Dictyostelium macroautophagy mutants vary in the severity of their developmental defects

Grant P. Otto\textsuperscript{1}, Mary Y. Wu\textsuperscript{1}, Nevzat Kazgan\textsuperscript{1}, O. Roger Anderson\textsuperscript{2}, and Richard H. Kessin\textsuperscript{1}\*  

\textsuperscript{1}Department of Anatomy and Cell Biology  
Columbia University  
630 West 168th St.  
New York, NY 10032  
USA

\textsuperscript{2}Department of Biology  
Lamont-Doherty Earth Observatory  
Columbia University  
Palisades, NY 10964  
USA

*To whom correspondence should be addressed:

Department of Anatomy and Cell Biology, P&S 12-517  
Columbia University  
630 W168th Street  
New York, NY 10032  
USA  
Telephone: 212-305-5653  
Fax: 212-305-3970  
Email: rhk2@columbia.edu

Running Title: Macroautophagy in Dictyostelium.

Key Words: Autophagy, proteolysis, slime mould, D. discoideum.
SUMMARY

Macroautophagy is the major mechanism that eukaryotes use to recycle cellular components during stressful conditions. We have previously shown that the Atg12-Atg5 conjugation system, required for autophagosome formation in yeast, is necessary for Dictyostelium development. A second conjugation reaction, Aut7/Atg8 lipidation with phosphatidylethanolamine, as well as a protein kinase complex and a phosphatidylinositol 3’-kinase complex, are also required for macroautophagy in yeast. In this study, we characterize mutations in the putative Dictyostelium discoideum orthologues of budding yeast genes that are involved in one of each of these functions, ATG1, ATG6 and ATG8. All three genes are required for macroautophagy in Dictyostelium. Mutant amoebae display reduced survival during nitrogen starvation and reduced protein degradation during development. Mutations in the three genes produce aberrant development with defects of varying severity. As with other Dictyostelium macroautophagy mutants, development of atg1-1, atg6- and atg8- is more aberrant in plaques on bacterial lawns than on nitrocellulose filters. The most severe defect is observed in the atg1-1 mutant, which does not aggregate on bacterial lawns and arrests as loose mounds on nitrocellulose filters. The atg6- and atg8- mutants display almost normal development on nitrocellulose filters, producing multi-tipped aggregates that mature into small fruiting bodies. The distribution of a green fluorescent protein fusion of the autophagosome marker, Atg8, is aberrant in both atg1-1 and atg6- mutants.
INTRODUCTION

In the social amoeba *D. discoideum*, starvation is a signal for the initiation of multicellular development. Starving amoebae aggregate in response to cAMP to form mounds. Within these cell aggregates, intercellular signals direct the formation of a multicellular slug that migrates to a suitable location for formation of a fruiting body. The fruiting body is composed of a spore mass held aloft on a stalk composed of cells that vacuolate and die. Development is an energy-intensive process, and requires that amoebae cease production of growth-related proteins and lipids, and initiate a developmental program (reviewed in (1)). One mechanism employed by *Dictyostelium* and other eukaryotes to mobilize resources required for development is macroautophagy. Macroautophagy is required for sporulation in *Saccharomyces cerevisiae* (2), differentiation in the yeast *Podospora anserina* (3), metamorphosis in *Drosophila melanogaster* (4), and dauer development in *Caenorhabditis elegans* (5). In this transport process, bulk cytoplasm and organelles are sequestered in double-membrane vesicles (autophagosomes/autophagic vacuoles) that fuse with and deliver their content to the lytic compartment of the cell, the lysosome or vacuole. Genetic studies in *S. cerevisiae* have identified 15 *ATG* genes that are required for the formation of these double membrane autophagosomes (2,6,7). A new unified nomenclature for autophagy-related genes was introduced recently (8), and we will use the *atg* designation henceforth. The genes can be grouped into several classes that function in the localization of autophagy components to the site(s) of autophagosome biogenesis, termed the preautophagosomal structure (PAS) in yeast, and in the generation of double-membrane vesicles (reviewed in (9)). Three
signaling complexes are required for macroautophagy: a Tor-kinase complex, an Atg1-phosphoprotein kinase complex, and a phosphatidylinositol-3’ kinase (PI3K) complex. Tor is a phosphotidylinositol kinase-related Ser/Thr kinase that functions in the control of protein metabolism in response to nutritional status (reviewed in (10)). In addition to increasing protein synthesis by multiple mechanisms, Tor also functions in macroautophagy by signaling to Atg1, a Ser/Thr kinase that forms a signaling complex with Atg13 and Atg17 (11,12). Tor influences the phosphorylation status of Atg13, which in turn alters the affinity of the Atg13-Atg1 association. During starvation, Tor is inactive, Atg13 is hypophosphorylated and binds tightly to and activates Atg1 kinase. However, enhanced Atg1 kinase activity during starvation is apparently dispensable for induction of macroautophagy, suggesting that Atg1 function in autophagy may be structural (13).

Macroautophagy in yeast also requires a protein complex containing a class III phosphatidylinositol 3’-kinase (PI3K), Vps34, a membrane bound Ser/Thr kinase, Vps15, and a coiled-coil protein Vps30/Atg6 (14,15). This core complex functions both in macroautophagy and vacuolar protein sorting. Pathway specific components are required for the localization or targeting of the PI3K activity: Vps38 is sorting-specific, whereas the small coiled-coil protein Atg14 functions in macroautophagy (15), and is thought to target the PI3K complex to the PAS in yeast (16,17). The human homologue of ATG6, beclin 1, encodes a Bcl-2-interacting protein that plays a role in negatively regulating mammalian cell growth and tumorigenesis (18,19). Beclin 1 binds to the human Vps34 homologue, PtdIns 3-kinase, and both proteins localize to the trans-Golgi network, suggesting that the macroautophagy function of Beclin 1 may be related to protein sorting
Two novel post-translational modifications are essential for efficient autophagosome formation. Atg12 is conjugated to Atg5 in an ubiquitination-like reaction requiring the E1-like enzyme Atg7 (21,22) and the E2-like enzyme Atg10 (23). In yeast, the Atg12-Atg5 conjugate binds a coiled-coil protein, Atg16, that mediates formation of a 350-kDa oligomeric complex (24), and localization of the complex to the PAS (17). In mammals and other eukaryotes (including Dictyostelium), the Atg16 orthologue, Atg16-like protein (Atg16L), contains both a coiled-coil motif and WD repeats (25). Mouse Atg16L functions similarly to Atg16 in yeast, forming 800-kDa oligomers with the Atg12-Atg5 conjugate, and targeting the complex to the forming autophagosome, the cup-shaped isolation membrane.

In the second conjugation reaction, Aut7/Atg8 is lipidated by phosphotidylethanolamine, through the action of the E1-like and E2-like enzymes, Atg7 and Aut1/Atg3, respectively (26,27). Lipidated Atg8, Atg8-PE, is localized to the PAS in yeast, but in addition is incorporated into the forming autophagosome membrane and completed autophagosomes (28). Atg8 synthesis is strongly induced by starvation, whereas disrupting Atg8 synthesis or function results in the production of small, physiologically inefficient autophagosomes (29). There are three Aut7/Atg8 homologues in humans: GATE-16 (Golgi-associated ATPase enhancer of 16kDa), GABARAP (GABA receptor-associated protein), and MAP-LC3 (microtubule-associated protein light chain 3). Only MAP-LC3 has been demonstrated to function in autophagy (30).
Finally, a membrane protein complex composed of the binding partners Atg9 and Atg2 (31) is required for autophagosome formation. These two proteins localize to the PAS in yeast, but otherwise their function is poorly understood.

