

REVIEW

Autophagy in *Dictyostelium*: Mechanisms, regulation and disease in a simple biomedical model

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ABSTRACT

Autophagy is a fast-moving field with an enormous impact on human health and disease. Understanding the complexity of the mechanism and regulation of this process often benefits from the use of simple experimental models such as the social amoeba *Dictyostelium discoideum*. Since the publication of the first review describing the potential of *D. discoideum* in autophagy, significant advances have been made that demonstrate both the experimental advantages and interest in using this model. Since our previous review, research in *D. discoideum* has shed light on the mechanisms that regulate autophagosome formation and contributed significantly to the study of autophagy-related pathologies. Here, we review these advances, as well as the current techniques to monitor autophagy in *D. discoideum*. The comprehensive bioinformatics search of autophagic proteins that was a substantial part of the previous review has not been revisited here except for those aspects that challenged previous predictions such as the composition of the Atg1 complex. In recent years our understanding of, and ability to investigate, autophagy in *D. discoideum* has evolved significantly and will surely enable and accelerate future research using this model.

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Introducing *D. discoideum*, a simple model for autophagy with striking similarities to animal cells

Dictyostelium discoideum is a protist that belongs to the Amoebozoa, a sister group of animals and fungi. They are known as social amoebae since the motile cells are able to aggregate and form a simple multicellular organism. Under starvation, groups of approximately 100,000 cells form a mound that goes through different stages of development to give rise to a fruiting body, in which vacuolated dead cells form a stalk that supports the spores (Fig. 1). Vegetative *D. discoideum* cells are more similar to animal cells than fungi or plant cells in many respects. For example, they lack rigid cell walls that might restrict movement, allowing the cells to perform typical animal-like processes such as phagocytosis, macropinocytosis, pseudopod-based cell motility and chemotaxis. The parallels with animal cells extends to the evolutionary conservation of many genes that have been lost during fungal evolution.¹


The similarity to animal cells makes *D. discoideum* a suitable model to address the study of genes or processes relevant to human disease. These include the study of pathogen infections,² cell motility-related pathologies,³ mitochondrial

diseases,⁴ the study of the mechanism of action of certain drugs,⁵ cancer,⁶ neurodegenerative disorders⁷ and lysosomal-related disorders⁸ among others. *D. discoideum* also provides useful ways to study the mechanisms and the physiological roles of autophagy.⁹ As in other organisms, autophagy is required for *D. discoideum* to survive starvation, for the turnover of proteins, to remove protein aggregates and is also fundamental during infection by intracellular pathogens. The first description of autophagic vacuoles in *D. discoideum* (defined at that time simply as “bodies containing partially degraded cytoplasmic material”) comes from transmission electron microscopy (TEM) studies of germinating spores in 1969,¹⁰ shortly after Christian de Duve coined the term autophagy.¹¹ We have come a long way from these initial morphological observations and now begin to understand the significance and the mechanisms of this crucial cellular process in the context of a whole organism.

During starvation, autophagy is responsible for the liberation of nutrients to maintain viability. Therefore, defects in autophagy drastically reduce the ability of cells to survive prolonged periods of nutrient deprivation. Typically, wild-type

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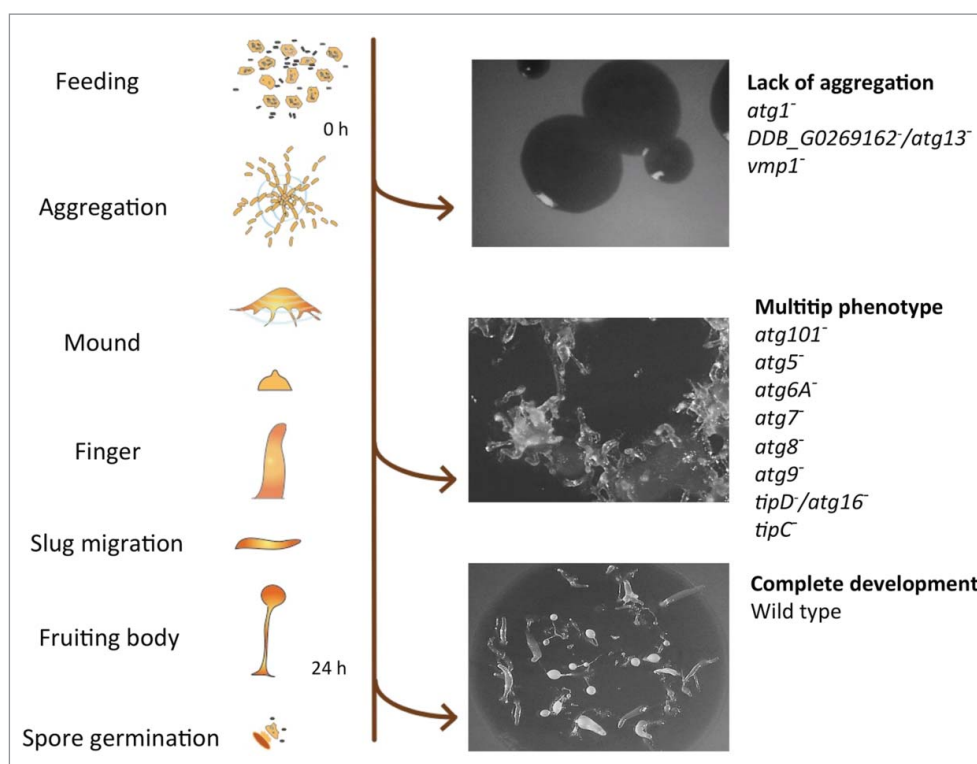


Figure 1. *D. discoideum*'s life cycle and representative phenotypes associated with the lack of autophagy. Mutant strains have been described for the genes depicted at the right side (see details in the main text). The pictures correspond from top to bottom to the strains *DDB_G0269162⁻/atg13⁻*, *tipC⁻* and wild-type AX4.

D. discoideum cells survive for more than 10 d under amino acid starvation, whereas autophagy-deficient mutants start losing viability after just 24 h.^{12,13} During starvation, autophagy is also responsible for a reduction in both cell volume and total protein.^{12,13} The *D. discoideum* developmental program takes place in the absence of nutrients; therefore, the intracellular degradation and recycling of the cell's own material by autophagy becomes essential as a source of energy and simple metabolites required for the biosynthetic pathways taking place during development. Thus, it is not surprising that autophagic dysfunction leads to abnormal development, which facilitates the identification of autophagy mutants in the laboratory. The severity of the phenotype ranges from complete lack of aggregation to the formation of multi-tipped mounds, which are unable to form normal stalks and viable spores (Fig. 1).¹³ It is unclear how much of the defects in development are simply the result of imbalanced nutrient homeostasis or whether more specific signaling events are involved. However, the signaling peptide SDF-2 (spore differentiation factor 2) is essential for spore formation, and its precursor protein, AcbA, is secreted by an unconventional mechanism that requires autophagy.¹⁴ Moreover, terminal stalk differentiation also seems to require autophagy for the formation of the vacuoles that accompany stalk cell differentiation.¹⁵ Nonrecycling roles for autophagy are therefore important for the terminal differentiation of both spores and stalk cells.

Three forms of autophagy have been described that differ in the mechanism by which the cargo is delivered to the lysosome. Chaperone-mediated autophagy,¹⁶ microautophagy,¹⁷ and macroautophagy. Macroautophagy (denoted here as autophagy for simplicity) is the best known, the most conserved autophagic pathway and the only one described in *D. discoideum* so far.

Although autophagy was initially thought to simply be a mechanism to recycle nutrients by nonselective self-degradation (so called bulk autophagy), the enormous importance of selective autophagy is now becoming clear. The selective process is devoted to the specific degradation of abnormal protein aggregates (aggrephagy), organelles (mitophagy, pexophagy, ribophagy, reticulophagy, nucleophagy) or pathogens (xenophagy). Impaired selective autophagy is therefore thought to underlie the etiology of numerous diseases.¹⁸

The hallmark of autophagy is the formation of intracellular double-membrane vesicles (autophagosomes), which eventually fuse with lysosomes, leading to degradation of the cargo and the inner membrane. The basic molecular machinery that controls autophagy was initially discovered in *Saccharomyces cerevisiae*, in which the proteins involved were named Atg, for autophagy-related. These proteins form different complexes that are required for the induction, elongation and completion of the autophagic process. This has been extensively reviewed in the past few years.¹⁹⁻²¹ Most of these proteins and complexes can be recognized in *D. discoideum* by sequence homology as summarized in Fig. 2. A more detailed description of the putative *D. discoideum* Atg protein homologs was included in a previous review.⁹ The generation and analysis of a number of mutants from each complex have subsequently confirmed the functional conservation of the autophagic machinery in *D. discoideum* (the available mutants and their phenotypes are depicted in Fig. 1). Lack of aggregation is the phenotype observed for *atg1⁻*,²² *DDB_G0269162⁻/atg13⁻*,²³ and *vmp1⁻*.²⁴ Formation of multiple tips in mounds is the phenotype observed in *atg101⁻*,²³ *atg5⁻*,¹³ *atg6A⁻*,²² *atg7⁻*,¹³ *atg8⁻*,²² *atg9⁻*,²⁵ *tipD⁻/atg16⁻*²⁶⁻²⁸ and *tipC⁻*.²⁷ Importantly, additional non-Atg proteins have been found to be involved in

