Loss of Dictyostelium ATG9 results in a pleiotropic phenotype affecting growth, development, phagocytosis and clearance and replication of Legionella pneumophila

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Summary

Infection of Dictyostelium discoideum with Legionella pneumophila resulted in a large number of differentially regulated genes among them three core autophagy genes, ATG8, ATG9 and ATG16. Macroautophagy contributes to many physiological and pathological processes and might also constitute an important mechanism in cell-autonomous immunity. For further studies we selected the highly conserved ATG9. In colocalization studies with GFP-tagged ATG9 and different organelle marker proteins we neither observed colocalization with mitochondria, the ER nor lysosomes. However, there was partial colocalization with the Golgi apparatus and many ATG9-GFP-containing vesicles localized along microtubules and accumulated around the microtubule organizing centre. ATG9-deficient cells had pleiotropic defects. In addition to growth defects they displayed severe developmental defects, consistent with the known role of autophagy in Dictyostelium development. Unexpectedly, the ATG9 mutant also had a strong phagocytosis defect that was particularly apparent when infecting the cells with L. pneumophila. However, those Legionellae that entered the host could multiply better in mutant than in wild-type cells, because of a less efficient clearance in the early and a more efficient replication in the late phase of infection. We conclude that ATG9 and hence macroautophagy has a protective role during pathogen infection.

Introduction

The social amoeba Dictyostelium discoideum is a well-established model organism for the study of basic biological processes such as signal transduction, cytokinesis, chemotaxis and cell motility as well as basic aspects of development (Noegel and Schleicher, 2000; Chisholm and Firtel, 2004; Robinson and Spudich, 2004; King and Insall, 2009). All stages of the life cycle are motile and upon starvation the amoebae enter a developmental program, in which up to 100 000 cells aggregate by chemotaxis towards cAMP. The aggregate transforms via distinct morphological states into a mature fruiting body, composed of a ball of spores supported by a thin, long stalk made of vacuolized dead cells (Eichinger, 2003). In the vegetative phase the amoebae grow as separate, independent cells and feed on bacteria and yeast. Uptake of the food occurs by phagocytosis, a complex and evolutionarily highly conserved mechanism. The ingested microorganism is trapped in a phagosome and, via the phagolysosomal pathway, is ultimately delivered to a lysosome where it is degraded by an intricate cocktail of hydrolytic enzymes (Duhon and Cardelli, 2002; Cosson and Soldati, 2008). However, despite its impressive armory to kill bacteria, Dictyostelium can be infected with a number of medically relevant pathogenic bacteria (Skiwian et al., 2002; Steinert et al., 2007).

In higher eukaryotes phagocytosis is also a means to fight invading pathogens. This task is performed by...
specialized cells such as macrophages or neutrophils that have the ability to rapidly and efficiently internalize a variety of microorganisms and particles and degrade them. These cells represent professional phagocytes that are important for innate and adaptive immunity in metazoan. In recent years it became also clear that the basic mechanisms of host pathogen interactions are conserved in lower eukaryotes (Dorer and Isberg, 2006; Bozzaro et al., 2008; Jin et al., 2009). Thus, the professional phagocyte D. discoideum has become an attractive model system for the study of the infection with human pathogens in particular the investigation of bacterial virulence traits and the analysis of the roles of host gene products in phagocytosis and killing (Steinert and Heuner, 2005; Hilbi et al., 2007; Cosson and Soldati, 2008; Isberg et al., 2009). For the study of the host side a large number of D. discoideum mutants are available from the Dictyostelium stock centre, additional genes of interest can be easily disrupted and a complete and well-curated genome sequence is on-hand (Eichinger, 2003; Eichinger et al., 2005).

In order to survive bacterial pathogens manipulate host cell processes to avoid phagolysosomal fusion and to establish a replicative niche (Isberg et al., 2009). The host on the other hand initiates elaborate defence processes of which one appears to be macroautophagy (hereafter autophagy) (Huang and Klionsky, 2007). Autophagy is an ancient cellular pathway that is conserved from yeast to man. It presumably evolved to enable cells to survive periods of starvation. More than 30 autophagy (ATG) genes have been identified, mainly in yeast, of which 18 constitute the core machinery for starvation induced autophagy. Cytosolic material is captured into double membrane-bound vesicles that mature into autophagosomes and then, after fusion with lysosomes, become autophagolysosomes. There the cargo is degraded and then recycled for further use (Nakatogawa et al., 2009). Autophagy contributes to many physiological and pathological processes, including cell differentiation and development, programmed cell death, cancer and neurodegenerative disorders (Xie and Klionsky, 2007). Previous work in Dictyostelium showed that autophagy is required for normal development. Autophagy mutants were generated in five core autophagy genes, and all mutants displayed developmental defects albeit of variable severity (Otto et al., 2003; 2004a). There is accumulating evidence that autophagy is also an important mechanism in the complex interactions that occur between host and pathogen. Interestingly, autophagy appears to be a defence mechanism that is able to degrade some pathogens while others apparently modulate and subvert it (Huang and Klionsky, 2007). In the case of Salmonella typhimurium or Mycobacterium tuberculosis cells target bacteria for degradation through autophagy. (Gutierrez et al., 2004). Shigella flexneri on the other hand escapes autophagy, while Staphylococcus aureus even utilize the autophagosome for replication (Ogawa et al., 2005; Schnaith et al., 2007). In the case of Dictyostelium it has been reported that Legionella pneumophila is able to replicate in autophagy mutants (Otto et al., 2004b). However, the authors did not examine if disruption of the autophagic machinery might be advantageous for the replication of the pathogen. Such a possibility is supported by data from macrophages where autophagy apparently constitutes a defence mechanism that L. pneumophila must modulate to establish infection (Amer and Swanson, 2005).

