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Autophagy in Dictyostelium

Genes and pathways, cell death and infection

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The use of simple organisms to understand the molecular and cellular function of complex processes is instrumental for the rapid development of biomedical research. A remarkable example has been the discovery in *S. cerevisiae* of a group of proteins involved in the pathways of autophagy. Orthologues of these proteins have been identified in humans and experimental model organisms. Interestingly, some mammalian autophagy proteins do not seem to have homologues in yeast but are present in Dictyostelium, a social amoeba with two distinctive life phases, a unicellular stage in nutrient-rich conditions that differentiates upon starvation into a multicellular stage that depends on autophagy. This review focuses on the identification and annotation of the putative Dictyostelium autophagy genes and on the role of autophagy in development, cell death and infection by bacterial pathogens.

Introducing Dictyostelium, A Suitable Model to Study Autophagy

Dictyostelium discoideum is a simple eukaryote that lives in the soil and feeds on bacteria by phagocytosis. The individual cells divide by binary fission as long as food is present, however, when bacteria are exhausted, starvation triggers a process of chemotaxis driven by cyclic-AMP (cAMP).¹⁻³ The resulting cell aggregate is surrounded by a complex extracellular matrix of protein, cellulose and polysaccharides that isolates it from the environment. This cellular association behaves like a true multicellular organism undergoing different stages of development accomplished by the coordination of morphogenesis and cellular differentiation. Eventually, the aggregates give rise to fruiting bodies, each formed by a cellular stalk that supports a mass of spores. The latter will germinate when environmental conditions are adequate.⁴⁻⁶ The life cycle of the experimental model species *Dictyostelium discoideum* and representative pictures of each stage are illustrated in Figure 1.

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Since Dictyostelium cells undergo development in the absence of any source of external nutrients they need to mobilize resources to be able to respond to the high cell activity required for aggregation and morphogenesis. This mobilization is in part achieved by glycogenolysis and autophagy, the degradation and turnover of the cells' own biomolecules. Autophagy is essential for development in many different systems.⁷⁻⁹ Three types of autophagy have been described, chaperone-mediated autophagy, microautophagy and macroautophagy. In the first one, specific proteins are recognized by chaperones that mediate their translocation across the limiting membrane of the lysosome into the lumen for their degradation.¹⁰ This form of selective autophagy plays an important role in the cell's response to stress and the presence of damaged proteins. In contrast, microautophagy consists of the invagination or protrusion/septation of the lysosome membrane, thus capturing the cargo and delivering it into the lysosomal lumen, again for degradation.¹¹ We will focus our review on the third type, macroautophagy (referred to as autophagy hereafter), a mechanistically different degradative process characterized by the formation of double-membrane vesicles called autophagosomes that engulf part of the cytosol or even organelles. The outer membrane of the autophagosomes subsequently fuses with lysosomes, forming autolysosomes where the contents and inner membrane of the autophagosome are degraded and the simple molecular constituents recycled. This form of autophagy is essential for temporary cell survival under starvation conditions. Autophagy is also induced in other circumstances such as for the elimination of protein aggregates or defective organelles or in response to intracellular bacteria, and it is therefore of immense importance in diverse pathological processes as well as in aging.¹²⁻¹⁴ The origin of the autophagosomal membrane and the mechanism mediating its expansion and maturation are not yet completely understood.

In mammals and Dictyostelium, nascent autophagosomes originate in the cytoplasm from multiple origins, in contrast with *S. cerevisiae*, where these structures are concentrated in a single location of the cytoplasm (named the PAS or phagophore assembly site). These autophagosomes appear in Dictyostelium and higher organisms as a punctate pattern in the cytoplasm when they are analyzed by fluorescence microscopy using specific autophagosome markers like GFP-Atg8/LC3.^{15,16} At the molecular level, several proteins involved in autophagosome formation

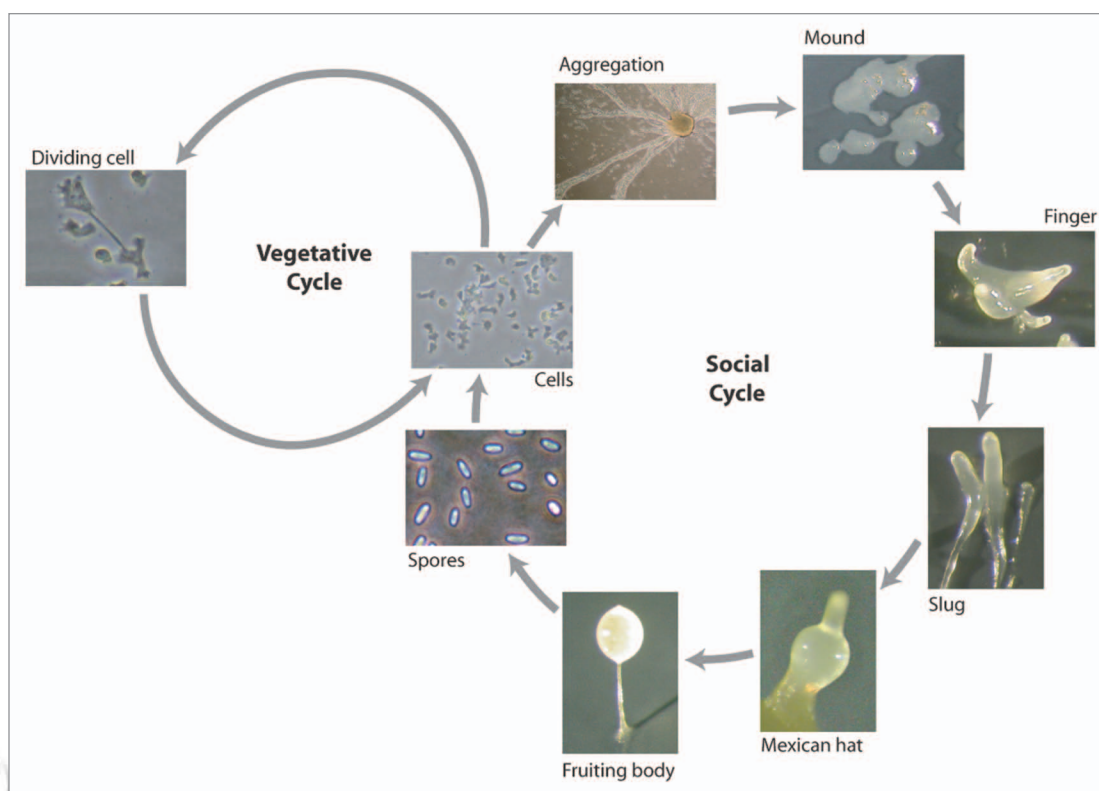


Figure 1. Dictyostelium life cycle. Representative pictures of vegetative and developmental stages are shown. In the wild, Dictyostelium amoeba feed on soil bacteria by phagocytosis but most laboratory strains are also able to grow in liquid axenic media by macropinocytosis. It is important to point out that cells are haploid throughout these vegetative and developmental cycles and this facilitates the generation of knockout strains.

(named Atg for autophagy-related) have historically been identified, primarily in the yeast *S. cerevisiae*. They are grouped in functional complexes required for the origin, elongation, completion and degradation of the autophagosome membrane, although the precise mechanisms of action of many of these proteins and the way in which they are regulated temporally are not yet completely understood (reviewed in ref. 12, 17–19). Two different complexes containing the protein kinase Atg1 and the lipid kinase Vps34 are necessary for induction and nucleation of autophagosomes and to recruit other proteins to the assembly site. Vesicle expansion and completion require two ubiquitin-like conjugation systems involving Atg8 and Atg12. Other proteins like Atg2, Atg9 and Atg18 play a role in membrane traffic and the biogenesis of the autophagosome. Many of these autophagy proteins are conserved in evolution and can be recognized in Dictyostelium by sequence homology analysis as described in detail below.

Despite its simplicity, Dictyostelium shows striking similarities with higher eukaryotes in many biological aspects including chemotaxis,^{2,3,20-22} developmental signaling pathways,^{4,23,24} the response to bacterial infections,²⁵⁻²⁸ the response to therapeutic drugs²⁹⁻³² and programmed cell death including autophagic cell death (reviewed in ref. 33). The Dictyostelium genome has been fully sequenced³⁴ and carefully annotated (<http://dictybase.org/>) and it is amenable to a wide range of molecular genetic techniques including the generation of mutants by homologous recombination and random genetic screens,^{6,33,35-38} that have facilitated the

use of comparative genomics to identify relevant genes conserved in the human genome.^{37,39}

General Autophagy Mechanisms and Evolutionarily Conserved Autophagy Genes: Induction of Autophagy and the Atg1 Complex

We will now examine the potential of Dictyostelium as a model for autophagy by describing the proteins that are known to be involved in this complex process in other systems and the extent to which they have been conserved in Dictyostelium. **Figure 2** shows a scheme of autophagosome formation and conserved proteins that can be identified in the Dictyostelium genome by comparison with the available information in yeast and mammalian systems.