Suzuki and co-workers examined the localization of GFP-Atg8 and Atg5-GFP in different autophagy mutants in an attempt to order the function of the groups of autophagy proteins (17). The Atg6-containing PI3K complex and Atg9 are required for localization of Atg5 to the PAS. The Atg5-Atg12 conjugation system is required for recruitment of Atg8-PE to the PAS, while the Atg1-containing phosphoprotein kinase complex and Atg2 function after the recruitment of Atg8-PE to the PAS. Two distinct autophagy protein complexes exist at the PAS, one containing the Atg1 complex and Atg9, the other containing the conjugation proteins and Aut7/Atg8 (16).

*Dictyostelium* provides a simple yet powerful model for studying the role of macroautophagy in multicellular development. We have used *Dictyostelium* to study the requirement for macroautophagy in the establishment of a replicative vacuole for the intracellular bacterial pathogen, *Legionella pneumophila* (32). In addition, mutations in two *Dictyostelium* macroautophagy genes representative of the Atg12 conjugation system, *atg5* and *atg7*, produce severe developmental defects (33). The mutants produce aberrant fruiting bodies that are devoid of mature, detergent-resistant spores. Both mutations produce macroautophagy defects and developmental phenotypes of similar severity. We asked whether mutations in macroautophagy genes representative of the other functional classes produce an arrest at a similar stage in multicellular development. We generated mutations in representatives of the phosphoprotein kinase complex (*atg1*), the PI3K-signaling complex (*atg6*), and the lipidation system (*atg8*). We chose *atg1* and
atg6 because these two genes are the only representatives of the cognate complexes that have been identified in D. discoideum. We chose atg8 because it is the only structural component identified in the Atg8 lipidation system. We show that atg1, atg6 and atg8 genes are also required for macroautophagy in Dictyostelium. The three mutations produce defects in survival, recycling of cytoplasm and organelles during nitrogen starvation, and protein degradation during development. Additionally, development is aberrant in all three mutants. Mutation of atg1 produces the most severe macroautophagy and developmental defects, whereas mutations in atg6 and atg8 permit a further progression in development.
EXPERIMENTAL PROCEDURES

Strains

All mutations were created in the strain DH1, which is a uracil auxotroph. Strains were grown in HL5 medium or on lawns of Klebsiella pneumoniae (34).

Development and spore production assays

Multicellular development was examined on nitrocellulose (NC) filters, on non-nutrient agar, or in plaques on bacterial lawns. For NC filters, axenically grown cells in mid-log phase (2-4 x 10^6 cells/ml) were washed twice in cold Sorensen C (SorC) buffer (16.7 mM Na_2H/KH_2PO_4, 50 µM CaCl_2, pH 6.0). The cells were resuspended in SorC buffer and plated on 25mm, 0.45 µM nitrocellulose filters (Millipore Corp.), which rested on SorC-soaked Whatman Grade 17 filter pads, at a density of ~3.3 x 10^6 cells/cm^2, or 1.6 x 10^7 cells/filter (34). For development on non-nutrient agar, cells were resuspended at 10^7 cells/ml, and 100 µl of these cells were plated on 35-mm SorC-1% Phytigel (Sigma) agar plates and allowed to dry. For development on bacterial lawns, 25-50 amoebae were mixed with 150 µl of an overnight culture of K. pneumoniae, and plated on SM plates. Phenotype was examined after 5 days incubation at 22 °C. For spore production assays, the same protocol was followed as for development on NC filters. Filters were transferred to 5 ml SorC in 15 ml Falcon tubes 28-32 hours after initiating development, and all developing structures and cells dislodged by vigorous vortexing. The harvested structures were incubated for 5 minutes in 0.3% Triton X-100 to kill amoebae but leave
mature spores intact. Spores were counted with a haemacytometer and appropriate dilutions were plated on *K. pneumoniae* lawns to determine viability.

**Gene disruption**

Gene disruptions were created by insertion of a selectable marker into the coding sequence of the genes. A 2316 bp fragment of *atg1* lacking only the C-terminal 18 bp was obtained by PCR with primers 1-1 (ATAAATGAAACGAGTAGGAG) and 1-4 (ACTATTGGTATTCAAAAACTG), and ligated into pGEM-T Easy (Promega). The blasticidin resistance cassette was removed from pBsR519 with *BamHI*, and cloned into the *Bgl*II site (position 705) of the *atg1* PCR product.

A 5’ 1646 bp portion of *atg6* obtained by a PCR reaction with primers KO6-1A (ACTCACACCCCTTTCAACTGTC) and 6-6 (GTATCTCTGTTAAATCCTT) was ligated into pGEM-T Easy (Promega). The blasticidin-resistance cassette was removed from pBsR519 with *BamHI*, and was cloned into the *BamHI* site (position 435) of *atg6*.

A 1454 bp portion of the *atg8* locus, encompassing the entire *atg8* coding sequence, 367 bp upstream of the translational start codon, and 490 bp downstream of the translational stop codon, was obtained by PCR reactions with primers 8-5 (CCAAACCAGTTAATAAAAAA) and 8-4 (ACAAAGAAGGATAATTGAAC), and was ligated into pGEM-T Easy (Promega). The blasticidin-resistance cassette was removed from pBsR519 with *HindIII*, and was cloned into the *HindIII* site of *atg8*, 12 bp after the translational start codon. The three gene-targeting constructs were linearized with *ApaI* prior to electroporation into DH1 cells by the method of Kuspa *et al.* (35). Transformants were selected with blasticidin (5 µg/ml) in HL5 medium for 1 week,
harvested from Petri dishes, plated onto SM plates, and clones of mutant or wild-type phenotype were selected for further analysis. Homologous recombination of the targeting construct with the endogenous locus was confirmed by PCR or by Southern blot.

**Fusion constructs**

The GFP-Atg8 fusion was constructed as previously described (33). Briefly, the full-length coding sequence of *atg8* was obtained by PCR from a genomic DNA template. The PCR product was cloned into pGEM-T Easy (Promega), cut from pGEM-T Easy with *Sac*I, and ligated into *Sac*I-digested, dephosphorylated pTX-GFP. The expression of *atg8* is under control of the constitutively active *actin 15* promoter.

Cyan fluorescent protein obtained by PCR of pECFP (Clontech) with primers XFP-5’ (GGTACCATGGTGAGCAAGGGCGAG) and XFP-3’ (GAGCTCCTTTGTACAGCTCGTCCAT) was cloned into pGEM-T Easy. The insert was removed from pGEM-T Easy with *Kpn*I and *Sac*I and cloned into *Kpn*I/*Sac*I-digested pDXA-HC (36) to produce pDXA-HC-CFP. The *atg1* coding sequence was generated by PCR from genomic DNA with primers 1-5GFP (GAGCTCATGAAACGAGTAGGAGAT) and 1-3GFP (GAGCTCTGTATTATTGGAATACT), cloned into pGEM-T Easy, and digested with *Sac*I for cloning into *Sac*I-digested, dephosphorylated pDXA-HC-CFP. Amoebae were transfected by electroporation (35), and transformants were selected with G418 (5 µg/ml) in HL5 medium for 1 week. Transformants were harvested from Petri dishes, plated onto SM + G418 (60 µg/ml) plates, and individual clones were selected for further analysis. We confirmed that a protein of the correct size was produced in transformed strains by
western blotting with rabbit polyclonal antiserum against GFP (Molecular Probes).