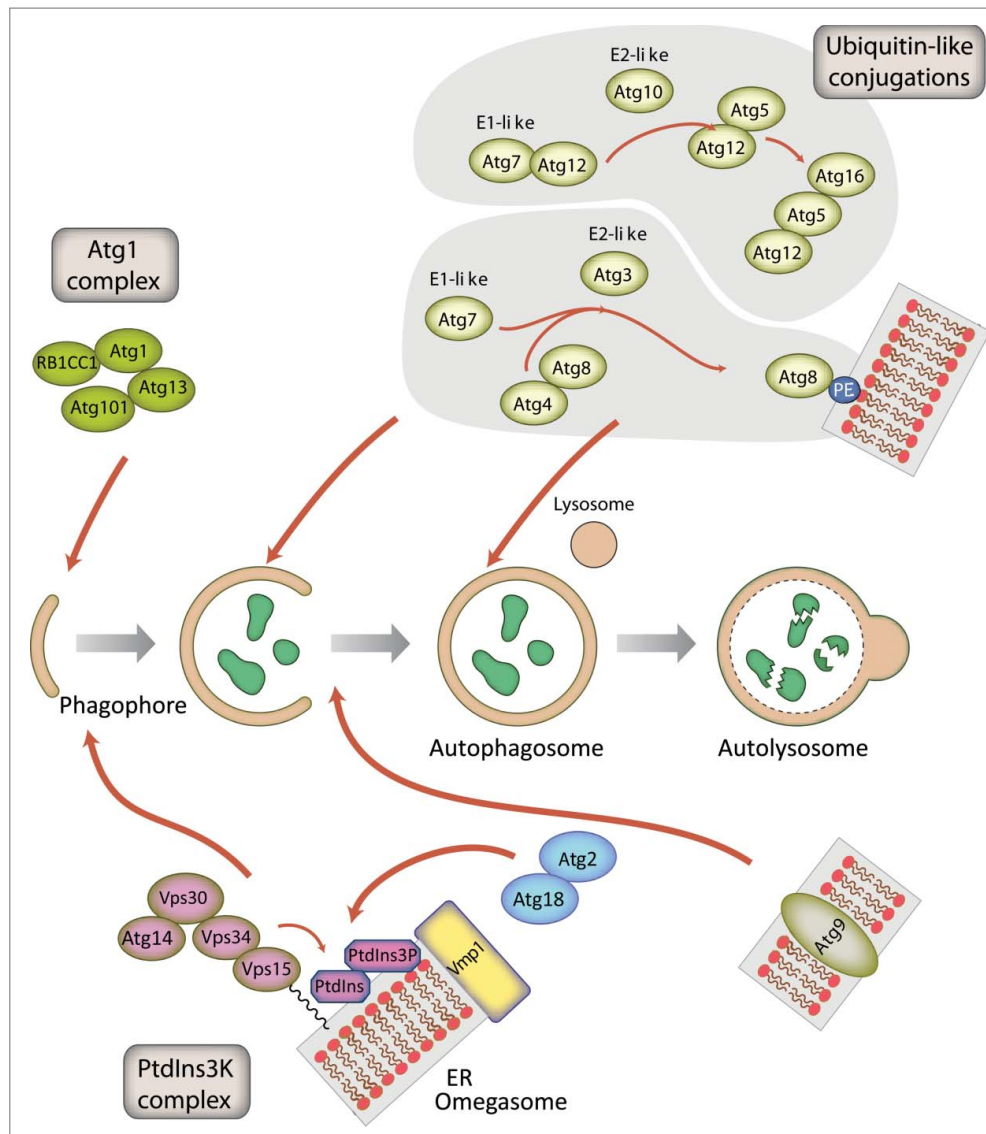


Figure 2. Schematic representation of autophagosome formation and the proteins involved in *D. discoideum*. The autophagic proteins were identified by sequence homology with those described in yeast and mammalian cells. The predicted complexes have been organized using the information available from the yeast *S. cerevisiae*, mammalian cells and *D. discoideum*. The inductive stage depends on the serine/threonine Atg1 kinase complex and the class III PtdIns3K complex. The latter complex generates the signaling lipid PtdIns3P, which is essential for the recruitment of Atg18 and Atg2. The elongation of the phagophore membrane requires 2 ubiquitin-like (Ubl) conjugation systems (upper right). In the first conjugation reaction Atg12 is covalently bound to Atg5. Atg12–Atg5 interacts with Atg16 and localizes to the phagophore membrane, from which it regulates the second conjugation reaction that attaches the protein Atg8 (known as LC3 in mammals) to phosphatidylethanolamine (PE) of the expanding phagophore membrane. Atg8 initially localizes to both sides of the phagophore and remains inside the completed autophagosome until fusion with the lysosome occurs; Atg8 on the cytosolic side of the autophagosome is cleaved from PE by Atg4 and recycled, whereas Atg8–PE of the luminal leaflet of the bilayer is degraded. Atg9-containing vesicles supply membrane for phagophore expansion. Recent advances have clarified the composition of the Atg1 complex and the role of Vmp1 as a key protein in the origin of the autophagosomes from the ER.

Dictyostelium autophagy such as the ER transmembrane protein Vmp1 (vacuole membrane protein 1), which demonstrates the usefulness of *Dictyostelium* as an alternative simple genetic system to study autophagy.

Methods and tools to study autophagy in *D. discoideum*

Over recent years the number of tools available to study autophagy in *D. discoideum* has expanded greatly. TEM remains the gold standard for identifying the double-membrane autophagic structures, but it is difficult and time-consuming to obtain quantitative data. In particular, as each *D. discoideum* cell only contains approximately 5 autophagosomes under

conditions of amino acid starvation,²⁹ very few are present in each of the thin sections required for TEM.¹³ However, with recent advances in 3D electron microscopy it should now be possible to reconstruct complete amoeba, although quantification of autophagosomes has yet to be demonstrated. In contrast, light microscopy has been extensively used, allowing rapid quantification of large numbers of cells, and dynamic information to be captured.

It is often experimentally desirable to induce autophagy in a defined manner. Typically in mammalian cell cultures autophagy is induced using either starvation e.g. Hank's balanced salt solution or drug treatment, e.g., rapamycin, which inhibits MTORC1 (mechanistic target of rapamycin [serine/threonine

kinase] complex 1) function. In *D. discoideum*, complete starvation can be induced using non-nutrient buffers but this initiates development, complicating interpretation. To avoid this, it is preferable to use a defined medium such as SIH medium lacking arginine and lysine (available from ForMedium for use in SILAC). Within 5 min this gives a comparable induction of autophagy to complete starvation, without cells entering development.²⁹ Although *D. discoideum* has a functional rapamycin-sensitive TORC1 complex,³⁰ short-term rapamycin treatment is unable to activate autophagy.

Several markers have been used to study autophagy in *D. discoideum*. In mammalian systems the most commonly used markers are MAP1LC3/LC3 (microtubule associated protein 1 light chain 3) family proteins, the ubiquitin-like (Ubl) orthologs of yeast Atg8 that associate with autophagic entities from initiation until recycling.³¹ *D. discoideum* possess 2 Atg8 paralogs (Atg8a and b), which are both good markers of autophagosomes although they play partially nonredundant roles (discussed below).³² Another useful marker is Atg18, the ortholog of mammalian WIPI2. Atg18 localizes to omegasome-anchored phagophores (the precursors to autophagosomes) at initiation events but, in contrast to Atg8, dissociates immediately after autophagosome formation is completed.^{29,33,34} Atg18 is therefore specific for the formation of new autophagosomes.

