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Development by Self-Digestion: Molecular Mechanisms and Biological Functions of Autophagy

Review

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Autophagy is the major cellular pathway for the degradation of long-lived proteins and cytoplasmic organelles. It involves the rearrangement of subcellular membranes to sequester cargo for delivery to the lysosome where the sequestered material is degraded and recycled. For many decades, it has been known that autophagy occurs in a wide range of eukaryotic organisms and in multiple different cell types during starvation, cellular and tissue remodeling, and cell death. However, until recently, the functions of autophagy in normal development were largely unknown. The identification of a set of evolutionarily conserved genes that are essential for autophagy has opened up new frontiers for deciphering the role of autophagy in diverse biological processes. In this review, we summarize our current knowledge about the molecular machinery of autophagy and the role of the autophagic machinery in eukaryotic development.

Introduction

The word "autophagy" is derived from the Greek and means to eat ("phagy") oneself ("auto"). Two modes of autophagy that involve dynamic rearrangement of the sequestering membrane have been identified in eukaryotic cells, microautophagy and macroautophagy. These two modes differ with respect to the pathway by which cytoplasmic material is delivered to the lysosome but share in common the final steps of lysosomal degradation of the cargo with eventual recycling of the degraded material. Microautophagy involves the engulfment of cytoplasm directly at the lysosomal surface, by invagination, protusion, and/or septation of the lysosomal limiting membrane. In contrast, macroautophagy involves the formation of cytosolic double-membrane vesicles that sequester portions of the cytoplasm. Fusion of the completed vesicle, an autophagosome, with the lysosome results in the delivery of an inner vesicle or autophagic body into the lumen of the degradative compartment.

In this review, we will focus on macroautophagy (herein referred to as autophagy), an evolutionarily conserved process that occurs in virtually all eukaryotic cells, ranging from yeast to mammals. Whereas the ubiquitin-proteosomal system is the major cellular pathway for the degradation of short-lived proteins, autophagy is the primary intracellular catabolic mechanism for degrading and recycling long-lived proteins and organelles. It occurs as a cellular response to both extracellular stress conditions (e.g., nutrient starvation, hypoxia, overcrowding, high temperature) and intracellular stress conditions (e.g., accumulation of damaged or superfluous organelles and cytoplasmic components) and allows lower eukaryotic organisms such as yeast to survive nutrient starvation conditions by recycling. In mammalian systems, autophagy is thought to be involved in many physiological processes, including the response to starvation, cell growth control, antiaging mechanisms, and innate immunity, whereas the deregulation of autophagy has been proposed to play a role in certain diseases, including cancer, cardiomyopathy, muscular diseases, and neurodegenerative disorders.

For many years, it has been presumed that autophagy is involved in cellular architectural changes that occur during differentiation and development, presumably via its role in organelle and protein turnover. In a review article published in 1966, it was noted by Christian de Duve (1974 Nobel laureate and discoverer of the lysosome) and Robert Wattiaux that autophagy "is enhanced in cells undergoing remodeling in the course of differentiation or other induced changes, as in newborn kidney, lung, intestine, fetal duodenum, metamorphosing insect salivary glands, regressing Mullerian ducts, amphibian erythrocytes, keratinizing skin, and rat prostate after castration" (de Duve and Wattiaux, 1966). Yet, until recently, studies on the role of autophagy in differentiation and development have been confined largely to morphologic correlations. Consequently, definitive proof of a mechanistic role of autophagy in differentiation and development has been lacking.

With the landmark discovery of the autophagy-related (*ATG*) genes in yeast (many of which have counterparts in other organisms), it has now become possible to use genetic approaches in model organisms to pinpoint precise events in differentiation and development that require the cellular autophagic machinery. This review will summarize our current knowledge about the molecular components of the autophagic machinery and the biological functions of this machinery in development in different model organisms as well as highlight the critical unanswered questions in the field.

The Molecular Machinery of Autophagy in Yeast

While the morphology of autophagy was first characterized in mammalian cells, the molecular components of autophagy were, for the most part, initially described in yeast due in large part to the facile application of genetic analyses (reviewed in Reggiori and Klionsky, 2002; Wang and Klionsky, 2003). Several different approaches led to the identification of the *Au*T*opha*G*y-related, or *ATG*, genes (Klionsky et al., 2003). Most of the *ATG* genes were originally identified in *Saccharomyces cerevisiae*, *Hansenula polymorpha*, and *Pichia pastoris*, and their roles in autophagy have been covered in other

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recent reviews (for example, Habibzadegah-Tari and Dunn, 2004; Kiel and Veenhuis, 2004; Noda and Ohsumi, 2004; Stromhaug and Klionsky, 2004). Accordingly, we present a very brief summary of the yeast autophagy proteins as a background for a more detailed discussion of autophagy in differentiation and development in higher eukaryotes.

Analyses of the yeast *atg* mutants have provided a framework for dissecting the autophagic process into distinct steps as illustrated in Figure 1. These include:

1. Induction

The degradative autophagy pathway is induced upon starvation through a mechanism that involves the protein kinase Tor, a negative regulator of autophagy (Schmelzle and Hall, 2000). The starvation-induced kinase, Gcn2, and its downstream targets, eIF2α, and the Gcn4 transcriptional transactivator, also play a role in autophagy induction (Talloczy et al., 2002). Tor kinase may inhibit autophagy through two general mechanisms (reviewed in Abeliovich, 2004). First, Tor acts in a signal transduction cascade through various downstream effectors to control both translation and transcription. Second, Tor appears to directly or indirectly cause the hyperphosphorylation of Atg13. This highly phosphorylated form of Atg13 has a lower affinity for the Atg1 kinase, and the reduced interaction may inhibit autophagy (Kamada et al., 2000). Atg1 and Atg13 are part of a putative complex (Figure 1); the target of Atg1, however, has not been identified and the functions of most of the other components of the complex are not known. Of note, both the regulatory kinases, Tor and Gcn2, and the Atg1 component of the induction complex play a conserved role in autophagy in higher eukaryotes (reviewed in Abeliovich, 2004; Codogno and Meijer, 2004; Petiot et al., 2002).

2. Cargo Selection and Packaging

In *S. cerevisiae*, many of the components of the autophagic machinery are used for a specific and constitutive biosynthetic transport route termed the cytoplasm to vacuole targeting (Cvt) pathway (reviewed in Stromhaug and Klionsky, 2004). The Cvt pathway is one example of how autophagy can become a specific process through utilization of additional components that are involved in the recognition and packaging of cargo. The Atg11 and Atg19 proteins are not required for autophagy, but play critical roles in the delivery of biosynthetic cargo such as the hydrolase aminopeptidase I to the vacuole (Figure 1). There is as of yet no evidence for the Cvt pathway in any organism other than *S. cerevisiae*. Accordingly, homologs of *ATG11* and *ATG19* have not been identified in higher eukaryotes (Table 1).