Northern blot analysis

Northern blot analysis was conducted as previously described (37). Cells were deposited on nitrocellulose filters for development, and a filter was harvested every 4 hours for RNA extraction. Five µg total RNA was glyoxylated, size fractionated on 1% agarose gels, transferred to nitrocellulose, and hybridized with random primer-labeled DNA probes. Probes were obtained by isolation of appropriate restriction fragments following separation on low-melting temperature agarose gels. The \textit{atg1} probe consisted of an \(~700\) bp \textit{Bgl}II-\textit{Sac}I fragment of the \textit{1-1/1-4} PCR product in pGEM-T Easy. The \textit{atg8} probe consisted of the \(~800\) bp \textit{Cla}I-\textit{EcoRI} fragment of the \textit{8-5/8-4} PCR product in pGEM-T Easy.

Protein turnover assays

Protein turnover assays were based on those of White and Sussman (38). Developing cells were recovered from Millipore filters in SorC buffer, pelleted by centrifugation, and resuspended in 50 mM Tris-HCl (pH 6.9) containing protease inhibitors (Complete, Mini-Roche). Cells were lysed by freeze-thaw before determining protein levels using the Pierce Coomassie® Plus Protein Assay Reagent, following the manufacturer’s instructions.

Electron microscopy
Electron microscopy procedures were as described (39). Briefly, cells were fixed with 2% phosphate-buffered glutaraldehyde (pH 7.2), followed by 0.5-2% osmium tetroxide fixation in the same buffer, sedimented by centrifugation and enrobed in agar, dehydrated with an aqueous/acetone series, and embedded in TAAB epon resin (Energy Beam Sciences, Agawam, MA).

**Fluorescence microscopy**

To examine the localization of GFP fusion proteins, axenic cells were incubated overnight in HL5 medium supplemented with 10 µg/ml G418 in 35 mm glass bottom microwell dishes (MatTEK Corporation). The following day, the medium was replaced with SorC 1-6 hours prior to visualization (starving cells) or visualized directly (growing cells). GFP-expressing cells were viewed under a Nikon Eclipse TE300 microscope, images were captured with a cooled CCD camera, and processed using Metamorph 5 imaging software (Universal Imaging Corporation).

Chimaeric development was conducted as previously described (33).
RESULTS

Structural features of atg1, atg6 and atg8 genes in Dictyostelium discoideum

We previously identified putative Dictyostelium orthologues for Atg1 (AY191011), Atg6 (AY191013), and Atg8 (AY191015) (33). In addition, a putative open reading frame with homology to Atg6, which we call atg6B, was recently identified on chromosome 5 by the Dictyostelium Genome Sequencing Project at Baylor College of Medicine (http://dictybase.org/db/cgi-bin/dictyBase/locus.pl?locus=apg6B).

Atg1 is a serine-threonine kinase initially identified in budding yeast (40). Atg1p is composed of an N-terminal kinase domain (see narrow arrowheads in Figure 1A) and a C-terminal domain containing no recognizable motifs. Protein kinases contain twelve conserved subdomains that mediate ATP-binding, binding of the substrate, and phosphotransfer (41). All twelve subdomains are present in the Atg1 orthologues of S. cerevisiae, Arabidopsis thaliana, and D. discoideum. The putative Atg1 orthologues from mammalian cells (42,43) and Arabidopsis (44) have not been shown experimentally to function in autophagy, whereas the C. elegans Atg1 orthologue, unc-51, does function in autophagy (5). The homology between the Atg1 orthologues is highest in the N-terminal kinase domain, and lower in the poorly-conserved C-terminus. The predicted 668 residue Dictyostelium Atg1 has an asparagine-rich stretch immediately following the kinase domain. Poly-asparagine repeats are common in Dictyostelium proteins, but their function is unknown. Additionally, a large 29 residue glutamine-rich stretch is present in the C-terminal half of the protein. The Dictyostelium Atg1 kinase domain shares highest homology with its Arabidopsis counterpart (44% identity over 268 residues). The
homology of the C-terminal domain is also highest with the *Arabidopsis* protein, (19% over 331 residues). The *Dictyostelium* protein lacks the predicted coiled-coil motif present at the C-terminus of *S. cerevisiae* Atg1 (13), as do the *C. elegans* and *A. thaliana* Atg1 orthologues when analyzed using the COILS server (http://www.ch.embnet.org/software/COILS_form.html (45)).

*Dictyostelium* contains two putative Atg6 orthologues, *atg6* and *atg6B*. The predicted 1368 residue Atg6 and 855 residue Atg6B proteins are significantly larger than the 450-550 residue proteins in other organisms, due to an extended N-terminus (Figure 1B shows an alignment of the C-termini of Atg6 proteins). The N-terminus of Atg6 contains numerous stretches of asparagine residues of unknown function that are widespread of *D. discoideum* proteins (190/671 N-terminal residues (28%) are asparagine). This part of the protein also contains many serine residues (85/671 residues or 13%). Atg6B contains 3 poly-glutamine stretches, and is rich in serine and threonine residues (12% and 10% by frequency, respectively). Both *Dictyostelium* Atg6 orthologues are more similar to the plant (31%/303 and 38%/331 residues) and human (32%/309 and 39%/327 residues) orthologues than to the yeast orthologue (24%/425 and 25%/419 residues) or each other (24%/643 residues). Atg6 is predicted to contain a single coiled-coil (using the COILS server at http://www.ch.embnet.org/software/COILS_form.html), a feature described for orthologues in other organisms (14,18), whereas Atg6B contains 2 predicted coiled-coils.

The predicted 122 residue *Dictyostelium* Atg8 orthologue shares a remarkably high degree of sequence identity to Atg8 proteins from *Arabidopsis* (72%) and budding yeast (63%), whereas identity to the human MAPLC3 is lower (35%). The glycine
residue required for conjugation to PE is conserved, and corresponds to G119 in Dictyostelium Atg8, G117 in AtAtg8a, G120 in HsAtg8/MAP1LC3, and G116 in ScAut7/Atg8 (asterisk in Figure 1C). Presumably the 3 amino acids following G119 must be cleaved by the putative Dictyostelium Atg4 orthologues previously identified (33), a process that is conserved in other organisms.

**Generation of mutations in atg1, atg6 and atg8**

We generated mutations in atg1, atg6, and atg8 by insertional mutagenesis (described in Experimental Procedures). atg6B was only identified recently, and has not been studied. The insertion in the atg1 gene is in a BamHI site at position 705 (wide arrowhead in Figure 1A), in subdomain IX of the kinase domain, leaving most of the kinase domain intact. Consistent with the site of insertion, a truncated transcript of ~1.2 kb is detected in the atg1-1 mutant strain by northern blot analysis. This transcript appears to be more stable during development than the full-length endogenous ~2.3 kb atg1 transcript in the parental DH1 strain (Figure 2, upper panel). This truncated transcript may produce a protein that functions as a dominant negative, although the significant complementation of atg1 mutant defects in the atg1-1 (act15/cfp-atg1) strain argues against this possibility. Alternatively, a truncated protein may produce a gain-of-function. However, the severe autophagy defects we observe in this mutant (see below) suggest that atg1-1 is a strong loss-of-function allele.