Immunolabeling of endogenous autophagy proteins allows a variety of markers to be observed, and several good antibodies against *D. discoideum* Atg8 have been generated.^{32,35} While these antibodies are useful for many experiments, fixed samples lack dynamic information. It is therefore often useful to perform live-cell imaging and track markers in real-time to dissect the different phases of autophagosome formation. GFP-Atg8 fusions permit visualization from initiation through expansion, closure and fusion with the lysosome, whereupon the GFP fluorescence becomes quenched. Movement in the Z-axis, however, makes following individual autophagosomes over time challenging. To reduce this problem, sheets of agarose can be placed upon the cells to compress them, reducing cell height and improving image quality. This approach allows individual autophagosomes to be followed from an initial punctum, to a cup and then a complete circular autophagosome (Fig. 3 and Movie S1). The timing of each phase can be quantified from such movies, as well as the time it takes for the GFP fluorescence to be quenched, giving an indirect combined measure of acidification and proteolysis. This is a powerful method to directly quantify autophagosome dynamics, but does not represent basal autophagy levels, as the compression itself potentially induces autophagosome initiation.²⁹

An additional way to differentiate autolysosomes (the product of autophagosomes fusing with lysosomes) is by fusing Atg8 to both GFP and RFP in tandem.³⁶ As RFP is less sensitive to quenching by low pH, only the red signal can be observed in mammalian autolysosomes, whereas phagophores and autophagosomes emit both green and red fluorescence. Unfortunately, *D. discoideum* lysosomes have a much lower pH than their mammalian counterparts (3.5 compared to 4.5-5.0), which is sufficient to quench both GFP and RFP. Treatment with lysosomotropic agents such as ammonium chloride (NH₄Cl), however, can be used to partially elevate lysosomal pH, and reveal autolysosomes, with the caveat that this

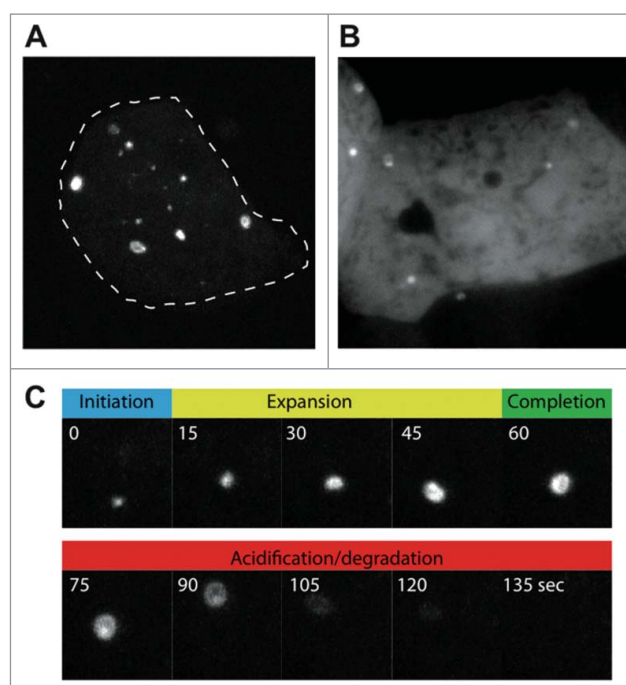


Figure 3. Live-cell imaging of autophagosome formation in *D. discoideum*, using (A) GFP-Atg8a, and (B) GFP-Atg18. Images were obtained under compression to improve clarity, and to allow individual autophagosomes to be followed over time. (C) Shows the different stages of autophagosome formation from the cell in (A) marked by GFP-Atg8a. The autophagosome shown in (C) has been marked by an arrow in Movie S1.

treatment itself will induce autophagy and lead to osmotically distended compartments with dilute content.^{37,38}

Autophagic flux refers to the net amount of material that is captured and degraded by this pathway over time. This is an important but complex and dynamic measurement and is not a simple reflection of the number of autophagosomes. Currently, the best method to measure flux is based on observing the autophagic cleavage of GFP from its fusion with cytosolic proteins.³⁸ Although GFP fluorescence is rapidly quenched within acidic lysosomes, the GFP protein itself is relatively resistant to lysosomal proteolysis. When GFP is fused to cytosolic proteins, captured and delivered to lysosomes by autophagy, GFP fragments accumulate while the rest of the fusion protein is degraded.³⁹ GFP cleavage fragments can therefore be quantified by western blot analysis of whole cell lysates probed with anti-GFP antibodies. As *D. discoideum* are professional phagocytes, they possess an extremely efficient proteolytic machinery and free GFP is barely detectable due to rapid degradation. Therefore NH₄Cl treatment is again required to inhibit proteolysis sufficiently for cleaved GFP fragments to accumulate.^{37,38} Band intensities for GFP-marker and free-GFP can then be quantified to give a measure of autophagic flux. It is again important to note that the concentrations of NH₄Cl required are very high (0.2-0.3 M), and likely to induce autophagy themselves. Nonetheless, this system provides a good measure of maximum autophagic capacity, and is useful to demonstrate gross effects of mutations on the pathway.^{37,38}

The array of techniques available, coupled with orthologs of most mammalian autophagy genes, makes *D. discoideum* a robust model for the study of autophagy. While a system for studying autophagic flux in noninduced conditions is yet to be

established, significant progress has been made and *D. discoideum* provides many useful means in which to study this pathway.

Phagophore nucleation: The Atg1 complex in *D. discoideum* is closely related to the mammalian ULK1 complex

The Atg1/ULK1 protein kinase complex works at the initiation step in both selective and nonselective autophagy.⁴⁰ In mammals, ULK1 phosphorylates BECN1 to activate the PIK3C3/VPS34 lipid kinase,⁴¹ and phosphorylation of the transmembrane protein Atg9 by its budding yeast homolog Atg1 plays a key role in the recruitment of Atg18 and Atg8 to the phagophore assembly site (PAS).⁴² Initial studies of nonselective autophagy in yeast identified the different components of the Atg1 complex: the Atg1 protein kinase itself, the regulatory subunit Atg13 and the Atg17-Atg31-Atg29 scaffolding subcomplex.⁴³ Selective autophagy processes, such as the cytoplasm-to-vacuole targeting (Cvt) pathway, involve a different scaffold protein, Atg11 (Fig. 4). Although not absolutely required in nonselective autophagy, Atg11 interacts with Atg29⁴⁴ and, thus, it is still unclear whether Atg11 and the Atg17-Atg31-Atg29 subcomplex form mutually exclusive complexes with Atg1. Dephosphorylation of the TOR kinase substrate Atg13 upon nutrient starvation promotes Atg1 complex assembly in nonselective autophagy.⁴⁵ In contrast, under nutrient-rich conditions, selective autophagy receptor-bound targets activate Atg1 via the scaffold protein Atg11.⁴⁶ Autophagy receptors have been identified in mitophagy (Atg32), pexophagy (Atg36), nucleophagy (Atg39), reticulophagy (Atg40) and the Cvt pathway (Atg19 and Atg34).⁴⁷

The ULK1 protein complex in mammals differs from the budding yeast Atg1 complex in several aspects. This complex consists of the ULK1 protein kinase and 3 proteins, ATG13, ATG101 and the scaffold protein RB1CC1/FIP200 (Fig. 4). ATG101, which is absent in budding yeast, binds to and stabilizes Atg13.^{48,49} The crystal structure of the Atg13-Atg101 complex revealed that the HORMA domains present in these 2 proteins heterodimerize.^{50,51} In budding yeast, Atg13

contains a 3-strand β -sheet insertion in the HORMA domain that stabilizes this protein in the absence of ATG101. The scaffold protein RB1CC1 is the functional counterpart of both Atg17 and Atg11 in yeast, and results from phylogenetic analysis suggest that Atg17 arose via gene duplication of Atg11 and loss of the C-terminal region.⁵² This gene duplication event and functional specialization appears to be essentially restricted to fungi. In mammals, the HTT (huntingtin) protein also shares structural similarity to yeast Atg11 and, like RB1CC1, functions as a scaffold protein in the ULK1 complex.⁵³

The Atg1 complex in *D. discoideum* is evolutionarily closer to the mammalian ULK1 complex than its budding yeast counterpart. In addition to the Atg1 protein kinase,⁵⁴ Atg13 and Atg101 homologs have been identified²³ and interaction studies have shown that Atg13, like its mammalian homolog, binds to both Atg1 and Atg101 (Fig. 4). The likely RB1CC1/Atg11 homolog in *D. discoideum* is DDB_G0285767.⁵² A putative Atg17 homolog has also been reported but functional studies argue against a role in autophagy,²³ thus supporting the idea that Atg17 proteins are restricted to fungi. Similarities between mammals and *D. discoideum* are not limited to the core components of the complex, as shown by the conservation of the mammalian selective autophagy receptor and Atg8-binding protein Sqstm1/p62 in *D. discoideum*.^{23,55} Interestingly, *D. discoideum* Atg1 kinase also interacts with the pentose phosphate pathway (PPP) enzyme transketolase (Tkt).²³ Although no direct phosphorylation has been reported, the activity of TKT is altered in strains lacking or overexpressing Atg1, suggesting a possible crosstalk between autophagy and the PPP. Interestingly, a recent report shows that another component of the PPP, the ribose-5-phosphate isomerase (RpiA), regulate autophagy in HeLa cells.⁵⁶

The origin of the autophagosomal membrane, function of Vmp1 and regulation of PtdIns3P

The origin and elongation of the phagophore membrane are still the subject of intense research. In mammalian cells the ER, establishing close contacts with other organelles such as mitochondria and lipid droplets, forms a specialized cradle-like

