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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

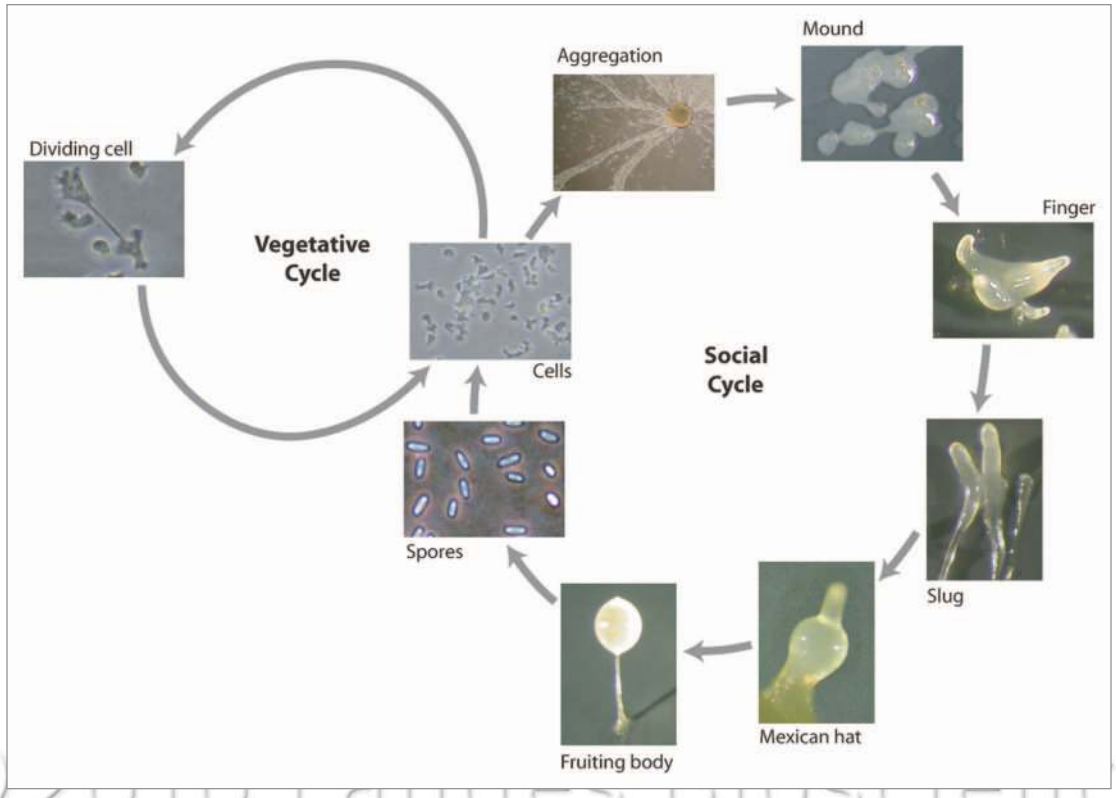

Figure 1. Dictyostelium life cycle. Representative pictures of vegetative and developmental stages are shown. In the wild, Dictyostelium amoeba feed on soil bacteria by phagocytosis but most laboratory strains are also able to grow in liquid axenic media by macropinocytosis. It is important to point 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 drugs[29-32] and programmed cell death including autophagic cell death (reviewed in ref. 33). The Dictyostelium genome has been fully sequenced[34] 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

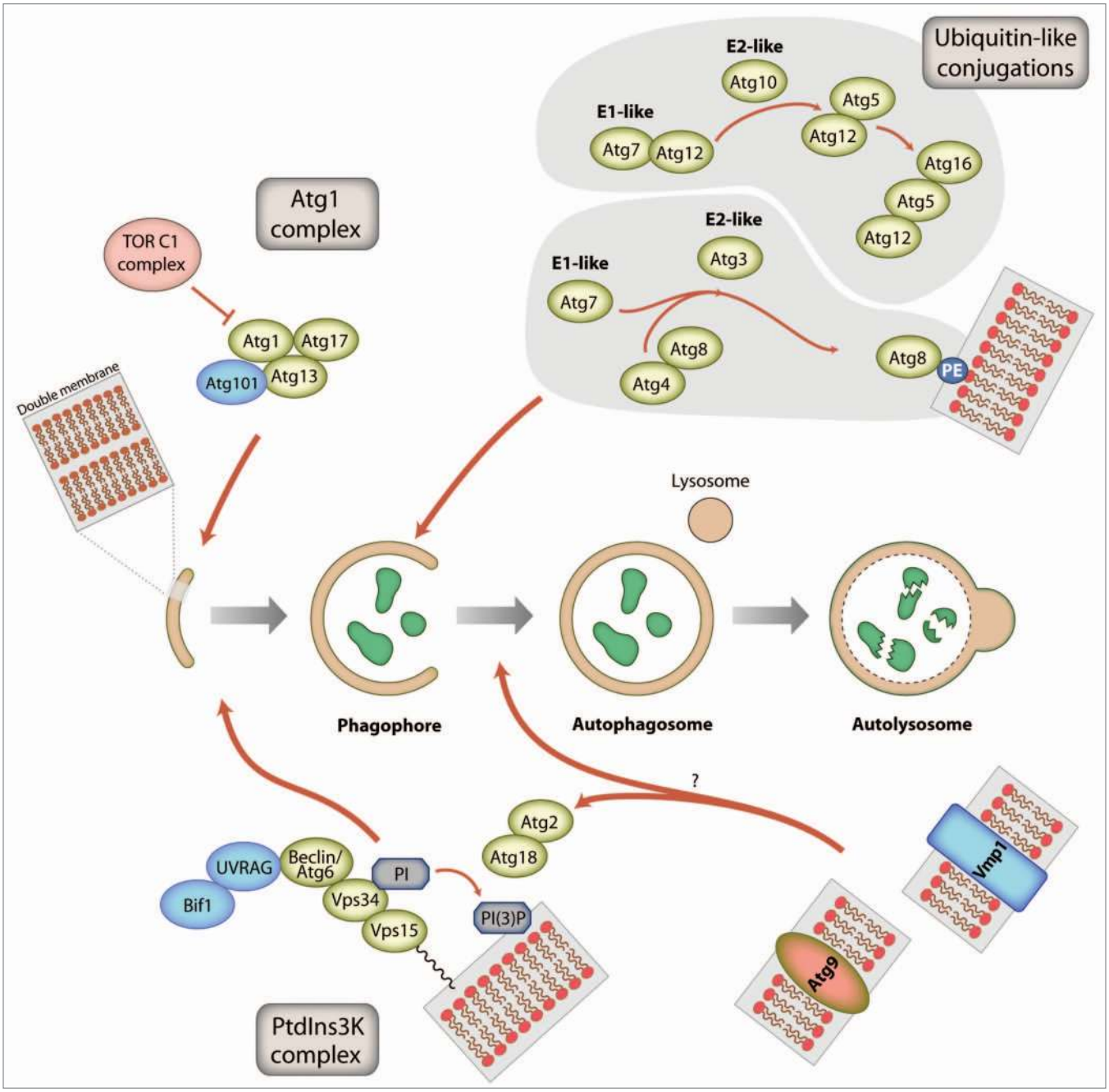

Figure 2. Autophagosome formation and putative signaling pathways in Dictyostelium. The phagophore is a double membrane whose origin is still a matter of debate. This membrane enlarges and finally engulfs parts of the cytoplasm. After fusion with lysosomes the content is degraded and recycled. The predicted 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 systems[43-49] including