3. Vesicle Nucleation

The preautophagosomal structure (PAS) may act as the site of vesicle formation during both autophagy and the Cvt pathway (Kim et al., 2002; Suzuki et al., 2001). This structure, and the entire process of vesicle nucleation, is one of the least understood aspects of autophagy. Unlike vesicle formation throughout the endomembrane system (involving the endoplasmic reticulum, Golgi complex, etc.), autophagic vesicles do not appear to bud off from preexisting organelles. Rather, the vesicle is thought to form de novo. An ongoing question, however, concerns the source of the vesicle membrane. The most common consensus is that the endoplasmic reticulum plays a significant role in supplying membrane during vesicle formation, although the mechanism that would be required to divert a portion of the endoplasmic reticulum away from its normal function and into the process of sequestering vesicle formation is not clear (reviewed in Fengsrud et al., 2004). One set of proteins that may play a fundamental role in vesicle nucleation is the phosphatidylinositol (PtdIns) 3-kinase complex I, which includes the PtdIns 3-kinase Vps34, along with Vps15, Vps30/Atg6, and Atg14 (Kihara et al., 2001). The transmembrane protein Atg9 also appears to act early in the process of vesicle formation. Atg9, Vps15, Vps34, and Vps30/Atg6 (herein referred to as Atg6) are conserved in higher eukaryotes, whereas Atg14 is not (Table 1).

4. Vesicle Expansion and Completion

The majority of identified proteins that play a role in the autophagy and Cvt pathways act at the step of vesicle formation. Included in this group are two sets of components involving ubiquitin-like (Ubl) proteins that participate in novel conjugation reactions (reviewed in Ohsumi, 2001). Atg8 is a Ubl that undergoes proteolytic processing prior to modifying the lipid phosphatidylethanolamine. Atg12, a second Ubl, is covalently attached to Atg5. The purpose of the conjugation reactions is not known. However, one of the major questions concerning sequestering vesicle formation is what supplies the driving force for deformation/curvature of the membrane. In most vesicle budding processes, a central role is played by protein components that form a transient coat. The Atg12-Atg5 proteins are the best candidates for a transient coat complex involved in autophagy. Interestingly, almost all of the components that act at this stage of the pathway have orthologs in at least some higher eukaryotes (Table 1).

5. Retrieval

Most characterized protein targeting pathways utilize a retrograde trafficking process to retrieve certain components for reutilization. Recent evidence suggests that this type of recycling may also occur during autophagy (Reggiori et al., 2004). With the exception of Atg8, other autophagy proteins that are thought to act at the stage of vesicle formation are not associated with the completed autophagosome, suggesting that they are retrieved at some point prior to, or upon, vesicle completion. (The association of Atg8 with the completed autophagosome has rendered it a useful marker for autophagy not only in yeast, but also in higher eukaryotes [Kabeya et al., 2000; Suzuki et al., 2001; Kim et al., 2002; Melendez et al., 2003; Mizushima et al., 2003].) An example of autophagy protein retrieval is seen with Atg9, the only transmembrane protein involved in vesicle formation. The recycling of Atg9 requires the action of Atg2 and Atg18 (Figure 1). Atg18 and, as noted above, Atg9 have orthologs in higher eukaryotes (Table 1), suggesting that this type of retrieval pathway may be evolutionarily conserved.

6. Vesicle Targeting, Docking, and Fusion

It is essential to regulate the timing of vesicle fusion with the vacuole. Molecular genetic studies have indicated that the machinery required for the fusion of autophagosomes and Cvt vesicles with the vacuole includes the SNARE proteins Vam3, Vam7, Vti1, and Ykt6; the NSF, SNAP, and GDI homologs Sec17, Sec18, and Sec19; the rab protein Ypt7; and members of the class

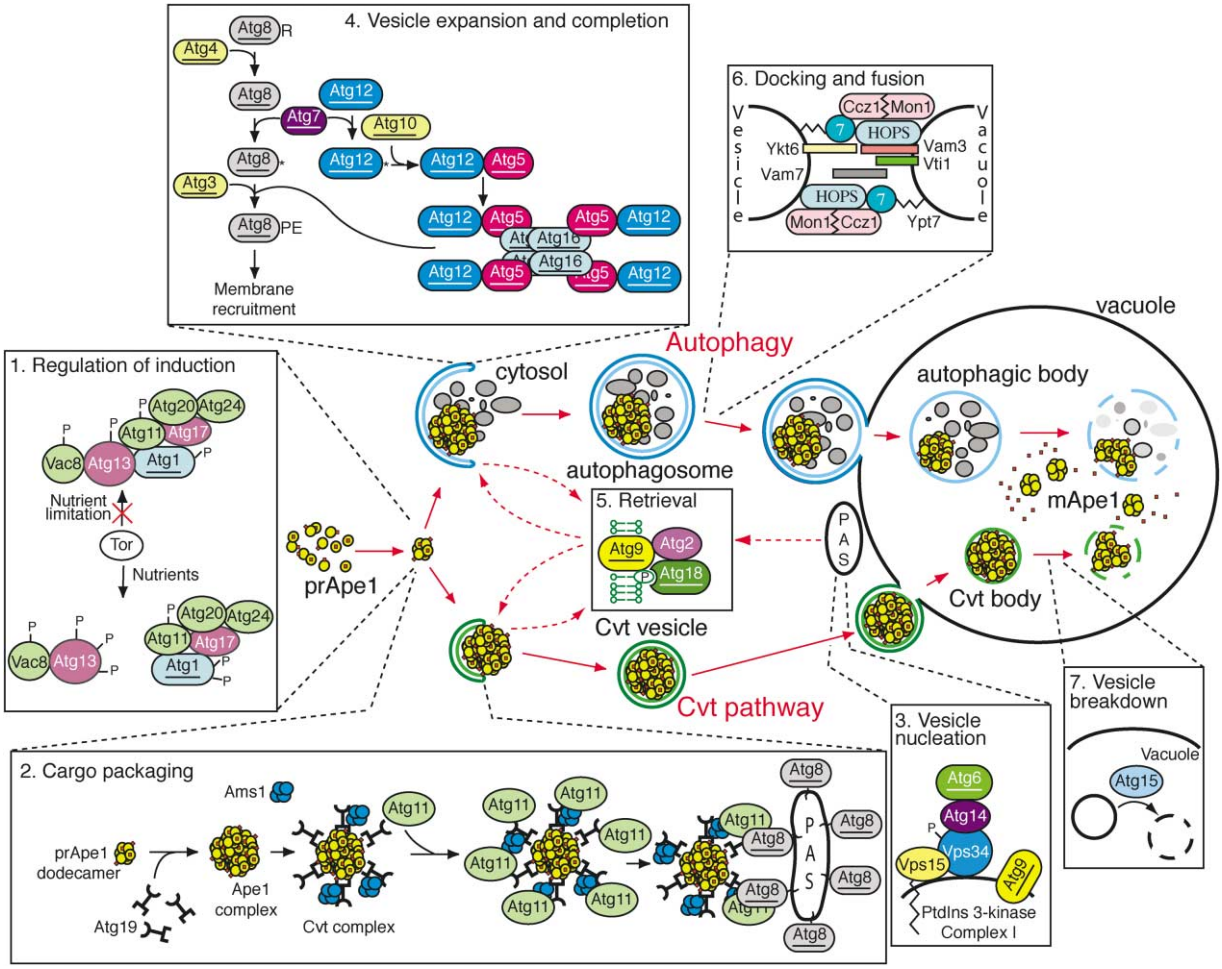

Figure 1. Schematic Representation of the Autophagy and Cytoplasm to Vacuole Targeting Pathways in Yeast

The two processes can be divided into several discrete steps as indicated. Proteins that are involved in each step are shown in the insets. Most of the molecular machinery overlaps between the two pathways, except that the cytoplasm to vacuole targeting (Cvt) pathway uses components that are required for packaging of specific cargo. In addition, some factors that interact with Atg1 are specific for only one pathway. Proteins that have been shown to have functional orthologs in higher eukaryotes are shown in oblong boxes with the protein name underlined. The major steps of autophagy are as follows:

1. Regulation of induction. Tor kinase and downstream effectors regulate induction of autophagy and conversion between autophagy and the Cvt pathway. Tor directly or indirectly causes hyperphosphorylation of Atg13, resulting in reduced affinity for Atg1 kinase. Modulation of Atg1 kinase activity may result in a conversion between the two pathways. Other proteins that interact with the putative Atg1 complex are shown. Proteins in green are required only for the Cvt pathway, and proteins in purple are required only for autophagy.