Here we describe the subcellular localization of Dictyostelium ATG9 and its function in growth, development and during phagocytosis and infection. ATG9 is so far the only known integral membrane protein of the core autophagic machinery and is thought to deliver membrane lipids to the site of autophagosome formation (Reggiori et al., 2005; Mari and Reggiori, 2007; Webber et al., 2007). We show in immunofluorescence experiments and live cell imaging that ATG9-containing vesicles (ACVs) randomly appeared at the cell periphery, travelled, presumably along microtubules, to the perinuclear region and accumulated around the microtubule organizing centre (MTOC). Deletion of atg9 resulted in a pleiotropic phenotype with defects in growth, development, phagocytosis, and during infection with L. pneumophila. Expression of GFP-tagged ATG9 rescued the mutant phenotype. The strong phagocytosis defect points to an unexpected link between phagocytosis and autophagy. The results of the infection experiment support a protective function of ATG9 during L. pneumophila infection.

Results

ATG9 is highly conserved and upregulated during Legionella infection

In previous work we studied the global transcriptional regulation of Dictyostelium cells upon infection with L. pneumophila. Comparative analysis of the 24 h time point post infection revealed 240 differentially regulated genes, which are thought to be involved in the pathogenic response (Farbrother et al., 2006). Bioinformatic analysis showed that three of the more than 50 human orthologues among the 240 genes, namely ATG8, ATG9 and ATG16, encoded proteins of the core autophagy machinery (Table 1). Because the role of autophagy in cell-autonomous immunity is still a matter of debate, we decided to further study this process with a particular emphasis on phagocytosis and infection. We selected ATG9 for further study because: (i) it was upregulated during infection, (ii) it is highly conserved and (iii) it is so far the only known integral membrane autophagy protein.
whose study could provide information about the unknown membrane source for the autophagosome. *Dictyostelium atg9* encodes a protein of 699 amino acids. It contains the conserved APG9 (autophagy 9) domain of approximately 300 amino acids. The program TMHMM (http://www.cbs.dtu.dk/services/TMHMM/) predicted six transmembrane domains between amino acids 75 and 500 (Fig. 1A). Pairwise alignments of the APG9 domain of *Sacharomyces cerevisiae* (yeast), *D. discoideum*, *Arabidopsis thaliana* (plant) and *Homo sapiens* (human) showed highest similarity of the *Dictyostelium* APG9 domain with the yeast orthologue and slightly lower similarities with plant and with human. However, comparison of the human APG9 domain with those of yeast, plant and *Dictyostelium* revealed highest similarity with the latter sequence (Fig. 1B). We next performed sequence alignments with orthologous proteins from animals, plants and fungi and generated an evolutionary tree, which showed that the *Dictyostelium* protein is most closely related to the fungal orthologues and that the ATG9 proteins from animals and plants cluster in two distinct groups (Fig. 1C).

**ATG9 is upregulated during development**

Autophagy is an important pathway in eukaryotes for recycling cellular material during developmental processes. We therefore investigated the developmental regulation of ATG9. Real-time polymerase chain reaction (PCR) showed an approximately fivefold upregulation at the mRNA level after 4 and 8 h, eightfold after 12 and 16 h

### Table 1. Differential regulation of *Dictyostelium* autophagy (ATG) genes 24 h post infection with *L. pneumophila.*

| **Dictyostelium** | **Human orthologue** |
|------------------|---------------------|
| **DDB ID**       | **Description**     | **Regulation Log2 ratio** | **Protein size (aa)** | **Entrez gene ID** | **Protein size (aa)** | **Identity (%)** | **Aligned with *Dictyostelium* (%)** |
| DDB0191423       | ATG9                | 0.609                       | 699                    | 79065               | 839                     | 34               | 73                      |
| DDB0191413       | ATG8                | −0.968                      | 122                    | 11337               | 117                      | 54               | 93                      |
| DDB0215341       | ATG16               | −0.704                      | 612                    | 55054               | 607                      | 30               | 93                      |

Positive values indicate upregulation, negative downregulation.

Fig. 1. The ATG9 protein is conserved from yeast to man. A. Domain structure of the 699-amino-acid protein. Blue rectangles indicate putative transmembrane domains. APG9, autophagy 9 domain. B. Sequence similarity of human, yeast (*S. cerevisiae*), plant (*A. thaliana*) and *Dictyostelium* ATG9. The percent similarity between the selected proteins was determined by successively aligning the APG9 domains of two proteins. C. Evolutionary tree of ATG9 members from diverse phyla. A CLUSTALX alignment of the APG9 domain of *Dictyostelium* ATG9 (red, D.d.) and of selected APG9 domains from other organisms was used to create a phylogenetic tree with the TreeView programme. The scale bar indicates 10% divergence. UniProt accession numbers are provided. C.e., *C. elegans*; D.m., *Drosophila melanogaster*; H.s., *H. sapiens*; M.m., *Mus musculus*; F.r., *Fugu rubripes*; D.r., *Danio rerio*; S.c., *S. cerevisiae*; N.c., *Neurospora crassa*; A.t., *A. thaliana*; O.s., *Oryza sativa*.

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and sevenfold after 20 h of Dictyostelium development (Fig. 2A). On the protein level we observed an approximately eightfold upregulation during the first 6 h of development. The slight decrease at the 8 h time point is probably due to inefficient transfer of the protein (Fig. 2B).

The need to recycle cellular components during starvation is apparently reflected in the strong upregulation of ATG9. A similar developmental regulation was observed for Dictyostelium ATG1 and ATG8 (Otto et al., 2004a).