Autophagy induction and its regulation must be tightly controlled by the energy and nutritional status of the cell. The nutrient sensor TOR (target of rapamycin) belongs to a protein family of conserved serine/threonine kinases known as phosphatidylinositol kinase-related kinases. TOR receives a wide variety of intra- and extracellular signals such as nutrients, energy, growth factors, calcium and amino acids.^{40,41} TOR associates with different proteins to form two complexes and only one of them, TORC1, is primarily involved in autophagy. The *S. cerevisiae* TORC1 contains Tor1 or 2, Kog1, Tco89 and Lst8 and is sensitive to rapamycin. As in higher eukaryotes, the Dictyostelium genome codes for proteins highly similar to Tor, Kog1 (also known as Raptor) and

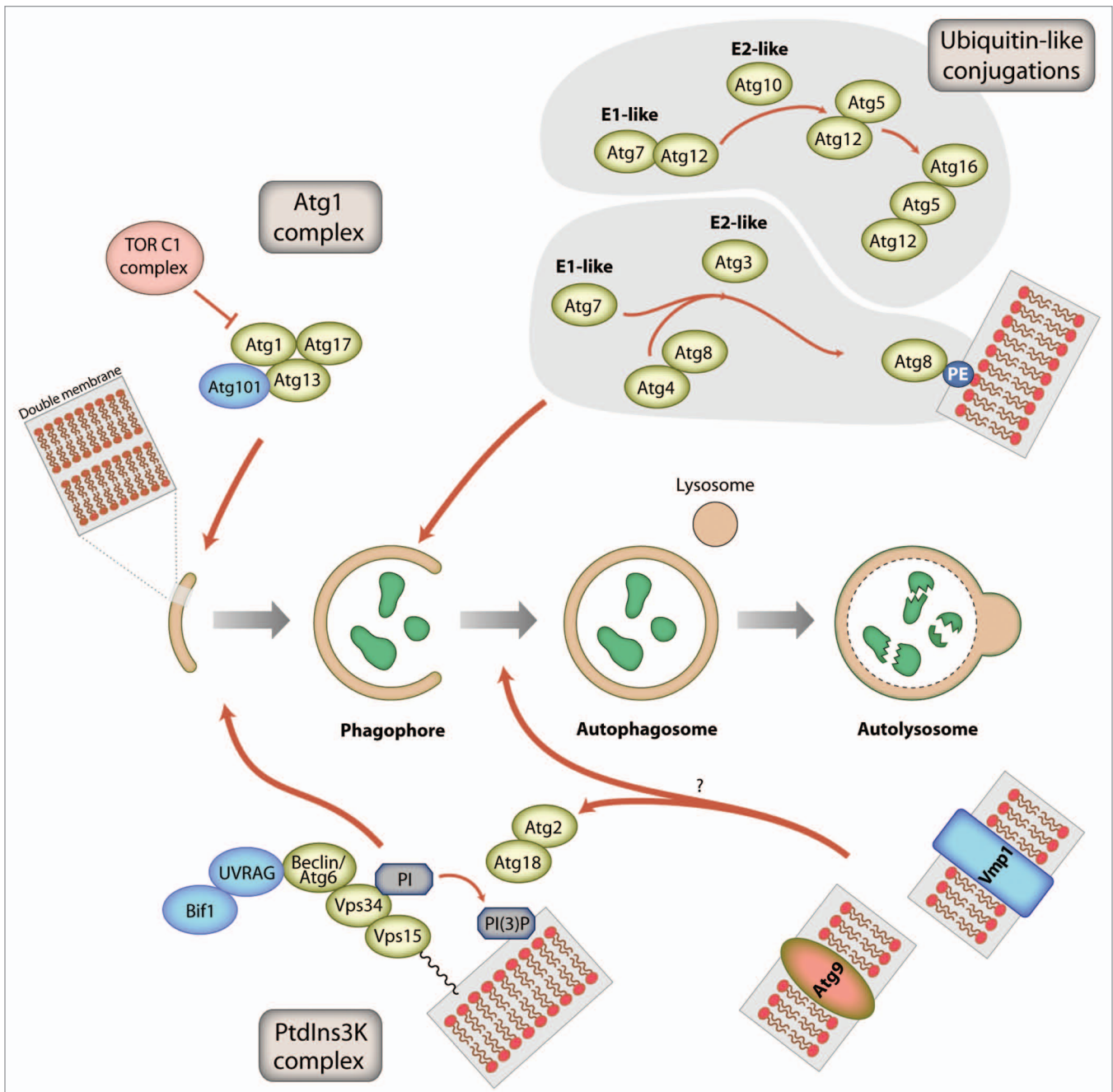


Figure 2. Autophagosome formation and putative signaling pathways in *Dictyostelium*. The phagophore is a double membrane whose origin is still a matter of debate. This membrane enlarges and finally engulfs parts of the cytoplasm. After fusion with lysosomes the content is degraded and recycled. The predicted *Dictyostelium* autophagic proteins have been organized in hypothetical functional complexes using the information available from the yeast *S. cerevisiae* and mammalian cells. Some proteins such as Atg101, UVRAG, Bif-1 and Vmp1 seem to be present in *Dictyostelium* and higher eukaryotes but are absent in *S. cerevisiae*. Vmp1 and Atg9 are transmembrane proteins whose functions are not completely characterized and have been proposed to be involved in membrane trafficking during autophagosome formation.

Lst8. TORC1 regulates many different aspects of cell growth and metabolism and functions upstream of the Atg1 complex, a protein complex containing the kinase Atg1 that plays a central role in the regulation of autophagy by integrating signals from the cellular nutrient status (via its regulation by TOR) and recruiting other autophagy proteins to the site of autophagosome origin (reviewed in ref. 41 and 42). The protein subunit composition

of the Atg1 complex and the interplay among these subunits is tightly regulated and depends on TOR activity. *Dictyostelium* codes for proteins with significant similarity to several Atg1 complex subunits (Table 1) and functional analyses have been carried out on Atg1, as described below.

Atg1 is a serine/threonine kinase whose activity is required for autophagy in many different model systems⁴³⁻⁴⁹ including

Table 1. Atg1 protein complex subunits

	Function/features	Dictyostelium	Human	<i>S. cerevisiae</i>	E-value Dd-Hu	E-value Dd-Sc
Atg1	Serine/threonine-kinase. (Ma; Cvt)*	Atg1 (DDB_G0292390)	ULK1 (G.ID: 8408) ULK2 (G.ID: 9706)	Atg1 (YGL180W)	1e-38 7e-39	7e-42
Atg13	Atg1 regulator. (Ma; Cvt)	DDB_G0269162	Atg13 related protein (NP_001136145.1)	Atg13 (YPR185W)	n.s.**	n.s.
Atg17	Scaffold protein. (Ma)	Atg17 (DDB0237867)	-	Atg17 (YLR423C)		2e-3
Atg101	Atg1 complex-interacting protein. (Ma)	DDB_G0288287	Atg101 (G.ID: 60673)	-	2e-8	
FIP200	Atg1 complex-interacting protein. (Ma)	DDB_G0268498	FIP200 (G.ID: 9821)	-	1e-3	
Atg29	Atg17-interacting protein. (Ma)	-	-	Atg29 (YPL166W)		
Atg31	Atg17/Atg29-interacting Protein. (Ma)	-	-	Atg31 (YDR022C)		

*Ma, Macroautophagy; Cvt, Cytoplasm-to-vacuole targeting. **The putative Dictyostelium Atg13 shows no significant homology (n.s.) with yeast and human homologues but it contains a conserved pfam Atg13 superfamily domain (as determined by a search of conserved domains at NCBI: <http://www.ncbi.nlm.nih.gov/>).

Dictyostelium.¹⁶ Dictyostelium Atg1 has an N-terminal kinase domain that shares a high degree of similarity with its counterparts in other organisms, and a poorly conserved C-terminal region. Both domains are separated by an asparagine-rich sequence.¹⁵ The kinase domain is essential for the function of the protein in autophagy, as kinase-dead DdAtg1 has a dominant-negative effect, resulting in a mutant phenotype similar to that observed in the null strain.¹⁶ Interestingly the C terminus contains a short region of significant similarity with human Ulk2 that is also present in other Atg1 proteins such as in *Arabidopsis* and *C. elegans* but is absent from the *S. cerevisiae* Atg1. The precise function of this domain is unknown, but its presence is required for autophagy.¹⁶

In *S. cerevisiae*, Atg1 interaction with Atg13 and Atg17 is essential for autophagy induction. This interaction is prevented under nutrient-rich conditions by TOR-dependent phosphorylation of Atg13.^{50,51} Starvation conditions inhibit TOR activity and Atg13 becomes rapidly dephosphorylated allowing Atg13 and Atg17 to interact with Atg1 and to activate its kinase activity.⁵¹ Rapamycin, an inhibitor of TOR, is a classic activator of autophagy even under nutrient-rich conditions. A putative Dictyostelium Atg13 homologue has been annotated in the Dictyostelium database (Dicty-base: <http://www.dictybase.org/>). Although it contains a conserved Atg13 domain that is present in the pfam database (<http://pfam.sanger.ac.uk/>), Atg13 shows a very low level of similarity between species, suggesting that this protein has largely diverged during evolution.⁵²

As seen in Table 1, the mammalian Atg1 complex also contains FIP200 (focal adhesion kinase (FAK) family interacting protein of 200 kDa), also known as RB1CC1 (Retinoblastoma 1 inducible coiled coil-1). FIP200 is a multifunctional protein involved in multiple cellular processes besides autophagy such as cell adhesion, migration, cell death and proliferation. It interacts with many different proteins and it is believed to be a functional homologue of Atg17 although they do not share sequence

similarity.^{53,54} Dictyostelium has putative Atg17 and FIP200, but their level of similarity is too low to decide with some confidence whether or not they are real homologues without any further experimental evidence.