2. Cargo packaging. The Cvt pathway and peroxisome degradation are specific autophagic processes requiring recognition and packaging of cargo. During the Cvt pathway, dodecamers of precursor aminopeptidase I (Ape1) form into large complexes that bind the receptor Atg19. Subsequent interaction with Atg11 brings the cargo into contact with the ubiquitin-like protein Atg8 and allows inclusion into the sequestering vesicle that is formed from the preautophagosomal structure (PAS).

3. Vesicle nucleation. The yeast PtdIns 3-kinase, Vps34, forms part of the core machinery of two distinct lipid kinase complexes. Complex I is required for the Cvt and autophagy pathways and generates PtdIns(3)phosphate at the PAS.

4. Vesicle expansion. Two ubiquitin-like (Ubl) proteins are required for autophagy-related pathways. Atg8 undergoes two posttranslational processing events resulting in conjugation to phosphatidylethanolamine (PE) and recruitment to the PAS membrane. The Atg7 protein is homologous to the E1 ubiquitin activating enzyme and is required for activation of Atg8 and the second Ubl protein Atg12. The C-terminal glycine of Atg12 is linked to an internal lysine of Atg5. The Atg12-Atg5 conjugate binds Atg16, and oligomerization of Atg16 allows the formation of a multimeric complex. Atg4 is a cysteine protease, while Atg3 and Atg10 are analogs of E2 ubiquitin conjugating enzymes.

5. Retrieval. Atg8 is the only structural protein known to remain with the completed autophagosome or Cvt vesicle. Other peripheral membrane components involved in vesicle formation presumably cycle on and off the membrane, while the integral membrane protein Atg9 is removed through a specific retrieval process involving Atg2 and Atg18.

6. Docking and fusion. The components required for fusion of the autophagosome or Cvt vesicle with the vacuole are common to all pathways that terminate at the vacuole. Two components, Ccz1 and Mon1, have recently been shown to act at the tethering or docking stage that brings the vesicles into proximity with the vacuole prior to fusion (Wang and Klionsky, 2003).

7. Vesicle breakdown. The vacuole is the terminal site for many subcellular trafficking processes, yet little is known about turnover of membranes in this compartment. Atg15 is a putative lipase that traffics to the vacuole through the multivesicular body pathway, and that is required for intralumenal breakdown of Cvt and autophagic bodies.

Table 1. Orthologs of Yeast Autophagy-Related Genes in Higher Eukaryotes

Gene Designation									
ATG	*At*[a]	*Ce*	*Dd*	*Dm*	*Hs*	*Mm*	*Rn*	References[c]	Protein Characteristics
Regulation of induction									
1	---	*unc-51*	*DdATG1*	---	---	---	---	1–3	Protein kinase
Vesicle nucleation									
6	---	*bec-1*	*DdATG6*	---	*beclin 1*	*beclin 1*	---	1; 2; 4–7	Component of PtdIns 3-kinase complex
Vesicle expansion and completion									
3	---	---	---	*DrAUT1*	*hAPG3*	---	---	8–10	E2-like enzyme conjugates PE to Atg8
4	---	---	---	*APG4/AUT2*	---	*APG4B*	---	11–13	Cysteine protease cleaves at C terminus of Atg8
5	---	---	*DdATG5*	---	*hAPG5*	*APG5*	---	14–17	Conjugated to Atg12 through internal lysine
7	*AtAPG7*	*M7.5*	*DdATG7*	---	*HsGSA7* *hAPG7*	*mAPG7*	---	1; 15; 18–21	E1-like enzyme activates ubiquitin-like proteins Atg8 and Atg12
8	---	*lgg-1*	*DdATG8*	---	*MAP1LC3*[b]	*mAPG8* *APG8L*	*LC3*	1; 2; 12; 13; 22; 23	Ubiquitin-like protein conjugated to PE
10	---	---	---	---	---	*mAPG10*	---	24; 25	E2-like enzyme conjugates Atg12 to Atg5
12	---	---	*DdATG12*	---	*hAPG12*	*APG12* *mAPG12*	---	14–16; 26; 27	Ubiquitin-like protein conjugated to Atg5
16	---	---	---	---	---	*APG16L*	---	28; 29	Component of Atg12-Atg5 complex
Recycling									
9	*AtAPG9*	---	---	---	---	---	---	30; 31	Integral membrane protein
18	---	*F41E6.13*	---	---	---	---	---	1; 32	Peripheral membrane protein, binds PtdIns(3)P

[a] Only genes that have been mutated and shown to function in autophagy or that have been shown to interact with other autophagy-related proteins in published papers have been included in this table. The abbreviations are: *At, Arabidopsis thaliana*; *Ce*, *Caenorhabditis elegans*; *Dd, Dictyostelium discoideum*; *Dm, Drosophila melanogaster*; *Hs, Homo sapiens*; *Mm*, *Mus musculus*; *Rn, Rattus norvegicus,* PE, phosphatidylethanolamine.

[b] There are three homologs of human *MAP1LC3* that are designated A, B, and C.

[c] (1) Melendez et al., 2003; (2) Otto et al., 2004; (3) Matsuura et al., 1997; (4) Liang et al., 1999; (5) Kametaka et al., 1998; (6) Qu et al., 2003; (7) Yue et al., 2003; (8) Juhasz et al., 2003; (9) Tanida et al., 2002b; (10) Ichimura et al., 2000; (11) Thumm and Kadowaki, 2001; (12) Hemelaar et al., 2003; (13) Kirisako et al., 2000; (14) Mizushima et al., 2001; (15) Otto et al., 2003; (16) Mizushima et al., 1998a; (17) Kametaka et al., 1996; (18) Doelling et al., 2002; (19) Tanida et al., 2001; (20) Yuan et al., 1999; (21) Kim et al., 1999; (22) He et al., 2003; (23) Tanida et al., 2002c; (24) Mizushima et al., 2002; (25) Shintani et al., 1999; (26) Tanida et al., 2002a; (27) Mizushima et al., 1998b; (28) Mizushima et al., 2003; (29) Mizushima et al., 1999; (30) Hanaoka et al., 2002; (31) Noda et al., 2000; (32) Guan et al., 2001.

C Vps/HOPS complex (reviewed in Wang and Klionsky, 2003). While it is speculative at this time, it is possible that the presumed coat proteins function in part to conceal the fusion machinery until the sequestering vesicle is completed, thus providing a means of regulating the timing of fusion.

7. Vesicle Breakdown

To successfully recycle cytoplasmic components, it is necessary to break down the single-membrane autophagic body that results from fusion of the double-membrane autophagosome with the vacuole. The vesicle lysis step is known to depend on an acidic pH of the vacuole lumen, and proteinase B; however, the action of the latter may be to activate vacuolar zymogens that play a direct role in the breakdown process. Along these lines, the Atg15 protein has homology to a family of lipases and seems likely to function in this manner (Teter et al., 2001).

