of PGL-1 and PGL-3. SEPA-1 interacts directly with PGL-3 and is an integral component of PGL granules in autophagy mutants [21]. Thus the normal role of SEPA-1 is to function in somatic cells as a receptor that recruits PGL-1 and PGL-3 into aggregates for degradation.

In genetic screens, mutations in *epg-2* were identified that cause a specific defect in degradation of PGL granules, but not in other autophagy-regulated processes [23]. In *epg-2* mutants, PGL granules are formed, but fail to be removed. *epg-2* encodes a coiled-coil domain-containing protein. EPG-2 itself forms aggregates that co-localize with SEPA-1 aggregates in wild-type and PGL granules in autophagy mutants [23]. EPG-2 is also removed by autophagy, and this process is independent of *pgl-1*, *pgl-3* and *sepa-1*. Loss of function of *epg-2* disrupts the association of PGL granules with LGG-1/Atg8 puncta, which are markers for autophagic structures [23]. Therefore, although SEPA-1 interacts directly with the autophagy protein LGG-1/Atg8, removal of PGL granules also requires EPG-2, which may function as an adaptor linking PGL granules to the autophagic machinery. Thus components of PGL granules act in a hierarchical order to facilitate their removal by autophagy during embryogenesis [23].

Degradation of *C. elegans* SQST-1 (sequestosome 1) during embryogenesis

Human p62/SQSTM1 (sequestosome 1) is a common component of ubiquitinated protein aggregates, including Lewy bodies in Parkinson's disease, neurofibrillary tangles in Alzheimer's disease, huntingtin aggregates in Huntington's disease, and Mallory bodies in alcoholic hepatitis [24]. p62 and its fruitfly homologue Ref(2) function as a receptor for the formation of polyubiquitinated protein aggregates and mediate their autophagic degradation [25–27]. p62 contains a self-oligomerization PB1 domain at its N-terminus, an LC3 (light chain 3)-interacting LIR motif, and a UBA domain at its C-terminus [25,28,29]. p62 is an autophagy substrate and its degradation requires the self-oligomerization and LC3-binding motifs [28,29]. The *C. elegans* p62 homologue SQST-1 is also degraded by autophagy during embryogenesis [23]. In wild-type nematodes, SQST-1 is weakly expressed and diffusely localized in the cytoplasm (Figure 1G). However, in autophagy mutants, SQST-1 accumulates and forms numerous aggregates (Figure 1H). SQST-1 aggregates and PGL

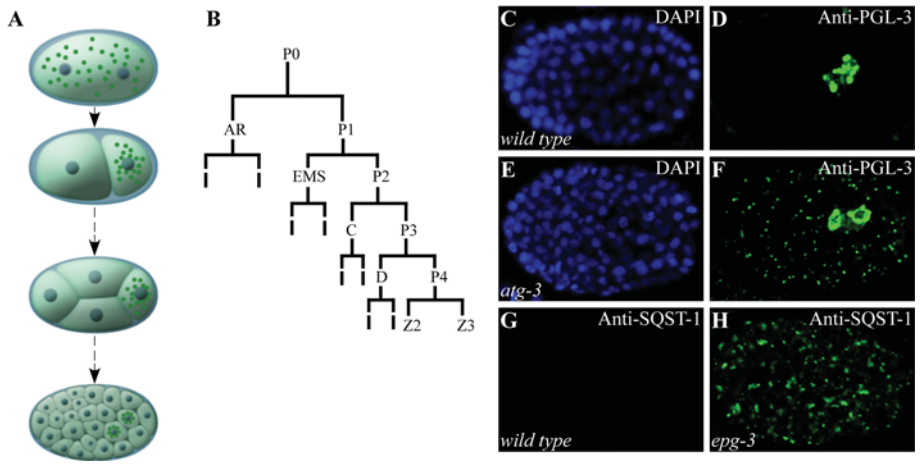

Figure 1 Degradation of P granules and SQST-1 during embryogenesis

(**A**) Cartoon illustrating the process by which P granules are segregated into germ blastomeres, from the germline blastomere P1 to germ precursor cells Z2 and Z3 during embryobiogenesis. (**B**) Lineage diagram showing the generation of germ precursor cells. (**C** and **D**) Endogenous P granules, detected by anti-PGL-3 antibody, are restricted to germ precursor cells Z2 and Z3 in wild-type embryos. (**C**) DAPI (4′,6-diamidino-2-phenylindole) image of the embryo shown in (**D**). (**E** and **F**) PGL-3-positve granules ectopically accumulate in somatic cells in *atg-3* mutant embryos. (**E**) DAPI image of the embryo shown in (**F**). (**G** and **H**) SQST-1, detected by anti-SQST-1 antibody, is weakly expressed and diffusely localized in a wild-type embryo (**G**), but accumulates into a large number of aggregates in an *epg-3* mutant embryo. See [23] for a description of experiments shown in (**C**–**H**).

granules are distinct in various autophagy mutants [23]. Therefore autophagy activity is required for selective degradation of a variety of aggregate-prone proteins during *C. elegans* embryogenesis, a process referred to as aggrephagy.