To generate an atg6 mutant, the blasticidin cassette was inserted into a BamHI site at position 435 in the 4104 bp atg6 open reading frame, leaving the possibility that a truncated 145 amino acid residue protein is produced. We have confirmed by PCR that
the \textit{atg6} mutant phenotype is a result of homologous recombination (data not shown). We could not detect an \textit{atg6} transcript by northern blot analysis or RT-PCR in the parental strain DH1, and so could not confirm that a truncated mRNA is present in the \textit{atg6} mutant. However, this insertion most likely creates a null mutation, for the following reasons: the phenotype of the \textit{atg6} mutant cells with this insertion is consistent with that of a macroautophagy mutant. Additionally, the insertion is at the 5’ end of the gene, upstream of the presumed functional part of the protein, based upon homology of the \textit{Dictyostelium} protein with other orthologues (see Figure 1B). Therefore, we refer to the resultant mutant strain as \textit{atg6}.

To create the \textit{atg8} mutant, we inserted the blastidin cassette into a \textit{Hind}III site 15 base pairs into the \textit{atg8} coding sequence. We cannot detect an \textit{atg8} transcript in the \textit{atg8} mutant strain with a probe to the 3’ 318 bp of the \textit{atg8} gene, whereas a developmentally regulated ~1.1-1.2 kb transcript (not 0.9 kb as previously described (33)) is detected in the parent DH1 (Figure 2, lower panel). The insertion creates a null mutation, and thus we refer to the \textit{atg8} mutant as \textit{atg8}.

\textbf{Autophagy mutants show developmental defects of varying severity}

We tested the growth of autophagy mutants and the parental strain in both shaking axenic culture (by haemacytometer counts) and on bacterial lawns (by plaque size). The \textit{atg1-1} mutant grows slightly slower than the parental strain under both conditions (data not shown). The mutations in \textit{atg6} and \textit{atg8} produce no discernable growth defects. All three mutants show defective development when developing within plaques on bacterial lawns, and when developed on nitrocellulose filters (see Figure 3). \textit{Dictyostelium}
amoebae form plaques on lawns of *K. pneumoniae*, where the amoebae at the edge actively engulf bacteria, while those at the center starve and initiate development (Figure 3D). The *atg1-1* mutant does not aggregate when developing in plaques on *K. pneumoniae* lawns, but forms loose mounds on nitrocellulose filters (Figure 3B, E). The *atg6* and *atg8* mutants are the least severe of the autophagy mutants we have characterized thus far. Development is almost normal on nitrocellulose filters, where multi-tipped aggregates are formed that mature into small but otherwise normal fruiting bodies for *atg8* (Figure 3H), or small fruiting bodies with truncated stalks for *atg6* (Figure 3G). The developmental defect is more severe on bacterial lawns, where both strains aggregate mainly at the center of plaques, and not at the periphery as the plaque grows, and the multi-tipped aggregates that form produce very small fruiting bodies (Figure 3J, K).

The *atg1-1* mutant phenotype is significantly rescued on both nitrocellulose filters and bacterial lawns by complementation with CFP-Atg1 (Figure 3C, F). The effects of complementing the *atg8* mutation by expressing GFP-Atg8 are not as obvious because the *atg8* phenotypic defect is relatively mild (Figure 3I, L). We could not maintain the full-length *atg6* gene stably in bacteria, and so could not perform complementation experiments.

Since the *atg6* and *atg8* mutants have the mildest developmental defect of the macroautophagy mutants we have examined to date, we determined whether the defective fruiting bodies produced by these mutants contained mature, detergent-resistant, viable spores. The *atg1-1* mutant produces no spores, consistent with the early block in development prior to cell-type differentiation and morphogenesis (Table 1).
Complementation with CFP-Atg1 rescues spore production to ~10-25% of wild-type levels, consistent with the significant phenotypic improvement. The \textit{atg6}\textsuperscript{-} and \textit{atg8}\textsuperscript{-} mutants produce some detergent-resistant spores, although significantly fewer than wild-type. The majority of spores formed by \textit{atg8}\textsuperscript{-} are round (83-92%), and not the elliptical shape of mature DH1 spores. The \textit{atg6}\textsuperscript{-} mutant produces fewer spores than \textit{atg8}\textsuperscript{-}, but most are elliptical. Expression of GFP-Atg8 in \textit{atg8}\textsuperscript{-} yields an increase in spore production, and a greater proportion of the spores that are produced are elliptical (27-38%).

\textbf{Autophagy mutations are cell autonomous}

The \textit{atg6}\textsuperscript{-} and \textit{atg8}\textsuperscript{-} strains will produce spores when developed on nitrocellulose filters, but they produce fewer spores than wildtype, and many of the \textit{atg8}\textsuperscript{-} spores appear to be immature. To test whether development in the presence of wild-type cells may rescue the spore production or maturation defect of these two mutants, we performed mosaic development experiments. Mutant and wild-type cells were mixed in a ratio of 1:3, and placed on nitrocellulose filters or non-nutrient agar for development. When GFP-expressing mutant cells mixed with unmarked parental DH1 are developed on non-nutrient agar, both \textit{atg6}\textsuperscript{-} and \textit{atg8}\textsuperscript{-} amoebae, but not \textit{atg1}-1, are observed in the sorocarps of chimaeric fruiting bodies by fluorescence microscopy (Figure 4). The \textit{atg1}-1 mutant cells, like \textit{atg5}\textsuperscript{-} and \textit{atg7}\textsuperscript{-} amoebae, are found in the basal disc, a structure that supports the fruiting body (Figure 4D). We developed mixed populations on nitrocellulose filters and examined detergent-resistant spores produced by chimaeric fruiting bodies. \textit{atg6}\textsuperscript{-} and \textit{atg8}\textsuperscript{-} amoebae produce plaques that are clearly distinguishable from parental plaques (see
Figure 3). We observed no plaques with the atg8− phenotype of 223 examined. If sorocarps were harvested with a sterile metal loop into SorC, and the spores plated with bacteria on SM plates without detergent treatment, then 3% (17/587) of the resulting plaques had the atg8− phenotype. With detergent treatment, chimaeras of atg6− and DH1 yielded 6/217 (3%) plaques with the atg6− phenotype, and this value did not change significantly if spores were plated without detergent treatment. Since the mutant input is 25%, it appears that the two mutants are predominantly excluded from the fruiting structures during mosaic development. In conclusion, all three mutations are cell autonomous, because production of mature, viable spores by mutant cells cannot be rescued by mixing with wild-type cells.