In *S. cerevisiae*, under nutrient-replete growth conditions, Atg1 also regulates the autophagy-dependent cytoplasm-to-vacuole targeting (Cvt) pathway, a mechanism that targets specific hydrolases to the vacuole of *S. cerevisiae*.⁵⁵ The hydrolases are packed into autophagosome-like vesicles and delivered to the vacuole in a manner similar to that used during autophagy.⁵⁶ This specific and biosynthetic form of autophagy has only been described in *S. cerevisiae* and related yeasts.^{57,58} Atg1 and Atg13 are required for both autophagy and the Cvt pathway, but Atg17 is specific to autophagy. A number of other Atg1 complex subunit proteins are known to have specific roles in these pathways. Atg29 and Atg31 are specific for autophagy while Atg11, Atg20 and Atg24 (Suppl. Table 1) are only required for the Cvt pathway.^{59,60} As in humans, no protein similar to any of these proteins can be recognized by sequence similarity in Dictyostelium (see Table 1 and Suppl. Table 1), except for Atg24.

Interestingly, a putative homologue of the mammalian protein Atg101, absent in yeast, can be found in the Dictyostelium genome. Atg101 is a recently described protein essential for autophagy that interacts with Ulk1 in an Atg13-dependent manner. Additionally, it contributes to Atg13 function by protecting Atg13 from proteasomal degradation.^{52,61}

Nucleation and the Phosphatidylinositol 3-Kinase (PtdIns3K) Complex

In *S. cerevisiae*, the class III PtdIns3K Vps34 (vacuolar protein sorting 34) is a lipid kinase necessary for autophagy and the Cvt pathway.⁶² Its activity generates phosphatidylinositol-3-P (PtdIns3P), believed to be required for binding of other autophagic proteins to the autophagosome nucleation site, such as the

Table 2. PtdIns3K protein complex subunits

	Function/features	Dictyostelium	Human	<i>S. cerevisiae</i>	E-value Dd-Hu	E-value Dd-Sc
Atg6/Beclin 1	Subunit of the PtdIns3K complex. (Ma; Cvt)	Atg6B (DDB_G0288021) Atg6A (DDB_G0269244)	BECN1 (G.ID: 8678) BECN1L1 (G.ID: 441925)	Atg6 (YPL120W)	1e-51 2e-27	5e-22 1e-10
Vps34	Class III-phosphatidylinositol 3-kinase. (Ma; Cvt)	PikE (DDB_G0289601) Lower homology to Class I PI3Ks (PikA-H)	PIK3C3 (G.ID: 5289)	Vps34 (YLR240W)	1e-99	1e-121
Vps15	Myristoylated serine/threonine protein kinase. (Ma; Cvt)	Vps15 (DDB_G0282627) Lower homology at the kinase domain of other proteins	PIK3R4 (G.ID: 30849)	Vps15 (YBR097W)	8e-73	2e-62
UVRAG	Regulates the Beclin1-PtdIns3K complex. (Ma)	DDB_G0288175 DDB_G0283825	UVRAG (G.ID: 7405)	-	1e-28 3e-12	
Bif-1	BAR and SH3-containing protein. (Ma)	DDB_G0284997	SH3GLB1/Bif1 (G.ID: 51100)	-	0.014*	
Atg14	Regulates PtdIns3K. (Ma; Cvt)	DDB_G0278351	KIAA0831	Atg14 (YBR128C)	0.03	n.s.

*The possible Dictyostelium homologue for Bif1 has low homology but contains the expected C-terminal SH3 domain and an N-terminal BAR domain.

phosphoinositide interacting proteins Atg18 and Atg21.⁶³⁻⁶⁵ Besides autophagy, Vps34 is also implicated in other signaling pathways such as the TOR pathway and G-protein signaling to MAPK.⁶² Vps34 interacts with Vps15, a myristoylated protein kinase that seems to regulate Vps34.^{66,67} This interaction and the kinase domain of Vps15 are necessary for Vps34 activity, although Vps15 does not seem to phosphorylate Vps34 directly.^{62,68} A third protein, Atg6 (known as Beclin 1 or Vps30) is also part of the complex.⁶⁹ Atg6/Beclin 1 was first identified as a Bcl-2-interacting protein and it is a mammalian tumor suppressor involved in different cancers.^{70,71} The complex containing Vps34, Vps15 and Atg6 additionally interacts with two mutually exclusive proteins in *S. cerevisiae*, Vps38 and Atg14. The first one is involved in the Vps pathway and the second one is required for autophagy and the Cvt pathway.

Similar proteins to Vps34, Vps15 and Atg6 can be easily recognized in Dictyostelium and human (Table 2). In contrast, Atg14 appears to be present only in close relatives of *S. cerevisiae* and no highly similar proteins can be found in Dictyostelium and higher eukaryotes. However, it should be noted that recently, a distantly related mammalian Atg14 protein has been identified by computational analysis.⁷²⁻⁷⁴ This mammalian Atg14 and UVRAG (UV-radiation resistance-associated gene), another PtdIns3K complex subunit interact with Beclin 1 and Vps34 in a mutually exclusive way. UVRAG has been proposed to be the functional homologue of Vps38 although they do not show significant identity. Therefore, as described in yeast (concerning Atg14 and Vps38), the mammalian cells might also have two different PtdIns3K complexes containing either Atg14 or UVRAG and their mutually exclusive presence might account for the specific functions of this complex in autophagy and other membrane trafficking processes.^{73,74} Interestingly, a putative homologue of UVRAG can be detected in the Dictyostelium genome as shown in Table 2 with a fairly good e-value score. Sequence comparison with Atg14 did not detect any similar protein in Dictyostelium when compared with *S. cerevisiae* Atg14, but identified a protein with a low score when compared with the human Atg14 (Table 2).

Besides UVRAG, the mammalian complex might contain additional proteins not identified in yeast such as Ambra1 and Bif-1 whose functions are being characterized.^{75,76} Bif-1 interacts with UVRAG and promotes the activation of Vps34. Bif-1 contains two characteristic domains, an amino-terminal N-BAR (Bin-Amphiphysin-Rys) domain, and a carboxy-terminal SH3 (Src-homology 3) and has been proposed to be involved in the biogenesis of the autophagosome membrane due to its membrane binding and bending activities.^{77,78} While no similar proteins can be recognized in Dictyostelium for Ambra1, a putative Bif-1 can be identified and, although it shows a low level of similarity, the predicted sequence has the characteristic BAR and SH3 functional domains.

Vesicle Expansion and Ubiquitin-Like Conjugation Systems

Membrane expansion into a fully developed autophagosome requires the function of two ubiquitin-like protein conjugation reactions.⁷⁹ In the first conjugation system Atg12 is covalently bound to Atg5, a reaction catalyzed by the E1-type enzyme Atg7 and the E2 enzyme Atg10.^{80,81} Atg16 interacts noncovalently with Atg12-Atg5 to form a complex that multimerizes.^{82,83} This reaction and the localization of the Atg12-Atg5-Atg16 complex may facilitate the second conjugation reaction, and/or dictate in part where this reaction occurs. In the second reaction the ubiquitin-like protein Atg8 (commonly known as LC3 in mammals) is attached to the expanding autophagosome membrane by conjugation to phosphatidylethanolamine.^{84,85} Atg8 is first processed by the protease Atg4 to uncover a conserved glycine at the C terminus that is then used for the covalent binding to the phospholipid with the aid of the E1-type enzyme Atg7, also used in the first conjugation reaction, and the E2-type enzyme Atg3.