PGL granules and SQST-1 aggregates are degraded by the basal level of autophagy

The *C. elegans* embryo, which remains the same size from the one-cell stage to the end of embryogenesis (558 cells), is enclosed by a tough eggshell that is impermeable to most solutes. The development of *C. elegans* embryos therefore relies mainly on the degradation of maternally loaded yolk rather than external nutrients. Consistently, autophagic degradation of PGL granules and SQST-1 aggregates is not regulated by TOR (target of rapamycin) signalling, which integrates nutrient status into the autophagic machinery [30,31].

Maternally contributed P granule components that remain in somatic cells during early embryonic divisions may also provide a nutrient resource for embryogenesis. Autophagic degradation of PGL granule components also prevents the formation of aggregates that could be toxic for animal development, as the defect in embryonic development in autophagy mutants is suppressed by mutation of *sepa-1* [32].

Genetic screens for genes essential for aggrephagy

C. elegans is a relatively simple animal, with 959 somatic cells in the hermaphrodite. It is transparent with an invariant cell lineage, self-fertilizing, easy to culture and has a short reproductive cycle (~3 days). These features, together with the wealth of knowledge about *C. elegans* developmental biology, as well as powerful genetic tools such as forward and reverse genetic screens to identify new genes, make *C. elegans* an ideal model system to identify essential autophagy genes [20]. Approximately 160 mutants were identified from genetic screens that exhibit defects in degradation of both PGL granules and SQST-1 aggregates [23]. Further characterization of these mutants indicated that mutations in yeast *Atg* gene homologues, including *Atg1–Atg10*, *Atg12*, *Atg16* and *Atg18*, were isolated from the screens, demonstrating high conservation of the autophagic machinery between yeast and *C. elegans* [23]. Genes that have no yeast counterparts or that are distantly related homologues of Atg genes are named as *epg* genes (ectopic PGL granules or p62 aggregates). *epg-1* and *epg-8* encode highly divergent functional homologues of Atg13 and Atg14 respectively [30,33]. *epg-3*, *epg-4*, *epg-5*, *epg-6* and *epg-9* encode autophagy components that are absent from budding yeast, but exist in higher eukaryotes [23,31,34].

CONSERVED *ATG* GENES ESSENTIAL FOR AGGREPHAGY IN *C. ELEGANS*

The two ubiquitin-like conjugation systems

The two ubiquitin-like proteins (Atg8 and Atg12) undergo ubiquitin-like conjugation processes, which are essential for the expansion and closure of the autophagosome [35]. The Atg8 precursor is processed by the cysteine protease Atg4 to expose its C-terminal glycine residue, which is then activated by Atg7 (an E1 ubiquitin-activating enzyme), transferred to Atg3 (an E2 ubiquitin-conjugating enzyme) and finally conjugated to PE (phosphatidylethanolamine) [36,37]. Atg8–PE forms oligomers that may mediate tethering and fusion of autophagosomal precursors [38]. Atg8–PE localizes to both the outer and inner membrane of the isolation membrane. Upon completion of the autophagosome, Atg4 acts as a deconjugation enzyme that cleaves Atg8 from PE on the outer membrane, whereas Atg8–PE bound to the inner membrane of the autophagosome is delivered to the lysosome [36,39]. Under nutrient-rich conditions, Atg8 mainly exists in the cytosol; under autophagy-inducing conditions, lipidated Atg8 associates with autophagic structures [36,38]. Thus Atg8–PE is widely used as a marker to monitor autophagy induction and autophagosome formation. In the Atg12-conjugation system, the C-terminal glycine residue of Atg12 is conjugated to the lysine residue of Atg5 via the actions of the E1 enzyme Atg7 and a specific E2 enzyme, Atg10 [40]. The Atg12–Atg5 conjugate interacts further with Atg16 to form a multimeric complex via Atg16 oligomerization that localizes to the isolation

membrane [41–43]. The Atg12–Atg5–Atg16 complex exerts an E3-like enzyme activity on the conjugation of Atg8 with PE [44].

C. elegans has two Atg8 homologues, encoded by *lgg-1* and *lgg-2*. The role of *lgg-1* in the aggrephagy pathway has been characterized [23]. As in *atg-3* and *atg-7* mutants, loss of function of *lgg-1* causes accumulation of PGL granules and SQST-1 aggregates, which are spherical, dispersed in the cytoplasm and are separable [23]. Both the unlipidated form (LGG-1-I) and lipidated form (LGG-1-II) are present in embryonic extracts, whereas LGG-1 precursors are absent [23]. LGG-1 exhibits a dynamic expression pattern during embryogenesis. In wild-type embryos, LGG-1 forms distinct punctate structures at the ~64- to 100-cell stage (Figure 2A), which largely disappear from late-stage embryos [23]. LGG-1 puncta are absent from *atg-3* and *atg-7* mutants, indicating that LGG-1-II is required for the formation of LGG-1 puncta [23]. LGG-1 puncta exhibit distinct morphologies and distribution patterns in different autophagy mutants. For example, in *epg-1* and *epg-9* mutant embryos, LGG-1 forms big aggregates in a few cells [23,31] (Figure 2B). In *epg-3*, *epg-4* and *epg-6* mutants, LGG-1 accumulates into cluster-like structures during embryogenesis [23,34] (Figure 2C).