Table 1. Atg1 protein complex subunits

	Function/features	Dictyostelium	Human	*S. cerevisiae*	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	*S. cerevisiae*	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	*S. cerevisiae*	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/ GABARAPL2, 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	*S. cerevisiae*	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) Wdr45l (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]

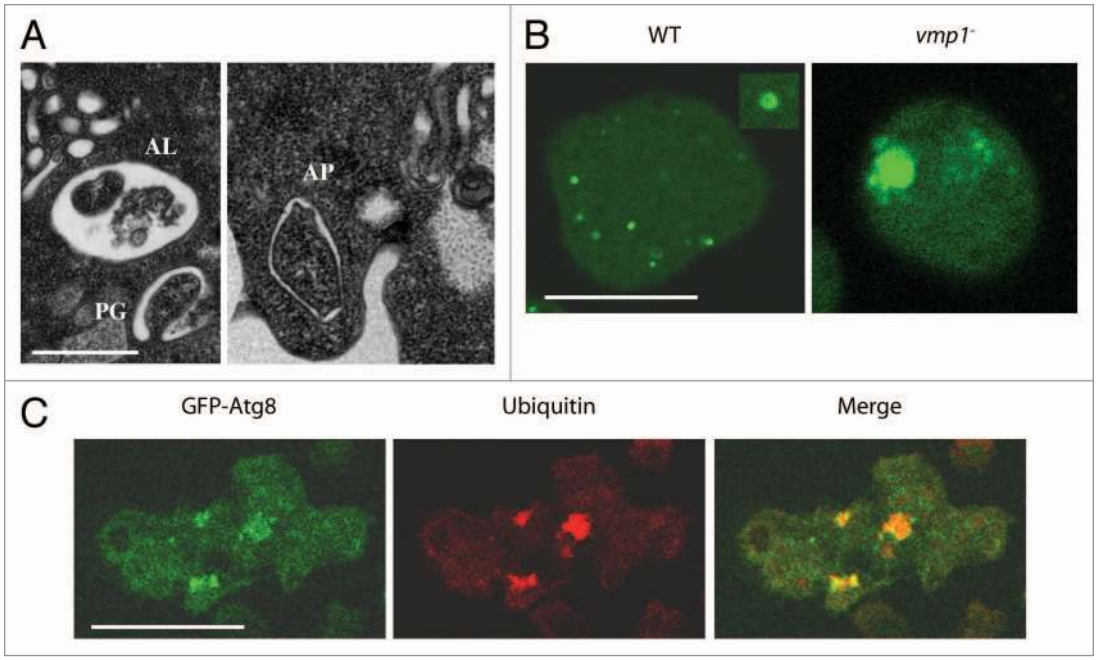

Figure 3. Monitoring autophagy in Dictyostelium. (A) Examples of autophagic structures in Dictyostelium as seen by transmission electron microscopy. PG, AP and AL correspond to putative phagophore, autophagosome and autolysosome respectively. Bar: 0.5 μm. (B) Fluorescence microscopy of wild-type (WT) and *vmp1*- cells expressing the autophagosome 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 determine 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, Atg9[92] 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 Vmp1[98] 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
atg1$^-$ (DH1)	Aggregation/mound arrest	Slow growth	affected	Presence of large aggregates	Otto et al. 2004 (15)
atg5$^-$ (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)
atg6$^-$ (DH1)	Multi-tipped aggregates/small fruiting bodies	Normal growth	affected	Not detected	Otto et al. 2004 (15)
atg7$^-$ (DH1)	Aggregation/Multi-tipped aggregates/ aberrant fruiting bodies	Normal growth	affected	Presence of small aggregates	Otto et al. 2003 (87)
atg8$^-$ (DH1)	Multi-tipped aggregates/small fruiting bodies	Normal growth	affected	Not detected	Otto et al. 2004 (15)
atg9$^-$ (AX2)	Aggregation/Multi-tipped aggregates/ aberrant fruiting bodies	Slow growth	Not analyzed	Not analyzed	Tung et al. 2010 (92)
TipD/atg16- (AX4)	Multi-tipped aggregates/small fruiting bodies	Not analyzed	Not analyzed	Not analyzed	Stege at al. 1999 (86)
vmp1$^-$ (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 cells[134-136] 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

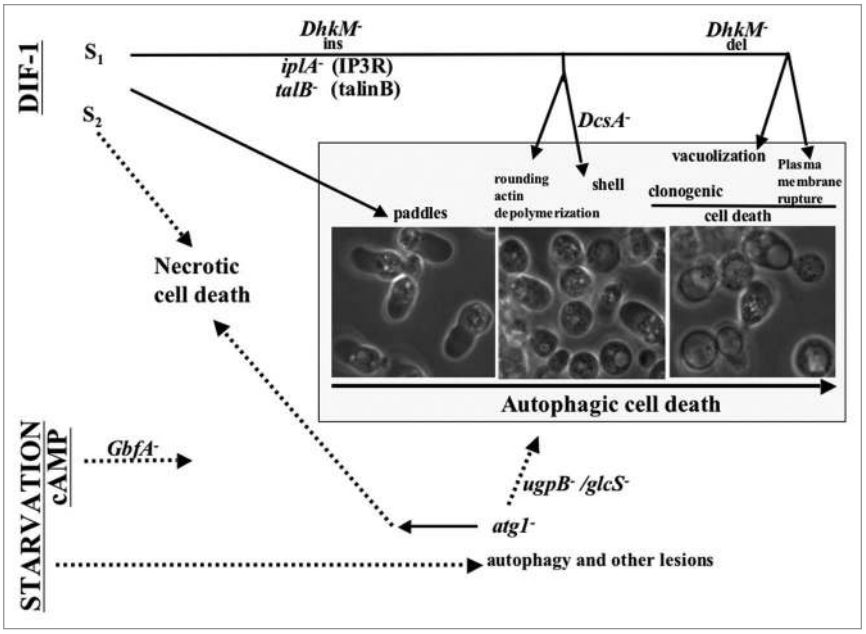

Figure 4. Pathways to cell death in Dictyostelium and mutational analysis thereof. In a first stage (lower half), starvation and cAMP lead to autophagy and sensitize cells to induction by DIF-1 (upper half) of either autophagic (right part) or necrotic (left part) cell death depending on whether the *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 NFkappaB 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-nonself discrimination[165] and innate immunity[166] 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 vacuole[178] 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 autophagic 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 *slrA* 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