The proteins involved in these reactions are very well conserved during evolution and can be easily recognized by sequence similarity in Dictyostelium as shown in Table 3. Of note, two Atg8-like proteins are present in Dictyostelium, whereas only one

Table 3. Ubiquitin-like conjugation systems

	Function/features	Dictyostelium	Human	<i>S. cerevisiae</i>	E-value Dd-Hu	E-value Dd-Sc
Atg3	E2-like enzyme. (Ma; Cvt)	Atg3 (DDB_G0277319)	Atg3 (G.ID: 64422)	Atg3 (YNR007C)	1e-39	7e-19
Atg4	Cysteine protease. (Ma; Cvt)	Atg4 (DDB_G0273443) DDB_G0283753	Atg4B (G.ID: 23192) Other homologues (Atg4A, C, D)	Atg4 (YNL223W)	3e-19 5e-24	2e-11 1e-12
Atg5	Conjugates with Atg12. (Ma; Cvt)	Atg5 (DDB_G0289881)	Atg5 (G.ID: 9474)	Atg5 (YPL149W)	1e-15	5e-6
Atg7	E1-like enzyme. (Ma; Cvt)	Atg7 (DDB_G0271096)	Atg7 (G.ID: 10533)	Atg7 (YHR171W)	1e-148	1e-116
Atg8	Ubiquitin-like protein that conjugates with phosphatidylethanolamine (PE). (Ma; Cvt)	Atg8 (DDB_G0286191) DDB_G0290491	GABARAP (G.ID: 11337) Other homologues (LC3/ MAP1LC3A; GATE16/ GABARAP2, etc.,)	Atg8 (YBL078C)	3e-30 2e-21	1e-35 3e-29
Atg10	E2-like enzyme. (Ma; Cvt)	Atg10 (DDB_G0268840)	Atg10 (G.ID: 83734)	Atg10 (YLL042C)	4e-18	0.97
Atg12	Conjugates with Atg5 (Ma; Cvt)	Atg12 (DDB_G0282929)	Atg12 (G.ID: 9140)	Atg12 (YBR217W)	1e-14	8e-7
Atg16	Interaction with Atg12-Atg5 conjugates (Ma; Cvt)	TipD (DDB_G0275323)	Atg16L1 (G.ID: 55054)	Atg16 (YMR159C)	1e-68	1e-4

Table 4. Other autophagic proteins

	Function/features	Dictyostelium	Human	<i>S. cerevisiae</i>	E-value Dd-Hu	E-value Dd-Sc
Atg2	Peripheral membrane protein involved in Atg9 cycling (Ma; Cvt)	DDB_G0277419	Atg2A (G.ID: 23130) Atg2B (G.ID: 55102)	Atg2 (YNL242W)	7e-11 4e-24	4e-29
Atg9	Transmembrane protein (Ma; Cvt)	Atg9 (DDB_G0285323)	Atg9A (G.ID: 79065) Atg9B (G.ID: 285973)	Atg9 (YDL149W)	9e-67 1e-26	3e-88
Atg15	Lipase (Ma; Cvt)	-	-	Atg15 (YCR068W)		
Atg18	WD repeat domain phosphoinositide-interacting protein (Ma; Cvt)	Atg18 (DDB_G0285375) Wdr451 (DDB_G0282581)	WIPI-3 (56270) Other homologues (WIPI-1; WIPI-3; WDR45L/WIPI-3)	Atg18 (YFR021W)	1e-37 8e-81	4e-35 8e-29
Atg22	Amino acid export from vacuole (Ma; Cvt)	-	-	Atg22 (YCL038C)		
Atg23	Peripheral membrane protein. (Ma; Cvt)	-	-	Atg23 (YLR431C)		
Atg27	Type I membrane protein. (Ma; Cvt)	-	-	Atg27 (YJL178C)		
Vmp1	Transmembrane protein (Ma)	Vmp1 (DDB_G0285175)	TMEM49 (G.ID: 81671)	-	6e-61	

is present in yeast. Remarkably, the level of similarity between the Dictyostelium and the human proteins is generally higher than that observed between Dictyostelium and *S. cerevisiae* homologues. Another striking similarity is the presence of an extended C terminus in Atg16 containing multiple WD-40 repeats, a feature typically found in the Atg16 homologues of animals but absent in fungi. This domain is probably involved in additional protein-protein interactions that might have been conserved between Dictyostelium and animals. The putative Atg16 homologue (named TipD) was targeted by insertional mutagenesis in a genetic screen for a multi-tipped phenotype but its requirement in autophagy was not addressed.⁸⁶ Interestingly the developmental phenotype observed in the *tipD* mutant is similar to that described in other Dictyostelium mutants affecting both conjugation reactions, such as *atg7*, *atg5* and *atg8*.^{15,87}

Other Autophagy-Related Proteins

A number of autophagy proteins not included in the above-mentioned functional clusters are involved in other less known

processes such as the transport and recycling of components from the autophagosome. As shown in Table 4, several of these proteins, such as Atg2, Atg9 and Atg18, can be recognized in the *S. cerevisiae*, Dictyostelium and human genomes. Vmp1 on the other hand is absent in fungi but present in Dictyostelium and higher organisms, another example of the evolutionary proximity of Dictyostelium and animals.

Atg9 is a multispanning membrane protein involved in membrane traffic from not well-defined cellular compartments to the autophagosome and is therefore believed to play a role in the origin and elongation of the autophagic membrane.^{88,89} The subcellular localization of Atg9 depends on the organism under study. In *S. cerevisiae*, Atg9 appears to be located on the surface of mitochondria or in vesicles in very close proximity to these organelles.^{90,91} In mammalian cells, Atg9 traffics between the Golgi and endosomes suggesting an involvement of the Golgi complex in the autophagic pathway. In Dictyostelium, Atg9 resides in small vesicles that travel from the cell's periphery to the microtubule-organizing center. Its deletion leads to a pleiotropic phenotype including autophagy defects.⁹²

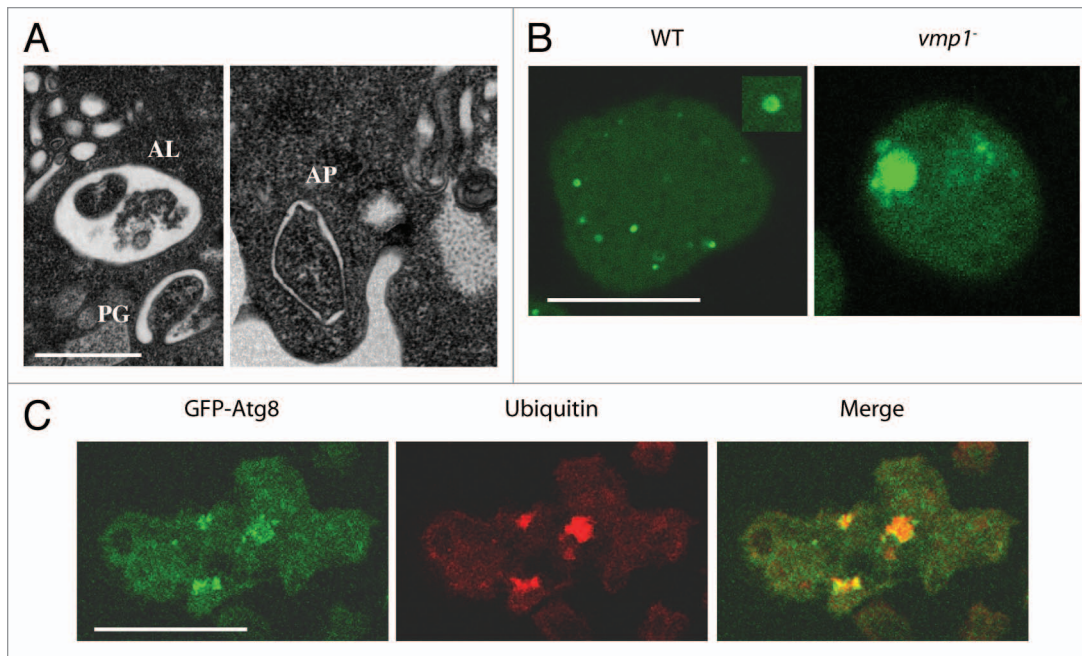


Figure 3. Monitoring autophagy in Dictyostelium. (A) Examples of autophagic structures in Dictyostelium as seen by transmission electron microscopy. PG, AP and AL correspond to putative phagophore, autophagosome and autolysosome respectively. Bar: 0.5 μm . (B) Fluorescence microscopy of wild-type (WT) and *vmp1*⁻ cells expressing the autophagosomal marker GFP-Atg8. Close examination of the punctate pattern (see the inset) reveals a vesicle-like appearance of the autophagosomes. In the mutant the marker appears aggregated. Bar: 10 μm . (C) These protein aggregates observed in *vmp1*⁻ organisms (and other autophagic mutants, see the text) colocalize with ubiquitin as determined by immunofluorescence with anti-ubiquitin antibody and contain the scaffold protein p62 as observed in many different protein aggregation diseases.