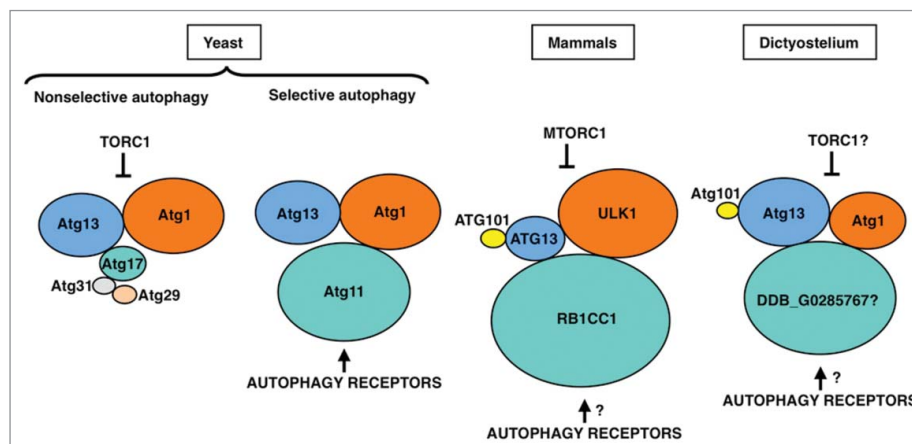


Figure 4. The Atg1/ULK1 complex in yeast, mammalian cells and *D. discoideum*. Proteins are colored according to their conservation, and protein size is proportional to the molecular weight. The inhibition of the Atg1/ULK1 kinase activity by TORC1, and its activation through binding of the autophagy receptors to the Atg11 subunit, are indicated.

structure known as the omegasome.⁵⁷ This structure is enriched in the signaling lipid PtdIns3P, generated by the class III phosphatidylinositol 3-kinase (PtdIns3K) PIK3C3/VPS34. It is thought that the phagophore expands by fusing with vesicles that originate from different cellular compartments. Some of these vesicles contain the transmembrane protein ATG9 and emanate from the Golgi and recycling endosomes,^{58,59} whereas others can originate from the ER-Golgi intermediate compartment (ERGIC).⁶⁰⁻⁶²

D. discoideum autophagosomes form simultaneously in different locations of the ER²⁹ and are thus, in this respect, more similar to those in mammalian cells than yeast, in which autophagosomes originate from the PAS, a single spot near the vacuole. It has been proposed that *S. cerevisiae* has become highly specialized during evolution. As a result, some processes such as autophagy have diverged from a more universally conserved mechanism, which has remained unaltered in other organisms such as *D. discoideum*.⁶³ A valuable example of these differences is the presence in *D. discoideum* of the conserved protein Vmp1 that is required for correct formation of the omegasome, but absent in *S. cerevisiae* and other fungi.

The phenotype of Vmp1-deficient cells differs from that of cells lacking classical Atg proteins because it affects a wide array of processes apparently not connected to autophagy as described in *D. discoideum* and *Chlamydomonas reinhardtii*.^{24,64} Autophagic flux is blocked in Vmp1-deficient *D. discoideum* but PtdIns3P production and subsequent recruitment of the autophagy machinery to the ER still occurs. Indeed, PtdIns3P is abnormally high in the mutants and correlates with the accumulation of enlarged omegasomes and LC3-containing structures in *D. discoideum*,⁶⁵ *C. elegans*⁶⁶ and mammalian cells.⁶⁷ Taken together, these studies suggest that Vmp1 might be required for the correct structure of the omegasome and/or

the capacity of the phagophore to elongate and become a functional autophagosome. Since Vmp1 is an ER-resident protein it is tempting to speculate that Vmp1 generates an ER microenvironment that orchestrates the autophagic machinery to allow the correct expansion of the phagophore rather than the recruitment of Atg proteins (Fig. 5).

Another interesting phenotype of the Vmp1 mutant is the defect in macropinocytosis, a mechanism of nutrient uptake that allows *D. discoideum* to grow in liquid media. The abnormal accumulation of PtdIns3P in the mutant impairs macropinocytosis indirectly and prevents growth in liquid media. This conclusion is based on experiments that preclude the accumulation of PtdIns3P. Atg1 is upstream of the PtdIns3K and therefore a double mutant in Vmp1 and Atg1 lacks this abnormal signaling.⁶⁵ Remarkably, macropinocytosis and cell growth in liquid media is recovered in the double mutant, suggesting that additional nonautophagy-related defects may occur as a result of an abnormal regulation of PtdIns3P signaling at the omegasome.⁶⁵ It will be interesting to determine if such a phenomenon also occurs in mammalian cells.

The 2 ubiquitin-like (Ubl) conjugation systems are highly conserved in *D. discoideum*

The proteins involved in the 2 Ubl conjugation systems are highly conserved from yeast to mammalian cells and very likely function in an analogous manner in all eukaryotes including *D. discoideum*.⁹ However, while yeast harbors only one isoform for each of the 8 different proteins of the 2 Ubl conjugation systems there are 2 paralogs for ATG16 (ATG16L1, L2), 4 for ATG4 (ATG4A, B, C, D) and 7 for ATG8/LC3 in mammals.^{9,68,69} In particular, the large number of ATG8/LC3 paralogs in humans and mice complicates

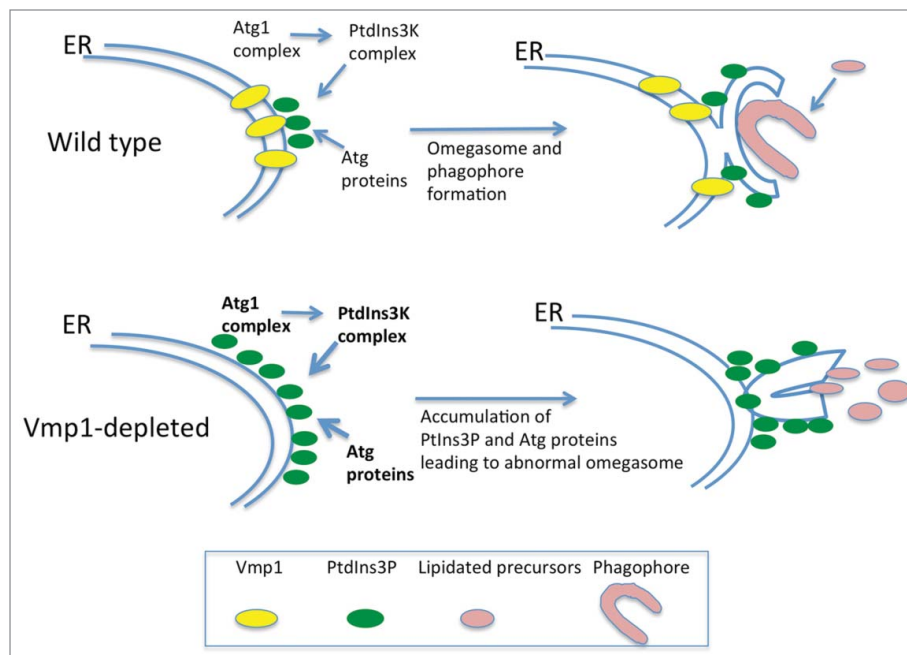


Figure 5. Vmp1 is essential for correct PtdIns3P signaling and omegasome formation in *D. discoideum* and mammalian cells. Vmp1 accumulates in subdomains of the ER where the autophagic machinery is recruited. PtdIns3P is formed at these domains to regulate omegasome formation and phagophore elongation. In the absence of Vmp1, PtdIns3P is aberrantly generated leading to persistent recruitment of autophagy proteins and unproductive autophagosome formation.

functional studies in these organisms. These proteins are grouped into the MAP1LC3 (microtubule associated protein 1 light chain 3) subfamily with 4 members (LC3A, B, B2 and C), and the GABARAP (GABA type A receptor-associated protein) subfamily with 3 members (GABARAP, GABARAPL1 and GABARAPL2/GATE16) protein.⁶⁸ The GABARAPL2 protein is approximately equally distant from the LC3 and the GABARAP subfamilies (Fig. 6) and the precise functions of the individual proteins are largely unresolved.⁷⁰ In contrast, fungi possess only a single *ATG8* gene, while plants, insects, nematodes and amoebzoa, like *D. discoideum*, usually have 2 genes for *Atg8*.

Phylogenetic analysis showed that *D. discoideum* *Atg8a* and *Acanthamoeba castellanii* *Atg8* are situated in the same evolutionary branch as the orthologs from fungi and plants. In contrast, the 2 *Atg8b* proteins from *D. discoideum* and *A. castellanii* appear evolutionarily more closely related to the LC3 subfamily from animals (Fig. 6). Live cell imaging of *D. discoideum* cells expressing RFP-*Atg8a* and GFP-*Atg8b* showed that *Atg8b* associates with autophagosomes before *ATG8a*.³² In mammals, LC3 subfamily members are involved in the elongation of the phagophore, whereas the GABARAP subfamily seem to be essential for a later stage in maturation.^{71,72} Thus, *Atg8b* is likely the functional ortholog of the mammalian LC3 subfamily, which is also supported by the grouping in the

evolutionary tree (Fig. 6), and *Atg8a* the ortholog of the GABARAP subfamily.³² It appears therefore, that duplication of *Atg8* early in eukaryotic evolution allowed specialization of the paralogs during autophagosome maturation. Expansion of the respective subfamilies in mammals likely led to the acquisition of further specialized functions in more complex animals.⁷⁰ Similarly, the *Atg4* paralogs and the *Atg16* paralog in the 2 Ubl conjugation systems may enable the fine-tuning of the activation of the different *Atg8/LC3* family members.