The Atg1 kinase complex

The Atg1 complex consists of the serine/threonine protein kinase Atg1 and its regulator Atg13. The Atg1–Atg13 complex in yeast also associates with Atg17–Atg29–Atg31 during starvation-induced autophagy [45–49]. The kinase activity of Atg1 is essential for autophagy [50]. The composition of the Atg1 complex is divergent in mammalian cells. The Atg1 homologue ULK1 forms a complex with mAtg13 (the highly divergent homologue of Atg13), FIP200 (200 kDa focal adhesion kinase family-interaction protein) and Atg101 [51–53]. Homologues of FIP200 and Atg101 are widely conserved in eukaryotes, but not in *S. cerevisiae*. Both Atg13 and FIP200 are essential for the stability of ULK1 as well as its correct localization to pre-autophagosomes [51,54–56]. Furthermore, Atg101 regulates the stability and basal phosphorylation of Atg13 and ULK1 [52]. The physiological function of Atg101 has yet to be determined.

The Atg1–ULK1 complex acts at multiple steps in autophagosome formation. It is essential for the induction step of autophagy by integrating nutrient status via TOR kinase [57,58]. Under growth conditions, yeast Atg13 is hyperphosphorylated in a TOR-dependent manner and dissociates from Atg1, whereas nutrient depletion results in hypophosphorylation of Atg13, which binds to Atg1 with high affinity and activates its kinase activity [59]. In mammalian cells, the ULK–mAtg13–FIP200 complex is stable and not altered by nutrient conditions. mTORC1 [mTOR (mammalian TOR) complex 1] associates directly with the ULK1 complex in a nutrient-dependent manner and mTOR phosphorylates ULK1 and mAtg13 [54–56]. The Atg1–ULK1 complex also recruits other Atg proteins to the PAS in yeast and the punctate structures consisting of Atg proteins in mammalian cells and regulates the cycling of Atg9 between autophagosomes and other punctate structures [57,58,60]. The molecular composition and regulatory mode of the Atg1 complex differ substantially between yeast and higher eukaryotes, and may have evolved to respond to integration of various developmental signals during animal development.

The *C. elegans* Atg1 kinase complex consists of UNC-51/Atg1, EPG-1 and EPG-9 [30,31]. *unc-51* encodes the *C. elegans* Atg1 kinase homologue. Mutations in *epg-1* and *epg-9* were identified that cause defects in degradation of PGL granules and SQST-1 aggregates [30,31]. EPG-1 is highly conserved in other nematode species, including *Caenorhabditis briggsae* and *Caenorhabditis remanei*. The N-terminus of EPG-1 shows similarity to mammalian Atg13 and is more distantly related to yeast Atg13. EPG-1 interacts directly with UNC-51 [30]. EPG-1 thus encodes a highly divergent Atg13 homologue. *epg-9* encodes a protein with significant homology with mammalian ATG101 [31]. EPG-9 associates directly with EPG-1. Formation of the UNC-51–EPG-1–EPG-9 complex is not required for the stability of the individual proteins [31]. Besides abnormal autophagy, loss of function of *unc-51* causes other defects, including loss of co-ordination, small body size and abnormal axonal growth and guidance. These defects are not observed in *epg-1* and *epg-9* mutants [30,31]. Therefore UNC-51 forms a complex with EPG-1 and EPG-9 that specifically functions in the autophagy pathway.

Genetic analysis also revealed that UNC-51, EPG-1 and EPG-9 act at the same step in the aggrephagy pathway. Mutants of *unc-51*, *epg-1* and *epg-9* exhibit the same autophagy phenotypes: the PGL granules and SQST-1 aggregates are round, evenly dispersed in the cytoplasm and separable, whereas the LGG-1 puncta exhibit a characteristic distribution pattern in which they are absent from most embryonic cells, but accumulate into large aggregates in a few cells [23,31].

The Vps (vacuolar protein sorting) 34 complex

The class III PI3K Vps34 phosphorylates PtdIns to produce PtdIns3*P* [11]. PtdIns3*P* is highly enriched on autophagosomal membranes and also near the tips of elongating isolation membranes [61]. In yeast, Vps34 forms two distinct complexes. Complex I contains Vps34, Vps15 (a serine/threonine kinase required for Vps34 activity) and two accessory proteins, Vps30/Atg6 and Atg14, whereas complex II contains Vps38 instead of Atg14 [62,63]. The complex containing Atg14 is involved in autophagy, whereas the complex containing Vps38 functions in the endocytic pathway [62,63]. In mammalian cells, the PI3K complex I includes hVps34, Beclin 1 (homologue of Atg6), p150 (homologue of Atg15) and the highly divergent Atg14 homologue, Atg14L [64–67]. Multiple factors, including Bcl2, UVRAG (UV radiation resistance-associated gene) and Ambra1 (activating molecule in Beclin 1-regulated autophagy), regulate autophagy activity via their interaction with Beclin 1 [68]. PtdIns3*P* plays essential roles in the autophagy pathway [11]. The PtdIns3*P*-enriched subdomain of the ER (endoplasmic reticulum), which is called the omegasome and can be labelled by the ER-localized PtdIns3*P*-binding protein DFCP1 (double FYVE-domain-containing protein 1), provides a platform for accumulation of Atg proteins, expansion of autophagosome membranes and generation of autophagosomes [69–72]. PtdIns3*P* also regulates autophagosome maturation and controls autophagosome size [34].