The fact that Vmp1 and Atg9 are transmembrane proteins known to be required for autophagosome formation in mammalian cells raises interesting questions about their possible role membrane traffic during autophagy.⁹³⁻⁹⁵ In contrast to Atg9, Vmp1 is an endoplasmic reticulum-resident protein in Dictyostelium. It is not yet clear to what extent the autophagosome membrane originates de novo or from pre-existing organelles. The localization of Vmp1 to the ER and its partial colocalization with autophagosomes are in line with other studies that suggest the involvement of the ER in autophagy.⁹⁶⁻⁹⁹

Atg18 interacts with Atg2, and this complex is localized to the autophagosome membrane via Atg18 binding to PtdIns(3)P, through the novel conserved motif FRRG.^{64,65} This localization is essential for the recruitment of other autophagic proteins and for autophagy, although the precise function of these proteins is unknown.

There are a number of proteins specifically involved in selective autophagy in *S. cerevisiae*. Most of these proteins do not have clear homologues in Dictyostelium and human except for Atg21, a homologue of Atg18 and Atg24, a member of the sorting nexin family with a phosphoinositide binding Phox domain and a BAR domain (Suppl. Table 1). This protein is also involved in endosomal protein sorting. The lack of similar proteins is not surprising taking into account that most of these proteins are involved in the Cvt pathway, a process not present in Dictyostelium.

In summary, the Dictyostelium genome codes for most of the basic components that have been described to regulate autophagy. Moreover, the strong similarity with animals and the presence of

certain proteins conserved in Dictyostelium and humans that are absent in yeast emphasize the high level of conservation of the basic autophagy machinery between this simple social amoeba and man.

Monitoring Autophagy in Dictyostelium

Transmission electron microscopy (TEM) has been a classical method to monitor autophagy although interpretation of the structures is difficult since autophagosome formation is a very dynamic process with morphologically different stages of maturation. Clear criteria must be applied to determine if a given structure is a bona fide autophagosome, such as the presence of double-membrane vesicles containing organelles or material similar in density to the cytoplasm. This double membrane might have a cup-shape when the formation of the autophagosome has not been completed. When the autophagosome is fused with the lysosomes, the internal membrane and the cytoplasmic material might appear partially degraded. Figure 3 shows an example of such structures in Dictyostelium. During vegetative growth, TEM images of Dictyostelium cells show very few double membrane autophagosomes, and most of the vacuoles are single membrane and contain spongy material that is believed to correspond to different degrees of digestion of the axenic medium that has been taken up by macropinocytosis.^{100,101} Other vesicles are electron-lucent and probably correspond to contractile vacuoles.¹⁰⁰ However, during starvation the number of food vacuoles decreases and double-membrane autophagosomes become

abundant reaching a maximum around 4–5 hours after the initiation of starvation,¹⁰⁰ confirming the activation of autophagy by starvation in *Dictyostelium*, as described in other organisms. The absence of autophagosomes has been determined by TEM in several *Dictyostelium* autophagic mutants including *atg1*, *atg6*, *atg8*, *atg7*, *atg5* and *vmp1*.^{15,87,93} Another characteristic feature of TEM images is the progressive disappearance of cytoplasm and organelles during starvation in wild type as a consequence of autophagy. Conversely, the autophagic mutants show dense cytoplasm with little degradation.^{15,87}

Molecular markers of autophagy are proteins involved in the autophagy process that can be used to monitor autophagy. The most common marker is Atg8/LC3 that becomes lipidated and attached to the autophagosome membrane, and participates in its elongation. The use of GFP-Atg8/LC3 allows in vivo visualization of autophagy by confocal fluorescence microscopy. In mammalian cells and *Dictyostelium*, this marker appears as a punctate pattern, as illustrated in **Figure 3**. Since autophagy is a dynamic process involving induction, maturation and degradation, a defect in a particular stage affects the Atg8/LC3 pattern in different ways. For example, a suppression of an early step of autophagosome formation will decrease the number of puncta, but a blockage of late stages might leave the induction unaffected, resulting in an accumulation of puncta.^{102,103} In *Dictyostelium*, the use of the GFP-Atg8 marker reveals some specific features of the system that must be taken into account. Although TEM analysis showed that starvation increases the number of autophagosomes, a number of GFP-Atg8 puncta are present during growth conditions and this number does not seem to be significantly affected during starvation. However, closer examination shows differences in the morphology of puncta. During growth, most of the puncta appear as simple dots. Conversely, during starvation the number of structures showing a cup-like or vesicle-like shape increases (**Fig. 3**). A possible interpretation is that during growth there are many initial autophagosome origins that do not progress in their elongation probably because they require additional signaling events. This signaling would be triggered by starvation to promote the activation of autophagy, and therefore the vesicle-like puncta, reflecting bona fide autophagosomes, become more evident. Alternatively, the dot-like structures observed during growth might represent artifactual aggregation of Atg8/LC3 as described in other systems.¹⁰⁴

Interestingly, when autophagy is blocked by genetic ablation of Atg1 or Vmp1 in *Dictyostelium*, the GFP-Atg8 marker colocalizes with large ubiquitinated protein aggregates together with p62 (**Fig. 3**). This phenomenon is less pronounced in other mutants such as Atg7 and Atg5. These aggregates have been described in many other systems where autophagy has been inhibited.^{105–107} The accumulation of these aggregates suggests a role for autophagy in their clearance. Other markers that associate with the phagophore have been used in other systems to monitor autophagy, such as Atg5, Atg12, Atg16 and Atg18.^{108–110} As described above, *Dictyostelium* possess proteins highly similar to each of them. They could potentially be used as additional markers to overcome some of the problems observed with GFP-Atg8.

The use of certain substrates to monitor autophagy-dependent protein degradation allows asking whether or not autophagy reaches its last stages, providing information about the autophagic flux. Since Atg8/LC3 and p62 are degraded by autophagy the total amount of these proteins decreases upon autophagy induction despite the expected transcriptional activation. Therefore, the total amount of these markers inversely correlates with autophagic flux.^{103,111,112} *Dictyostelium* cells expressing GFP-Atg8 can be used to monitor the degradation of this marker by western blot using anti-GFP antibodies. As expected we found that the amount of this marker decreases in the first hours of starvation and this decrease is prevented in the autophagic mutant *atg1* (unpublished observation), suggesting that a similar mechanism operates in *Dictyostelium* and could be used to monitor autophagy.

The conservation of autophagy genes and the mechanisms involved make us believe that some other techniques used to monitor autophagy in other systems might be applied to *Dictyostelium* in the future as more research teams join the field and use this model system to study autophagy.

Dictyostelium Autophagy Mutants are Affected in Development

Insertional and knockout mutants have been generated for several *Dictyostelium* autophagy genes as shown in **Table 5**. They comprise at least one component of each of the described functional complexes: Atg1 from the Atg1 complex,¹⁵ Atg6/Beclin 1 from the PtdIns3K complex,¹⁵ Atg5, Atg7, Atg8 and Atg16^{15,86,87} from the ubiquitin-like conjugation systems. Similarly, the two transmembrane proteins identified in mammalian cells to have an essential role in autophagosome formation, Atg9⁹² and Vmp1^{93,99} have also been ablated in *Dictyostelium*.

Autophagy is required for multicellular development in *Dictyostelium* and, interestingly, the severity of the phenotypes depends on the mutated gene. Mutants affected in the ubiquitin-like conjugation systems and Atg6/Beclin 1 have a defect at the mound/finger stage with the formation of multi-tipped structures leading to small or abnormal fruiting bodies.^{15,86,87} As described above in **Table 2**, the *Dictyostelium* genome codes for two homologous Atg6 proteins (Atg6A and Atg6B) and only the first one has been disrupted. As a consequence, the phenotype observed might be affected by partial redundancy.

Stronger phenotypes have been observed in the mutants affecting Atg1 or the transmembrane proteins Atg9 and Vmp1. They show vegetative growth defects, and development is partially or totally arrested at the aggregation or mound stages, depending on the experimental conditions. It should be noted that whereas the proteins involved in ubiquitin-like conjugation reactions seem to play specific roles in autophagy, the Atg1 complex,⁴² the PtdIns3K complex,⁶² Atg9 and Vmp1⁹⁸ might be involved in other membrane trafficking processes. The strong phenotype observed in some of these mutants might therefore be attributed in part to other possible additional defects not directly related to autophagy.