Atg9 studies in *D. discoideum* reveal new functions

The multimembrane spanning *Atg9* protein is the only known integral membrane protein of the core autophagic machinery and thought to be involved in the delivery of membrane lipids to the growing autophagosome.⁷³ *Atg9* orthologs exist in all eukaryotic species so far examined and the protein is essential for autophagy. For example, in *Drosophila melanogaster*, *Atg9* depletion reduces both the number and size of autophagosomes, and blocks the fusion of autophagosomes with lysosomes.⁷⁴ Modulation of *Atg9* levels in yeast strains directly correlates with the frequency of autophagosome formation and autophagic activity.⁷⁵

The PAS in yeast originates from *Atg9*-positive clusters, called *Atg9* peripheral sites.⁷⁶ The origin of these clusters,

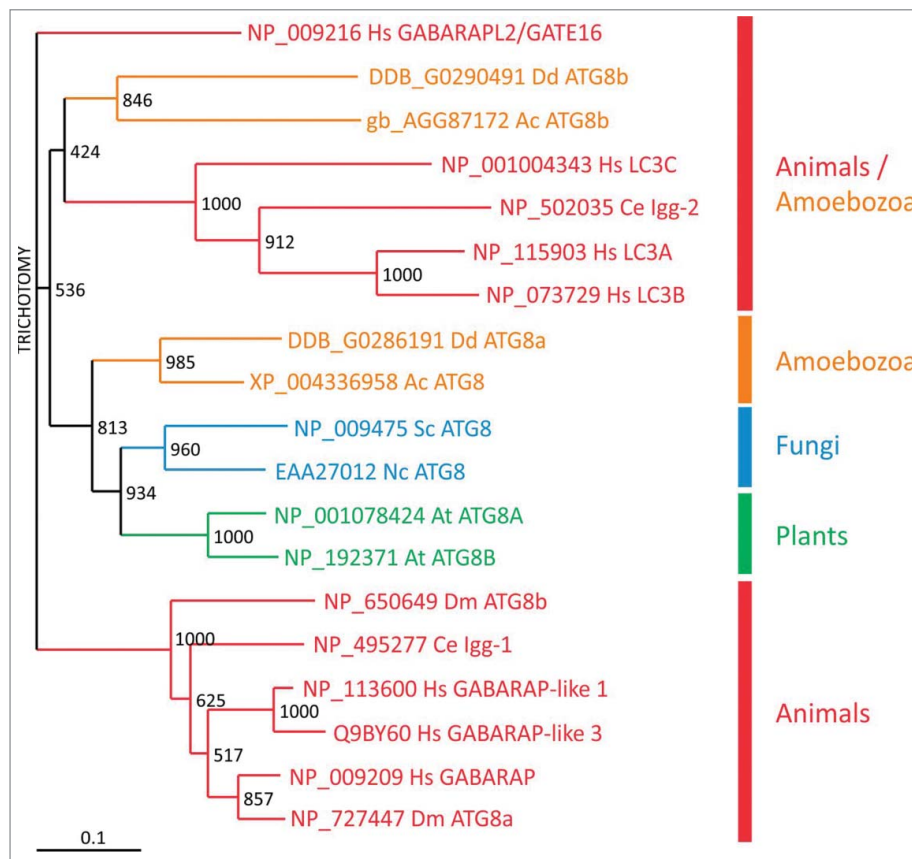


Figure 6. Evolutionary relationship of *Atg8/LC3* family members. Phylogenetic analysis of *Atg8/LC3* family proteins from animals (red), amoebzoa (orange), fungi (blue) and plants (green). A CLUSTALX alignment was used to create a phylogenetic tree with the TreeView program. The scale bar indicates amino acid substitutions per site. Bootstrap values are provided at the node of each branch. GenBank, SwissProt or dictyBase (<http://dictybase.org/>) accession numbers are provided on the right of the tree. Hs, *Homo sapiens*; Dm, *Drosophila melanogaster*; Ce, *Caenorhabditis elegans*; At, *Arabidopsis thaliana*; Nc, *Neurospora crassa*; Sc, *Saccharomyces cerevisiae*; Ac, *Acanthamoeba castellanii*; Dd, *D. discoideum*. LC3, microtubule-associated protein 1 light chain 3; GABARAP, GABA type A receptor-associated protein; GABARAPL2/GATE16, GABA type A receptor associated protein like 2; Igg: LC3, GABARAP and GATE-16 family.

however, has remained poorly understood. They localize in proximity to the mitochondrial membrane⁷⁷ and may represent vesicles derived from mitochondrial-ER contact sites. Alternatively, based on partial colocalization between Atg9 and the late Golgi protein marker Sec7, the Atg9 peripheral sites could emerge as a new organelle through the delivery of newly synthesized Atg9 to these sites via the secretory pathway.⁷⁶ Upon delivery of the lipids, Atg9 is no longer required and may be retrieved to the peripheral sites by an as yet unknown mechanism.

In mammals, there are 2 ATG9 paralogs, namely ATG9A/ATG9L1 and ATG9B/ATG9L2. ATG9A (ATG9 hereafter) is ubiquitously expressed in human adult tissues, whereas ATG9B is only expressed in placenta and pituitary gland.⁷⁸ Mammalian ATG9 is localized at the ER, the trans-Golgi network and early, late and recycling endosomes.^{79,80} Recently, it was shown that in *D. melanogaster* and in human cells the Atg1/ULK1 protein kinase phosphorylates a MYLK/myosin light chain kinase, which in turn activates MYL/myosin II regulatory light chain. This is followed by myosin II activation, which apparently drives transport of ATG9-containing membranes to the phagophore.⁸¹ *D. discoideum* Atg9 does not colocalize with mitochondria, the ER or lysosomes; however, there is a partial colocalization with the Golgi apparatus and many Atg9-GFP-containing vesicles localize along microtubules and accumulate around the microtubule organizing center.²⁵ Disruption of *Atg9* results in a pleiotropic phenotypes with severe impairments in development, slug migration, vegetative growth, phagocytosis, clearance of *Legionella pneumophila* and proteasomal activity.²⁵ A similar pleiotropic phenotype is observed in *tipD*⁻/*atg16*⁻ cells.²⁶

Unexpectedly, some of the defects of the *atg9* knockout mutant can be partially or fully restored by expression of wild-type or point-mutated CdcD/p97, an evolutionarily highly conserved member of the AAA-ATPase family.⁸² VCP/p97, the mammalian homologue, is a key player in ER-associated protein degradation, in the ubiquitin-proteasome system for protein degradation system, in aggresome formation and also in autophagosome maturation.⁸³⁻⁸⁵ Heterozygous mutations in the human *VCP/p97* gene cause autosomal-dominant inclusion body myopathy with early onset Paget disease of bone and frontotemporal dementia and further neurological disorders;^{86,87} however, the molecular basis of the pathogenesis remains unknown. VCP is essential for autophagosome maturation in mouse embryonic fibroblasts and, moreover, disease-causing VCP point mutations impair autophagy.⁸⁸

Interestingly, *atg9*⁻, *tipD*⁻/*atg16*⁻ and *atg9*⁻ *tipD*⁻/*atg16*⁻ double mutant *D. discoideum* cells have a severe and counterintuitive defect in proteasomal activity. Since the amounts of proteasomal subunits are not altered in knockouts of *atg9* or *tipD/atg16*, this result suggests that intact autophagy is required for optimal proteasomal activity.^{26,82} Furthermore, this phenotype is fully or partially suppressed by overexpression of wild-type or point-mutated *cdcD/p97* in *atg9*⁻ cells. This result provides evidence that in *D. discoideum* CdcD/p97 and Atg9 are functionally directly or indirectly linked and supports the existence of a delicate balance between the 2 major protein degradation pathways, proteasomal degradation and autophagy. Disruption of this balance causes severe impairment of essential cellular

functions in lower eukaryotes such as *D. discoideum* but ultimately may cause late-onset complex diseases in humans. How exactly the ubiquitin-proteasome system and autophagy are interconnected needs to be unraveled in the future.