**Autophagy mutants are hypersensitive to amino acid starvation**

A hallmark of macroautophagy mutants in budding yeast (2) and in *Dictyostelium* (33) is an inability to survive amino acid starvation. We tested whether the mutations we created result in the reduction in viability expected of macroautophagy mutants, and tested atg5− in the same experiment as a representative of the Atg conjugation system. We starved amoebae in amino acid-free FM medium, and plated aliquots with *K. pneumoniae* on SM/5 plates every 2 days to test for viability, as previously described (33). The parental strain DH1 survives well over the 8-day duration of the experiment (Figure 5), whereas all three mutants show severely reduced viability after only 4 days. The severity of the viability defect correlates with that of the developmental defect: the atg1-1 mutant is most severe, whereas atg6- and atg8- show a less significant reduction in viability, similar to that of the atg5- mutant.
Bulk protein turnover is reduced in macroautophagy mutants

Macroautophagy is the major mechanism of bulk protein turnover in starving yeast cells (46), and Dictyostelium macroautophagy mutants degrade significantly less protein than wild-type cells during development (33). Therefore, we tested whether atg1-1, atg6- and atg8- had a similar defect in protein degradation. Total protein measured by the Bradford assay drops by 30-45% in wild-type cells developed for 24 hours on nitrocellulose filters (Table 2). The three macroautophagy mutants contain only 5-15% less total protein than at the start of development, and the severity of the defect correlates with that of the developmental phenotype. The complemented atg1-1 strain, atg1-1 (act15/cfp-atg1), displays protein degradation comparable to the parent DH1, whereas rescue in atg8- (act15/gfp-atg8) is poorer.

Transmission electron microscopy of amino acid-starved amoebae

A diagnostic feature of amino acid-starved Dictyostelium macroautophagy mutants is an absence of the significant cytoplasmic degradation observed in starved wild-type cells by transmission electron microscopy (TEM). We undertook TEM studies to confirm that our new mutations produce the ultrastructural changes expected for macroautophagy mutants. Growing mutant amoebae are indistinguishable from their growing wild-type counterparts (data not shown). We also examined amoebae starved of amino acids for 36 hours. As we have observed for the atg5- and atg7- mutants (33), atg1-1, atg6- and atg8- mutants all show little evidence of turnover of cytoplasmic constituents compared to wild-type DH1 cells (Figure 6). Again, as we have observed for
other measures of autophagic efficiency, the ultrastructural phenotype of *atg1-1* is more severe than the *atg6* and *atg8* mutants. The cytoplasm of *atg1-1* amoebae is invariably more dense than that of *atg6* or *atg8* amoebae (compare Figure 6C to B and E). The starved *atg1-1* amoebae commonly show profiles of mitochondria encircled by rough endoplasmic reticulum (arrowheads in Figure 6C; see also Figure 7). We do not observe any structures that could be definitively identified as autophagosomes in any of the mutants examined in this study.

We commonly observe profiles of large vesicle clusters in the *atg1-1* mutant that are absent in the parent DH1 (Figure 7). The clusters consistently contain three vesicle classes: larger vesicles with empty lumina (narrow arrow in Figure 7B), vesicles of intermediate size with electron-dense lumina (wide arrow in Figure 7B), and very small vesicles that are sometimes linearly arrayed (arrowheads in Figure 7B). We have observed similar profiles in *atg5* and *atg7* mutants, although the clusters are smaller than in *atg1-1* (data not shown). The reduced size of these clusters in *atg5* and *atg7* may explain why we observe them less frequently than in *atg1-1*.

We also examined whether expression of GFP fusions could rescue the mutant phenotype of *atg1-1* and *atg8* mutants. In both cases, we observed a gradation in the degree of complementation. A minority of starved cells are similar in appearance to mutant cells, and have dense cytoplasm and many organelles. A majority of cells had the phenotype expected for a complemented mutant, and were similar to starved wild-type cells in that the cytoplasm contained large electron-lucent regions and few organelles (Figure 7D, F). We believe that the variable phenotype is a result of variable expression of the fusion protein, which has been documented for the pDXA vector backbone (47),
and which we have observed by fluorescence microscopy for the pTX-GFP vector (data not shown).

**The localization of GFP-Atg8 in macroautophagy mutants**

Mutations in the genes of the Atg5-Atg12 conjugation system affect the localization of the GFP-Atg8 fusion protein in *D. discoideum*. We wanted to know whether mutations in the *Dictyostelium* orthologues of macroautophagy genes encoding components of signaling complexes, like Atg1 or Atg6, also affect the distribution of GFP-Atg8. We transformed *atg1-1, atg6* and *atg8* amoebae with an expression vector containing GFP-Atg8 under control of the constitutive *actin15* promoter. In the parent DH1, we observe diffuse cytoplasmic fluorescence, small dots, and semi-circular and round structures (Figure 8A, B) that are presumably the isolation membranes and autophagosomes, respectively, described in mammalian cells (48). In the *atg1-1* mutant, GFP-Atg8 labels a similar structure to that previously observed for strains bearing mutations in the Atg5-Atg12 conjugation system. However, the labeled structure is even larger, and appears diffuse with brighter areas in the *atg1-1* mutant (Figure 8C, D). Often, in addition to the larger structure, a few small diffuse dots are observed in the cytoplasm of the cell (Figure 8D). In contrast, in the *atg6* mutant we observe diffuse cytoplasmic localization and small dots similar to those seen in DH1. However, it appears that there are fewer of these dots than in wild-type (Figure 8E), and often we observe a greater proportion of *atg6* amoebae containing semicircular fluorescent structures, which may be isolation membranes (arrowhead and inset in Figure 8F). In the *atg8* strain complemented by expression of the GFP-Atg8 fusion, we see structures that
we interpret as isolation membranes (arrowheads in Figures 8G and H) and near-complete or completed autophagosomes (arrow in Figure 8G), just as in GFP-Atg8-expressing DH1.

We also examined the subcellular localization of CFP-Atg1 expressed from the constitutive actin 15 promoter. We observe diffuse cytoplasmic localization in DH1, atg1-1, atg5-, atg6-, atg7-, and atg8- strains, under both growth and starvation conditions (data not shown).
DISCUSSION

In the present work, we continue our characterization of macroautophagy in the social amoeba *D. discoideum*. We previously identified putative orthologues of budding yeast Atg1, Atg6 and Atg8, and here undertake more detailed analysis of them. The *Dictyostelium* proteins share significant sequence similarity and similar domain organization with their counterparts in *S. cerevisiae, A. thaliana*, and mammals (Figure 1). To confirm the function of these genes, we generated mutations in *atg1, atg6* and *atg8* by insertional mutagenesis. All three mutants have developmental defects consistent with those of *Dictyostelium* macroautophagy mutants such as *atg5* and *atg7* (33). First, development is less robust in plaques on *K. pneumoniae* lawns than on nitrocellulose (NC) filters (Figure 3). Second, *atg6* and *atg8* form multi-tipped aggregates on both NC filters and bacterial lawns, whereas *atg5* and *atg7* form multi-tipped aggregates on NC filters only. The *atg1-1* mutant commonly arrests at the loose mound stage on NC filters (Figure 3B), but we have observed rare progression beyond this arrest to form multi-tipped aggregates (data not shown). Spore production mirrors the severity of the developmental defect (Table 1). The three mutants also display other attributes of macroautophagy mutants: reduced protein degradation during development (Table 2), reduced survival under amino acid starvation (Figure 5), and the ultrastructural features of starving mutant cells, including cytoplasm displaying little evidence of degradation compared to the parental strain (Figure 6). Therefore, we conclude that the *Dictyostelium* *atg1, atg6* and *atg8* genes are genuine orthologues of their budding yeast counterparts.