Table 5. Dictyostelium autophagic mutants and related phenotype

Mutant and parental strain	Developmental phenotype	Growth	Survival to starvation	Ubiquitin ⁺ aggregates	References
<i>atg1</i> ⁻ (DH1)	Aggregation/mound arrest	Slow growth	affected	Presence of large aggregates	Otto et al. 2004 (15)
<i>atg5</i> ⁻ (DH1)	Aggregation/Multi-tipped aggregates/aberrant fruiting bodies	Normal growth	affected	Presence of small aggregates	Otto et al. 2003 (87) Calvo-Garrido and Escalante. 2010 (99)
<i>atg6</i> ⁻ (DH1)	Multi-tipped aggregates/small fruiting bodies	Normal growth	affected	Not detected	Otto et al. 2004 (15)
<i>atg7</i> ⁻ (DH1)	Aggregation/Multi-tipped aggregates/aberrant fruiting bodies	Normal growth	affected	Presence of small aggregates	Otto et al. 2003 (87)
<i>atg8</i> ⁻ (DH1)	Multi-tipped aggregates/small fruiting bodies	Normal growth	affected	Not detected	Otto et al. 2004 (15)
<i>atg9</i> ⁻ (AX2)	Aggregation/Multi-tipped aggregates/aberrant fruiting bodies	Slow growth	Not analyzed	Not analyzed	Tung et al. 2010 (92)
<i>TipD/atg16</i> ⁻ (AX4)	Multi-tipped aggregates/small fruiting bodies	Not analyzed	Not analyzed	Not analyzed	Stege et al. 1999 (86)
<i>vmp1</i> ⁻ (AX4)	Aggregation/mound arrest	Slow growth	affected	Presence of large aggregates	Calvo-Garrido and Escalante. 2010 (99)

A similar argument, that autophagy may be required during all stages of the Dictyostelium developmental program, arises from the study of temperature-sensitive Atg1 mutants.¹⁶ Development is arrested when the mutant is shifted to the restrictive temperature even after 16 hours of development when the structures are at the slug stage. Development is then resumed when they are set back to the permissive temperature.¹⁶ It seems that a constant turnover of cellular material might be required at all stages of Dictyostelium development. However, as stated before, since Atg1 has been proposed to play additional roles besides autophagy, the Atg1 requirement during development might also involve other functional aspects that have not yet been characterized.

At the cellular level, dysfunction in protein degradation pathways such as in the ubiquitin-proteasome system and autophagy might lead to the persistence of ubiquitin-positive protein aggregates, a hallmark of many degenerative diseases. Interestingly, Dictyostelium *vmp1*⁻ mutants show accumulation of enormous ubiquitin-positive protein aggregates containing the autophagy marker GFP-Atg8 and the putative Dictyostelium p62 homologue as described in many degenerative human diseases.⁹⁹ In mammalian cells, p62 functions as a scaffold protein that provides a link between ubiquitinated aggregates and the autophagy machinery via the direct interaction of p62 with ubiquitin and the autophagosome protein Atg8/LC3. The presence of p62 in these ubiquitinated aggregates suggests that a similar mechanism functions in Dictyostelium. The inability of *vmp1*⁻ cells to clear these aggregates by autophagy would explain their accumulation, as described in mutant mice where the autophagy genes *Atg5* and *Atg9* have been knocked out.^{113,114}

The analysis of other Dictyostelium autophagic mutants (*atg1*⁻, *atg5*⁻, *atg6*⁻, *atg7*⁻ and *atg8*⁻) show a correlation between the severity of their corresponding phenotypes and the presence of ubiquitin-positive protein aggregates.⁹⁹ An attractive hypothesis is that the phenotypes are aggravated by the presence of

aggregates that might function as a sink for interacting proteins altering their normal localization or concentration. This phenomenon has been recently described in Dictyostelium with the formation of actin inclusions in cells by mistargeting VASP, an actin-binding protein, to endosomes. These actin aggregates are reminiscent of Hirano bodies that are often present in neurodegenerative diseases and, in Dictyostelium, are found to sequester other actin-binding proteins and endosomal proteins, promoting their disappearance from the cytoplasm.¹¹⁵ These Hirano body-like aggregates can also be induced in Dictyostelium by the overexpression of a truncated form of a 34 kDa actin-binding protein.¹¹⁶ A recent report shows that both autophagy and the proteasome pathway contribute to the degradation of Hirano bodies in Dictyostelium. Moreover, the autophagosome marker protein GFP-Atg8 colocalizes with model Hirano bodies in wild-type Dictyostelium cells, but not in *atg5*⁻ or *atg1*⁻ cells.¹¹⁷

Dictyostelium Autophagic Cell Death

Cell death with autophagy has been observed in particular in development and in pathology.¹¹⁸ Importantly, in recent years a causative role for autophagy in cell death could be demonstrated in certain cases through the decrease of cell death upon inactivation of an autophagy gene, often with no accompanying causative apoptotic or necrotic cell death.¹¹⁹⁻¹³² The question, then, becomes not whether autophagy is causative in some cases of animal cell death (it clearly can be), but how.

The protist Dictyostelium shows, when starved, developmental formation of a fruiting body consisting of viable spores and dead stalk cells.¹³³ Stalk cell formation can be mimicked in vitro under monolayer culture conditions, where Dictyostelium cells can differentiate from vegetative into “stalk” vacuolated cells¹³⁴⁻¹³⁶ showing signs of autophagy (see below) and undergoing cell death. This monolayer model shows many advantages for the study of autophagic cell death (ACD)³³ including the absence of

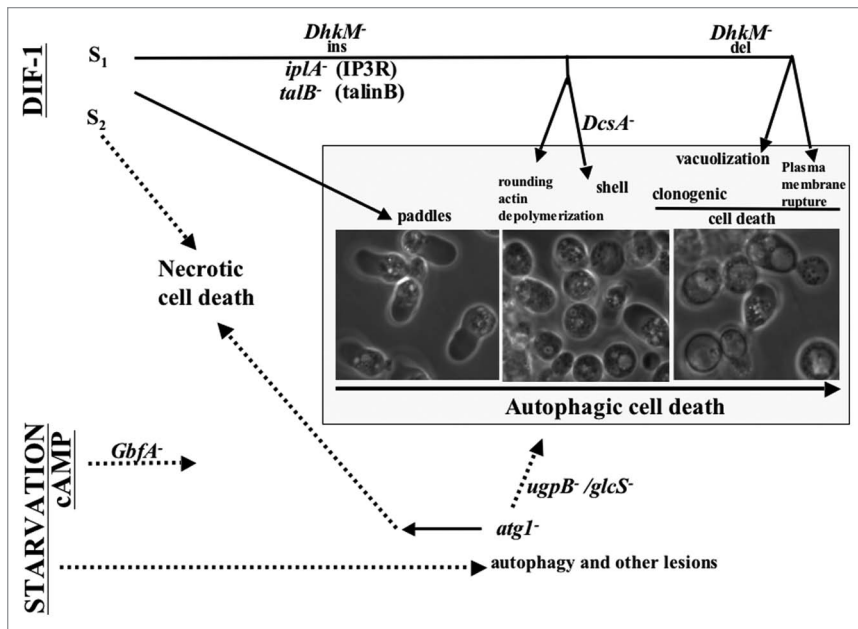


Figure 4. Pathways to cell death in Dictyostelium and mutational analysis thereof. In a first stage (lower half), starvation and cAMP lead to autophagy and sensitize cells to induction by DIF-1 (upper half) of either autophagic (right part) or necrotic (left part) cell death depending on whether the *atg1* gene is wild type or inactivated by mutation. The indicated other mutations allowed the dissection of particular autophagic cell death (see main text).

the main members of the apoptosis machinery that could interfere with it.^{137,138}

Importantly, triggering ACD in monolayers requires at least two distinct stimuli. The first stimulus is starvation together with cAMP.^{135,139,140} These induce, on the one hand, autophagy, as manifested by the appearance of autophagosomes,^{136,141,142} and on the other hand, major mitochondrial lesions.¹⁴³ Starvation, cAMP and the resulting alterations including autophagy do not by themselves lead to ACD. To induce cell death, a second stimulus is required, namely the main stalk differentiation-inducing factor DIF-1, a small dichlorinated molecule.¹⁴⁴⁻¹⁴⁷ DIF-1 is naturally synthesized during starvation-induced development. In monolayer experiments, ACD can be induced by addition of cAMP and DIF-1 to starved cells undergoing autophagy.¹³⁴⁻¹³⁶ ACD includes first the emergence of polarized “paddle” cells, then their rounding and acquisition of a cellulose shell. Small vacuoles then fuse to form large vacuoles ultimately occupying most of the cell volume (Fig. 4). Plasma membrane rupture occurs later (50% at around 40 hours of treatment) as judged by propidium iodide staining.^{134,136} The whole sequence of ACD subcellular events is shown in Figure 4.