Autophagy and disease: New leads from the social amoeba in human genetic disorders

Autophagic dysfunction may lead to defects in protein and organelle homeostasis associated with numerous human diseases.⁸⁹ A very relevant example is the connection of autophagy with major neurodegenerative diseases (including Alzheimer, Parkinson and Huntington diseases), often associated with the accumulation of abnormal protein aggregates or nonfunctional organelles. Autophagy dysfunction in *D. discoideum* often leads to the accumulation of large ubiquitinated protein aggregates.⁵⁵ Ubiquitin is a small regulatory protein that can be covalently linked to proteins to target them for proteasomal or autophagic degradation. These protein aggregates can be observed by both immunofluorescence microscopy using anti-ubiquitin antibodies and by western blot. As these aggregates are insoluble in 1% Triton X-100, they can rapidly be separated from whole cell lysates by centrifugation, separated by SDS-PAGE and detected using commercially available anti-ubiquitin antibodies. Ubiquitinated proteins are dramatically increased in the Triton-insoluble fraction of autophagy-deficient mutants.⁵⁵ The size and number of these aggregates correlates well with the severity of the phenotypes of the different autophagy mutants.⁵⁵ Interestingly, the protein aggregates formed in the Vmp1 mutant also accumulate the receptor protein Sqstm1,⁵⁵ which is involved in the clearance of several ubiquitinated cargos in mammalian cells and has been implicated in aggregation disorders and infectious diseases.⁹⁰

Packing proteins into aggregates, if properly degraded by autophagy, could function as a regulated cellular mechanism to handle abnormal proteins that otherwise would be toxic. However, several studies in *D. discoideum* suggest that the protein aggregation phenotype might be deleterious for cell function. Overexpression of the actin-binding protein VasP fused to an endosomal targeting signal leads to the formation of huge actin aggregates reminiscent of Hirano bodies,⁹¹ structures that are often present in neurodegenerative diseases. These actin aggregates sequester a number of actin binding and endosomal proteins promoting their disappearance from their normal location in the cytoplasm⁹¹ leading to cytoskeletal defects. These findings open the possibility that protein sequestration might also contribute to neuronal malfunction in these pathologies. Hirano body-like aggregates can also be induced in *D. discoideum* by the overexpression of a truncated form of a 34-kDa actin-binding protein.⁹² Another report showed that both autophagy and the proteasome pathway contribute to the degradation of Hirano bodies in *D. discoideum*.⁹¹ The autophagosome marker protein GFP-Atg8 colocalizes with Hirano bodies in wild-type *D. discoideum* cells, but not in cells deficient in the autophagic proteins Atg5 or Atg1.⁹³ Interestingly, Hirano bodies can be released by exocytosis following autophagy, which could explain the presence of these aggregates in both intracellular and extracellular spaces in the brain.⁹³

Another example using *D. discoideum* to study autophagy-related pathologies was the discovery of a possible connection of the rare disease Chorea-acanthocytosis (ChAc) with autophagy. ChAc is a neurodegenerative disease associated with abnormally shaped erythrocytes known as acanthocytes, leading to disability and premature death. ChAc is caused by loss-of-function mutations in *VPS13A*, most of which lead to a decrease or absence of the *VPS13A* protein (also known as Chorein).^{94,95} The molecular function of this protein is not known and the literature provides fragmented and nonconclusive results. Proposed functions for *VPS13A* include membrane trafficking, membrane morphogenesis, phagocytosis and actin cytoskeleton regulation.⁹⁶⁻⁹⁹ There are 3 additional *VPS13* proteins in humans (*VPS13B,C* and *D*)¹⁰⁰ and mutations in 2 of them, *VPS13B* and *C*, lead to Cohen syndrome and Parkinson disease, respectively.^{101,102} *D. discoideum* has 6 *VPS13*-related proteins, which are more similar to human *VPS13A* and *C* than to *VPS13B* or *D*. One of these proteins was initially named TipC since the phenotype of an insertional mutant was the formation of multiple tips in large mounds, a typical phenotype of autophagy mutants as pointed out above.²⁸ Several years later this mutant was revisited and the presence of a strong autophagy blockade was confirmed.²⁷ Subsequently, additional studies of the autophagic pathway in *VPS13A*-depleted human HeLa cells confirmed that autophagy could play a role in the etiology of this devastating disease.²⁷

Defects in either the retromer or WASH (WAS protein family homolog) complexes also lead to endosomal trafficking and autophagy defects associated with human diseases. The WASH complex regulates the formation of actin filaments on endosomes, driving multiple endocytic protein sorting and recycling events,¹⁰³ and mutations in the gene encoding KIAA0196/strumpellin, a component of the WASH complex, cause a form of autosomal dominant hereditary spastic paraplegia.¹⁰⁴ Furthermore, an aspartate to asparagine substitution (D620N) in *VPS35*, a component of the retromer sorting complex that recruits WASH to endosomes, results in autosomal dominant familial Parkinson disease.¹⁰⁵ *D. discoideum* have all components of the WASH complex, and mutants in WASH also have a defect in autophagy. However, while defects either in the retromer or WASH lead to an early inhibition of autophagosome formation due to defective trafficking of ATG9-containing vesicles and deficient BECN1 activation in mammals,^{105,106} *D. discoideum* WASH null cells do not have defects in the induction of autophagosome formation.¹² The observed blockade in autophagy is due to impaired recycling of vacuolar-type H⁺-translocating ATPase (V-ATPase) subunits and lysosomal hydrolases that leads to a late defect in lysosome recycling that blocks degradation of the cargo.¹²

Mitochondrial diseases are complex degenerative disorders caused by mutations in mitochondrial proteins encoded in the nuclear or mitochondrial genome. *D. discoideum* has been used to study the cytopathology involved in mitochondrial disease, specially those involving alterations in pathways that affect AMP-activated protein kinase (AMPK), the kinase required to sense cellular energy levels and regulate autophagy through MTOR, ULK1 and BECN1.^{4,107,108} Unexpected connections with autophagy are observed in *D. discoideum* cells deficient in components of the NADH:ubiquinone oxidoreductase or

complex I, the first complex of the respiratory chain. Mutants affecting complex I assembly factors Ndufaf5 and MidA/Ndufaf7 have a strong activation of bulk autophagy,^{109,110} but not mitophagy, which, in the case of MidA could be mediated by a chronic activation of AMPK. Given the importance of autophagy in cellular homeostasis, this connection might have relevance in human pathology and subsequently a similar phenomenon has been observed in skin fibroblast cultures derived from patients with mitochondrial disease.^{111,112}

Autophagy and bacterial infectious diseases in *D. discoideum*

Autophagy also represents an efficient mechanism for eukaryotic cells to capture and kill invading pathogens. The killing of intracellular pathogens by autophagy, termed “xenophagy,” is therefore emerging as a major regulator of both cytosolic and vacuolar pathogens. Many bacterial pathogens are able to manipulate, either by inhibition or induction, the host xenophagic response to take advantage of autophagy for their own benefits.¹¹³ Since *D. discoideum* lives in the soil and feeds on bacteria and yeasts, it likely developed mechanisms to discriminate between food and pathogenic organisms early during evolution. Due to its easy experimental manipulation, *D. discoideum* has become a useful model to study host-pathogen interactions and xenophagy⁹ and in the past 5 y has been used to study interactions of the host autophagic machinery with pathogenic microbes such as *Salmonella enterica* serovar Typhimurium (*S. enterica*), *L. pneumophila*, *Francisella noatunensis*, *Staphylococcus aureus* and *Mycobacterium marinum*.^{26,35,114-118} Furthermore, *D. discoideum* has also been used to identify and dissect the mechanisms of action of drugs with potential utility in medical therapy against pathogens.¹¹⁹⁻¹²²

M. marinum infects mainly fish and frogs, but it can also produce skin lesions in humans. Due to safety reasons and to the faster growth rate than its close relative *M. tuberculosis*,¹²³ *M. marinum* represents an easier tool to research mycobacterial pathogenesis. In mammalian cells and zebrafish, *M. marinum* induces an autophagic response that includes upregulation of some autophagy-related genes and LC3 recruitment to the mycobacteria-containing vacuole (MCV).¹²⁴⁻¹²⁸ This recognition by the autophagy machinery requires the activity of the mycobacterial pore-forming toxin ESAT-6, and engagement of the host autophagy-related proteins SQSTM1, MYD88 (myeloid differentiation primary response 88), DRAM1 (DNA damage regulated autophagy modulator protein 1) and TMEM173/STING (transmembrane protein 173).^{124,125,129,130} ESAT-6 is secreted by the type VII secretion system ESX-1, and induces permeabilization of the MCV membrane, exposing *M. marinum* to the cytosol.¹³¹ As a consequence, the ESX-1-dependent membrane damage allows the host autophagy machinery to recognize bacterial products including DNA, and to induce an autophagic response dependent on the cytosolic DNA-sensing TMEM173/STING pathway.^{125,129} However, apart from its indirect role in MCV permeabilization, ESX-1 seems to be dispensable for ubiquitination of *M. marinum* in macrophages.¹³⁰

It has been recently shown that, in *D. discoideum*, the autophagy machinery is not only recruited to cytosolic *M. marinum*, but it also targets the bacteria escaping out of

hosts cell by the nonlytic mechanism called “ejection.”^{35,132} During this process, ubiquitin, Atg8, Sqstm1 and Atg18 localize at the distal pole of the ejecting bacteria, with a proposed function in sealing the plasma membrane wound.³⁵ This recruitment of autophagy to the ejecting bacteria seems to be independent on ESX-1, as well as on other *M. marinum* virulence factors. However, host Atg1, Atg5, Atg6A and Atg7 are all required for Atg8 association with the ejecting bacterium suggesting that this process may be entirely driven by the host. Furthermore, even though Atg1 is not necessary for ejectosome formation, it is essential for the tight membrane sealing during ejection, avoiding cytosolic leakage and, thereby, death upon bacterial egress.³⁵

Interestingly, disruption of *Sqstm1*, encoding the only selective autophagy receptor protein described so far in *D. discoideum*, seems to have no effect on Atg8 recruitment to *M. marinum*. Gerstenmaier and collaborators thus suggest that other receptor proteins must exist in this amoeba.³⁵ Ubiquitin-binding CUE-domain containing proteins have been recently described to act as autophagy receptors for protein aggregates in yeast and humans. Consistent with the evolutionary conservation of these proteins, 2 *D. discoideum* CUE domain-

containing proteins, CnrD and DDB_G0274365/CueA also localize to MCVs (Drs. J. King and T. Soldati, unpublished results). In addition to the domain for ubiquitin binding, these proteins present LIR motifs in their sequences (Fig. 7), making them ideal selective autophagy receptor candidates.