Homologues of Vps34, Beclin 1 and Vps15 are readily identified in *C. elegans* and their loss of function causes defects in the degradation of PGL granules and SQST-1 aggregates. The complex also contains a highly divergent functional homologue of Atg14, encoded by *epg-8* [33]. EPG-8 is highly conserved in nematodes, but obvious EPG-8 homologues are not present outside the nematode phylum. EPG-8 has slight similarity to the mammalian Atg14 protein. EPG-8 is predicted to possess two coiled-coil domains within the N-terminus. The first coiled-coil domain of EPG-8 binds to BEC-1 [33]. Formation of the BEC-1–EPG-8 complex is not required for the stabilities of EPG-8 and BEC-1, which is different from the situation in yeast and mammalian cells, where Atg14 is not stable in the absence of interactions with Vps30/Beclin 1 [63,64]. In addition,

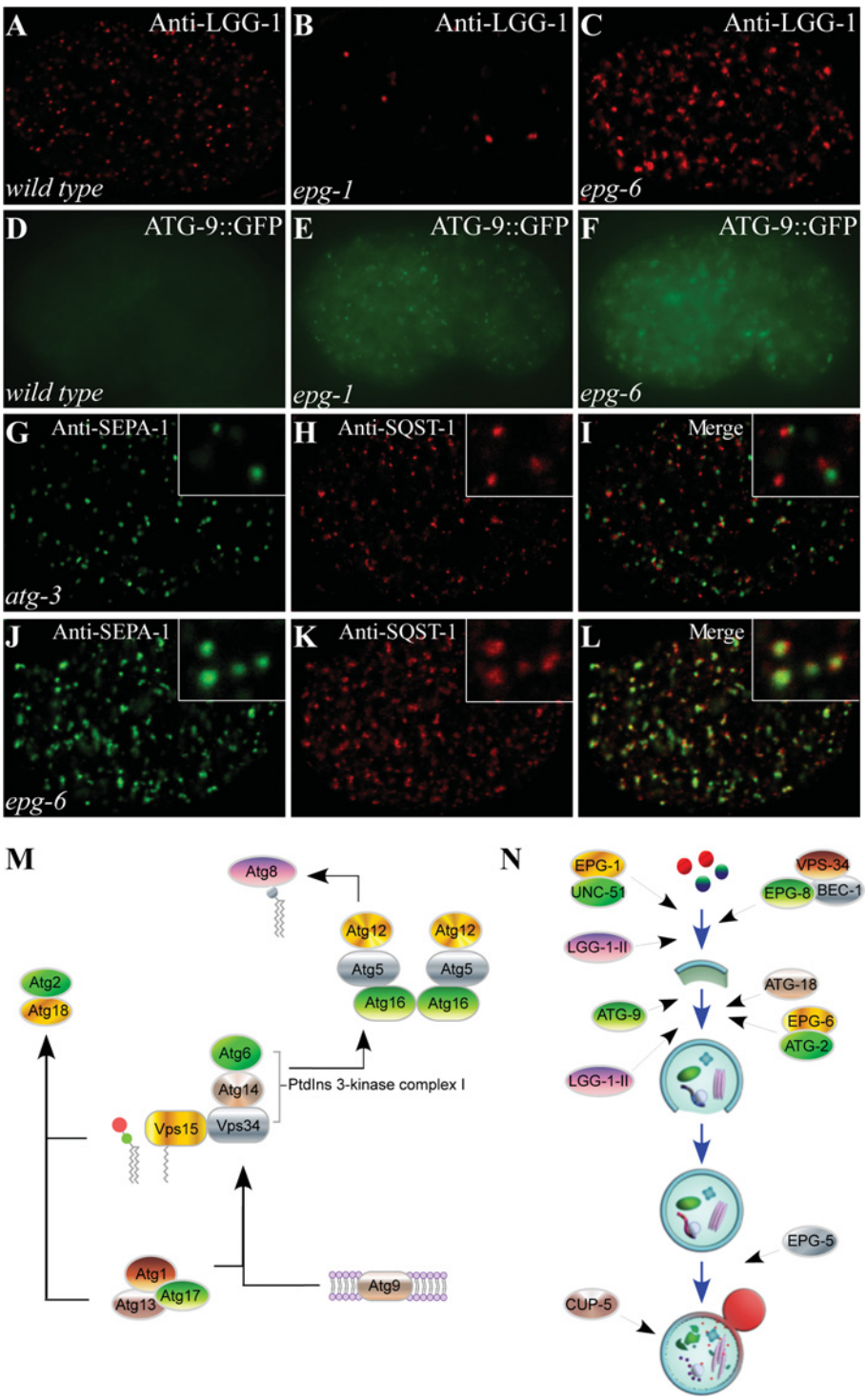

Figure 2 Distinct phenotypes in autophagy mutants (LGG-1 puncta and protein aggregates, DFCP1 and ATG-9 puncta) and the hierarchy in yeast and *C. elegans*