ACD has been further investigated in this model, mostly using random insertional mutagenesis,³³ and here its requirements and genetic control are briefly reviewed (summarized in Fig. 4). Starvation-induced events are blocked by mutations of *gbfA* (G-box binding factor; a transcription factor)³³ and of *ugpB* (UDP-glucose pyrophosphorylase)/*glcS* (glycogen synthase).¹⁴¹ The DIF-1-triggered pathway leading to ACD was also studied by insertional mutagenesis. The correct functioning of this pathway from its triggering by DIF-1 to the induction of ACD

requires the following genes: *iplA* (IP3 receptor),¹⁴⁸ *talB* (talin B),³³ and *DhkM* (receptor histidine kinase M).¹⁴⁹ Mutation of these genes dissociates the autophagic cell death phenotype into several subcellular traits under various molecular controls.^{33,149}

In this system, is autophagy itself mechanistically required for or only accompanying ACD? If autophagy were required for ACD in the Dictyostelium model, mutation of one of the *atg* genes essential for the autophagic pathway should prevent not only autophagy, but also most or all of the signs of ACD. Indeed, an *atg1* mutation decreases autophagy^{15,150} and suppresses ACD.¹⁵⁰ Cell death, however, still occurs upon addition of DIF-1 to starved *atg1* cells, but as necrotic cell death (NCD), quite distinct from ACD. NCD involves immediate and massive mitochondrial uncoupling, perinuclear clustering of mitochondria, lysosomal permeabilization and rapid plasma membrane rupture.¹⁵⁰⁻¹⁵² Several mutations that inhibit the pathway leading to ACD do not affect or affect much less, the pathway leading to NCD,^{33,141,148} and NCD and ACD differ as to the specificity of their DIF-1 signaling.¹⁵³ These data are compatible with the interpretation that a mutation of the autophagy gene *atg1* could at the same time affect two distinct types of cell death, leading to NCD and preventing ACD. However, in this model NCD occurs much sooner and quicker than ACD and may thus preempt the occurrence of ACD, leading to the alternative interpretation that the *atg1* mutation would just favor the more rapid NCD, without any significance as to an Atg1 requirement for ACD. Current investigations aiming at rigorously checking an Atg1 requirement for ACD are based on drastic suppression of NCD. A first approach includes the differential reversibility of ACD and NCD upon early removal of the inducer. Specifically, removal of DIF-1 15 min after its addition led, in *atg1* cells to full reversal of early signs of NCD and ultimately to no or little death, but in wild-type cells to almost no reversal of ACD, which proceeded to vacuolization and death (reviewed in ref. 136, 151). A second approach is to use as a death-inducer not DIF-1, but a given DIF-1 derivative called 107 or desmethyl-DIF-1, which induces ACD, but almost no NCD.¹⁵³ Preliminary experiments using early removal of 107, or yet other approaches, or combinations of these, to prevent induction and/or completion of NCD strongly suggest that ACD is indeed dependent on Atg1 in this model.

Altogether, in this Dictyostelium monolayer model, autophagic cell death triggering requires a first signal, starvation/cAMP, leading to autophagy and a second signal, DIF-1, leading from autophagy to ACD. Autophagy is not directly causative of death (since autophagy is not sufficient) but primes for a mechanism that is (the DIF-1 pathway to ACD can occur only if *atg1* is intact). We think that such a second signal or something homologous to it, may well exist to trigger ACD in less simple eukaryotes,

where it is still buried in complexity. While we do not know yet to what extent this mechanism is conserved for instance in mammalian cells, in some cases the latter can show vacuolizing ACD morphologically very similar to that seen in *Dictyostelium*.¹⁵⁴ On the pathway triggered by DIF-1, some mutations specifically affect ACD, not autophagy. These mutations dissociate ACD into distinct, separately controlled subcellular lesions. To pursue this genetic analysis of ACD in this very favorable model, a search for further ACD mutants is ongoing.

Autophagy and Infection in *Dictyostelium*

The first line of defense against invading bacteria is comprised of phagocytic cells of the innate immune system. These cells are specialized in the recognition of invading pathogens and respond by activating antimicrobial immune responses (reviewed in ref. 155). These cells recognize and contain microbes early during infection via complement activation, phagocytosis, autophagy and immune activation by families of pattern recognition receptors (PRRs). The response relies on recognition of evolutionarily conserved structures of commensals and pathogens, termed pathogen-associated molecular patterns (PAMPs). The family of TLRs is the major and most extensively studied class of PRRs. The main bactericidal strategy relies on phagocytosis, the process by which cells engulf particles, which is conserved during evolution. In organisms such as amoebae, “phagotrophy” is used for feeding and appears as a distinguishing feature in the last common ancestor of eukaryotes.¹⁵⁶ In immune phagocytes, the bactericidal and degradation machineries have been harnessed to meet the needs for presentation of antigenic peptides.

Studies of autophagy identified important functions in the regulation of innate immunity and inflammation (reviewed in ref. 157). Xenophagy refers to the use of the autophagy pathway to digest foreign rather than self-constituents. The PRR-triggered pathways and the autophagy process intersect at many different levels: TLRs can regulate autophagy induction, the autophagy machinery can be used to deliver pathogen genetic material for binding to endosomal TLRs, and TLRs may act in the recruitment of autophagy proteins to phagosomal membranes. Indeed, Atg proteins have been identified in the major proteomic investigations of phagosomal components.¹⁵⁸ The pathways leading from bacterial sensing to xenophagy are very complex and have not been completely deciphered yet. Nevertheless, a picture is emerging with a central axis of signaling making use of the general nutrient-sensing cascade involving the energy sensor AMP-activated protein kinase (AMPK) that, in response to high AMP/ATP ratios, inhibits TORC1 and leads to induction of autophagy (reviewed in ref. 159). In addition, during evolution, before the NFκB pathway emerged as the central coordinator of the immune response, the p38 mitogen-activated protein kinase (MAPK) cascade served as the ancestral antimicrobial defense-coordinating pathway.¹⁶⁰

Facing the evolution of ever more efficient bacterial sensing and killing mechanisms, microorganisms subject to predation were under strong selective pressure to develop the traits needed to survive phagocytic cells, including passive (resistant capsule)

or active (toxin secretion) defense mechanisms, but also the ability to replicate directly within the predator cell. This results in a paradox: many microorganisms, although they only accidentally infect mammals, have evolved sophisticated mechanisms to do so.¹⁶¹ One of the clearest examples is *Legionella* that did not infect humans before the invention of air conditioning. Indeed, the virulence traits of *Legionella* and pathogens such as *Chlamydia* and waterborne *Mycobacteria*¹⁶² were probably selected to fight amoebae long before the appearance of metazoans. Despite evolutionary perfection, phagocytic cells can be hijacked by intracellular pathogens that overcome their killing mechanisms and establish themselves a vacuolar or cytosolic niche to survive and/or proliferate. Upon cell invasion, bacteria must confront xenophagy, an efficient intracellular defense machinery. Beside bacteria that are completely controlled by autophagy as part of the innate surveillance mechanism, several bacterial pathogens have evolved virulence strategies to either inhibit autophagy to establish a persistent infection or even to take advantage of autophagy to generate a replication niche and to succeed in colonization and spreading (reviewed in ref. 163).

The amoeba *Dictyostelium* is an attractive model system to study host-pathogen interactions.^{25,164} Recent reports suggest that self-nonsel discrimination¹⁶⁵ and innate immunity¹⁶⁶ already evolved in amoebae. *Dictyostelium* cells feed on soil bacteria and, throughout their life, ingest, kill and digest microorganisms at a rate of at least one per minute. Thus, *Dictyostelium* is likely to have evolved mechanisms that enable it to discriminate and respond appropriately to various bacteria to optimize feeding and to avoid subversion by pathogens. Indeed, genome-wide mutagenesis screening reveals pathways of uptake and killing mechanisms specific to Gram⁺ or Gram⁻ bacteria.¹⁶⁷ Several transcriptomic analyses of *Dictyostelium*'s reaction to different bacterial species have been carried out and reveal strong modulation of thousands of transcripts.¹⁶⁸⁻¹⁷⁰ Many of these genes belong to a set of “innate immunity-related” genes that bear homologies to plant and insect innate immune defenses, as well as to the mammalian pathways,^{27,168} confirming that *Dictyostelium* can recognize bacteria and modulate its response.²⁶ In the multicellular slug, a special cell-type, the sentinel cell, patrols in search of xenobiotics and bacteria.¹⁶⁶

Because of its ease of manipulation and the conservation of cell-autonomous defense pathways, *Dictyostelium* has been successfully used and instrumental in the study of virulence mechanisms of *Pseudomonas*, *Legionella* and *Vibrio cholera*.¹⁷¹⁻¹⁷⁴ Most interesting in the view of autophagy, *Dictyostelium* is an experimental host to pathogens that interact and interfere with xenophagy such as *Salmonella*, mycobacteria and especially *Legionella*.¹⁷⁵

Salmonella enterica serovar Typhimurium is a food-borne pathogen that is usually restricted to the gastrointestinal tract, but can cause severe extra-intestinal diseases in the elderly. In epithelial and other cell types, *Salmonella* escapes the phagosome pathway and establishes a replication compartment that retains some characteristics of the endosomal pathway. Contrary to the fate of many intracellular pathogens for which the course of infection in *Dictyostelium* is similar to the one in macrophages, *Salmonella* is killed and degraded hours after ingestion by the

amoeba.¹⁷⁶ Interestingly, Salmonella appears to evade the common fate of nonpathogenic bacteria such as *E. coli* and escapes phagosome maturation. But, even though Salmonella does not succumb to the bactericidal activities of the phagosomal pathway, it is nevertheless surrounded by GFP-Atg8-positive membranes about 2 hours post-infection and finally is degraded in autolysosomes.¹⁷⁷ Confirming the restrictive role of autophagy, infection of *atg1*-, *atg6*- and *atg7*-null mutants results in the formation of a standard Salmonella-containing vacuole (SCV) and bacteria proliferation. This is finally detrimental to these autophagy-defective Dictyostelium mutants, which die within 1–3 days of infection.¹⁷⁷