To investigate the cellular function of ATG9 we generated two independent knockout mutants and two mutants that express ATG9 fused to GFP in the mutant background. AGA expresses GFP fused N-terminally to ATG9 while in the AAG mutant GFP is fused to the C-terminus of ATG9. Southern blot and PCR verification of the knockout mutant is shown in Fig. 3A and B. No ATG9 protein is detected in the ATG9 mutant while AGA and AAG express the GFP-ATG9 or, respectively, ATG9-GFP fusion protein of approximately 105 kDa (Fig. 3C). Both strains significantly overexpress the fusion protein in comparison with the expression of ATG9 in wild-type AX2 cells. In the case of the AAG mutant a degradation product is seen with the polyclonal ATG9-specific antibodies but not with the GFP-specific antibody (Fig. 3C and D).

ACVs localize along microtubules

The membrane source for the growing autophagosome is still unknown. A possible candidate for the unidentified membrane carrier is ATG9, the only integral membrane protein required for autophagosome formation. In yeast, ATG9 is indispensable for pre-autophagosomal structure (PAS) formation and part of its peripheral pool appears to cycle between the mitochondria and the PAS. The remaining part of the peripheral ATG9 pool resides in a hitherto unknown cellular compartment (Reggiori et al., 2005; He

Fig. 2. Expression of ATG9 during development. A. Quantification of developmental regulation by real-time PCR. B. Western blot of protein extracts from cells that were developed in Soerensen buffer using the polyclonal ATG9 antibody. The time of development in hours is shown below the blot.

Fig. 3. Generation and verification of mutant strains. A. Southern blot of AX2 and two independent ATG9 gene replacement mutants. Genomic DNA was digested with EcoRI and BglII, separated in 0.7% agarose, transferred to nitrocellulose and probed with the 3′-arm of the gene replacement construct. #1, ATG9-#1; #2, ATG9-#2. B. Reverse transcriptase PCR of AX2 and ATG9-#1. No addition of cDNA (blank) was used as a negative control and AX2 genomic DNA as a positive control. C. and D. Western blot of wild-type AX2, the ATG9-#1 replacement mutant and two strains that either express GFP fused N-terminally (AGA) or C-terminally (AAG) to ATG9 in the gene replacement mutant ATG9-#1. (C) Polyclonal anti-ATG9 antibody; (D) monoclonal anti-GFP antibody.
and Klionsky, 2007). In mammalian cells part of the peripheral pool localizes to the trans-Golgi network (TGN) and endosomes (Young et al., 2006). We made use of the generated AGA and AAG strains and performed colocalization studies using markers for different subcellular organelles that could supply membrane material for autophagosome formation. We used porin as marker for mitochondria, the lysosomal antigen to label lysosomes, protein disulfide isomerase (PDI) as marker for the ER, vata for the contractile vacuole and comitin for the Golgi apparatus. ATG9-GFP expressing cells contained a large number of dot-like structures that presumably represented small vesicles (Fig. 4, left panel). We did not detect ATG9-GFP at mitochondria, lysosomes, the ER and at the contractile vacuole (Fig. 4A–C, middle and right panels; vata staining not shown). However, for some cells we observed partial colocalization with the Golgi marker comitin (Fig. 4D, arrows), reminiscent of the TGN localization of ATG9 in mammalian cells (Young et al., 2006). Another interesting observation was that ACVs were strongly enriched in the perinuclear region. We therefore performed colocalization studies with the centrosomal marker protein CP250 and microtubules (Fig. 4E). Interestingly, ACVs were enriched around the MTOC, in addition, we frequently observed them in inter-phase cells in close proximity to microtubules, suggesting a possible role for microtubules in their transport (Fig. 4E, inset). Transport along microtubules could also explain the accumulation around the MTOC. This enrichment was lost in dividing cells, and ACVs were evenly distributed throughout the cytoplasm (Fig. 4F, arrow). Immunofluorescence studies with cells that were starved for 3 h showed similar colocalization and subcellular distribution of ACVs (data not shown). We also studied the dynamics of ACVs by life cell imaging. In a number of instances we saw random appearance of ACVs at the cell periphery. The newly generated ACVs usually remained briefly at the ‘birth site’ and then travelled swiftly in direction of the MTOC, suggesting transport along microtubules (Movie S1).

The ATG9- mutant displays severe developmental defects

It has already been shown that autophagy is required for a number of developmental processes (Otto et al., 2003; 2004a). We therefore investigated the development of AX2, the ATG9- mutant and the AGA and AAG mutants by plating cells on phosphate agar. Development was severely impaired in the ATG9- knockout mutant, and we observed four main developmental defects (Movie S2). (i) ATG9- aggregates were considerably larger than wild-type AX2 aggregates, and the mutant displayed a multi-tip phenotype. While AX2 aggregates produce only one tip each, mutant aggregates produced generally three tips per aggregate (Fig. 5A). (ii) Slug formation in the mutant was very efficient. Moreover, they were usually thinner and much longer than AX2 slugs (Fig. 5B). (iii) In addition the ATG9- slugs frequently broke into several pieces (Fig. 5B, orange arrows). Furthermore, some slugs migrated normally in the case of the mutant (Fig. 5B, white and yellow arrows); others, however, did not migrate and only elongated (Fig. 5B, green arrow, back of the slug, and blue arrow, front of the slug). This observation prompted us to investigate the phototactic ability of the ATG9- mutant. Strikingly, in contrast to AX2 the mutant slugs generally did not move at all into the direction of the light source making it impossible to measure a phototactic index (Fig. S1). Occasionally there appeared to be a very small shift of some slugs in the inoculation spot into direction of the light source, indicating that phototactic ability might still be intact while slug migration is defective (Fig. S1). (iv) Finally, only a minority of the mutant slugs proceeded through terminal differentiation and produced fruiting bodies. The few fruiting bodies that were produced in the ATG9- mutant were misshapen. They possessed a thickened stalk, were much smaller than wild-type fruiting bodies and generated only a tiny spore head. Re-expression of ATG9 fused to GFP in the AGA or AAG strains rescued the developmental phenotype (Fig. 5C). These results clearly indicate that autophagy is required at several steps during Dictyostelium development. Disturbance of the process leads to severe impairments in aggregation, the tipped aggregate stage, the slug stage and fruiting body generation.