The Molecular Machinery of Autophagy in Higher Eukaryotes

Given the high degree of evolutionary conservation of autophagy genes, the advances made in the study of yeast autophagy have formed the foundation for the identification of the molecular machinery and biological functions of the autophagic machinery in higher eukaryotes. Many (but not all) of the yeast autophagy genes have candidate orthologs in higher eukarytoes, and a number of these candidate orthologs now have proven roles in autophagy in plants, nematodes, slime mold, flies, and mammals (see underlined Atg proteins in Figure 1 and see Table 1). Furthermore, inactivation of these orthologs in higher eukaryotes has revealed not only conservation of autophagy function, but also potentially important roles for the autophagic machinery in many aspects of development, including normal reproductive growth, stress-induced adaptations, aging, and, more preliminarily, in cell death and cell growth control (Table 2).

Evolutionarily conserved components of the yeast autophagy machinery have been identified in virtually all of the stages of general autophagy described in the previous section in yeast (see Figure 1, Table 1, Table 2). A component of the autophagy induction complex, the serine-threonine kinase Atg1, is required for autophagy and development in *C. elegans* and *Dictyostelium*.

Table 2. Summary of Known Effects of *ATG* Gene Mutations on Differentiation and Development

Organism	Gene Designation in Organism	Yeast *ATG* Ortholog	Mutant Phenotype	Reference(s)[a]
S. cerevisiae	most *ATG* genes		defective sporulation	1
A. thaliana	*AtAPG7*	*ATG7*	premature leaf senescence, enhanced chlorosis	2; 3
	AtAPG9	*ATG9*	premature leaf senescence, enhanced chlorosis	2; 3
D. discoideum	*DdATG1*	*ATG1*	defective fruiting body formation	4
	DdATG5	*ATG5*	" " " "	5
	DdATG6	*ATG6*	" " " "	4
	DdATG7	*ATG7*	" " " "	5
	DdATG8	*ATG8*	" " " "	5
D. melanogaster	*CG10967*	*ATG1*	larval/pupal lethality	6
	DrAUT1	*ATG3*	" "	7
	APG4/AUT2	*ATG4*	modifies Notch signaling pathway mutants	8
C. elegans	*unc-51*	*ATG1*	defective dauer development	9
	*bec-1**	*ATG6*	" " "	9
	*M7.5**	*ATG7*	" " "	9
	*lgg-1**	*ATG8*	" " "	9
	*F41E6.13**	*ATG18*	" " "	9
	*bec-1**	*ATG6*	larval lethality	9
	*lgg-1**	*ATG8*	" "	9
	*F41E6.13**	*ATG18*	" "	9
	*bec-1**	*ATG6*	decreased life span extension	9
M. musculus	*beclin 1*	*ATG6*	embryonic lethality; increased tumor formation (in $^{+/-}$ mice)	10; 11

* = RNAi mutation; phenotype varies according to dose of RNAi; doses used to assess dauer development and life span extension do not affect reproductive development (see Melendez et al., 2003).

[a] (1) Deutschbauer et al., 2002; (2) Doelling et al., 2002; (3) Hanaoka et al., 2002; (4) Otto et al., 2003; (5) Otto et al., 2004; (6) Spralding et al., 1999; (7) Juhacz et al., 2003; (8) Thumm and Kadowaki, 2001; (9) Melendez et al., 2003; (10) Yue et al., 2003; (11) Qu et al., 2003.

A component of the PtdIns 3-kinase complex, Atg6, involved in vesicle nucleation, is known to be involved in autophagy and development in *C. elegans*, *Dictyostelium*, and mice. Several components of the two novel protein conjugation systems have been identified in higher eukaryotes, including Atg3, Atg4, Atg7, and Atg8 in the Atg 8 system, and Atg5, Atg7, Atg10, Atg12, and Atg16 in the Atg12 system. Some of these components, including Atg5, Atg7, and Atg8, have been shown to function in development in *Arabidopsis*, *C. elegans*, and/or *Dictyostelium*. Furthermore, the integral membrane protein Atg9 and the peripheral membrane protein Atg18 that are involved in retrieval play a role in autophagy and development in *Arabidopsis* and *C. elegans*, respectively.

Despite the striking evolutionary conservation of biochemical and autophagy functions of several yeast autophagy genes in higher eukaryotes, it is also important to consider possible differences between the yeast autophagic machinery and the autophagic machinery in higher eukaryotes. Components of the autophagic machinery may function in cellular pathways other than autophagy (in yeast or in higher eukaryotes), and these precise roles may differ in different organisms. For example, the component of the Class III PtdIns 3-kinase complex, Atg6, was originally discovered in yeast as a protein required for vacuolar protein sorting (Vps30) (Seaman et al., 1997), but it is not clear that this function is conserved in Atg6 orthologs in higher eukaryotes (Melendez et al., 2003). A genetic null or RNAi mutation of the *C. elegans* ortholog of yeast *ATG1*, *unc-51*, results in an uncoordinated phenotype (Ogura et al., 1994); however, this phenotype is not observed with other RNAi mutations of *C. elegans* autophagy genes (Melendez et al., 2003), suggesting a distinct additional role for *unc-51* in axonal elongation. Some autophagy genes may participate in other pathways that regulate development which are present in higher eukaryotes, but not in yeast. Two examples include the identification of Beclin 1 (mammalian ortholog of yeast Atg6) as a protein that interacts with antiapoptotic members of the Bcl-2 family (Liang et al., 1998) and the identification of *Drosophila* Atg4 in a genetic screen for modifiers of developmental phenotypes of Notch signaling mutants (Thumm and Kadowaki, 2001).

Of note, functional redundancy as well as functional divergence has also been observed for components of the autophagic machinery in higher eukaryotes. As an example of functional redundancy, two homologs appear to exist for yeast *ATG4* in *Drosophila*, and both of these can complement autophagy in *atg4* mutant yeast (Thumm and Kadowaki, 2001). As an example of functional divergence, many higher eukaryotic organisms have multiple homologs of yeast *ATG8*, although only one of these may function in autophagy in each organism (e.g., *lgg-1* but not *lgg-2* in *C. elegans* [Melendez et al., 2003], and LC-3 but not GATE-16 and GABARAP in mammalian cells [Kabeya et al., 2000]). Furthermore, it also seems likely that many of the molecular functions of yeast autophagy genes may be executed in higher eukaryotes by genes with no apparent structural homology to components of the yeast autophagic machinery.

It is tempting to speculate that this is the case not only for the "missing" components of putative complexes that include known Atg protein orthologs in higher eukaryotes, but also for molecular determinants of specificity for cargo recognition, such as those identified in the yeast Cvt pathway and the yeast pathway for selective degradation of peroxisomes. Until we understand the specificity, if any, of cargo recognition in higher eukaryotes, it will be difficult to fully unravel the underlying mechanisms by which autophagy mediates different biological processes.

In interpreting the developmental phenotypes of organisms with autophagy gene mutations, it is important to keep these considerations in mind. While such considerations certainly underscore the need for caution, there is also increasingly convincing evidence for an important role for autophagy genes in a wide spectrum of differentiation and developmental processes. This evidence will be discussed in the following sections.

Autophagy in Stress-Induced Differentiation and Development

In both yeast and mammalian cells, nutrient limitation, high population density, and increased temperature are potent inducers of autophagy (Dunn, 1994; Gordon et al., 1987). Not surprisingly, differentiation and developmental events that require the autophagic machinery are triggered by some of these same forms of environmental stress. From an evolutionary perspective, autophagy may have originally arisen as a mechanism to protect unicellular organisms against starvation or other forms of environmental stress. However, the stimulus to degrade organelles may have created a cellular milieu favoring the acquisition of other evolutionary advantages, including the ability to undergo differentiation and development. Differentiation and development both require cells to undergo significant phenotypic changes and, presumably, must entail a mechanism for the breakdown and recycling of obsolete cellular components. Thus, it may not be a coincidence that certain differentiation and developmental processes, especially in lower eukaryotic organisms, are triggered by environmental stressors that positively regulate autophagy. Rather, it seems likely that autophagy is mechanistically involved in these processes.