All of the phenotypes described above are more severe in the *atg1-1* mutant than in *atg6* or *atg8*. The *atg1-1* amoebae die more rapidly when nitrogen-starved, they
degrade even less protein during development than the other mutants, and by TEM they have a denser cytoplasm after 36 hours amino acid starvation. The mild phenotype of atg8\textsuperscript{c} compared to atg1-1 in Dictyostelium parallels the severity of the macroautophagy defect in S. cerevisiae mutants, where aut7\Delta (atg8\Delta) cells survive for longer than atg1\Delta mutants during nitrogen-starvation (29). Consistent with this result, loss of atg8 in yeast does not completely block autophagosome formation, but results in production of very small autophagosomes (29).

Two proteins with homology to S. cerevisiae Atg6 exist in Dictyostelium, which is unique among the organisms in which autophagy has been studied. Both Dictyostelium orthologues are larger than Atg6 in other eukaryotes, and have a unique, extended N-terminus. We show here that atg6 is required for autophagy, whereas the second gene, atg6B, was only identified recently and its function is uncharacterized. The mild autophagic and developmental defects in atg6\textsuperscript{c} may occur because of functional redundancy with atg6B. Interestingly, the pathway-specific components of the two yeast PI3K complexes, Atg14 and Vps38, have yet to be identified in the D. discoideum genome. Therefore, it is possible that the two functions ascribed to Atg6 in yeast and mammalian cells, namely autophagy and protein sorting, are performed by distinct polypeptides in Dictyostelium, obviating the need for specific targeting proteins. A test of this hypothesis would be to abrogate atg6B function and determine whether it is required for autophagy, and determine the requirement (if any) for either Atg6 homologue in protein sorting. Of the remaining components of the autophagy-specific PI3K complex, an orthologue of the yeast class III PI3K, Vps34, has been studied in Dictyostelium (49). The ddvps34 gene is duplicated in the genome, and is essential for
growth. Reducing \textit{ddvps34} mRNA levels by antisense technology results in reduced growth and aberrant development on bacterial lawns, but little effect on growth in liquid medium or development on non-nutrient agar (49). \textit{Dictyostelium} autophagy mutants also display a more severe developmental defect in plaques on bacterial lawns compared to non-nutrient agar or nitrocellulose filters. Whether either of the Atg6 proteins interacts with DdVps34 remains to be tested. A putative orthologue of the remaining PI3K complex component, Vps15/p150, is present in the \textit{Dictyostelium} genome (unpublished observation).

The \textit{atg1-1} amoebae contain profiles indicative of large clusters of vesicles by TEM (Figure 7). We have observed similar profiles in the \textit{atg5} and \textit{atg7} mutants, although in these mutants the clusters are smaller and they are observed less frequently (data not shown). We do not observe these clusters in the \textit{atg6} mutant, suggesting that they are rare or absent in this genetic background. The occurrence and size of these clusters observed by TEM correlates with the frequency and size of the structure that is fluorescently labeled in the \textit{atg1-1}, \textit{atg5} and \textit{atg7} mutants expressing GFP-Atg8 (Figure 8). Therefore, we speculate that these vesicle clusters are the same structures that are labeled with GFP-Atg8. These large vesicle clusters are unique to \textit{Dictyostelium}, and to our knowledge have not been described in budding yeast macroautophagy mutants.

We suggest that the vesicle clusters represent a stalled or unresolved site of autophagosome biogenesis and/or a membrane source for autophagosomes. In the absence of the Atg5-Atg12 conjugation system and Atg1 function, the target for membrane delivery may be absent and/or non-functional, resulting in an accumulation of vesicle traffic either at the membrane source or the membrane target. The absence of the
vesicle clusters in \textit{atg6} cells suggests that Atg6 may function in the generation of these vesicles. These speculations presuppose a spatially distinct source of membrane and cellular address for membrane delivery (the \textit{Dictyostelium} equivalent of the PAS). Our future studies aim to address the subcellular localization of the vesicle clusters as a possible membrane source for autophagosomes, and to characterize the composition of this structure by biochemical isolation.
ACKNOWLEDGEMENTS

We thank all members of the Kessin lab for helpful discussions. We thank Howard A. Shuman for the use of his microscope. This work was supported by NIH grant # GM33136 to RHK. This is Lamont-Doherty Earth Observatory Contribution Number XXXX.
**FIGURE LEGENDS**

**Figure 1.** Amino acid alignment of *Dictyostelium discoideum* (Dd) Atg1, Atg6 and Atg8 orthologues with those from *Arabidopsis thaliana* (At), *Homo sapiens* (Hs) and *Saccharomyces cerevisiae* (Sc). (A) Alignment of Atg1. The kinase domain is delimited with narrow arrowheads. The blasticidin cassette was inserted at the site indicated with a broad arrowhead. The solid and dashed lines indicate the ATP-binding site and the catalytic loop, respectively. Accession numbers: *S. cerevisiae* - P53104, *D. discoideum* - AAO39074, *A. thaliana* - AAK59554. (B) Alignment of Atg6. Only the C-terminal portion of the alignment is shown. Accession numbers: *A. thaliana* - AAK62668, *H. sapiens* - NP_003757, *S. cerevisiae* - Q02948, *D. discoideum* Atg6 - AAO39076. Atg6B sequence was obtained from the Dictybase website (http://dictybase.org/db/cgi-bin/dictyBase/locus.pl?locus=apg6B). (C) Alignment of Atg8. The C-terminal glycine is indicated with an asterisk. The blasticidin cassette was inserted at the site indicated with a broad arrowhead. Accession numbers: *S. cerevisiae* - P38182, *D. discoideum* - AAO39078, *A. thaliana* - AAM70188, *H. sapiens* - NP_073729. Alignments for A and C were performed using the ClustalW 1.8 algorithm at the BCM Search Launcher (http://searchlauncher.bcm.tmc.edu/multi-align/multi-align.html), and at the Protein Information Resource (http://pir.georgetown.edu/pirwww/search/multaln.html) (50) for B. The resulting alignment was shaded using the Boxshade server (http://www.ch.embnet.org/software/BOX_form.html). Identical residues are shaded black and similar residues are shaded grey.
Figure 2. Northern blot analysis of atg1 and atg8 expression. Wild-type or mutant cells were harvested for RNA extraction during multicellular development at the time points indicated (in hours) above each lane. 5 µg total RNA was size fractionated on 1% agarose gels, transferred to nitrocellulose, and hybridized with random primer-labeled DNA probes. Upper panel, northern blot of DH1 and atg1-1 RNA hybridized with a probe to the 5’ 700 bp of the atg1 gene. A truncated ~1.2 kb atg1 transcript is produced in atg1-1, and the transcript levels show similar developmental regulation as in the parent DH1. Lower panel, northern blot of DH1 and atg8- RNA hybridized with a probe to the 3’ 318 bp of the atg8 gene. The atg8 transcript accumulates rapidly in DH1 in response to the initiation of development.