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

References

1. Willard SS, Devreotes PN. Signaling pathways mediating chemotaxis in the social amoeba, *Dictyostelium discoideum*. Eur J Cell Biol 2006; 85:897-904.
2. Janetopoulos C, Firtel RA. Directional sensing during chemotaxis. FEBS Lett 2008; 582:2075-85.
3. King JS, Insall RH. Chemotaxis: finding the way forward with Dictyostelium. Trends Cell Biol 2009; 19:523-30.
4. Strmecki L, Greene DM, Pears CJ. Developmental decisions in *Dictyostelium discoideum*. Dev Biol 2005; 284:25-36.
5. Annesley SJ, Fisher PR. *Dictyostelium discoideum*-a model for many reasons. Mol Cell Biochem 2009; 329:73-91.
6. Escalante R, Vicente JJ. *Dictyostelium discoideum*: a model system for differentiation and patterning. Int J Dev Biol 2000; 44:819-35.
7. Meléndez A, Levine B. Autophagy in *C. elegans*. WormBook 2009; 1-26.
8. McPhee CK, Baehrecke EH. Autophagy in *Drosophila melanogaster*. Biochim Biophys Acta 2009; 1793:1452-60.
9. Cecconi F, Levine B. The role of autophagy in mammalian development: cell makeover rather than cell death. Dev Cell 2008; 15:344-57.
10. Kon M, Cuervo AM. Chaperone-mediated autophagy in health and disease. FEBS Lett 2009; 284:1399-404.
11. Uttenweiler A, Mayer A. Microautophagy in the yeast *Saccharomyces cerevisiae*. Methods in molecular biology. Clifton NJ 2008; 445:245-59.
12. Levine B, Kroemer G. Autophagy in the pathogenesis of disease. Cell 2008; 132:27-42.
13. Marino G, Lopez-Otin C. Autophagy and aging: new lessons from progeroid mice. Autophagy 2008; 4:807-9.
14. Mellen MA, de la Rosa EJ, Boya P. The autophagic machinery is necessary for removal of cell corpses from the developing retinal neuroepithelium. Cell Death Differ 2008; 15:1279-90.
15. Otto GP, Wu MY, Kazgan N, Anderson OR, Kessin RH. Dictyostelium macroautophagy mutants vary in the severity of their developmental defects. J Biol Chem 2004; 279:15621-9.
16. Tekinay T, Wu MY, Otto GP, Anderson OR, Kessin RH. Function of the *Dictyostelium discoideum* Atg1 kinase during autophagy and development. Eukaryot Cell 2006; 5:1797-806.
17. Xie Z, Klionsky DJ. Autophagosome formation: core machinery and adaptations. Nat Cell Biol 2007; 9:1102-9.
18. Uchiyama Y, Shibata M, Koike M, Yoshimura K, Sasaki M. Autophagy-physiology and pathophysiology. Histochem Cell Biol 2008; 129:407-20.
19. Reggiori F. Membrane origin for autophagy. Curr Topics Dev Biol 2006; 74:1-30.
20. Van Haastert PJ, Veltman DM. Chemotaxis: navigating by multiple signaling pathways. Sci STKE 2007; 2007:40.
21. Stephens L, Milne L, Hawkins P. Moving towards a better understanding of chemotaxis. Curr Biol 2008; 18:485-94.
22. Kay RR, Langridge P, Traynor D, Hoeller O. Changing directions in the study of chemotaxis. Nat Rev 2008; 9:455-63.
23. Harwood AJ. Dictyostelium development: a prototypic Wnt pathway? Meth Mol Biol 2008; 469:21-32.
24. Fountain SJ. Neurotransmitter receptor homologues of *Dictyostelium discoideum*. J Mol Neurosci 2010; 41:263-9.
25. Steinert M, Heuner K. Dictyostelium as host model for pathogenesis. Cellular Microbiology 2005; 7:307-14.
26. Bozzaro S, Bucci C, Steinert M. Phagocytosis and host-pathogen interactions in Dictyostelium with a look at macrophages. Int Rev Cell Mol Biol 2008; 271:253-300.
27. Cosson P, Soldati T. Eat, kill or die: when amoeba meets bacteria. Curr Opin Micro 2008; 11:271-6.
28. Jin T, Xu X, Fang J, Isik N, Yan J, Brzostowski JA, Hereld D. How human leukocytes track down and destroy pathogens: lessons learned from the model organism *Dictyostelium discoideum*. Immunol Res 2009; 43:118-27.
29. Shaulsky G, Escalante R, Loomis WF. Developmental signal transduction pathways uncovered by genetic suppressors. Proc Natl Acad Sci USA 1996; 93:15260-5.
30. Escalante R, Loomis WF. Whole-mount in situ hybridization of cell-type-specific mRNAs in Dictyostelium. Dev Biol 1995; 171:262-6.
31. Alexander S, Min J, Alexander H. *Dictyostelium discoideum* to human cells: pharmacogenetic studies demonstrate a role for sphingolipids in chemoresistance. Biochim Biophys Acta 2006; 1760:301-9.
32. Williams RS. Pharmacogenetics in model systems: defining a common mechanism of action for mood stabilisers. Prog Neuropsychopharmacol Biol Psychiatry 2005; 29:1029-37.
33. Giusti C, Tresse E, Luciani M-F, Golstein P. Autophagic cell death: analysis in Dictyostelium. Biochim Biophys Acta 2009; 1793:1422-31.
34. Eichinger L, Pachebat JA, Glockner G, Rajandream MA, Sucgang R, Berriman M, et al. The genome of the social amoeba *Dictyostelium discoideum*. Nature 2005; 435:43-57.
35. Kuspa A, Loomis WF. Tagging developmental genes in Dictyostelium by restriction enzyme-mediated integration of plasmid DNA. Proc Natl Acad Sci USA 1992; 89:8803-7.
36. Kuspa A, Loomis WF. Transformation of Dictyostelium—Gene disruptions, insertional mutagenesis and promoter traps. Meth Mol Genet 1994; 3:3-21.
37. Torija P, Robles A, Escalante R. Optimization of a large-scale gene disruption protocol in Dictyostelium and analysis of conserved genes of unknown function. BMC Microbiol 2006; 6:75.
38. Urushihara H. The cellular slime mold: eukaryotic model microorganism. Exp Anim 2009; 58:97-104.
39. Torija P, Vicente JJ, Rodrigues TB, Robles A, Cerdan S, Sastre L, et al. Functional genomics in Dictyostelium: MidA, a new conserved protein, is required for mitochondrial function and development. J Cell Sci 2006; 119:1154-64.
40. Schmelzle T, Hall MN. TOR, a central controller of cell growth. Cell 2000; 103:253-62.
41. Mizushima N. The role of the Atg1/ULK1 complex in autophagy regulation. Curr Opin Cell Biol 2010; 22:132-9.
42. Chan EY, Tooze SA. Evolution of Atg1 function and regulation. Autophagy 2009; 5:758-65.
43. Matsuura A, Tsukada M, Wada Y, Ohsumi Y. Apg1p, a novel protein kinase required for the autophagic process in *Saccharomyces cerevisiae*. Gene 1997; 192:245-50.
44. Melendez A, Tallóczy Z, Seaman M, Eskelinen EL, Hall DH, Levine B. Autophagy genes are essential for dauer development and life-span extension in *C. elegans*. Science 2003; 301:1387-91.
45. Scott RC, Schuldiner O, Neufeld TP. Role and regulation of starvation-induced autophagy in the Drosophila fat body. Dev Cell 2004; 7:167-78.
46. Chan EY, Kir S, Tooze SA. siRNA screening of the kinome identifies ULK1 as a multidomain modulator of autophagy. J Biol Chem 2007; 282:25464-74.
47. Chang YY, Neufeld TP. An Atg1/Atg13 complex with multiple roles in TOR-mediated autophagy regulation. Mol Biol Cell 2009; 20:2004-14.
48. Ganley IG, Lam du H, Wang J, Ding X, Chen S, Jiang X. ULK1·ATG13·FIP200 complex mediates mTOR signaling and is essential for autophagy. J Biol Chem 2009; 284:12297-305.