This hypothesis is supported by genetic data in three model systems, including *S. cerevisiae*, *Dictyostelium discoideum*, and *C. elegans*, that demonstrate an essential role for *ATG* genes in stress-induced differentiation and development (Figure 2). Autophagy genes are dispensable for vegetative growth in yeasts, but are required for survival during starvation and for starvation-induced differentiation (Figure 2; Table 2). During nutrient-limiting conditions, yeasts undergo a differentiation process of sporulation, in which haploid gametes are produced by two meiotic divisions from a parental diploid (Figure 2). This process has selective advantages, since the spores are relatively resistant to environmental conditions and are also able to undergo sexual reproduction (meiotic variation). Yeasts with homozygous mutations in *ATG* genes are unable to sporulate (Tsukada and Ohsumi, 1993), and some *ATG* genes (e.g., *AUT1, AUT3*) were originally isolated in screens for genes that complement sporulation defects (Schlumpberger et al., 1997; Straub et al., 1997). Genes involved in autophagy and vesicle transport were also found to be an important class of molecules required for efficient sporulation in a whole-genome analysis of sporulation mutant strains of *S. cerevisiae* (Deutschbauer et al., 2002).

Upon nutrient deprivation or overcrowding, the soil amoeba, *Dictyostelium discoideum*, undergoes a complex developmental cycle to produce a multicellular organism (Kessin, 2001) (Figure 2). Starving amoebae aggregate using a cyclic AMP signaling system to form mounds of about 100,000 cells, and each mound undergoes morphogenesis to produce a mature fruiting body composed of a sphere of spores supported by a cellular stalk. This multicellular developmental process is postulated to have a selective advantage by protecting developing spores from the noxious environment of the soil and by facilitating maximal dispersal of the organism when the spores germinate. Insertional mutagenesis of the *Dictyostelium* orthologs of yeast *ATG5*, *ATG6*, *ATG7*, and *ATG8* has minimal effects on cellular viability and growth in the presence of nutrients, but results in loss of cellular viability and aberrant multicellular development during starvation (Otto et al., 2003, 2004) (Figure 2; Table 2). While the severity of the developmental defect varies somewhat according to the specific *ATG* gene mutation and the growth substrate, most mutants are arrested somewhere between the mound and slug stage. They form multi-tipped aggregates that fail to complete normal morphogenesis and instead become aberrant fruiting bodies with thickened stalks and few viable mature spores. This abnormal phenotype cannot be rescued by codevelopment with wild-type cells in a chimeric organism, indicating that the autophagy mutations are cell autonomous.

Autophagy genes are also essential for another stress-induced developmental process, the formation of dauer larvae in the nematode, *Caenorhabditis elegans*. Under conditions of limited food, high population density, increased temperature, or exposure to "dauer" pheromone, *C. elegans* nematodes reversibly arrest in an alternative third larval stage, the dauer diapause, that is metabolically and morphologically specialized to survive in an unfavorable environment (Riddle, 1997) (Figure 2). Dauer larvae undergo radial constriction and elongation of the body and pharynx, have increased fat storage, are resistant to SDS detergent treatment, survive long-term in a dauer state, and resume normal reproductive development when returned to favorable conditions. Recently, it has been shown that autophagy is enhanced during dauer development in lateral hypodermal seam cells (Melendez et al., 2003), a cell type that is responsible for formation of the specialized dauer cuticle and radial constriction of the body (Albert and Riddle, 1988; Liu and Ambros, 1989; Sulston and Horvitz, 1977). Furthermore, inactivation of *C. elegans atg* genes (e.g., *C. elegans* orthologs of yeast *ATG1*, *ATG6*, *ATG7*, *ATG8*, and *ATG18*) does not affect dauer initiation, but blocks morphogenetic and physiological features of dauer development, inhibits seam cell autophagy, and prevents dauer survival (Melendez et al., 2003).

While these data support a role for autophagy genes in stress-induced differentiation and development in both unicellular and multicellular organisms, many important

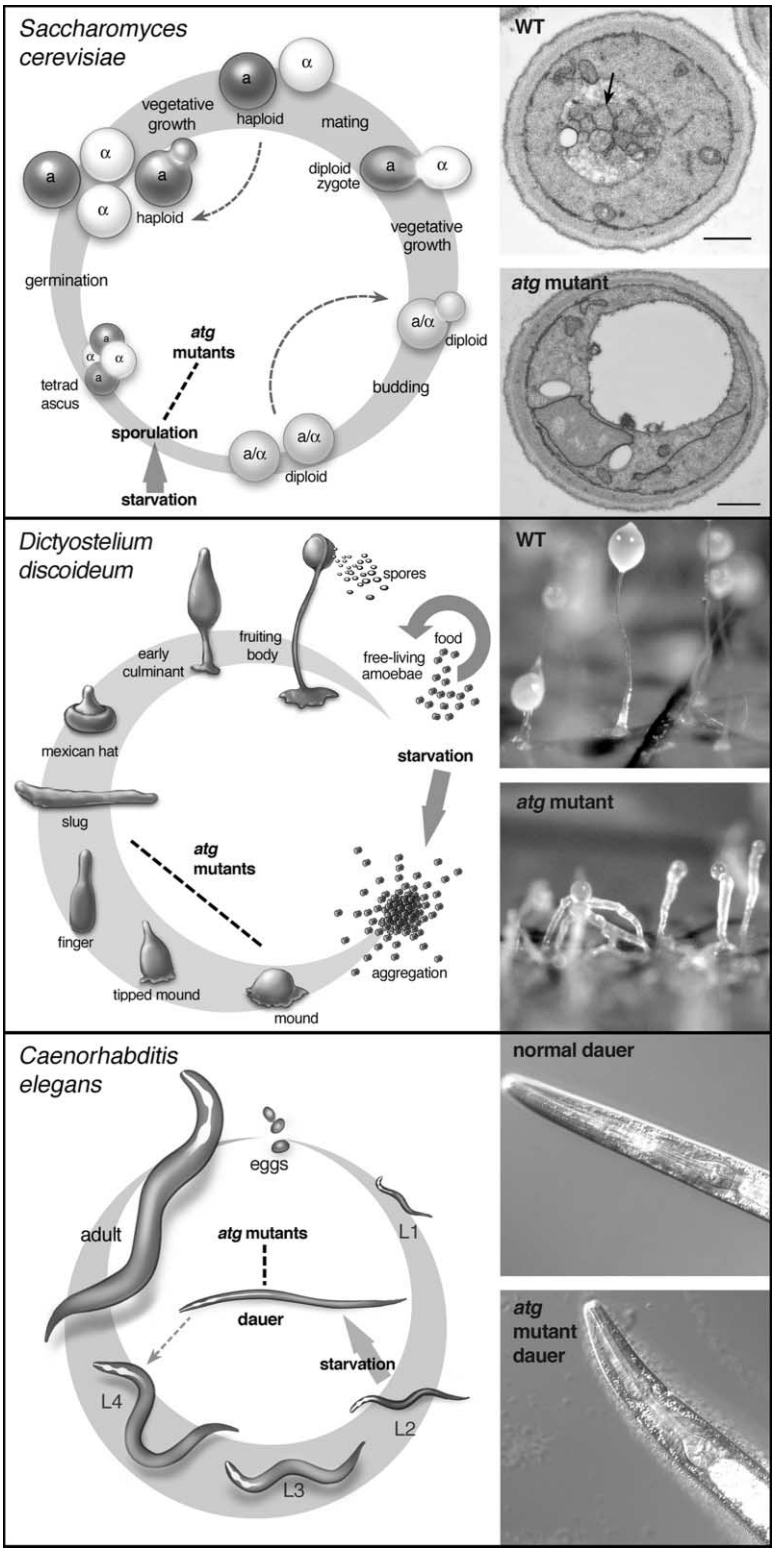

Figure 2. Autophagy Gene Mutations Block Stress-Induced Differentiation and Development in *S. cerevisiae*, *D. discoideum*, and *C. elegans*