(**A**–**C**) Immunostaining of LGG-1 shows distinct patterns in wild-type, *epg-1* mutants and *epg-6* mutants during embryogenesis. (**D**–**F**) ATG-9::GFP shows different patterns in wild-type, *epg-1* mutants and *epg-6* mutants during embryogenesis. (**G**–**I**) SEPA-1 and SQST-1 aggregates, detected by specific antibodies, are largely separable in *atg-3* mutant embryos. (**J**–**L**) SEPA-1 and SQST-1 aggregates accumulate into cluster-like structures and co-localize well in *epg-6* mutant embryos. See [23,34] for a description of experiments shown in (**A**–**L**). (**M**) The hierarchy of the Atg proteins, based on genetic studies, in organizing the PAS in yeast. Modified by permission from Macmillan Publishers Ltd: *Nature Reviews Molecular Cell Biology* [Nakatogawa, H., Suzuki, K., Kamada, Y., and Ohsumi, Y. (2009) Dynamics and diversity in autophagy mechanisms: lessons from yeast. Nat. Rev. Mol. Cell Biol. **10**, 931–937], © 2009. (**N**) The hierarchical order of autophagy proteins, based on genetic analyses, in the aggrephagy pathway in *C. elegans*.

Atg14L enhances the kinase activity of Vps34 in mammalian cells [66,67]. However, the level of PtdIns3*P* remains unchanged in *epg-8* mutants [33]. *epg-8* mutants exhibit autophagy phenotypes identical with those in *bec-1* mutants: PGL granules and SQST-1 aggregates are spherical and separable, and levels of LGG-1-I and LGG-1-II are dramatically elevated compared with wild-type embryos, whereas LGG-1 puncta are weaker [33]. Despite the conservation of common components of the Vps34 complex, including Vps34, Vps30/Beclin 1 and Vps15, Atg14 is highly divergent in yeast and higher eukaryotes, suggesting that it may have evolved to elaborate various signalling pathways in metazoans.

The Atg2–Atg18 complex

PtdIns3*P* regulates autophagy, at least partially, by recruiting WD40 repeat PtdIns3*P*-binding effectors to autophagic membranes. The WD40 repeat PtdIns3*P* effector Atg18 forms a complex with Atg2 and is recruited to the PAS in yeast via its binding to PtdIns3*P* [73]. The mammalian PtdIns3*P* effectors include the WIPI (WD40 repeat protein interacting with phosphoinositides) family proteins, which have been classified into two large paralogue groups, WIPI1/2 and WIPI3/4 [74]. PtdIns3*P* recruits WIPI1 and WIPI2 to early autophagic structures, and WIPI1 and WIPI2 were originally thought to act as mammalian Atg18 homologues [73–75].

Mutations in *atg-18* and *epg-6* that cause defects in the aggrephagy pathway were isolated [34]. EPG-6 and ATG-18 are WD40 repeat proteins with PtdIns3*P*-binding activity. Phylogenetic analysis indicated that ATG-18 is related to WIPI1/2, whereas EPG-6 is more closely related to WIPI3/4. EPG-6, but not ATG-18, interacts directly with ATG-2 [34]. WIPI4 also interacts with Atg2 [76]. Taken together with the genetic data below, these results strongly argue that EPG-6/WIPI4 is the functional orthologue of yeast Atg18.

Consistent with the finding that EPG-6 forms a complex with ATG-2, *epg-6* and *atg-2* mutants exhibit the same phenotypes: PGL granules and SQST-1 aggregates form clusters that largely co-localize, and LGG-1 puncta and DFCP1 punctate structures are also enlarged and accumulate into clusters that co-localize with PGL aggregates [34]. This suggests that *epg-6* and *atg-2* control the size of omegasomes and autophagosomes. EM (electron microscopy) analysis revealed that isolation membranes, mostly located between the RER (rough ER) cisterns, and also multilayered membrane-like structures (possibly phospholipid accumulations) that often associated with isolation membranes, accumulate in *epg-6* and *atg-2* mutants [34]. Therefore the formation of the isolation membrane and its further progression to the autophagosome stage (closure of the isolation membrane) might be disturbed in *epg-6* and *atg-2* mutants. The EPG-6/WIPI4 complex controls autophagosome size, perhaps by modulating the persistence of PtdIns3*P*, which would restrict the expansion of PtdIns3*P* along the ER strands, or by direct involvement in generating negative curvature at the inner surface of the isolation membrane, or in preventing homotypic fusion of the edges of the isolation membrane.

atg-18 mutants show distinct phenotypes from *epg-6* and *atg-2* mutants [34]. In *atg-18* mutants, PGL granules and SQST-1 aggregates are round and largely separable. DFCP1::GFP puncta accumulate in *atg-18* mutants, but are smaller in size than those in *epg-6* and *atg-2* mutants. In *atg-18* mutants, LGG-1 puncta are only weakly formed at early embryonic stages, but gradually accumulate in later-stage embryos, and are largely non-overlapping with PGL granules. ATG-18 may regulate the association of autophagosomal membranes with protein aggregates [34]. The distinct function of *epg-6* and *atg-18* in the aggrephagy pathway indicates that distinct autophagic PtdIns3*P* effectors mediate the role of PtdIns3*P* at different steps in the autophagy pathway.

Atg9

In yeast, the six-transmembrane protein Atg9 concentrates in a novel compartment comprising clusters of vesicles and tubules, movement of which to the PAS triggers the hierarchical recruitment of other Atg proteins that mediate fusion of the Atg9-positive vesicles and tubules, leading to the formation of isolation membranes [60,77,78]. The retrograde movement of Atg9 away from the PAS requires the Atg1 complex, the Vps34 complex and the Atg18–Atg2 complex [78,79].