Growth of the ATG9- mutant is impaired in shaking culture and on bacteria

We were then curious to find out if autophagy is also important during vegetative growth. First we checked growth of the ATG9- mutant and the AGA and AAG strains in comparison with wild-type AX2 cells in shaking culture. Log phase cells from a shaken axenic culture were adjusted to a density of 2 × 10^4 cells ml^-1, and the growth followed over 120 h. We observed a strong growth defect in the case of the ATG9- mutant. After 3 day growth at 21°C with shaking at 160 r.p.m. the cell titer of the mutant was about sixfold lower than that of AX2 wild-type cells. The growth defect was rescued through expression of ATG9 fused to GFP in the AGA or AAG strains and the cell titer increased only moderately (Fig. 6A). Final cell densities of AX2 wild-type and ATG9- cells were comparable (data not shown). We calculated the generation time in the logarithmic growth phase from days 1 to 3 and found an approximately 45% longer generation time (14.5 vs. ~10 h) of the ATG9- cells as compared with AX2.
**Fig. 4.** Subcellular localization of ATG9-GFP-labelled vesicles (ACVs). The AAG mutant expressing ATG9-GFP is shown. Cells were fixed with cold methanol and stained with antibodies against protein markers of different subcellular compartments.

A. Mitochondrial marker porin.
B. Lysosomal antigen.
C. ER marker PDI.
D. Golgi marker comitin, arrows indicate partial colocalization with ACVs.
E. Microtubule antibody YL1/2 (red) and centrosomal marker CP250 (white), inset shows localization of ACVs along microtubules.
F. Same as (E), arrow indicates dividing cell.

Left panel: DAPI staining and ATG9-GFP; middle panel: staining of subcellular organelles with different antibodies; right panel: merged images. Scale bars are 5 μm and 0.25 μm in inset.
and the AGA and AAG strains. The slower growth points either to a pinocytosis defect or to a defect in the intracellular utilization of nutrients in the case of the ATG9 mutant.

We then compared the growth of wild-type AX2 cells, the ATG9 mutant, the AGA and AAG strain on Klebsiella aerogenes and Escherichia coli B/2 bacteria. We quantified growth by measuring the plaque size of the different strains on both bacteria over several days. For both bacteria we found that the ATG9 mutant formed considerably smaller plaques in comparison with the other strains. The growth defect was not as dramatic as in shaking culture, and plaque size in case of the ATG9 mutant was reduced on average by about 30%. Again, expression of ATG9 fused to GFP in the mutant background (AGA and AAG strains) rescued this defect (Fig. 6B). The reduced plaque size of the ATG9 mutant could either be due to a decreased phagocytosis rate or to problems in intracellular digestion of consumed bacteria or to both. Figure 6C depicts examples of single plaques and of fruiting bodies of the four strains grown on Klebsiella. In addition to the smaller plaque size in case of the ATG9 mutant it is...
Fig. 6. Growth on K. aerogenes and in shaking culture.
A. Growth of AX2, ATG9\(^{-}\), AGA and AAG cells in shaking culture. Mean values and standard deviations of 12 growth curves from three independent experiments are shown. Please note the logarithmic scale of the y-axis.
B. Quantitative analysis of growth on a K. aerogenes lawn over 7 days. Mean values and standard deviations of 30 plaques for each strain are shown.
C. Growth on a lawn of K. aerogenes. The top row depicts single plaques after 6 days of growth, the middle row representative aerae with fruiting bodies (the insets show single spore heads) and the bottom row side views of representative fruiting bodies after 6 (AX2, AGA and AAG) and 9 days of growth on K. aerogenes (ATG9\(^{-}\)). The scale bars on the left side of the top and middle rows are valid for each series of images. Scale bar sizes: 2 mm top, middle, 0.25 mm and 0.05 mm for the inset. Scale bars for bottom row are 0.05 mm. Please note the different scale for the ATG9\(^{-}\) mutant.

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evident that only a very small fraction of the mutant cells in the aggregates were able to further develop and to produce fruiting bodies while most cells apparently did not proceed with development (Fig. 6C, top and middle rows). The few fruiting bodies that were generated were very tiny in comparison with AX2 wild-type and the rescued AAG and AGA strains (Fig. 6C, bottom row). In comparison with development on phosphate agar fruiting body generation was even more impaired upon growth on a lawn of Klebsiella. We also found that the viability of the mutant spores was strongly reduced (data not shown).

The ATG9- mutant displays a decreased phagocytosis rate

The decrease in plaque size on bacterial lawns in case of the ATG9- mutant indicated a phagocytosis defect. We therefore performed a number of phagocytosis assays. First we analysed the uptake of pHrodo™ E. coli by fluorescence measurements. The fluorescence of pHrodo™ E. coli strongly increases as the pH of its surrounding becomes more acidic. This is the case when phagosomes fuse with lysosomes, therefore phagolysosomal fusion of ingested particles can be followed. Using this tool we observed an approximately twofold lower fluorescence in the ATG9- mutant as compared with AX2 wild-type and the AGA and AAG strains (Fig. 7A). Next we analysed the uptake of fluorescent yeast cells. AX2 wild-type cells showed a strong increase in fluorescence after a lag phase of about 15 min. In the case of the ATG9- cells the increase in fluorescence was only moderate and final fluorescence was approximately threefold lower than for AX2 (Fig. 7B).