Shown are the life cycles for each organism and representative images of wild-type and autophagy gene (*atg*) mutant organisms. In *Saccharomyces cerevisiae*, starvation induces a differentiation process known as sporulation. *Atg* mutations block starvation-induced sporulation in diploid yeast. Shown are electron micrographs of a wild-type (WT) haploid yeast undergoing nitrogen starvation-induced autophagy, and an *atg6* null mutant yeast that is deficient in nitrogen starvation-induced autophagy. Autophagy is recognized by the presence of autophagic bodies (arrow) within the central vacuole of the yeast. Scale bars, 0.5 μm. Reproduced with permission from Talloczy et al. (2002).

In *Dictylstelium discoideum*, either starvation (shown in figure), overcrowding, or high temperature induces a multicellular developmental process, culminating in the formation of a mature fruiting body. *Atg* mutations block this multicellular developmental process, usually somewhere between the mound and slug stage of the life cycle. Shown are photomicrographs of normal fruiting body formation in wild-type (WT) *Dictyostelium discoideum* and defective fruiting body formation in *atg7* mutant *Dictyostelium discoideum*. Reproduced with permission from Otto et al. (2003).

In *Caenorhabdiitis elegans*, either starvation (shown in figure), overcrowding, high temperature, exposure to dauer pheromone, or loss-of-function mutations in the transforming growth factor-β, cyclic guanosine monophosphate, and insulin-like signaling pathways result in a reversible developmental arrest in an alternative L3 stage known as the dauer diapause. (Following return to normal growth conditions, dauer nematodes may resume reproductive development; see dotted arrow.) Dauer animals are distinguished morphologically by total body and pharyngeal elongation and constriction. *Atg* mutations result in abnormal dauer development. Shown are photomicrographs of a constricted, elongated pharynx in a normal dauer animal with a loss-of-function mutation in the insulin-like tyrosine kinase receptor, *daf-2(e1370)*, and a pharynx lacking these changes in an *atg6 RNAi; daf-2(e1370)* abnormal dauer animal.

questions remain unanswered about the role of autophagy in these processes. First, what are the extracellular signals that instruct cells to undergo autophagy and how are these extracellular signals transmitted within the cell? The environmental stress stimuli that trigger development are likely to activate conserved autophagy regulatory signals; for example, cyclic AMP, which mediates *Dictyostelium* multicellular development, is a regulator of autophagy in mammalian cells (Holen et al., 1996). In multicellular organisms, it seems likely that stress stimuli act via hormonal signaling pathways that are known to regulate development; for example, in

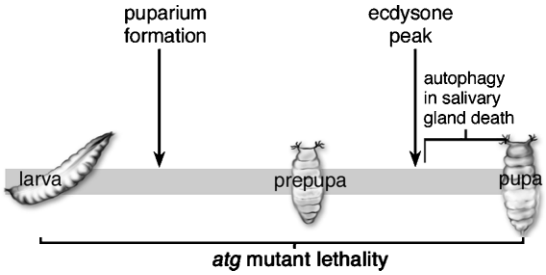

Figure 3. The Timing of Steroid-Regulated Salivary Gland Autophagy and of Autophagy Gene Mutant Lethality in the *Drosophila* Life Cycle

Autophagy is observed in dying salivary gland cells following the ecdysone peak, which occurs 12 hr after puparium formation. Mutation of *Drosophila ATG1* or *ATG3* results in lethality that ranges from the larval to pupal stage.

C. elegans, dauer development is negatively regulated by insulin-like and transforming growth factor-β signaling. Second, in the case of multicellular organisms, what determines the tissue specificity of autophagy induction? Is tissue specificity determined at the level of spatial localization of extracellular ligands and cell surface receptors and/or by cell-type differences in components of the autophagic machinery? At least in *C. elegans*, there is cell type-specific expression of autophagy genes that overlaps with the pattern of constitutive autophagy and dauer-associated increases in autophagy (Melendez et al., 2003), suggesting that cell type-specific differences in autophagy gene expression may govern tissue specificity of autophagy induction. Third, what are the precise mechanisms by which stress-induced autophagy results in the relevant phenotypic changes in unicellular and multicellular organisms? Presumably, the phenotypic changes require the breakdown of obsolete proteins and organelles that are replaced by more highly specialized ones. This process must have an exquisite level of specificity to result in the highly stereotypic phenotypic changes that occur during development. However, the molecular determinants of this presumed specificity have not been defined.

An intriguing further question is whether one can extrapolate from these findings to make predictions about the role of an "autophagy-like process" in stress-induced differentiation and developmental processes in prokaryotic organisms. For example, certain bacteria (e.g., different species of *Bacillus*), when challenged with stresses such as heat and starvation, undergo morphologic changes to produce a dormant cell type known as a spore, that is specialized to survive a wide range of assaults that would normally destroy a vegetative cell (reviewed in Driks, 1999). When reexposed to nutrients, the spore can convert to a growing cell through a process called germination. This process, i.e., the reversible entry into a dormant state specialized to survive harsh conditions following starvation or heat shock, has features reminiscent both of sporulation in yeast and of dauer development in *C. elegans*. Thus, although no apparent structural orthologs of yeast *ATG* genes have been identified in prokaryotes, this parallelism raises the question of whether structurally different but functionally similar components of a degradative "primitive autophagic-like machinery" are also present in sporulating bacteria. Similarly, the social behavior (i.e., fruiting body formation) of *Myxobacteria* has similarities to that of *Dictyostelium discoideum* (reviewed in Shimkets, 1990), suggesting the possible involvement of a process that resembles autophagy in this prokaryotic organism.

Role of Autophagy Genes in Normal Development

The role of the autophagic machinery in development is not confined to developmental changes that occur in adaptation to stress. Deletions, P element insertions, or RNAi-mediated silencing of some autophagy genes results in lethality during early development in different model organisms in normal growth conditions (Table 2). In general, however, the mechanisms of organismal death have not been defined in *ATG* gene mutant animals, and the precise role of autophagy in early development remains unknown. Furthermore, there are technical considerations that limit interpretation of studies done with *ATG* gene mutants in normal development, especially with respect to P element insertion or RNAi phenotypes. Accordingly, caution must be exercised in drawing conclusions about the phenotypes of such mutants. Nonetheless, the prediction—based on existing genetic data as well as the need for organelle and protein turnover in cellular and tissue remodeling—is that "bona fide" null mutations will reveal an essential role for "bona fide" autophagy genes in early development in diverse taxa.