49. Hosokawa N, Hara T, Kaizuka T, Kishi C, Takamura A, Miura Y, et al. Nutrient-dependent mTORC1 association with the ULK1-Atg13-FIP200 complex required for autophagy. Mol Biol Cell 2009; 20:1981-91.
50. Kamada Y, Funakoshi T, Shintani T, Nagano K, Ohsumi M, Ohsumi Y. Tor-mediated induction of autophagy via an Apg1 protein kinase complex. J Cell Biol 2000; 150:1507-13.
51. Kabeya Y, Kamada Y, Baba M, Takikawa H, Sasaki M, Ohsumi Y. Atg17 functions in cooperation with Atg1 and Atg13 in yeast autophagy. Mol Biol Cell 2005; 16:2544-53.
52. Mercer CA, Kaliappan A, Dennis PB. A novel, human Atg13 binding protein, Atg101, interacts with ULK1 and is essential for macroautophagy. Autophagy 2009; 5.
53. Hara T, Mizushima N. Role of ULK-FIP200 complex in mammalian autophagy: FIP200, a counterpart of yeast Atg17? Autophagy 2009; 5:85-7.
54. Hara T, Takamura A, Kishi C, Iemura S, Natsume T, Guan JL, Mizushima N. FIP200, a ULK-interacting protein, is required for autophagosome formation in mammalian cells. J Cell Biol 2008; 181:497-510.
55. Huang WP, Klionsky DJ. Autophagy in yeast: a review of the molecular machinery. Cell Struct Funct 2002; 27:409-20.
56. Khalfan WA, Klionsky DJ. Molecular machinery required for autophagy and the cytoplasm to vacuole targeting (Cvt) pathway in *S. cerevisiae*. Curr Opin Cell Biol 2002; 14:468-75.
57. Wang CW, Klionsky DJ. The molecular mechanism of autophagy. Mol Med 2003; 9:65-76.
58. Farre JC, Vidal J, Subramani S. A cytoplasm to vacuole targeting pathway in *P. pastoris*. Autophagy 2007; 3:230-4.
59. Kabeya Y, Noda NN, Fujioka Y, Suzuki K, Inagaki F, Ohsumi Y. Characterization of the Atg17-Atg29-Atg31 complex specifically required for starvation-induced autophagy in *Saccharomyces cerevisiae*. Biochem Biophys Res Commun 2009; 389:612-5.
60. Kawamata T, Kamada Y, Suzuki K, Kuboshima N, Akimatsu H, Ota S, et al. Characterization of a novel autophagy-specific gene, *ATG29*. Biochem Biophys Res Commun 2005; 338:1884-9.
61. Hosokawa N, Sasaki T, Iemura S, Natsume T, Hara T, Mizushima N. Atg101, a novel mammalian autophagy protein interacting with Atg13. Autophagy 2009; 5:973-9.
62. Backer JM. The regulation and function of Class III PI3Ks: novel roles for Vps34. Biochem J 2008; 410:1-17.
63. Krick R, Tolstrup J, Appelles A, Henke S, Thumm M. The relevance of the phosphatidylinositolphosphat-binding motif FRRGT of Atg18 and Atg21 for the Cvt pathway and autophagy. FEBS Lett 2006; 580:4632-8.
64. Obara K, Sekito T, Niimi K, Ohsumi Y. The Atg18-Atg2 complex is recruited to autophagic membranes via phosphatidylinositol 3-phosphate and exerts an essential function. J Biol Chem 2008; 283:23972-80.
65. Nair U, Cao Y, Xie Z, Klionsky DJ. The roles of the lipid-binding motifs of Atg18 and Atg21 in the cytoplasm to vacuole targeting pathway and autophagy. J Biol Chem 2010; 285:11476-88.
66. Herman PK, Stack JH, DeModena JA, Emr SD. A novel protein kinase homolog essential for protein sorting to the yeast lysosome-like vacuole. Cell 1991; 64:425-37.
67. Stack JH, Herman PK, Schu PV, Emr SD. A membrane-associated complex containing the Vps15 protein kinase and the Vps34 PI 3-kinase is essential for protein sorting to the yeast lysosome-like vacuole. EMBO J 1993; 12:2195-204.
68. Stack JH, Emr SD. Vps34p required for yeast vacuolar protein sorting is a multiple specificity kinase that exhibits both protein kinase and phosphatidylinositol-specific PI 3-kinase activities. J Biol Chem 1994; 269:31552-62.
69. Cao Y, Klionsky DJ. Physiological functions of Atg6/Beclin 1: a unique autophagy-related protein. Cell Res 2007; 17:839-49.
70. Aita VM, Liang XH, Murty VV, Pincus DL, Yu W, Cayanis E, Kalachikov S, Gilliam TC, Levine B. Cloning and genomic organization of *beclin 1*, a candidate tumor suppressor gene on chromosome 17q21. Genomics 1999; 59:59-65.
71. Miracco C, Cosci E, Oliveri G, Luzi P, Pacenti L, Monciatti I, Mannucci S, et al. Protein and mRNA expression of autophagy gene Beclin 1 in human brain tumours. Int J Oncol 2007; 30:429-36.
72. Sun Q, Fan W, Chen K, Ding X, Chen S, Zhong Q. Identification of Barkor as a mammalian autophagy-specific factor for Beclin 1 and class III phosphatidylinositol 3-kinase. Proc Natl Acad Sci USA 2008; 105:19211-6.
73. Itakura E, Kishi C, Inoue K, Mizushima N. Beclin 1 forms two distinct phosphatidylinositol 3-kinase complexes with mammalian Atg14 and UVRAG. Mol Biol Cell 2008; 19:5360-72.
74. Itakura E, Mizushima N. Atg14 and UVRAG: mutually exclusive subunits of mammalian Beclin 1-PI3K complexes. Autophagy 2009; 5:534-6.
75. Fimia GM, Stoykova A, Romagnoli A, Giunta L, Di Bartolomeo S, Nardacci R, et al. Ambra1 regulates autophagy and development of the nervous system. Nature 2007; 447:1121-5.
76. Takahashi Y, Coppola D, Matsushita N, Cualing HD, Sun M, Sato Y, et al. Bif-1 interacts with Beclin 1 through UVRAG and regulates autophagy and tumorigenesis. Nature Cell Biol 2007; 9:1142-51.
77. Takahashi Y, Meyerkord CL, Wang HG. Bif-1/Endophilin B1: a candidate for crescent driving force in autophagy. Cell Death Differ 2009; 16:947-55.
78. Takahashi Y, Meyerkord CL, Wang HG. BARgaining membranes for autophagosome formation: Regulation of autophagy and tumorigenesis by Bif-1/Endophilin B1. Autophagy 2008; 4:121-4.