Both results indicate a phagocytosis defect in the ATG9- mutant for microorganisms that are considered as ‘food’ by wild-type cells. Next we wanted to know whether there would also be a defect in the uptake of pathogenic bacteria. To analyse this we used S. aureus, a representative of the Gram-positive bacteria, and L. pneumophila, a Gram-negative bacterium. For both pathogens we found a reduction in uptake in case of the ATG9- mutant. Fluorescent activated cell sorting (FACS) analysis of Dictyostelium cells that had phagocytosed green fluorescent S. aureus showed an approximately twofold decrease for mutant cells (Fig. 7C). The uptake of L. pneumophila was measured by determining the colony-forming units (cfu) 3 h post infection. At this stage intracellular bacteria have not yet started to replicate. For the ATG9- mutant we observed a dramatic decrease in uptake rate that was approximately eightfold lower than for AX2 wild-type cells (Fig. 7D).

Clearance of L. pneumophila was less efficient and replication was better in ATG9- cells

Recently it was reported that autophagy was dispensable for intracellular L. pneumophila replication (Otto et al., 2004b). However, the authors did not address the question if autophagy could also be a means to fight the pathogen. In this case it would be expected that disruption of autophagy would be advantageous for the pathogen and its survival and/or replication would be favoured. We therefore performed time-course experiments with L. pneumophila infecting AX2 wild-type cells and the ATG9- mutant. To compensate for the reduced uptake rate of the mutant we used a multiplicity of infection (MOI) of 8 for the mutant and an MOI of 1 for the wild-type cells. This way similar numbers of cfu were obtained for both strains 3 h post infection (data not shown). We carried out 10 independent infection experiments and relative changes in intracellular bacteria at the different time points were calculated based on the cfu of the 3 h time point, which was considered one for both strains. In the early phase of infection, where L. pneumophila has to establish its replicative niche in the host, we observed a stronger decrease in intracellular bacteria in wild-type cells as compared with the ATG9- mutant (Table 2). Infection of D. discoideum with L. pneumophila resulted in an approximately twofold higher bacterial load in the case of the ATG9- mutant (Table 2, 48 hpi). The data support a model where autophagy has a protective role during infection of D. discoideum with L. pneumophila.

Discussion

Infection of D. discoideum with L. pneumophila resulted in the identification of 240 differentially regulated genes that were specific for the pathogenic response (Farbrother

Table 2. The ratio of cfu from ATG9- and AX2 cells of 10 independent experiments was calculated based on the normalized 3 hpi time point.

| Hours post infection | 3    | 8    | 18   | 24   | 28   | 38   | 48   |
|---------------------|------|------|------|------|------|------|------|
| Mean ratio ATG9-/AX2| 1.00 | 1.48 ± 0.15 | 1.52 ± 0.28 | 1.61 ± 0.16 | 2.28 ± 0.23 | 1.46 ± 0.21 | 1.95 ± 0.35 |
| P-value             | NA   | 1.28E-09 | 3.22E-05 | 7.26E-09 | 4.11E-06 | 2.72E-03 | 4.18E-06 |

Mean values ± SEM as well as P-values from a paired t-test are shown.
NA, not applicable.
et al., 2006). Of these more than 50 had clear orthologues in human including ATG8, ATG9 and ATG16 that encode core autophagy proteins (Table 1). We reasoned that their differential expression might either be part of the Dictyostelium defense mechanism or might be triggered by Legionella to support the establishment of a replicative niche or even both players might try to modulate autophagy during the infection process. To gain a better understanding of the highly conserved ATG9 protein we have investigated its cellular function in D. discoideum through the analysis of a gene deletion strain and the expression of GFP-tagged ATG9 in the mutant background. ATG9 is essential for autophagy in yeast, it is so far the only known integral membrane protein of the core autophagy machinery and a possible candidate for the unidentified membrane carrier of the growing autophagosome (Reggiori et al., 2005; He and Klionsky, 2007).

In immunofluorescence studies of GFP-tagged ATG9 with markers for different cellular organelles we found partial colocalization with the Golgi marker comitin (Fig. 4D). It is possible that this result may reflect close neighbourhood because we observed a strong enrichment of ACVs in a juxtanuclear position at the MTOC, the same region where the Golgi apparatus is found (Fig. 4E). A connection with the Golgi apparatus is however not excluded as a colocalization with the Golgi was observed in mammalian cells where ATG9 cycles between the TGN and endosomes (Young et al., 2006). In contrast in yeast,
detected 3 hpi in both strains, and intracellular replication was managed in ATG9 mutant, a phagocytosis defect (see below). If this were the case, the reported results would imply that L. pneumophila would indeed replicate better in the mutants. We found that clearance of L. pneumophila was less efficient in the ATG9 mutant during the first 18 h of infection and intracellular replication in the following 30 h slightly more efficient (Fig. 8 and Table 2). These results strongly support findings that are consistent with ATG9 and hence autophagy having a protective role during infection with L. pneumophila. In studies with Caenorhabditis elegans and Dictostelium it was shown that autophagy genes protect against S. typhimurium infection (Jia et al., 2009). In macrophages autophagy appears to be a defence mechanism that L. pneumophila and other pathogens must modulate to establish infection (Gutierrez et al., 2004; Amer and Swanson, 2005; Huang and Klionsky, 2007). Surprisingly, the ATG9 mutant also had a strong phagocytosis defect in the uptake of yeast, non-pathogenic and pathogenic bacteria (Figs 6C and 7). The defect was strongest with L. pneumophila where we observed an eightfold reduced uptake rate. To our knowledge this is the first report describing a phagocytosis defect upon deletion of a core autophagy gene. Interestingly, there appears to be a link between autophagy and phagocytosis in murine macrophages where it was shown that activation of toll-like receptors triggered recruitment of LC3/ATG8 and Beclin/ATG6, elements of the classical autophagy pathway, to the phagosome (Sanjuan et al., 2007). The authors also found that engaging the autophagy pathway via TLR signalling enhanced phagosome maturation and destruction of engulfed yeast cells. At present it is not clear how autophagy is linked to phagocytosis in Dictostelium. Apart from a possible direct link similar to macrophages it could also be that disruption of cellular recycling processes leads to a shortage of membranes and other components that are needed for efficient phagocytosis.