Like many other bacterial pathogens, *M. tuberculosis* can reside in various compartments of its host. As a facultative intracellular pathogen, it can reside outside cells, in the interstitial space or inside necrotic granulomatous lesions. After uptake by immune phagocytes and inducing an arrest of their phagocytic maturation pathway, it resides intracellularly, first inside a replication vacuole¹⁷⁸ and then in the cytosol.¹⁷⁹ In Dictyostelium, the establishment and course of an infection by *M. marinum* are similar to those observed for pathogenic mycobacteria in other host systems. Importantly, as is the case in animal macrophages, during infection of Dictyostelium, *M. marinum* escapes its vacuole and continues to proliferate in the cytosol.¹⁸⁰ It is worth noting that, in activated macrophages, autophagy appears to be able to overcome the phagosome maturation block imposed by mycobacteria and thus controls *M. bovis* BCG infection by directing the replication vacuole to fuse with lysosomes and kill the bacteria.¹⁸¹ Whether this might also be relevant for infections by *M. marinum* and *M. tuberculosis* still awaits further studies. However, recent studies point to a causality link between vacuole rupture, *M. marinum* exposure to the cytosol, ubiquitination and the spatial recruitment of Atg8-positive membranes, indicating the intervention of adapter proteins such as p62/sequestosome 1. Interestingly, for some cytosolic pathogens, the cell wall is a target for ubiquitination,¹⁸² whereas for others, the damaged vacuole is the target.¹⁸³ Furthermore, it is suggested that bactericidal peptides derived from ubiquitin and ribosomal proteins are brought in contact with the mycobacteria via p62-mediated autophagy.¹⁸⁴ Because most of these proteins and processes are conserved in Dictyostelium, including p62,⁹⁹ it will be exciting to investigate whether these mechanisms are also active during infection of Dictyostelium by *M. marinum*.

Legionella pneumophila is the prototype of an accidental pathogen for human, because its natural hosts are unicellular protozoa, such as Acanthamoeba. This explains why the use of the amoeba Dictyostelium to study the mechanisms of Legionella virulence and host resistance has been increasingly popular, and represents the “flagship” of host-pathogen studies in this model system. The many successes in this field of research have been very recently and comprehensively reviewed (see ref. 175), and here we will concentrate on the interactions of Legionella with autophagy. Studies in macrophages, mainly using pharmacological tools, had pointed to a potential positive involvement of autophagy in the biogenesis of the replication compartment.¹⁸⁵ For example, starvation-induced autophagy had a modest stimulatory effect on proliferation.¹⁸⁶ But this claim remained disputed,

until a seminal study using the genetic power of Dictyostelium demonstrated that the absence of either Atg1, Atg5, Atg6, Atg7 or Atg8 had little or no impact on the establishment of the replication compartment, and even slightly enhanced the proliferation of Legionella.¹⁸⁷ These findings were compatible with a role of autophagy in the control of Legionella infection, but this was not further examined until a few recent studies. The starting point was the finding that the global transcriptomic response to Legionella infection includes the prominent regulation of three autophagy genes encoding Atg8, Atg9 and Atg16.¹⁷² Among these, the multi-transmembrane protein Atg9 was chosen to study the impact of gene ablation in Legionella infection.⁹² First, surprisingly, the absence of Atg9 results in a significant decrease in phagocytic uptake, possibly reflecting a direct or indirect coupling between phagocytosis and autophagy. Then, a careful quantitative analysis of the early phase of infection reveals that, in wild-type Dictyostelium, Legionella is rapidly and strongly cleared from the amoeba in the first hours post uptake, and that this is strongly defective in *atg9* null cells.⁹² These findings confirm and extend the previous conclusions that autophagy plays a protective role to limit infection by Legionella.

But three recent studies indicate that the case is probably not definitively closed and that the interaction of Legionella with the autophagy pathway might be more complex than initially thought. First, it was recently discovered that an effector secreted by Legionella, AnkB, represents a case of molecular mimicry by which Legionella subverts the polyubiquitination machinery of its host, be it a macrophage or a Dictyostelium cell.¹⁸⁸ This protein contains a noncanonical F-box domain, the integrity of which is essential for rapid acquisition of polyubiquitinated proteins by the Legionella-containing vacuole and for bacteria proliferation. AnkB is proposed to act via a pathway including the SCF1 (RBX1-CUL1-SKP1) ubiquitin ligase complex that is highly conserved throughout eukaryotes.¹⁸⁸ Second, while studying the causes of increased susceptibility of patients with mitochondrial diseases to Legionella infection, Paul Fisher’s group highlighted the profound impact of an upregulation of the energy-sensing protein kinase AMPK.¹⁸⁹ Upregulation of AMPK is a primary response to the impaired energy production in such diseases, but the resulting dysfunction on the containment of Legionella infection was a relative surprise. Overexpression of AMPK in wild-type Dictyostelium phenocopied the situation in mutant cells, identifying AMPK as a dominant regulator of intracellular immunity to Legionella,¹⁸⁹ possibly via the TOR-autophagy or p38ERK-MAPK cascade pathways. More work is required to answer these exciting developments, but another study might point in that direction. High-throughput screening to identify host proteins that modulate Legionella growth in Dictyostelium reveal a pivotal role for DupA in the genesis of the replication niche.¹⁶⁹ DupA is a putative tyrosine/dual specificity phosphatase that appears to regulate ERK1 phosphorylation and activation of the MAPK cascade. Also of interest is the finding that many genes are regulated both in *dupA* null cells and upon infection with bacteria, including the *tirA* and *shrA* genes that encode proteins suggested to play an immune-like function in sentinel cells during development.¹⁶⁶

Concluding Remarks

Autophagy is a fast emerging field and although a big leap has been taken recently by identifying a group of proteins involved in the mechanism and regulation of autophagy, the molecular function of many of these Atg proteins is still poorly defined. It is very likely that a number of proteins involved in autophagy are still unknown and the use of simple experimental models should help us define these new components. Autophagy in the social amoeba *Dictyostelium* plays essential roles in its natural life that makes it a suitable model where autophagy can be studied in the context of a whole organism. The differences between *Dictyostelium* and the yeast model *S. cerevisiae* will enrich the possibilities of study while still maintaining the simplicity of the microorganisms. Its powerful molecular genetics, the availability of the genome sequence and the similarities with higher organisms will help shed light on many of the still unanswered questions and help discover new genes involved in this exciting field.

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Note

Supplementary materials can be found at: www.landesbioscience.com/supplement/CalvoGarridoAUTO6-6-Sup.pdf

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Sup. Table 1. Proteins involved in selective autophagy (Cvt pathway and Mitophagy)*

	Function/features	Dictyostelium	Human	<i>S. cerevisiae</i>	E-value Dd-Hu	E-value Dd-Sc
Atg11	Adapter protein.(Cvt)	-----	-----	Atg11(YPR049C)		
Atg19	Receptor protein. (Cvt)	-----	-----	Atg19(YOL082W)		
Atg20	Phosphoinositide binding Phox-domain protein.(Cvt)	-----	-----	Atg20(YDL113C)		
Atg21	WD repeat domain phosphoinositide-interacting protein (Cvt)	Atg18(DDB_G0285375) Wdr45l(DDB_G0282581)	WIPI-2(G.ID: 26100) Other homologues (WIPI-1; WDR45L/WIPI-3)	Atg21(YPL100W)	2e-51 4e-32	4e-12 2e-10
Atg24	Phosphoinositide binding Phox-domain protein. (Cvt)	Vps5(DDB_G0272989)	SNX30(G.ID401548) Sorting nexin family	Atg24/SNX4 (YJL036W)	1e-12	2e-4
Atg32	Mitochondrial-anchored transmembrane receptor. (Mit)	-----	-----	Atg32(YIL146C)		
Atg33	Mitochondrial mitophagy-specific protein. (Mit)	-----	-----	Atg33(YLR356W)		
Vac8	Vacuolar membrane protein that interacts with Atg13. Armadillo repeat protein. (Cvt)	-----**	-----**	Vac8(YEL013W)		
Vps38	Part of the PtdIns3K (Vps34)- complex (Cvt)	-----	-----	Vps38(YLR360W)		

*Atg genes not included: Atg25,Atg28 and Atg30 are required for pexophagy in other yeast species but are not conserved in *S. cerevisiae*. Atg26 is not involved in autophagy in *S. cerevisiae*.

** Low homology restricted to the armadillo-repeat domain can be detected in multiple proteins.