79. Geng J, Klionsky DJ. The Atg8 and Atg12 ubiquitin-like conjugation systems in macroautophagy. EMBO Rep 2008; 9:859-64.
80. Tanida I, Mizushima N, Kiyooka M, Ohsumi M, Ueno T, Ohsumi Y, Kominami E. Apg7p/Cvt2p: A novel protein-activating enzyme essential for autophagy. Mol Biol Cell 1999; 10:1367-79.
81. Shintani T, Mizushima N, Ogawa Y, Matsuura A, Noda T, Ohsumi Y. Apg10p, a novel protein-conjugating enzyme essential for autophagy in yeast. EMBO J 1999; 18:5234-41.
82. Mizushima N, Sugita H, Yoshimori T, Ohsumi Y. A new protein conjugation system in human. The counterpart of the yeast Apg12p conjugation system essential for autophagy. J Biol Chem 1998; 273:33889-92.
83. Mizushima N, Noda T, Ohsumi Y. Apg16p is required for the function of the Apg12p-Apg5p conjugate in the yeast autophagy pathway. EMBO J 1999; 18:3888-96.
84. Kirisako T, Baba M, Ishihara N, Miyazawa K, Ohsumi M, Yoshimori T, et al. Formation process of autophagosome is traced with Apg8/Aut7p in yeast. J Cell Biol 1999; 147:435-46.
85. Ichimura Y, Kirisako T, Takao T, Satomi Y, Shimonishi Y, Ishihara N, et al. A ubiquitin-like system mediates protein lipidation. Nature 2000; 408:488-92.
86. Stege JT, Laub MT, Loomis WF. Tip genes act in parallel pathways of early Dictyostelium development. Dev Genet 1999; 25:64-77.
87. Otto GP, Wu MY, Kazgan N, Anderson OR, Kessin RH. Macroautophagy is required for multicellular development of the social amoeba *Dictyostelium discoideum*. J Biol Chem 2003; 278:17636-45.
88. Webber JL, Young ARJ, Tooze SA. Atg9 trafficking in mammalian cells. Autophagy 2007; 3:54-6.
89. Mari M, Reggiori F. Atg9 trafficking in the yeast *Saccharomyces cerevisiae*. Autophagy 2007; 3:145-8.
90. He C, Klionsky DJ. Atg9 trafficking in autophagy-related pathways. Autophagy 2007; 3:271-4.
91. Reggiori F, Shintani T, Nair U, Klionsky DJ. Atg9 cycles between mitochondria and the pre-autophagosomal structure in yeasts. Autophagy 2005; 1:101-9.
92. Tung SM, Unal C, Ley A, Pena C, Tunggal B, Noegel AA, et al. Loss of Dictyostelium ATG9 results in a pleiotropic phenotype affecting growth, development, phagocytosis and clearance and replication of *Legionella pneumophila*. Cell Microbiol 2010; 12:765-80.
93. Calvo-Garrido J, Carilla-Latorre S, Lazaro-Dieguez F, Egea G, Escalante R. Vacuole membrane protein 1 is an endoplasmic reticulum protein required for organelle biogenesis, protein secretion and development. Mol Biol Cell 2008; 19:3442-53.
94. Vaccaro MI, Ropolo A, Grasso D, Iovanna JL. A novel mammalian trans-membrane protein reveals an alternative initiation pathway for autophagy. Autophagy 2008; 4:388-90.
95. Ropolo A, Grasso D, Pardo R, Sacchetti ML, Archange C, Lo Re A, et al. The pancreatitis-induced vacuole membrane protein 1 triggers autophagy in mammalian cells. J Biol Chem 2007; 282:37124-33.
96. Simonsen A, Stenmark H. Self-eating from an ER-associated cup. J Cell Biol 2008; 182:621-2.
97. Axe EL, Walker SA, Manifava M, Chandra P, Roderick HL, Habermann A, et al. Autophagosome formation from membrane compartments enriched in phosphatidylinositol 3-phosphate and dynamically connected to the endoplasmic reticulum. J Cell Biol 2008; 182:685-701.
98. Calvo-Garrido J, Carilla-Latorre S, Escalante R. Vacuole membrane protein 1, autophagy and much more. Autophagy 2008; 4:835-7.
99. Calvo-Garrido J, Escalante R. Autophagy dysfunction and ubiquitin-positive protein aggregates in Dictyostelium cells lacking Vmp1. Autophagy 2010; 6:100-9.
100. de Chastellier C, Ryter A. Changes of the cell surface and of the digestive apparatus of *Dictyostelium discoïdeum* during the starvation period triggering aggregation. J Cell Biol 1977; 75:218-36.
101. Neuhaus EM, Almers W, Soldati T. Morphology and dynamics of the endocytic pathway in *Dictyostelium discoideum*. Mol Biol Cell 2002; 13:1390-407.
102. Klionsky DJ, Abeliovich H, Agostinis P, Agrawal DK, Aliev G, Askew DS, et al. Guidelines for the use and interpretation of assays for monitoring autophagy in higher eukaryotes. Autophagy 2008; 4:151-75.
103. Mizushima N, Yoshimori T, Levine B. Methods in mammalian autophagy research. Cell 2010; 140:313-26.
104. Kuma A, Matsui M, Mizushima N. LC3, an autophagosome marker, can be incorporated into protein aggregates independent of autophagy: caution in the interpretation of LC3 localization. Autophagy 2007; 3:323-8.
105. Pankiv S, Clausen TH, Lamark T, Brech A, Bruun JA, Outzen H, et al. p62/SQSTM1 binds directly to Atg8/LC3 to facilitate degradation of ubiquitinated protein aggregates by autophagy. J Biol Chem 2007; 282:24131-45.
106. Kaniuk NA, Kiraly M, Bates H, Vranic M, Volchuk A, Brumell JH. Ubiquitinated-protein aggregates form in pancreatic β-cells during diabetes-induced oxidative stress and are regulated by autophagy. Diabetes 2007; 56:930-9.
107. Szeto J, Kaniuk NA, Canadien V, Nisman R, Mizushima N, Yoshimori T, et al. ALIS are stress-induced protein storage compartments for substrates of the proteasome and autophagy. Autophagy 2006; 2:189-99.
108. Mizushima N, Yamamoto A, Hatano M, Kobayashi Y, Kabeya Y, Suzuki K, et al. Dissection of autophagosome formation using Apg5-deficient mouse embryonic stem cells. J Cell Biol 2001; 152:657-68.