Multiple mutant alleles of *atg-9* were identified in our genetic screens [34]. In *atg-9* mutants, PGL granules and SQST-1 aggregates are spherical and separable. LGG-1 puncta are largely separable from PGL granules [34]. A functional translational reporter for *atg-9* shows that ATG-9::GFP diffusely localizes in the cytoplasm apart from a few tiny aggregates during *C. elegans* embryogenesis [34] (Figure 2D). In mutants of *unc-51*, *epg-1* and *epg-9*, ATG-9::GFP accumulates into numerous bright small punctate structures [31,34] (Figure 2E). In *epg-8* mutants, ATG-9::GFP accumulates into punctate structures that are larger in size and weaker in intensity than those in *unc-51* mutants. In *epg-6* and *atg-2* mutants, ATG-9::GFP accumulates into much larger puncta with irregular morphology that are co-localized with PGL granules and LGG-1 puncta (Figure 2F). *atg-18* mutants also accumulate ATG-9::GFP puncta, but these are weaker in intensity and appear to align closely with each other [34]. The distribution of ATG-9::GFP is thus regulated by the UNC-51 complex, EPG-8, ATG-18 and the EPG-6–ATG-2 complex, but displays distinct patterns in mutants for each of these factors.

THE DIFFERENTIAL FUNCTION OF MULTIPLE HOMOLOGUES OF THE SAME AUTOPHAGY GENE IN THE AGGREPHAGY PATHWAY

The presence of multiple homologues of the same yeast *Atg* genes confers another layer of complexity on the autophagic machinery in higher eukaryotes. At least eight Atg8 homologues are identified in mammals that are divided into LC3 and GATE-16 (Golgi-associated ATPase enhancer of 16 kDa)/GABARAP (γ-aminobutyric acid type A receptor-associated protein) subfamilies [80]. The single Atg4 in yeast has four mammalian homologues, Atg4A, Atg4B, Atg4C and Atg4D, which exhibit differential processing activity towards different Atg8 proteins [81–85]. HsAtg4B cleaves all Atg8s [83,84]. HsAtg4A efficiently cleaves GATE-16, but not other Atg8s, whereas HsAtg4C and HsAtg4D have minimal activity [81,85]. The physiological function of the individual homologues in the autophagy pathway remains poorly understood. The multiple homologues might act redundantly, function in a tissue-specific or temporal fashion, or accomplish other functions distinct from autophagy.

C. elegans has two Atg4 homologues, encoded by *atg-4.1* and *atg-4.2*. ATG-4.1 is closely related to human Atg4A and Atg4B, whereas *atg-4.2* is more closely related to human Atg4C and Atg4D [86]. Loss of *atg-4.1* activity causes defective degradation of a variety of protein aggregates, whereas *atg-4.2* mutants show no defects [86]. Both ATG-4.1 and ATG-4.2 process LGG-1 precursors, removing the C-terminal seven amino acids to expose the glycine residue. *In vitro* cleavage assays indicate that ATG-4.1 processes LGG-1 precursors approximately 100-fold more

efficiently than ATG-4.2 [86]. LGG-1 precursors, which are absent from wild-type animals, dramatically accumulate in *atg-4.1* mutants, but not in *atg-4.2* mutants. In *atg-4.1* mutants, lipidated LGG-1-II is present at a level similar to that in wild-type animals. Unlipidated LGG-1-I, however, is absent from *atg-4.1*-null mutants. In *atg-4.2* mutants, LGG-1 is properly processed and no LGG-1 precursors are found [86]. Consistent with the presence of the lipidated form of LGG-1, the temporal pattern of formation of LGG-1 puncta is similar to that of wild-type in *atg-4.1* and *atg-4.2* mutant embryos. LGG-1 puncta, however, are completely absent from *atg-4.1*; *atg-4.2* double mutants, indicating that *atg-4.1* and *atg-4.2* function redundantly, but ATG-4.1 plays a principal role in LGG-1 processing.

Both the processing and deconjugating activities of Atg4 are required for normal progression of autophagy in yeast [35]. Yeast Atg4 recycles Atg8–PE produced on inappropriate membranes to maintain a cytoplasmic pool of unlipidated Atg8 that can undergo lipidation and participate in autophagosome formation at the PAS [87]. A mutant form of LGG-1, in which the conjugated glycine residue is directly exposed, rescues defective degradation of protein aggregates in *atg-4.1* mutants and, to a lesser extent, in *atg-4.1*; *atg-4.2* double mutants. *atg-4.1* mutant animals expressing this mutant form of LGG-1 show no significant changes in levels of LGG-1-II, but accumulate unlipidated LGG-1, which is absent from *atg-4.1* mutants, implying that maintenance of an unlipidated pool of LGG-1 is essential for autophagy flux [86].

C. elegans has two Atg8 homologues, LGG-1 and LGG-2, and also two Atg16 homologues. The detailed functions of these genes in the aggrephagy pathway have yet to be characterized.