The WASH complex is also necessary for *M. marinum* ejection and is possibly involved in *D. discoideum* xenophagy.¹¹⁸ During infection, WASH-induced actin polymerization prevents MCV acidification by promoting recycling of the V-ATPase, rendering the compartment more permissive to infection.¹¹⁸ This correlates with previous results in mammalian cells, in which *M. marinum* modifies the composition of its MCV by depleting the V-ATPase and the lysosomal protease CTSD (cathepsin D), as also observed during infection of *D. discoideum*.^{124,133}

D. discoideum has also been recently established as a model host for another fish pathogen, *F. noatunensis*.¹¹⁶ This Gram-negative bacterium exploits autophagy in human and murine macrophages.^{134,135} In *D. discoideum*, the mRNA levels of *atg8* and *sqstm1* slightly increase during *F. noatunensis* infection, and Atg8, Sqstm1 and ubiquitin are recruited to the compartment containing this bacterium, suggesting an induction of

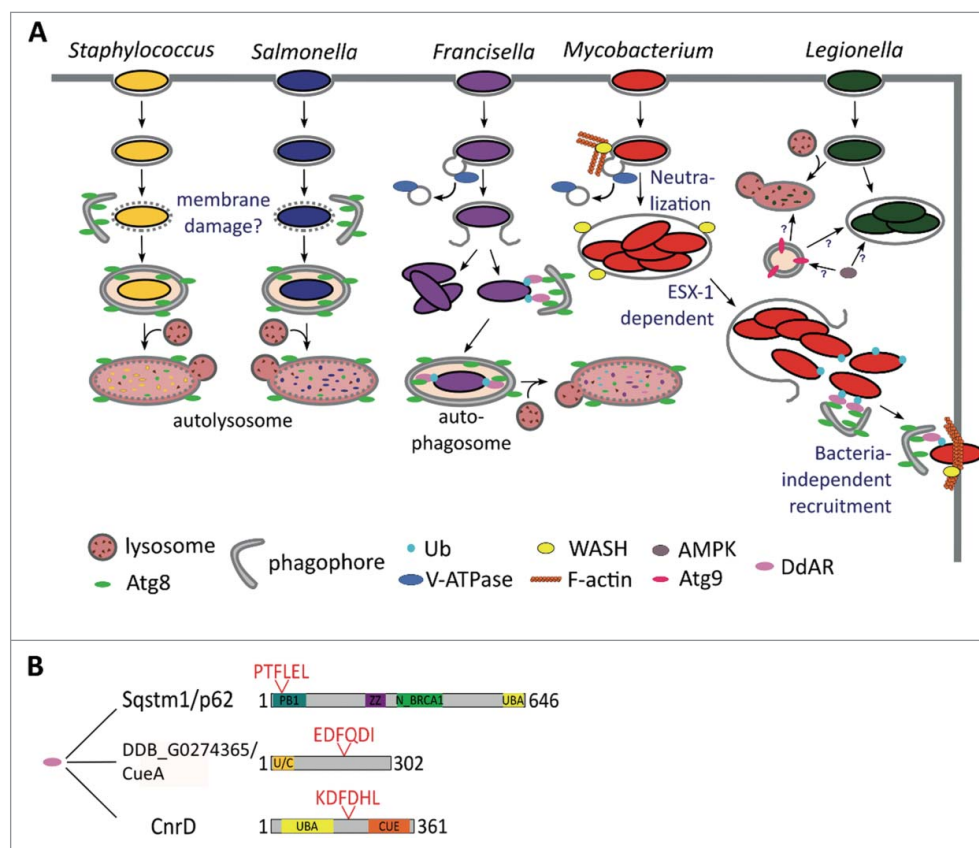


Figure 7. Xenophagy in *D. discoideum*. (A) The autophagosomal marker Atg8 is recruited to the compartment harboring both *S. aureus* and *S. enterica*, which might be a response to the putative membrane damage produced by these bacteria. As a consequence, the bacteria are engulfed in phagophores and killed in autolysosomes, where the luminal leaflet Atg8 is digested. After uptake by *D. discoideum*, *F. noatunensis* escapes phagosomal maturation and resides in a compartment lacking the V-ATPase. The majority of *F. noatunensis* replicates in the cytosol, while a small proportion of bacteria succumb to autophagy; *M. marinum* also prevents phagosomal acidification by the V-ATPase via WASH-induced actin polymerization. After proliferation inside the MCV, *M. marinum* is released to the cytosol, where it recruits the autophagy machinery, but does not seem to be fully engulfed in autophagosomes (unpublished observations). Finally, *M. marinum* is ejected through the *D. discoideum* plasma membrane in an autophagy-dependent manner; Atg9 controls *L. pneumophila* infection, whereas the autophagy activator kinase AMPK enhances its proliferation. Therefore, the function of autophagy during *L. pneumophila* in *D. discoideum* remains unclear. (B) Domain and motif organization of the already characterized *D. discoideum* autophagy receptor (DdAR) Sqstm1 and the 2 additional putative adaptors CnrD and DDB_G0274365/CueA. PB1, Phox and Bem1 domain; N_BRCA1, Next to BRCA1, central domain; UBA, ubiquitin-associated domain; CUE, ubiquitin system component Cue; U/C, UBA and CUE domains. Protein domains and LIR motifs were predicted with the web prediction resources InterPro (<http://www.ebi.ac.uk/interpro>) and iLIR, respectively.

autophagy. Furthermore, autophagy controls intracellular proliferation of *F. noatunensis*, since deletion of *atg1* in *D. discoideum* increases the bacterial burden after 24 h of infection.¹¹⁶

In contrast to growth of *F. noatunensis*, *L. pneumophila* shows similar proliferation kinetics when infecting wild-type and *atg1*⁻, *atg5*⁻, *atg6A*⁻, *atg7*⁻ and *atg8*⁻ *D. discoideum* cells. Likewise, this intracellular pathogen of amoebae and macrophages, rarely recruits Atg8, suggesting that xenophagy is not involved in amoebal immunity against *L. pneumophila*.¹³⁶ Whether *L. pneumophila* actively escapes autophagic capture in *D. discoideum* by uncoupling Atg8 from the phagophore membrane, via action of the RavZ toxin, as it does in human cells,¹³⁷ remains to be studied. Interestingly, Atg9 might be involved in controlling *L. pneumophila*, because its expression rises after 24 h of infection, and bacterial clearance decreases in *atg9*⁻ cells. Uptake of this bacterium is also decreased in *atg9*⁻, *tipD*⁻/*atg16*⁻ and *atg9*⁻ *tipD*⁻/*atg16*⁻ double-mutant *D. discoideum* cells.^{25,26} However, the mechanism is unclear, because, contrary to *atg9*, the expression of TipD/Atg16 and Atg8a is downregulated after infection with *L. pneumophila*.²⁵ Interestingly, the intracellular proliferation of *L. pneumophila* in *D. discoideum* increases in cells overexpressing the catalytic subunit of AMPK. Since AMPK regulates ATG9 localization via the activation of ULK1 in mammalian cells,¹³⁸ the different *L. pneumophila* proliferation rates observed during infection of *atg1*⁻, *atg9*⁻ and AMPK-overexpressing cells may indicate autophagy-independent functions for Atg9 and TipD/Atg16 as suggested by Xiong and collaborators.²⁶

Finally, it is controversial whether *S. enterica*, one of the Gram-negative bacteria more commonly used in laboratories to study the host innate immune responses at the molecular level,¹³⁹ is pathogenic or not for *D. discoideum*. *S. enterica* survives and proliferates inside very diverse human cell types,¹⁴⁰ but both its survival and death inside amoebae have been reported.^{114,141,142} Sillo and collaborators proposed that the discrepancies observed might be due to the use of the antibiotic gentamicin, normally employed in *S. enterica* infection protocols for mammalian cells. Gentamicin is likely to be taken up in large quantities by macropinocytosis during *D. discoideum* infection, thus affecting intracellular bacteria.¹¹⁴ Regardless, early during infection of *D. discoideum*, Atg8 is recruited to the compartment harboring *S. enterica*,¹⁴² and concomitant cleavage of GFP-Atg8 suggests an enhanced autophagic response.¹¹⁵ Together with the fact that *atg1*⁻, *atg6A*⁻ and *atg7*⁻ cells are more permissive for *S. enterica* growth,¹⁴² this observation implies a rapid autophagy-dependent killing of these bacteria by *D. discoideum* cells.