109. Mizushima N, Kuma A, Kobayashi Y, Yamamoto A, Matsubae M, Takao T, et al. Mouse Apg16L, a novel WD-repeat protein, targets to the autophagic isolation membrane with the Apg12-Apg5 conjugate. J Cell Sci 2003; 116:1679-88.
110. Proikas-Cezanne T, Pfisterer SG. Assessing mammalian autophagy by WIPI-1/Atg18 puncta formation. Methods Enzymol 2009; 452:247-60.
111. Bjørkøy G, Lamark T, Pankiv S, Øvervatn A, Brech A, Johansen T. Monitoring autophagic degradation of p62/SQSTM1. Methods Enzymol 2009; 452:181-97.
112. Mizushima N, Yoshimori T. How to interpret LC3 immunoblotting. Autophagy 2007; 3:542-5.
113. Rubinsztein DC. The roles of intracellular protein-degradation pathways in neurodegeneration. Nature 2006; 443:780-6.
114. Hara T, Nakamura K, Matsui M, Yamamoto A, Nakahara Y, Suzuki-Migishima R, et al. Suppression of basal autophagy in neural cells causes neurodegenerative disease in mice. Nature 2006; 441:885-9.
115. Schmauch C, Claussner S, Zoltzer H, Maniak M. Targeting the actin-binding protein VASP to late endosomes induces the formation of giant actin aggregates. Eur J Cell Biol 2009; 88:385-96.
116. Maselli AG, Davis R, Furukawa R, Fechheimer M. Formation of Hirano bodies in Dictyostelium and mammalian cells induced by expression of a modified form of an actin-crosslinking protein. J Cell Sci 2002; 115:1939-49.
117. Kim DH, Davis RC, Furukawa R, Fechheimer M. Autophagy contributes to degradation of Hirano bodies. Autophagy 2009; 5:44-51.
118. Bursch W. The autophagosomal-lysosomal compartment in programmed cell death. Cell Death Differ 2001; 8:569-81.
119. Yu SW, Baek SH, Brennan RT, Bradley CJ, Park SK, Lee YS, et al. Autophagic death of adult hippocampal neural stem cells following insulin withdrawal. Stem Cells 2008; 26:2602-10.
120. Yu L, Wan F, Dutta S, Welsh S, Liu Z, Freundt E, et al. Autophagic programmed cell death by selective catalase degradation. Proc Natl Acad Sci USA 2006; 103:4952-7.
121. Yu L, Alva A, Su H, Dutt P, Freundt E, Welsh S, et al. Regulation of an *ATG7-beclin 1* program of autophagic cell death by caspase-8. Science 2004; 304:1500-2.
122. Shimizu S, Kanaseki T, Mizushima N, Mizuta T, Arakawa-Kobayashi S, Thompson CB, Tsujimoto Y. Role of Bcl-2 family proteins in a non-apoptotic programmed cell death dependent on autophagy genes. Nat Cell Biol 2004; 6:1221-8.
123. Puyal J, Vaslin A, Mottier V, Clarke PG. Postischemic treatment of neonatal cerebral ischemia should target autophagy. Ann Neurol 2009; 66:378-89.
124. Pattingre S, Tassa A, Qu X, Garuti R, Liang XH, Mizushima N, et al. Bcl-2 antiapoptotic proteins inhibit Beclin 1-dependent autophagy. Cell 2005; 122:927-39.
125. Koike M, Shibata M, Tadakoshi M, Gotoh K, Komatsu M, Waguri S, et al. Inhibition of autophagy prevents hippocampal pyramidal neuron death after hypoxic-ischemic injury. Am J Pathol 2008; 172:454-69.
126. Hoyer-Hansen M, Bastholm L, Mathiasen IS, Elling F, Jaattela M. Vitamin D analog EB1089 triggers dramatic lysosomal changes and Beclin 1-mediated autophagic cell death. Cell Death Differ 2005; 12:1297-309.
127. Hofius D, Schultz-Larsen T, Joensen J, Tsitsigiannis DI, Petersen NH, Mattsson O, et al. Autophagic components contribute to hypersensitive cell death in Arabidopsis. Cell 2009; 137:773-83.
128. Espert L, Denizot M, Grimaldi M, Robert-Hebmann V, Gay B, Varbanov M, et al. Autophagy is involved in T cell death after binding of HIV-1 envelope proteins to CXCR4. J Clin Invest 2006; 116:2161-72.
129. Denton D, Shravage B, Simin R, Mills K, Berry DL, Baehrecke EH, Kumar S. Autophagy, not apoptosis, is essential for midgut cell death in Drosophila. Curr Biol 2009; 19:1741-6.
130. Chen Y, McMillan-Ward E, Kong J, Israels SJ, Gibson SB. Oxidative stress induces autophagic cell death independent of apoptosis in transformed and cancer cells. Cell Death Differ 2008; 15:171-82.
131. Berry DL, Baehrecke EH. Autophagy functions in programmed cell death. Autophagy 2008; 4:359-60.
132. Baek SH, Kim EK, Goudreau JL, Lookingland KJ, Kim SW, Yu SW. Insulin withdrawal-induced cell death in adult hippocampal neural stem cells as a model of autophagic cell death. Autophagy 2009; 5:277-9.
133. Whittingham WF, Raper KB. Non-viability of stalk cells in Dictyostelium. Proc Natl Acad Sci USA 1960; 46:642-9.
134. Levraud JP, Adam M, Luciani MF, de Chastellier C, Blanton RL, Golstein P. Dictyostelium cell death: early emergence and demise of highly polarized paddle cells. J Cell Biol 2003; 160:1105-14.
135. Kay RR. Cell differentiation in monolayers and the investigation of slime mold morphogens. Meth Cell Biol 1987; 28:433-48.
136. Cornillon S, Foa C, Davoust J, Buonavista N, Gross JD, Golstein P. Programmed cell death in Dictyostelium. J Cell Sci 1994; 107:2691-704.
137. Roisin-Bouffay C, Luciani MF, Klein G, Levraud JP, Adam M, Golstein P. Developmental cell death in Dictyostelium does not require paracaspase. J Biol Chem 2004; 279:11489-94.
138. Lam D, Levraud JP, Luciani MF, Golstein P. Autophagic or necrotic cell death in the absence of caspase and bcl-2 family members. Biochem Biophys Res Commun 2007; 363:536-41.
139. Yamada Y, Okamoto K. Cell-type-specific responsiveness to cAMP in cell differentiation of *Dictyostelium discoideum*. Dev Biol 1992; 149:235-7.
140. Berks M, Kay RR. Cyclic AMP is an inhibitor of stalk cell differentiation in *Dictyostelium discoideum*. Dev Biol 1988; 126:108-14.