The latter possibility could also be the basis for the severe developmental defect that is seen in Dictostelium autophagy mutants. Starving Dictostelium cells need to recycle cellular components to be able to proceed through development. When developed on phosphate agar, aggregates of the atg9 mutant were larger than from wild type, they produced multiple tips and practically all of

![Fig. 8. Analysis of time-course experiments with L. pneumophila infecting ATG9- and AX2 wild-type cells.](image)

**Legend:**
- **Relative Titer**
- **Time Post Infection [h]**
- **Fig. 8.** Analysis of time-course experiments with L. pneumophila infecting ATG9- and AX2 wild-type cells. The graph represents the cfu of the different time points post infection with the inset showing the early phase of infection. Using an MOI of 8 for ATG9- and an MOI of 1 for AX2 similar numbers of intracellular bacteria were detected 3 hpi in both strains, and intracellular replication was monitored over 48 h. The cfu 3 hpi for AX2 wild-type and ATG9- cells were separately considered as one. On this basis mean values and standard errors of the mean of 10 independent experiments were calculated for the later time points. Black circles AX2 wild-type cells, open circles ATG9- cells.
them entered the slug stage. Slugs were very long and thin and frequently broke. Finally, only a few, tiny and misshapen fruiting bodies were produced in the mutant (Fig. 5 and Movie S2). Previously it was reported that Dictyostelium mutants in ATG1, ATG6, ATG7, ATG8 and the ATG12–ATG5 conjugation system displayed developmental defects of varying severity (Otto et al., 2003; Otto et al., 2004a). The developmental phenotype of the ATG9 mutant was similar to the ATG5, ATG6, ATG7 and ATG8 mutants but clearly not identical. Its phenotype was stronger than in ATG6 and ATG8 and slightly weaker than in ATG5 and ATG7. As with other Dictyostelium autophagy mutants, development of ATG9 was more aberrant on bacterial lawns than on phosphate agar (Figs 5 and 6C). The fact that autophagy mutants considerably differ in the severity of their developmental defects, indicate additional and as yet largely unknown functions of these proteins.

In conclusion, disruption of ATG9 function in Dictyostelium cells resulted in a pleiotropic phenotype. Mutant cells not only displayed significant growth and severe developmental defects but, surprisingly, also had a strong phagocytosis defect. The latter phenotype indicates a hitherto unknown link between autophagy and phagocytosis. Whether the connection is direct or indirect remains to be determined. Last but not least infection experiments with L. pneumophila resulted in higher intracellular titers in mutant cells, supporting a model in which autophagy is part of a cell-autonomous defence system. Based on opposing differential regulation of three autophagy genes in response to infection we would like to postulate that both players, the host and the pathogen, try to modulate this ancient cellular process.

Experimental procedures

Bacterial culture

Legionella pneumophila Corby (Jepra et al., 1985) were cultured on BCYE-agar (10 g l⁻¹ yeast extract, 5 g l⁻¹ ACES, 15 g l⁻¹ agar adjusted to pH 6.9 with KOH) supplemented with sterile filtered 0.25 g l⁻¹ Fe(III)NO₃, 9H₂O and 0.4 g l⁻¹ L-cysteine for 3 days at 37°C in a humidified atmosphere with 5% CO₂. S. aureus NRS151 was obtained from NARS (network on antimicrobial resistance in S. aureus; http://www.narsa.net) and cultivated in Luria–Bertani medium.

Dictyostelium strains and growth conditions

D. discoideum strain AX2 was used as wild-type strain. The ATG9 mutant (ATG9⁻) was generated by gene inactivation of the atg9 gene in AX2 through homologous recombination with the Cre-loxP system (Faij et al., 2004). AGA and AAG were generated by transformation of ATG9 after transient expression of the Cre recombinase with expression constructs encoding GFP fused either N- or C-terminally to ATG9. Wild-type and mutant strains were grown at 21°C in liquid nutrient medium with shaking at 160 r.p.m. (Brink et al., 1990) or on SM agar plates with K. aerogenes (Williams and Newell, 1976). Generation times were calculated using the formula $n = \log N_t - \log N_0$ where $n$ is the number of generations, $N_t$ is the cell titer at time 0 and $N_0$ is the cell titer at time $t$. For development in suspension, axenically grown cells were washed twice with Soerensen buffer (17 mM Na-K-phosphate, pH 6.0) and resuspended in the same buffer at a concentration of $1 \times 10^7$ cells ml⁻¹ and shaken at 160 r.p.m. at 21°C.

Vector construction and transformation

The gene replacement construct was generated in the pLBLP vector where the Bsr resistance cassette is flanked by loxP sites (Faij et al., 2004). A PCR-amplified fragment of 434 bp (bases 34–468 of the coding sequence) was cloned into the BamHII and PstI sites and a 560 bp fragment (bases 1586–2146 of the coding sequence) into the HindIII and Sall sites of the vector. Vectors for expression of full-length ATG as GFP fusion proteins in D. discoideum under the control of the actin-15 promoter and actin-8-terminator were constructed using the pBsr-N-GFP N1 and pBsr-C-GFP C1 vectors (Blau-wasser et al., 2009). Proteins composed of either N- or C-terminal GFP fused to ATG9 were expressed in the ATG9 mutant. The constructs were generated by PCR and verified by sequencing. In all fusion proteins a linker of six amino acids with the sequence GGSGGS separated the GFP moiety from the full-length ATG9. The plasmids were introduced into AX2 wild-type cells and the ATG9 mutant by electroporation; transformants were selected in the presence of 5 µg ml⁻¹ Blasticidin S (ICN Biochemicals). The transformants were either identified by PCR screening of bsr resistant clones followed by Southern and Western blotting and/or by visual inspection under a fluorescence microscope followed by immunological detection of the expressed proteins in Western blots.