Figure 3. Phenotypes of Dictyostelium macroautophagy mutants. A-C, G-I: Development on nitrocellulose filters. (A) Wild-type cells aggregate and form fruiting bodies at the center of plaques. (B) atg1-1 forms loose mounds that rarely progress further. (G) atg6- forms multi-tipped aggregates that progress to form fruiting bodies with shortened stalks. (H) atg8- also forms multi-tipped aggregates, and these resolve to form sorocarps similar to but smaller than DH1. D-F, J-L: Development in situ on bacterial lawns. The parental strain, DH1, aggregates and forms fruiting bodies (D), whereas atg1-1 does not aggregate (E), and atg6- (J), and atg8- (K) only aggregate at the center of plaques, and not at the periphery when plaques expand. Expressing in-frame fusions of CFP to Atg1 [atg1-1 (act15/cfp-atg1); C and F] or GFP to Atg8 [atg8- (act15/gfp-atg8); I and L] in the respective mutant produces a more significant phenotypic improvement in the case of the complemented atg1-1 mutant.
Figure 4. Autophagy mutations are cell autonomous. Cells expressing GFP from an actin15 promoter were mixed 1:3 with unlabeled wild-type cells, and developed on nitrocellulose filters. Labeled atg6− and atg8− cells are found in the sorocarp (B and C), just as in the parent DH1 (A). atg1-1 cells are found only in the slime sheath discarded by the migrating slug when culmination begins, and in the basal disc (D).

Figure 5. Viability during nitrogen starvation. Amoebae starving in FM medium lacking amino acids were sampled at the indicated time points to determine viability by plaque formation on bacterial lawns. The atg1-1 mutant drops by almost 2 orders of magnitude in number after only 2 days. The atg6− and atg8− strains are as susceptible as the previously characterized atg5− mutant.

Figure 6. Electron microscopy studies of nitrogen-starved amoebae. Parental (A), atg6− (B), atg1-1 (C), atg1-1 (act15/cfp-atg1) (D), atg8− (E) and atg8− (act15/gfp-atg8) (F) amoebae were starved in FM medium lacking amino acids for 36 hours, and examined by transmission electron microscopy. Wild-type cells show significant cytoplasmic degradation, whereas atg1-1 and atg6− amoebae show little evidence of cytoplasmic turnover, similar to that of growing wild-type cells (not shown). Although atg8− shows some evidence of cytoplasmic depletion (D), the cytoplasm in all three mutants is significantly denser than in wild-type cells. Bar: 1 µm.

Figure 7. Profiles of vesicle clusters in atg1-1 amoebae. The amoebae were starved and examined by TEM as in Figure 5. (A) In addition to profiles of RER enclosing mitochondria, the atg1-1 mutant also contains a large cluster of vesicles. Bar: 1 µm. (B) A higher magnification view of the boxed area in (A). Three types of vesicle profiles are observed: larger vesicles with clear lumina (narrow arrow), smaller vesicles with
electron-dense lumina (wide arrow), and tiny vesicles sometimes arrayed linearly (arrowheads). Bar: 0.5 µm.

**Figure 8.** Localization of GFP-Atg8 in live, starving cells. (A, B) In wild-type cells, GFP-Atg8 labels small punctate structures (A) and autophagosomes (arrow in B). In *atg1-1* amoebae (C and D), a single large diffuse structure containing brighter areas is labeled in addition to other smaller diffuse dots (3 are barely visible in D). (E, F) *atg6* are similar to wild-type, except that cells contain fewer dots (E), and profiles of presumed isolation membranes are more common (F, arrow and inset). (G, H) *atg8* (*act15/gfp-atg8*) cells are similar to wild-type, and GFP-Atg8 seems to label autophagosomes (arrow in H) and isolation membranes (arrowheads in G and H). A and E are 2D-projections of a 20-step, 0.5 µm Z-series to show labeled structures in all planes of the cell. Inset in F is magnified 2-fold.
REFERENCES