141. Tresse E, Kosta A, Giusti C, Luciani MF, Golstein P. A UDP-glucose derivative is required for vacuolar autophagic cell death. Autophagy 2008; 4:680-91.
142. de Chastellier C, Ryter A. Changes on the cell surface and of the digestive apparatus of *Dictyostelium discoideum* during the starvation period triggering aggregation. J Cell Biol 1977; 75:218-36.
143. Kosta A, Luciani MF, Geerts WJ, Golstein P. Marked mitochondrial alterations upon starvation without cell death, caspases or Bcl-2 family members. Biochim Biophys Acta 2008; 1783:2013-9.
144. Town CD, Gross JD, Kay RR. Cell differentiation without morphogenesis in *Dictyostelium discoïdeum*. Nature 1976; 262:717-9.
145. Town C, Stanford E. An oligosaccharide-containing factor that induces cell differentiation in *Dictyostelium discoideum*. Proc Natl Acad Sci USA 1979; 76:308-12.
146. Sobolewski A, Neave N, Weeks G. The induction of stalk cell differentiation in submerged monolayers of *Dictyostelium discoideum*. Characterization of the temporal sequence for the molecular requirement. Differentiation 1983; 25:93-100.
147. Morris HR, Taylor GW, Masento MS, Jermyn KA, Kay RR. Chemical structure of the morphogen differentiation inducing factor from *Dictyostelium discoideum*. Nature 1987; 328:811-4.
148. Lam D, Kosta A, Luciani MF, Golstein P. The IP3 receptor is required to signal autophagic cell death. Mol Biol Cell 2008; 19:691-700.
149. Giusti C, Luciani MF, Ravens S, Gillet A, Golstein P. Autophagic cell death in Dictyostelium requires the receptor histidine kinase DhkM. Mol Biol Cell 2010; 21:1825-35.
150. Kosta A, Roisin-Bouffay C, Luciani MF, Otto GP, Kessin RH, Golstein P. Autophagy gene disruption reveals a non-vacuolar cell death pathway in Dictyostelium. J Biol Chem 2004; 279:48404-9.
151. Giusti C, Luciani MF, Klein G, Aubry L, Tresse E, Kosta A, Golstein P. Necrotic cell death: From reversible mitochondrial uncoupling to irreversible lysosomal permeabilization. Exp Cell Res 2009; 315:26-38.
152. Laporte C, Kosta A, Klein G, Aubry L, Lam D, Tresse E, et al. A necrotic cell death model in a protist. Cell Death Differ 2007; 14:266-74.
153. Luciani MF, Kubohara Y, Kikuchi H, Oshima Y, Golstein P. Autophagic or necrotic cell death triggered by distinct motifs of the differentiation factor DIF-1. Cell Death Differ 2009; 16:564-70.
154. Turcotte S, Chan DA, Sutphin PD, Hay MP, Denny WA, Giaccia AJ. A molecule targeting VHL-deficient renal cell carcinoma that induces autophagy. Cancer Cell 2008; 14:90-102.
155. Mogensen TH. Pathogen recognition and inflammatory signaling in innate immune defenses. Clin Microbiol Rev 2009; 22:240-73.
156. Cavalier-Smith T. Predation and eukaryote cell origins: a coevolutionary perspective. Int J Biochem Cell Biol 2009; 41:307-22.
157. Virgin HW, Levine B. Autophagy genes in immunity. Nat Immunol 2009; 10:461-70.
158. Dieckmann R, Gopaldass N, Escalera C, Soldati T. Monitoring time-dependent maturation changes in purified phagosomes from *Dictyostelium discoideum*. Methods Mol Biol 2008; 445:327-37.
159. Arsham AM, Neufeld TP. Thinking globally and acting locally with TOR. Curr Opin Cell Biol 2006; 18:589-97.
160. Irazoqui JE, Urbach JM, Ausubel FM. Evolution of host innate defence: insights from *Caenorhabditis elegans* and primitive invertebrates. Nat Rev Immunol 2010; 10:47-58.
161. Casadevall A, Pirofski LA. Accidental virulence, cryptic pathogenesis, martians, lost hosts and the pathogenicity of environmental microbes. Eukaryot Cell 2007; 6:2169-74.
162. Lamoth F, Greub G. Amoebal pathogens as emerging causal agents of pneumonia. FEMS Microbiol Rev 2009; In press.
163. Campoy E, Colombo MI. Autophagy in intracellular bacterial infection. Biochim Biophys Acta 2009; 1793:1465-77.
164. Dorer MS, Isberg RR. Non-vertebrate hosts in the analysis of host-pathogen interactions. Microbes Infec 2006; 8:1637-46.
165. Benabentos R, Hirose S, Sucgang R, Curk T, Katoh M, Ostrowski EA, et al. Polymorphic members of the lag gene family mediate kin discrimination in Dictyostelium. Curr Biol 2009; 19:567-72.
166. Chen G, Zhuchenko O, Kuspa A. Immune-like phagocyte activity in the social amoeba. Science 2007; 317:678-81.
167. Benghezal M, Fauvarque MO, Tournebize R, Froquet R, Marchetti A, Bergeret E, et al. Specific host genes required for the killing of Klebsiella bacteria by phagocytes. Cell Microbiol 2006; 8:139-48.
168. Sillo A, Bloomfield G, Balest A, Balbo A, Pergolizzi B, Peracino B, et al. Genome-wide transcriptional changes induced by phagocytosis or growth on bacteria in Dictyostelium. BMC Genom 2008; 9:291.
169. Li Z, Dugan AS, Bloomfield G, Skelton J, Ivens A, Losick V, Isberg RR. The amoebal MAP kinase response to *Legionella pneumophila* is regulated by DupA. Cell Host Microbe 2009; 6:253-67.
170. Carilla-Latorre S, Calvo-Garrido J, Bloomfield G, Skelton J, Kay RR, Ivens A, et al. Dictyostelium transcriptional responses to *Pseudomonas aeruginosa*: common and specific effects from PAO1 and PA14 strains. BMC Microbiol 2008; 8:109.
171. Pukatzki S, Ma AT, Sturtevant D, Krastins B, Sarracino D, Nelson WC, et al. Identification of a conserved bacterial protein secretion system in *Vibrio cholerae* using the Dictyostelium host model system. Proc Natl Acad Sci USA 2006; 103:1528-33.
172. Farbrother P, Wagner C, Na J, Tunggal B, Morio T, Urushihara H, et al. Dictyostelium transcriptional host cell response upon infection with Legionella. Cell Microbiol 2006; 8:438-56.