Development and phototaxis assays

Cells at a density of 2–3 × 10⁶ cells ml⁻¹ were washed twice with Soerensen buffer. A total of 1 × 10⁶ cells were then resuspended in 1 ml of Soerensen buffer and 500 µl evenly distributed onto a phosphate-buffered agar plate (9 cm in diameter) and incubated at 21°C. Different stages of development were observed, and the images were either captured manually at defined time points or automatically at 6 or 10 min intervals using a stereo microscope or an inverted microscope. Movies were processed with Image J. Phototaxis assays were essentially performed as described (Khaire et al., 2007). Approximately 48 h after inoculation, slime trails and cellular material were blotted onto nitrocellulose filter (BA85, Ø 82 mm, Schleicher and Schuell) by keeping the filter on the plate for 1 h. Filters were stained with 0.1% amido black in 25% isopropanol and 10% acetic acid (staining solution) for 10 min. To remove the excess stain two incubations in destaining solution (25% isopropanol, 10% acetic acid) were performed for 10–15 min, then the filters were washed with water and air-dried. The filters exhibiting the stained slime trails were used to determine the distance travelled by the slugs towards the source of light from the point of application.

Phagocytosis analysis

We analysed the phagocytosis of E. coli, S. aureus, L. pneumophila Corby (see below) and yeast cells in the different Dictyostelium strains and growth conditions. 

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Phagocytosis of *E. coli* was analysed with pHrodo™ *E. coli* Bioparticles (Invitrogen GmbH, Germany; http://www.invitrogen.com). The pH-sensitive pHrodo™ dye is a specific sensor of intracellular bacteria. The dye is non-fluorescent at neutral pH and fluoresces bright red in acidic environments. The assay was carried out essentially according to the instructions of the manufacturer (Invitrogen GmbH, Germany). Briefly, 2 × 10⁸ AX2 wild-type, ATG9⁻, AGA or AAG cells per well were transferred into the wells of a 96-well black microplate. The Dictyostelium cells were allowed to settle down, after 1 h the medium was removed and 100 µl of the pHrodo™ *E. coli* Bioparticles in phagocytosis buffer (20 mM Hepes pH 7.0, 2 mM MgCl₂, 10 mM NaCl) were added, corresponding to an approximately 150:1 ratio relative to Dictyostelium cells. Fluorescence measurements were performed with excitation at 544 nm and emission at 590 nm. Data were corrected by the fluorescence of bacteria alone. Each measurement was carried out in quadruplicate, and three independent experiments were performed. Data represent mean values with the fluorescence value of AX2 being set to 100%.

For quantifying the uptake of *S. aureus* by *D. discoideum* via FACS analysis, Dictyostelium cells were harvested and resuspended in a 1:1 mixture of Soerensen buffer and AX2 medium (AX/2) to a final concentration of 1 × 10⁶ cells ml⁻¹. From this suspension 0.3 ml were transferred into 12-well microtiter plates and incubated for 60 min at room temperature. *S. aureus NRS151* GFP were harvested from a preculture and resuspended in AX/2 medium to a concentration of 1 × 10⁶ cells ml⁻¹. The incubation medium in the plate wells was then replaced by 0.3 ml of the bacterial suspension, resulting in an MOI of 100. Dictyostelium and bacterial cells were incubated together for 3 h at room temperature, then washed once with Soerensen buffer and incubated with lysostaphin (0.2 mg ml⁻¹) for 15 min on ice in order to kill extracellular bacteria. Cells were then washed twice with ice-cold Soerensen buffer to remove excess lysostaphin and dead bacteria before being transferred into polystyrene FACS tubes. Using the CellQuest FACS software (BD Biosciences, Heidelberg, Germany) viable Dictyostelium cells were gated. Fluorescence intensity generated by intracellular GFP-expressing *S. aureus NRS151* was measured, and the acquired data were quantified in a histogram.

Quantitative phagocytosis of TRITC-labelled heat-killed yeast cells was performed as described (Rivero and Maniak, 2006). Data represent the mean values and standard errors of the mean of four independent experiments.