1. Kessin, R. H. (2001) *Dictyostelium - Evolution, cell biology, and the development of multicellularity.*, Cambridge Univ. Press, Cambridge, UK
2. Tsukada, M., and Ohsumi, Y. (1993) *FEBS Lett* **333**, 169-174
3. Pinan-Lucarre, B., Paolletti, M., Dementhon, K., Coulary-Salin, B., and Clave, C. (2003) *Mol Microbiol* **47**, 321-333
4. Juhasz, G., Csikos, G., Sinka, R., Erdelyi, M., and Sass, M. (2003) *FEBS Lett* **543**, 154-158
5. Melendez, A., Tallozcy, Z., Seaman, M., Eskelinen, E. L., Hall, D. H., and Levine, B. (2003) *Science* **301**, 1387-1391
6. Noda, T., Matsuura, A., Wada, Y., and Ohsumi, Y. (1995) *Biochem Biophys Res Commun* **210**, 126-132
7. Thumm, M., Egner, R., Koch, B., Schlumpberger, M., Straub, M., Veenhuis, M., and Wolf, D. H. (1994) *FEBS Lett* **349**, 275-280
8. Klionsky, D. J., Cregg, J. M., Dunn, W. A., Jr., Emr, S. D., Sakai, Y., Sandoval, I. V., Sibirny, A., Subramani, S., Thumm, M., Veenhuis, M., and Ohsumi, Y. (2003) *Dev Cell* **5**, 539-545
9. Melendez, A., Talloczy, Z., Seaman, M., Eskelinen, E. L., Hall, D. H., and Levine, B. (2003) *Science* **301**, 1387-1391
10. Raught, B., Gingras, A. C., and Sonenberg, N. (2001) *Proc Natl Acad Sci U S A* **98**, 7037-7044
11. Klionsky, D. J., Cregg, J. M., Dunn, W. A., Jr., Emr, S. D., Sakai, Y., Sandoval, I. V., Sibirny, A., Subramani, S., Thumm, M., Veenhuis, M., and Ohsumi, Y. (2003) *Dev Cell* **5**, 539-545
12. Huang, W. P., and Klionsky, D. J. (2002) *Cell Struct Funct* **27**, 409-420
13. Noda, T., and Ohsumi, Y. (1998) *FEBS Lett* **349**, 275-280
14. Kametaka, S., Okano, T., Ohsumi, M., and Ohsumi, Y. (1998) *Biochem Biophys Res Commun* **210**, 126-132
15. Kamada, Y., Funakoshi, T., Shintani, T., Nagano, K., Ohsumi, M., and Ohsumi, Y. (2000) *J Cell Biol* **150**, 1507-1513
16. Abeliovich, H., Zhang, C., Dunn, W. A., Jr., Shokat, K. M., and Klionsky, D. J. (2003) *Mol Biol Cell* **14**, 477-490
17. Kametaka, S., Okano, T., Ohsumi, M., and Ohsumi, Y. (1998) *J Biol Chem* **273**, 22284-22291
18. Kihara, A., Noda, T., Ishihara, N., and Ohsumi, Y. (2001) *J Cell Biol* **152**, 519-530
19. Kim, J., Huang, W. P., Stromhaug, P. E., and Klionsky, D. J. (2002) *J Biol Chem* **277**, 763-773
20. Suzuki, K., Kirisako, T., Kamada, Y., Mizushima, N., Noda, T., and Ohsumi, Y. (2001) *Embo J* **20**, 5971-5981
21. Liang, X. H., Kleeman, L. K., Jiang, H. H., Gordon, G., Goldman, J. E., Berry, G., Herman, B., and Levine, B. (1998) *J Virol* **72**, 8586-8596
22. Liang, X. H., Jackson, S., Seaman, M., Brown, K., Kempkes, B., Hibshoosh, H., and Levine, B. (1999) *Nature* **402**, 672-676
23. Kihara, A., Kabeya, Y., Ohsumi, Y., and Yoshimori, T. (2001) *EMBO Rep* **2**, 330-335
24. Yuan, W., Stromhaug, P. E., and Dunn, W. A., Jr. (1999) *Mol Biol Cell* **10**, 1353-1366
25. Tanida, I., Mizushima, N., Kiyooka, M., Ohsumi, M., Ueno, T., Ohsumi, Y., and Kominami, E. (1999) *Mol Biol Cell* **10**, 1367-1379
26. Shintani, T., Mizushima, N., Ogawa, Y., Matsuura, A., Noda, T., and Ohsumi, Y. (1999) *Embo J* **18**, 5234-5241
27. Mizushima, N., Noda, T., and Ohsumi, Y. (1999) *Embo J* **18**, 3888-3896
28. Mizushima, N., Kuma, A., Kobayashi, Y., Yamamoto, A., Matsubae, M., Takao, T., Natsume, T., Ohsumi, Y., and Yoshimori, T. (2003) *J Cell Sci* **116**, 1679-1688
29. Kirisako, T., Ichimura, Y., Okada, H., Kabeya, Y., Mizushima, N., Yoshimori, T., Ohsumi, M., Takao, T., Noda, T., and Ohsumi, Y. (2000) *J Cell Biol* **151**, 263-276
30. Ichimura, Y., Kirisako, T., Takao, T., Satomi, Y., Shimoni, S., Ishihara, N., Mizushima, N., Tanida, I., Kominami, I., Ohsumi, M., Noda, T., and Ohsumi, Y. (2000) *Nature* **408**, 488-492
31. Kirisako, T., Baba, M., Ishihara, N., Miyazawa, K., Ohsumi, M., Yoshimori, T., Noda, T., and Ohsumi, Y. (1999) *J Cell Biol* **147**, 435-446
32. Abeliovich, H., Dunn, W. A., Jr., Kim, J., and Klionsky, D. J. (2000) *J Cell Biol* **151**, 1025-1034
33. Tanida, I., Tanida-Miyake, E., Ueno, T., and Kominami, E. (2001) *J Biol Chem* **276**, 1701-1706
34. Wang, C. W., Kim, J., Huang, W. P., Abeliovich, H., Stromhaug, P. E., Dunn, W. A., Jr., and Klionsky, D. J. (2001) *J Biol Chem* **276**, 30442-30451
32. Otto, G. P., Wu, M. Y., Clarke, M., Lu, H., Anderson, O. R., Hilbi, H., Shuman, H. A., and Kessin, R. H. (2004) *Mol Microbiol* **51**, 63-72
33. Otto, G. P., Wu, M. Y., Kazgan, N., Anderson, O. R., and Kessin, R. H. (2003) *J Biol Chem* **278**, 17636-17645
34. Sussman, M. (1987) *Methods Cell Biol* **28**, 9-29
35. Kuspa, A., and Loomis, W. F. (1992) *Proc Natl Acad Sci USA* **89**, 8803-8807
36. Manstein, D. J., Schuster, H. P., Morandini, P., and Hunt, D. M. (1995) *Gene* **162**, 129-134
37. Podgorski, G. J., Franke, J., Faure, M., and Kessin, R. H. (1989) *Mol Cell Biol* **9**, 3938-3950
38. White, G. J., and Sussman, M. (1961) *Biochim. Biophys. Acta* **53**, 285-293
39. Anderson, O. R. (1994) *Journal of Eukaryotic Microbiology* **41**, 124-128
40. Straub, M., Bredschneider, M., and Thumm, M. (1997) *J Bacteriol* **179**, 3875-3883
41. Hanks, S. K., and Hunter, T. (1995) *Faseb J* **9**, 576-596
42. Yan, J., Kuroyanagi, H., Kuroiwa, A., Matsuda, Y., Tokumitsu, H., Tomoda, T., Shirasawa, T., and Muramatsu, M. (1998) *Biochem Biophys Res Commun* **246**, 222-227
43. Yan, J., Kuroyanagi, H., Tomemori, T., Okazaki, N., Asato, K., Matsuda, Y., Suzuki, Y., Ohshima, Y., Mitani, S., Masuho, Y., Shirasawa, T., and Muramatsu, M. (1999) *Oncogene* **18**, 5850-5859
44. Hanaoka, H., Noda, T., Shirano, Y., Kato, T., Hayashi, H., Shibata, D., Tabata, S., and Ohsumi, Y. (2002) *Plant Physiol* **129**, 1181-1193
45. Lupas, A., Van Dyke, M., and Stock, J. (1991) *Science* **252**, 1162-1164
46. Schlumpberger, M., Schaeffeler, E., Straub, M., Bredschneider, M., Wolf, D. H., and Thumm, M. (1997) *J Bacteriol* **179**, 1068-1076
47. Levi, S., Polyakov, M., and Egelhoff, T. T. (2000) *Plasmid* **44**, 231-238
48. Mizushima, N., Yamamoto, A., Hatano, M., Kobayashi, Y., Kabeya, Y., Suzuki, K., Tokuhisa, T., Ohsumi, Y., and Yoshimori, T. (2001) *J Cell Biol* **152**, 657-668
49. Zhou, K., Takegawa, K., Emr, S. D., and Firtel, R. A. (1995) *Mol Cell Biol* **15**, 5645-5656
50. Wu, C. H., Yeh, L. S., Huang, H., Arminski, L., Castro-Alvear, J., Chen, Y., Hu, Z., Kourtesis, P., Ledley, R. S., Suzek, B. E., Vinayaka, C. R., Zhang, J., and Barker, W. C. (2003) *Nucleic Acids Res* **31**, 345-347
Figure 2
Figure 5
**TABLE 1**  
*Macroautophagy mutants show reduced sporulation*

| Genotype                  | Sporulation (%)* |
|---------------------------|------------------|
| DH1                       | 100              |
| `atg1-1`                  | 0                |
| `atg6-`                   | 1-8              |
| `atg8-`                   | 1-15             |
| `atg1-1 (act15/cfp-atg1)` | 10-25            |
| `atg8- (act15/gfp-atg8)`  | 10-25            |

* Sporulation refers to the number of spores produced by a strain as a percentage of the number of spores produced by the parent DH1, expressed as the range of values observed for three independent experiments. For each experiment, the average number of spores produced by each strain on three filters was determined.
TABLE 2
Total protein levels after the 24 hour developmental cycle

| Genotype                  | Protein levels (%)* |
|---------------------------|---------------------|
| DH1                       | 55-70               |
| atg1-1                    | 90-95               |
| atg6-                      | 85-90               |
| atg8-                      | 85-90               |
| atg1 (act15/efp-atg1)     | 65-70               |
| atg8 (act15/gfp-atg8)     | 65-80               |

* Protein levels refer to the percentage of total protein measured in growing cells remaining after 24 hours of development on nitrocellulose filters. The range of values obtained from 3 experiments is shown.
Dictyostelium macroautophagy mutants vary in the severity of their developmental defects
Grant P. Otto, Mary Y. Wu, Nevzat Kazgan, O. Roger Anderson and Richard H. Kessin
J. Biol. Chem. published online January 21, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M311139200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC’s e-mail alerts