173. Cosson P, Zulianello L, Join-Lambert O, Faurisson F, Gebbie L, Benghezal M, et al. *Pseudomonas aeruginosa* virulence analyzed in a *Dictyostelium discoideum* host system. J Bact 2002; 184:3027-33.
174. Solomon JM, Isberg RR. Growth of *Legionella pneumophila* in *Dictyostelium discoideum*: a novel system for genetic analysis of host-pathogen interactions. Trends Microbiol 2000; 8:478-80.
175. Clarke M. Recent insights into host-pathogen interactions from Dictyostelium. Cell Microbiol 2010; 12:283-91.
176. Skriwan C, Fajardo M, Hagele S, Horn M, Wagner M, et al. Various bacterial pathogens and symbionts infect the amoeba *Dictyostelium discoideum*. Int J Med Microbiol 2002; 291:615-24.
177. Jia K, Thomas C, Akbar M, Sun Q, Adams-Huet B, Gilpin C, Levine B. Autophagy genes protect against *Salmonella typhimurium* infection and mediate insulin signaling-regulated pathogen resistance. Proc Natl Acad Sci USA 2009; 106:14564-9.
178. Russell DG. Who puts the tubercle in tuberculosis? Nat Rev 2007; 5:39-47.
179. van der Wel N, Hava D, Houben D, Fluitsma D, van Zon M, et al. *M. tuberculosis* and *M. leprae* translocate from the phagolysosome to the cytosol in myeloid cells. Cell 2007; 129:1287-98.
180. Hagedorn M, Soldati T. Flotillin and RacH modulate the intracellular immunity of Dictyostelium to *Mycobacterium marinum* infection. Cell Microbiol 2007; 9:2716-33.
181. Gutierrez MG, Master SS, Singh SB, Taylor GA, Colombo MI, Deretic V. Autophagy is a defense mechanism inhibiting BCG and *Mycobacterium tuberculosis* survival in infected macrophages. Cell 2004; 119:753-66.
182. Collins CA, De Maziere A, van Dijk S, Carlsson F, Klumperman J, Brown EJ. Atg5-independent sequestration of ubiquitinated mycobacteria. PLoS Path 2009; 5:1000430.
183. Dupont N, Lacas-Gervais S, Bertout J, Paz I, Freche B, Van Nhieu GT, et al. Shigella phagocytic vacuolar membrane remnants participate in the cellular response to pathogen invasion and are regulated by autophagy. Cell Host Microbe 2009; 6:137-49.
184. Ponpuak M, Davis AS, Roberts EA, Delgado MA, Dinkins C, Zhao Z, et al. Delivery of cytosolic components by autophagic adaptor protein p62 endows autophagosomes with unique antimicrobial properties. Immunity 2010; 32:329-41.
185. Dorn BR, Dunn WA Jr, Progulske-Fox A. Bacterial interactions with the autophagic pathway. Cell Microbiol 2002; 4:1-10.
186. Swanson MS, Isberg RR. Association of *Legionella pneumophila* with the macrophage endoplasmic reticulum. Infect Immun 1995; 63:3609-20.
187. Otto GP, Wu MY, Clarke M, Lu H, Anderson OR, Hilbi H, et al. Macroautophagy is dispensable for intracellular replication of *Legionella pneumophila* in *Dictyostelium discoideum*. Mol Microbiol 2004; 51:63-72.
188. Price CT, Al-Khodor S, Al-Quadan T, Santic M, Habyarimana F, Kalia A, Kwaik YA. Molecular mimicry by an F-box effector of *Legionella pneumophila* hijacks a conserved polyubiquitination machinery within macrophages and protozoa. PLoS Path 2009; 5:1000704.
189. Francione L, Smith PK, Accari SL, Taylor PE, Bokko PB, Bozzaro S, et al. *Legionella pneumophila* multiplication is enhanced by chronic AMPK signalling in mitochondrially diseased Dictyostelium cells. Dis Model Mech 2009; 2:479-89.