METAZOAN-SPECIFIC AUTOPHAGY GENES

epg-3

epg-3 encodes the homologue of mammalian VMP1 (vacuole membrane protein 1) [23], which is highly expressed in pancreas affected by acute pancreatitis [88]. EPG-3 is conserved in *Arabidopsis*, *Drosophila* and mammals, but no homologue is present in *S. cerevisiae*. In *epg-3* mutants, PGL granules, SQST-1 aggregates and LGG-1 puncta accumulate that are bigger in size and stronger in intensity than in wild-type. The LGG-1 puncta are co-localized with PGL granules. DFCP1-labelled omegasomes also dramatically accumulate and co-localize with LGG-1 puncta in *epg-3* mutant embryos [23]. EM analysis showed accumulation of isolation membranes in *epg-3* mutant animals, indicating that *epg-3* is essential for progression of omegasomes to autophagosomes [23].

epg-4

epg-4 encodes the homologue of human EI24 (etoposide-induced gene 24)/PIG8 (p53-induced gene 8), expression of which is strongly activated by the tumour-suppressor protein p53 and by etoposide, a genotoxic agent that activates p53 [89,90]. Both EPG-4 and EI24/PIG8 possess six transmembrane domains and localize to the ER [23,89]. EPG-4 homologues are present in higher eukaryotes, but absent from *S. cerevisiae*. The autophagy phenotype in *epg-4* mutants is similar to that in *epg-3* mutants, including accumulation of enlarged protein aggregates, LGG-1 puncta and DFCP1-labelled omegasomes [23]. In *epg-4* mutants, in addition to isolation membranes, structures with multiple membrane layers that surround cytoplasmic areas also accumulate [23]. Such lamellar structures have also been detected in *epg-3*, *epg-6* and *atg-2* mutants [23,34]. *epg-4* mutants also show a large number of ribosome-free double-membrane structures with wide clefts between the two membrane sheets, which appear to be continuous with the RER in some cases [23]. Therefore EPG-4 may be involved in transforming or reorganizing normal ER membranes into nascent isolation membranes.

Mice with neural-specific *Ei24* deficiency develop massive axon degeneration and extensive neuron loss in various brain and spinal cord regions [91]. Liver-specific depletion of *Ei24* causes severe liver injury with hepatomegaly with hepatocyte hypertrophy and development of tumour-like protrusions [91]. The behavioural and motor abnormalities and liver injury in *Ei24*-deficient mice are similar to those in *Atg5*- and *Atg7*-deficient mice [26,92–95]. *Ei24* deficiency impairs autophagic flux, resulting in accumulation of p62 aggregates and ubiquitin-positive inclusions in neurons and hepatocytes [91]. Therefore *Ei24* is an essential component of the basal autophagy pathway in mammals.

epg-5

epg-5 is the *C. elegans* homologue of the human *EPG5* gene, which is frequently mutated in breast tumours [96]. EPG-5 is highly conserved in *Drosophila* and mammals, but has no homologue in yeast. In *epg-5* mutants, PGL granules and SQST-1 aggregates are spherical and dispersed in the cytoplasm and are separable [23]. There is no evident accumulation of DFCP1 puncta. Thus *epg-5* mutants exhibit phenotypes distinct from those in *epg-3* and *epg-4* mutants.

cup-5

cup-5, encoding the *C. elegans* functional orthologue of MLN1/TRPML1 (transient receptor potential mucolipin 1), regulates lysosome biogenesis [97,98]. Reformation of lysosomes from endosome–lysosome hybrid organelles involves sorting and condensation of lysosomal contents, and budding and maturation of nascent lysosomes. The scission and maturation of nascent lysosomes is impaired in *cup-5* mutants, resulting in the formation of enlarged vacuoles with characteristics of late endosomes and lysosomes [97,98]. PGL granule components SQST-1 and LGG-1::GFP accumulate in enlarged vacuoles in *cup-5* mutants, indicating that the hybrid vesicles in these mutants also receive membranes and cargoes from autophagosomes [99]. Loss of autophagy activity partially suppresses the enlarged vacuole accumulation abnormality of *cup-5* mutants [99]. Therefore the basal level of autophagy activity regulates the size and number of lysosomes.

THE GENETIC HIERARCHY OF THE ATG PROTEINS IN ORGANIZING THE PAS IN YEAST AND IN THE AGGREPHAGY PATHWAY IN *C. ELEGANS*

The hierarchy in the formation of the PAS structure in yeast and Atg protein punctate structures in mammalian cells

All Atg proteins associate at least transiently with the PAS in yeast or with Atg-positive punctate structures in mammalian cells [58,60]. By using reporters for various Atg proteins, comprehensive analyses have been performed to establish the hierarchy of Atg proteins in organizing the PAS in yeast and the Atg-positive puncta in mammalian cells. The Atg1 kinase complex acts at the most upstream step, followed by the Atg14-containing PI3K complex [58,60]. Recruitment of Atg18/WIPI1 and formation of DFCP1-labelled omegasomes (in mammalian cells) depends on the Atg1–ULK1 complex and the PI3K complex

Table 1 Distinct genetic phenotypes in different autophagy mutants

NA, aggregates are absent from embryos; ND, not determined.