Recent data suggest an essential role for autophagy genes in early development in *Drosophila*, *C. elegans*, and mice. In *Drosophila*, a transgene that mediates RNA interference of *DrAUT1* results in an inability to induce autophagy in fat body cells before pupariation and death during insect metamorphosis (Juhasz et al., 2003) (Table 2; Figure 3). In *C. elegans*, higher concentrations of RNAi directed against *C. elegans* orthologs of yeast *ATG6*, *ATG8*, and *ATG18* (that result in more complete gene silencing than doses used for studies of dauer development) all result in an early developmental arrest during or before the first larval stage (Melendez et al., 2003) (Table 2). In mice, targeted disruption of *beclin 1* (ortholog of yeast *ATG6*) results in early embryonic lethality (Qu et al., 2003; Yue et al., 2003) (Table 2). Death occurs on approximately day E7.5, and is postulated to result from a defect in the visceral endoderm, an exchange system responsible for nutrition and waste product detoxification in the developing embryo (Yue et al., 2003). Interestingly, in both autophagy gene RNAi mutants in *C. elegans* and *beclin 1* null mouse embryos, there is widespread cell death, indicating a possible "pro-survival" role of autophagy during early development.

In plants, autophagy gene mutations (e.g., *AtAPG7* and *AtAPG9*) do not disrupt the completion of the plant life cycle, but they do result in several phenotypic alterations that may result from a defective ability to mobilize nutrients (Doelling et al., 2002; Hanaoka et al., 2002). Both *Atapg7* and *Atapg9* mutant plants appear to undergo normal embryogenesis, germination, cotyledon development, root and shoot elongation, and seed production under nutrient growth conditions. In contrast, during nitrogen or carbon starvation, *Atapg7* and *Atapg9* mutants exhibit significant developmental defects, including enhanced chlorosis (yellowing of leaves due to

a loss of chlorophyll) and accelerated senescence of cotyledons and rosette leaves. Upon close examination, however, more subtle defects are observed even in nutrient-rich conditions in plants with mutations in autophagy genes. *Atapg9* mutant plants exhibit an early flowering phenotype, resulting in earlier senescence and a reduction in the number of rosette leafs (Hanaoka et al., 2002). Following bolting (transition from a rosette form to one with an elongated stem), *Atapg7* mutant plants show acclerated senescence, characterized by premature chlorosis of the mature rosette leaves (Figure 4) (Doelling et al., 2002). Thus, the autophagic machinery appears to play a role even in normal plant development as well as in the successful adaptation to limited environmental nitrogen and carbon sources.

To understand the phenotypes of autophagy mutant plants, it should be noted that (1) the majority of cellular nitrogen is stored in chloroplasts and (2) leaf senescence represents the final stage of leaf development, in which a transition occurs from nutrient assimilation to nutrient remobilization in order to redirect nutrients from older leaves to the production of seeds. As a result of less efficient supply of nutrients through autophagic delivery (especially in the face of limited environmental nutrient supply), autophagy mutants may be forced to undergo enhanced catabolism of chlorophasts (e.g., chlorosis) and accelerated leaf senescence in order to be able to generate essential nutrients to maintain seed production. Although this conserved nutrient recycling function of autophagy in plant development is expected, the phenotypic analysis of autophagy mutant plants does not support earlier predictions (based upon morphologic observations) that autophagy is required for vacuole biogenesis. There are no obvious morphologic defects in the vacuoles in *Atapg7* and *Atapg9* mutant plants, suggesting that the mutant phenotypes are due to blocks in autophagic delivery rather than vacuolar function per se. Enhanced chlorosis in these mutants suggests that other proteolytic systems besides autophagy are required for the degradation of chloroplasts.

Autophagy in Developmental Cell Death: Innocent Bystander or Culprit?

During development, autophagy functions to remodel cells, but also is believed to represent a mechanism by which cells self-destruct. Autophagosomal structures were first noted during developmental cell death in regressing insect salivary glands (Schin and Clever, 1965) and have been observed in dying cells of developing animals of diverse taxa (reviewed in Bursch, 2001; Clarke, 1990; Schweichel and Merker, 1973). The consensus view is that autophagic cell death is activated primarily when the developmental program or homeostatic processes in adulthood require massive cell elimination. In such cases, autophagy has been proposed not only to contribute to cell death, but also to function as a mechanism for "self-disposal" during large-scale tissue histolysis, when the numbers of professional phagocytic or engulfment cells are insufficient to remove dead cell corpses (Yuan et al., 2003). However, as of yet, there is no definitive evidence that autophagy is necessary for developmental cell death or for the clearance of dead cells.

Insect metamorphosis represents perhaps one of the most extreme examples of extensive elimination of cells and tissues during developmental transitions. Accordingly, most of our knowledge about autophagic cell death in development has emerged from studies of steroid-hormone regulated autophagy in the fruit fly, *Drosophila melanogaster* (reviewed in Baehrecke, 2003; Neufeld, 2004). Paradoxically, the death of insect larval tissues has both served as the basis for the distinction of a form of nonapoptotic, programmed cell death known as "autophagic programmed cell death" and, in more recent analyses, has also highlighted the uncertainties that exist in separating apoptotic from autophagic cell death. Ultrastructural studies in the 1960s and the 1970s revealed that an early step in the destruction of most larval tissues is the accumulation of autophagic vacuoles (Nopanitaya and Misch, 1974; Sass and Kovacs, 1974; Schin and Clever, 1965). Based on morphologic criteria, this type of autophagic programmed cell death was thought to be distinct from apoptotic cell death (Schweichel and Merker, 1973). In classical apoptotic or Type I programmed cell death, there is early collapse of cytoskeletal elements but preservation of cytoplasmic organelles until late in the process. In contrast, in autophagic or Type II cell death, there is early degradation of cytoplasmic organelles by autophagy but preservation of cytoskeletal elements until late stages, presumably because of their role in autophagyocytosis (reviewed in Bursch et al., 2004; Clarke, 1990).

In studies of insect larval histolysis, features of both autophagic and apoptotic deaths have been shown to occur in the same cell. In degenerating prothoracic and labial glands of the tobacco hornworm *Manduca sexta*, there are morphologic signs of autophagy but the cells become TUNEL-positive with highly condensed chromatin (Dai and Gilbert, 1997; Jochova et al., 1997). Autophagic cell death of *Drosophila* salivary glands and midguts is also accompanied by markers of apoptosis such as DNA fragmentation. Furthermore, this process can be prevented by expression of the caspase inhibitor bacuolovirus p35 and is associated with transcriptional upregulation of proapoptotic molecules (e.g., *grim*, *reaper*, *hid*, the caspases, *dronc* and *dcp-1,* and the homolog of *ced-4/apaf-1*, *dark/ark*) and downregulation of the apoptosis inhibitor, *diap 2* (Gorski et al., 2003; Jiang et al., 1997; Lee et al., 2003; Lee and Baehrecke, 2001). Genome-wide analysis of dying *Drosophila* salivary glands has also revealed a marked transcriptional increase in a *Drosophila* ortholog of human death-associated protein kinase (*DAPk*) (Lee et al., 2003), a gene encoding a serine/threonine kinase that mediates both membrane blebbing (a feature of apoptosis) and cytoplasmic vesicle formation (a feature of autophagy) in dying human cell lines (Inbal et al., 2002).