**Uptake and infection assays with *L. pneumophila Corby***

For the uptake and infection assays Dictyostelium strains and *L. pneumophila Corby* were cultured and prepared as previously described (Hägele et al., 2000). Infections were performed in a 24-well set-up. Briefly, Dictyostelium cells were adjusted to 1 × 10⁶ cells ml⁻¹ in infection medium (a 1:1 mixture of HL5 medium and 1× Soerensen buffer; HL5 medium: 5 g of yeast, 10 g of glucose, 10 g of proteose peptone, 0.64 g of Na₂HPO₄, 0.48 g of KH₂PO₄ dissolved in 1 l of H₂O, pH 7.5). A total of 300 µl of this suspension was transferred into the wells of a 24-well plate, and cells were left 30 min at 25.5°C so that they could settle down. Bacteria were adjusted to 10⁷ cfu ml⁻¹ in infection medium as determined by OD₅₆₀. The supernatant and non-adhering Dictyostelium cells were removed from the wells and replaced by 300 µl of this bacterial suspension (MOI 10). After 3 h of infection extracellular bacteria were removed by two washing steps, first with 1× Soerensen buffer followed by infection medium containing 100 µg ml⁻¹ gentamicin. In order to kill remaining extracellular bacteria cells were incubated for additional 45 min with 300 µl of infection medium containing 100 µg ml⁻¹ gentamicin. Dead bacteria and gentamicin were removed by two washing steps with infection medium, and the cells were covered with 1 ml of infection medium for the remaining time of infection. At indicated time points cells were collected by resuspending, transferred into 1.5 ml of reaction tubes and mechanically lysed by centrifugation (16,000 g, 6 min) at room temperature and strong vortexing. Serial dilutions in ddH₂O were plated out on BCYE-agar for cfu determination. The time point 3 h corresponds to the cfu of intracellular bacteria after gentamicin treatment and was used to determine the uptake of *L. pneumophila*. To compensate for the differences in bacterial uptake rates between wild-type AX2 and the Δatg9 mutant time-course experiments were performed with MOI 8 for the mutant and MOI 1 for the wild type. In these infections comparable numbers of intracellular bacteria were detected 3 h post infection in both strains, and intracellular replication was monitored. Ten independent infections were performed, and cfu values were determined at the different time points post infection. The cfu value 3 h post infection was considered as 1 and used as a reference for calculating the relative changes at later infection time points. Mean values, standard errors of the mean and ratios of the mean values of the relative bacterial titers in AX2 and ATG9⁻ cells were calculated. The combined error ΔR of the ratio R was calculated based on the Gaussian error propagation using the formula: \( \Delta R/R = (\Delta A/A + \Delta B/B) \) with \( A \) and \( B \) being the mean values of the infections with AX2 and ATG9⁻, respectively, and \( \Delta A \) and \( \Delta B \) being the corresponding standard errors of the mean. A paired t-test was used to calculate the P-values of the different time points.

**Fluorescence microscopy**

Immunofluorescence microscopy was essentially done as described (Noegel et al., 2004). Briefly, axenically grown cells were harvested, washed with Soerensen buffer, resuspended in the same buffer, transferred to coverslips and fixed in cold methanol at −20°C. The coverslips were treated with PTB containing 1× PBS (phosphate-buffered saline), 0.1% Triton X100 and 0.1% BSA at room temperature. The following monoclonal antibodies either undiluted or diluted in PTB buffer (dilution in brackets): porin (Troll et al., 1992) (1:20), comitin (Weiner et al., 1993) (1:5), vαA (Jenne et al., 1998) (undiluted), PDI (Monnat et al., 1997) (1:5), lysosomal antigen (Neuhaus et al., 1998) (1:5), CP250 (Blau-wasser et al., 2009) (1:5) and YL1/2 (Kilmartin et al., 1982) (1:5). Secondary antibodies were Alexa-fluor 568 Goat anti-mouse (1:2000), Alexa-fluor 568 goat anti-rat (1:2000) and Alexa-fluor 647 donkey anti-mouse (1:2000) (all from Invitrogen GmbH, Germany). The nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI, Sigma-Aldrich, Munich, Germany) (Luke et al., 2008). Confocal images of live or fixed cells were taken with an inverted Leica DMI 6000B laser scanning microscope (Leica, Wetzlar, Germany) with a 100× HCX PL APO NA 1.40 oil immersion objective. Excitation of GFP was at 488 nm, of Alexa-fluor 568 at 568 nm, of Alexa-fluor 647 at 647 nm and of DAPI at 405 nm. Images were processed using the accompanying Leica Application Suite (LAS) software or Image J.
Antibody generation and Western blotting

For generation of ATG9-specific polyclonal antibodies (pAbs), sequences encompassing the APG9 domain (aa 188–567) were amplified and cloned into a pGEX-4T GST expression vector. The fusion protein was expressed in E. coli DH5α, purified using GST-Sepharose beads and used for the immunization of rabbits (BioGENES GmbH, Berlin, Germany). The resulting ATG9 pAbs were used for Western blotting at a 1:10 000 dilution. GFP was detected with monoclonal antibody K3-184-2 (Noegel et al., 2004). Secondary antibodies used were anti-mouse and anti-rabbit IgG POD (Sigma, Deisenhofen, Germany).

Miscellaneous methods

For SDS gel electrophoresis the proteins of 2 × 10⁶ cells were separated per lane. RNA isolation, northern transfer and real-time PCR were essentially done as described (Farbrother et al., 2006). For real-time PCR the amplified gene fragment from cDNA had a size of 1975 bp. Genomic DNA contains two short introns of 64 and 75 bp respectively. Control amplifications of the generated cDNAs of the different developmental time points with standard PCR resulted in a single band of the expected size after agarose gel electrophoresis. No cross contamination with standard PCR resulted in a single band of the expected size.

Acknowledgements

The authors would like to thank Sachin Kumar for bioinformatic analysis, Rui Tian for generating ATG9 knockout cells and the initial analysis of the mutant, Rolf Müller for cloning work, Rosemarie Blau-Wasser for help with immunofluorescence studies and Lorna Moll for phototaxis and development experiments. This work was supported by grants from the DFG (SFB670) and Köln Fortune.

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Supporting information

Additional Supporting Information may be found in the online version of this article.

Movie S1. Live cell imaging of AAG cells. ATG9-GFP was expressed in the ATG9 mutant background and the dynamics of ACVs recorded. Pictures were taken every 1.3 s.
**Movie S2.** Development of the ATG9\(^+\) mutant on phosphate agar plates. Development was followed over 30 h. Pictures were taken every 18 min.

**Fig. S1.** Phototaxis assay.  
A. AX2  
B. and C. ATG9\(^-\). (C) shows one of the rare cases where some mutant slugs display a very small shift into direction of the light source. The arrow indicates the light source. The phototaxis assay was performed as described in the experimental procedures.

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