Mutant	PGL granules pattern	SQST-1 pattern	Level of co-localization between PGL granules and SQST-1 aggregates	LGG-1 pattern	Level of co-localization between PGL granules and LGG-1 aggregates	DFCP-1::GFP pattern	ATG-9::GFP pattern
Wild-type	NA	NA	NA	Small punctate	NA	Diffuse	Diffuse
unc-51	Spherical	Spherical	Low	Big aggregates in a few cells	Low	Dispersed	Small punctate
epg-1	Spherical	Spherical	Low	Big aggregates in a few cells	Low	Dispersed	Small punctate
epg-9	Spherical	Spherical	Low	Big aggregates in a few cells	Low	Dispersed	Small punctate
vps-34	Spherical	Spherical	ND	Weak	ND	ND	ND
lgg-1	Spherical	Spherical	Low	NA	NA	Diffuse	Diffuse
atg-9	Spherical	Spherical	Low	Big punctate	Low	ND	ND
atg-2	Clustered	Clustered	High	Clustered	High	Big punctate	Big punctate
atg-18	Spherical	Spherical	Low	Spherical	Low	Small punctate	Weak punctate
epg-6	Clustered	Clustered	High	Clustered	High	Big punctate	Big punctate
epg-3	Clustered	Clustered	High	Clustered	High	Big punctate	Weak
epg-4	Clustered	Clustered	High	Clustered	High	Big punctate	Big punctate
epg-5	Spherical	Spherical	Low	Spherical	Low	Weak	Weak

[58,60]. Atg18–Atg2 is required for further recruitment of the Atg12–Atg5–Atg16 complex and Atg8–PE to the PAS. The order in which Atg proteins act during the formation of the PAS or Atg-positive puncta, however, might not reflect their sequential functions in autophagosome formation.

The hierarchical order of autophagy proteins in the aggrephagy pathway

During *C. elegans* embryogenesis, different autophagy mutants exhibit distinct phenotypes, including the formation, morphology and distribution of PGL granules, SQST-1 aggregates, and punctate structures formed by LGG-1, DFCP1 and ATG-9 (Table 1). In autophagy mutants for the two ubiquitin-like conjugation systems, the UNC-51–EPG-1–EPG-9 complex and the Vps34 complex, PGL granules and SQST-1 aggregates are spherical and dispersed in the cytoplasm, and these two types of protein aggregates are separable [23,31,33] (Figures 2G–2I). In *epg-3*, *epg-4*, *epg-6* and *atg-2* mutants, PGL granules, SQST-1 aggregates, LGG-1 puncta and DFCP1 puncta are enlarged and form irregularly shaped clusters that largely co-localize [23,34] (Figures 2J–2L). *cup-5* mutants display characteristic patterns of PGL granules and SQST-1 aggregates, which accumulate in enlarged vacuoles and are greater in size and weaker in intensity than those in other autophagy mutants [99]. On the basis of these genetic phenotypes in double mutants, the genetic hierarchical relationship of autophagy genes in the aggrephagy pathway were established. For example, in double mutants of *epg-5* with *epg-3*, *epg-4* and *atg-2*, the characteristic distribution and organization of PGL granules and LGG-1 puncta is the same as in *epg-3*, *epg-4* and *atg-2* single mutants, indicating that EPG-5 acts at a step downstream of these autophagy genes [23,34]. Loss of *atg-18* function largely suppresses accumulation of enlarged LGG-1 puncta in *epg-6* and *atg-2* mutants, so ATG-18 functions at an earlier step than EPG-6–ATG-2 in the autophagosome formation process [34]. Formation of DFCP1 puncta in *epg-6* and *atg-2* mutants requires the UNC-51/Atg1 complex and EPG-8/Atg14, and, unexpectedly, also requires the LGG-1 conjugation system [34]. Thus in the aggrephagy pathway, omegasome formation requires lipidated LGG-1. The presence of LGG-1–PE (LGG-1-II) on the protein aggregates, via direct interaction with aggregate components, may permit the autophagosome to build directly around the aggregates, devoid of bulk cytosol. LGG-1–PE may also function in the autophagosome expansion step. Mutation of *atg-9* does not suppress the accumulation of DFCP1::GFP in *epg-6* and *atg-2* mutants, indicating that *atg-9* functions in parallel to or downstream of omegasome formation in the aggrephagy pathway [34]. These genetic analyses demonstrate that in the aggrephagy pathway, the EPG-1–EPG-9–UNC-51 complex acts at the most upstream step, followed by the EPG-8–VPS34–BEC-1 PI3K complex [34]. *epg-5* functions at a downstream step in the autophagy pathway, probably in the formation of functional autolysosomes [23]. *cup-5* acts at the most downstream step of the aggrephagy pathway, and is required for proteolytic degradation in autolysosomes [99].

SUMMARY AND PERSPECTIVE

The mechanisms responsible for induction, formation and maturation of autophagosomes in multicellular organisms are poorly understood. Autophagic removal of a variety of protein aggregates during embryogenesis established *C. elegans* as a premiere genetic model to delineate the autophagy pathway. Using this model, the role of highly conserved and divergent Atg gene homologues and also the differential function of multiple homologues of the same yeast Atg genes in aggrephagy were revealed. Several autophagy genes specific to higher eukaryotes were also identified. The hierarchical relationship of autophagy genes in degradation of protein aggregates were established on the basis of the distinct genetic phenotypes in different autophagy mutants. Further investigation of the role of metazoan-specific genes in the autophagy pathway at the mechanistic level and also revealing the molecular basis of the puncta formed by LGG-1, DFCP1 and ATG-9 will provide insights into the molecular mechanisms of the autophagy pathway in higher eukaryotes.

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