Autophagy has also been implicated during infection of lipopolysaccharide-treated *D. discoideum* cells with *S. aureus*.¹¹⁵ The fate of this Gram-positive bacterium inside amoebae is affected by autophagy, since its uptake is reduced in *atg9*⁻ cells.^{25,115} Treatment with lipopolysaccharide, which also induces bacteria clearance in an Atg1- and Atg9-dependent manner, further increases the localization of Atg8 to the vacuoles containing *S. aureus*.¹¹⁵ Because *S. aureus* localizes to LC3-labeled compartments and subverts the autophagy pathway in mammalian cells,¹⁴³⁻¹⁴⁵ manipulating autophagy with protease inhibitors or compounds blocking autolysosome formation is

needed to confirm the induction or blockade of autophagy by these bacteria.¹⁴⁶

In conclusion, different bacterial species differentially subvert autophagy in *D. discoideum*, as occurs in mammalian cells. The *D. discoideum* autophagy machinery is recruited to *S. aureus*, *S. enterica*, *F. noatunensis* and *M. marinum* at different stages during infection (Fig. 7). This recruitment is normally induced by the damage or rupture of the bacteria-containing compartments and, as a consequence, the bacteria are killed in autolysosomes. However, both *F. noatunensis* and *M. marinum* are able to prevent phagosomal acidification by the V-ATPase, therefore, bypassing xenophagy.

Autophagy and cell death pathways in *D. discoideum*

In addition to caspase-mediated apoptosis in animal cells, an increasing number of nonapoptotic cell death types are being reported within and outside the animal kingdom. In these multiple different cell death types there might be common, conserved cell death core elements that can be analyzed through studying cell death in alternative model organisms¹⁴⁷ such *D. discoideum*.

The *D. discoideum* fruiting body includes a stalk made of vacuolized, cellulose-walled dead cells.¹⁴⁸ This developmental cell death can be mimicked in vitro in monolayer conditions¹⁴⁹⁻¹⁵¹ that include a stereotyped sequence of events^{9,150} such as vacuolization and cellulose encasing (Fig. 8). The induction of this process requires 2 exogenous signals. The first signal, starvation and cAMP, sensitizes the cells. Only cells which have received this first signal can be induced to die by a second signal, classically the polyketide DIF-1.¹⁵² In starved cells subjected to DIF-1, autophagy determines the type of cell

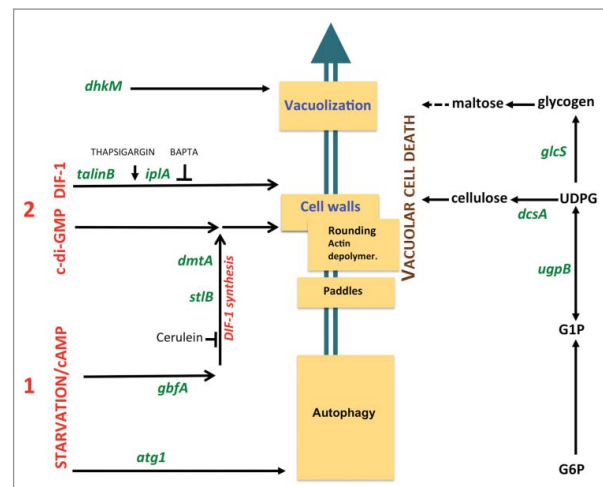


Figure 8. Genes and pathways governing *D. discoideum* vacuolar cell death. The cascade of subcellular cell death events is schematically depicted (*middle*). These include the formation of “paddle” cells, following by their rounding, biogenesis of a cellulose shell and extensive vacuolization. Random insertional mutagenesis and targeted mutagenesis identified a number of genes, here shown in green letters, encoding molecules required for this cell death. Details are given in the main text. These genes in turn helped define pathways to cell death, such as a polysaccharide pathway (*right*), a first signal pathway induced by starvation and cAMP (*lower left*) and second signal pathways (*upper left*). The latter include a DIF-1-induced autonomous pathway and a c-di-GMP pathway requiring endogenously synthesized or exogenous DIF-1. Ca²⁺-related drugs such as thapsigargin and BAPTA affect DIF-1 signaling leading to cell death.

death. In the presence of autophagy, DIF-1 induces a normal vacuolar cell death. However, in the absence of autophagy, DIF-1 induces necrotic cell death.^{153,154} This necrotic death includes mitochondrial uncoupling¹⁵⁵⁻¹⁵⁷ and depends not only on the absence of autophagy upon starvation, but also on given targets of the DIF-1 molecule, distinct from those that induce vacuolar cell death.¹⁵⁸ These results are in line with the known mitochondrial uncoupling effects of DIF-1 treatment¹⁵⁹ and with the recently demonstrated multiplicity of DIF-1-induced effects.¹⁶⁰ Importantly, although both pathways result in programmed death, while vacuolar cell death is part of normal development, necrotic cell death is incompatible with it.¹⁵³

Whether autophagy is also involved at the execution stage of vacuolar cell death has not been unambiguously proven. However, a large amount of debris is observed in the death-accompanying vacuoles indicating active degradation. Autophagy has also been proposed to have a structural role in terminal stalk differentiation.¹⁵ Stalk-cell vacuoles seem to originate from acidic vesicles and autophagosomes, which fuse to form autolysosomes. Their repeated fusion expands the vacuole, accompanied by cellulose wall formation enabling the stalk cells to make the stalk rigid and hold the spores aloft.¹⁵

DIF-1-induced vacuolar cell death in monolayers has been studied by random insertional mutagenesis. This led to the identification of a number of molecules required for this pathway, including the ITPR/IP3R homolog Ca^{2+} flux-controlling IplA,¹⁶¹ the UDP-glucose pyrophosphorylase UgpB, the glycogen synthase GlcS,¹⁶² the histidine kinase DhkM,¹⁶³ the transcription factor GbfA and the integrin-cytoskeleton intermediate TalB/talinB.¹⁵⁷ These and other studies have contributed to defining molecular pathways required for DIF-1-induced vacuolar cell death (Fig. 8).

An important recent discovery was the demonstration that not only DIF-1, but also the cyclic dinucleotide c-di-GMP are able to induce *D. discoideum* cell death.^{164,165} c-di-GMP is a universal bacterial second messenger¹⁶⁶ and can trigger innate immunity in bacterially-infected animal cells.¹⁶⁷ Unexpectedly, experiments using both inducers show marked synergy,¹⁶⁸ although c-di-GMP alone is insufficient to induce cell death in *D. discoideum* cell monolayers, requiring either DIF-1 or a DIF-1-related polyketide. The required DIF-1 can be endogenous, as shown by the inability of c-di-GMP to induce cell death when DIF-1 synthesis was blocked pharmacologically (by cerulenin), and by its rescue upon complementation by exogenous DIF-1.¹⁶⁸ This was confirmed genetically using mutants in the polyketide synthase *stlB* and the methylase *dmtA* genes that are required for DIF-1 biosynthesis.^{169,170} The corresponding *stlB*⁻ or *dmtA*⁻ mutant cells cannot be induced to die by exogenous c-di-GMP, and this can be complemented by exogenous DIF-1.¹⁶⁸ Thus, c-di-GMP can only trigger cell death in the presence of DIF1 or DIF-1-related polyketides, providing an additional level of control to this, and perhaps other c-di-GMP-dependent pathways.

How c-di-GMP synergizes with endogenous DIF-1, to drive cell death is not clear. However, at least part of the c-di-GMP pathway seems to be distinct from the autonomous DIF-1 pathway, since mutants of the latter, *talB*⁻ and *iplA*⁻, do not impair the former.¹⁶⁸ Among several cytosolic molecules able to sense c-di-GMP in animal cells, DDX41¹⁷¹ is especially well

conserved and has a clear *D. discoideum* ortholog. Attempts to inactivate *D. discoideum* DDX41 by targeted mutagenesis have not led to alterations of cell death so far (Y. Song, unpublished) although several other c-di-GMP binding proteins have been reported.¹⁷² Studying not only DIF-1- but also c-di-GMP-induced cell death in *D. discoideum* may provide important detail on the molecular mechanisms, including the role of autophagy, as well as suggestions as to how c-di-GMP acts in animal cells.

Concluding remarks

The strong conservation of the autophagic pathway between *D. discoideum* and mammalian cells justifies the use of this social amoeba as a model system for the study of the molecular mechanisms of autophagy and autophagy-related diseases. For basic studies *D. discoideum* can beautifully complement the enormous wealth of information obtained in *S. cerevisiae*, in particular those genes not conserved in yeast. In recent years, this potential has also become a reality in a wide range of biomedical conditions as exemplified in the use of *D. discoideum* as a surrogate host for studying the role of autophagy in infectious diseases.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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