The major signal that triggers entry into metamorphosis and activation of autophagic cell death in *Drosophila* is a high pulse titer of the steroid-hormone 20-hydroxyecdysone (ecdysone) that occurs during puparium formation (reviewed in Baehrecke, 2003; Neufeld, 2004; Thummel, 2001) (see Figure 3) The ecdysone-receptor complex (and the nuclear receptor competence factor BFTZ-F1) lead to salivary gland death through the transcriptional activation of a set of "early" genes (*BR-C*, *E74A*, and *E93*) which encode transcription factors that

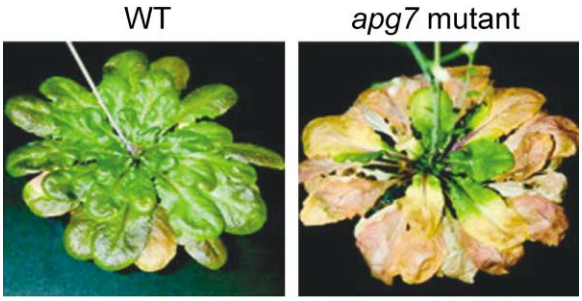

Figure 4. Premature Leaf Senescence in *Atapg7* Mutant Plants

Wild-type or *Atapg7-1* mutant plants were grown for 5 months under a short-day photoperiod on nutrient-rich soil. As discussed in the text, the *Atapg7-1* mutant plants show accelerated senescence. Reproduced with permission from Doelling et al. (2002).

in turn regulate the expression of "late" effector genes that appear to function more directly in apoptotic programmed cell death (reviewed in Baehrecke, 2003; Neufeld, 2004). Mutations in *BFTZ-F1*, *BR-C*, *E74A*, and *E93* all impair salivary gland degeneration during metamorphosis, although at different stages in the cell death process. E93 appears to be especially important in autophagy induction, as *E93* mutants exhibit an early block in autophagic vacuole formation in both salivary gland and midgut cells (Lee et al., 2002; Lee and Baehrecke, 2001). Both SAGE and microarray analyses have revealed that E93 is required for the transcriptional upregulation of many of the genes that are activated in dying salivary glands, including genes involved in autophagy, as well as apoptosis, noncaspase proteolysis, and cytoskeleton remodeling (Gorski et al., 2003; Lee et al., 2003).

These observations illustrate the complexity in deciphering the independent roles of autophagy and apoptosis in developmental cell death in the model organism *Drosophila*. One limitation is that the existing evidence for autophagic cell death in *Drosophila* is based primarily on morphologic evidence of autophagy as well as on the transcriptional upregulation of orthologs of yeast autophagy genes in dying cells. While the theory that autophagy is required for the massive histolysis that occurs during insect metamorphosis is both logical and consistent with the observations described above, it remains possible that autophagy is activated in dying cells (as a cellular strategy to survive stress by recycling intracellular components or as a "clean-up" mechanism) without being part of the death execution process. Consistent with this theory, mutations in the transcription factors *BR-C* and *E74A* block salivary gland death but do not appear to block autophagy (Lee and Baehrecke, 2001). Caspase inhibition completely blocks DNA fragmentation and salivary gland death, but only has mild effects on cellular autophagy (Jiang et al., 1997; Lee and Baehrecke, 2001). In addition, not all ecdysone-induced autophagy is associated with cell death; malphigian tubules, a form of insect nephric system, are extensively remodeled by autophagy during metamorphosis (Ryerse, 1979). Together, these findings suggest that, at least in *Drosophila*, autophagy per se is not sufficient to cause programmed cell death and some elements of the apoptosis program, or other as-of-yet-defined signals, are necessary for death to occur.

Not only does autophagy seem to be insufficient for developmental cell death in *Drosophila*, it also remains unknown whether autophagy is required for developmental cell death. Direct conclusive evidence will require the demonstration that loss-of-function mutations in autophagy genes block developmental cell death. With the identification of *Drosophila* strains with P element insertions in several *ATG* orthologs (Neufeld, 2004), it should now be possible to genetically assess the role of *ATG* genes in steroid-induced programmed cell death. Genetic studies in *C. elegans*, an organism that has a finite number of easily visualized developmental cell death events, are also likely to provide important insights regarding the role of *ATG* genes in programmed cell death.

It should be noted that the uncertainty about the role of autophagy in cell death is not confined to developmental programmed cell death but also extends to cell death that occurs during pathological settings. The best-studied example of this relates to neuronal death in neurodegenerative disorders, including Alzheimer's, Huntington's, and Parkinson's diseases in humans and cerebellar Purkinje degeneration in mice (reviewed in Larsen and Sulzer, 2002; Yuan et al., 2003). In both experimental models and human autopsy brain tissues, autophagosomal accumulation is often observed, leading to the concept that autophagy represents a mechanism of nonapoptotic neuronal cell death that contributes to neurodegeneration. However, increasing evidence indicates that autophagy plays a neuroprotective role by facilitating the removal of newly formed mutant protein aggregates that are characteristic of different neurodegenerative disorders (and that are too large to be removed by the proteasomal system). These two roles are not mutually exclusive, and it is possible that autophagy is initially activated as a housekeeping mechanism but eventually contributes to neuropathology. As in the case of developmental cell death, genetic studies in *ATG* null cells are needed to resolve this controversy.

Autophagy as an Antiaging Mechanism

In the developing organism, autophagy plays an essential role in the cellular and tissue remodeling that occurs during morphogenesis. In many tissues in the adult organism (especially postmitotic cells, e.g., neurons, cardiomyocytes), this function of autophagy is largely obsolete; however, protein and organelle turnover by autophagy plays an essential cellular homeostatic or housekeeping function, removing damaged or unwanted organelles and proteins. For many decades, it has been presumed that this homeostatic function of autophagy represents an antiaging mechanism, perhaps by reducing reactive oxidative species and other toxic intracellular substances that contribute to genotoxic stress. The conserved effects of protein caloric restriction (a dietary inducer of autophagy) on life span extension in diverse species has provided further fuel for this concept (reviewed in Bergamini et al., 2003). Recent biochemical and genetic studies provide more direct evidence for a role of both autophagy regulatory signals and components of the autophagic machinery in antiaging pathways.

The aging process has been analyzed genetically in different multicellular organisms, including *C. elegans*, *Drosophlia*, and mice. The best-characterized pathway is the insulin/insulin-like growth factor endocrine system that is causally linked to aging across taxa. Mutations in the insulin signaling pathway prolong life in worms, flies, and mice, but the most extensively studied model system has been *C. elegans* (reviewed in Bergamini et al., 2003; Guarente and Kenyon, 2000). Several of the components of the insulin-like signaling that affect adult life span in genetic studies in *C. elegans* are now known to regulate autophagy. Downstream of the insulin-like hormonal signal, the plasma-membrane associated Class I PtdIns 3-kinase converts PtdIns(4)P and PtdIns(4,5)P to PtdIns(3,4)P2 and PtdIns(3,4,5)P3, respectively, which bind to pleckstrin homology domains of Akt and its activator, PDK1 (reviewed in Brazil and Hemmings, 2001; Katso et al., 2001; Simonsen et al., 2001). Akt and PDK-1 phosphorylate several other protein kinases, including the autophagy inhibitor Tor. PTEN is a dual protein/lipid phosphatase that dephosphorylates the 3′ position of the Class I PtdIns 3-kinase products and, consequently, downregulates the PtdIns 3-kinase/Atg signaling pathway (reviewed in Blume-Jensen and Hunter, 2001). Loss-of-function mutations in the genes encoding the *C. elegans* insulin-like tyrosine kinase receptor (*daf-2*), the Class I PtdIns 3-kinase (*age-1*), the Akt molecules (*akt1, akt2*), the PDK (*pdk-1*), and the Tor kinase all extend life span (Vellai et al., 2003; reviewed in Guarente and Kenyon, 2000). Conversely, mutations in the *C. elegans* ortholog of PTEN (*daf-18*) suppress the life span extension of *daf-2* and *age-1* mutants.

In human colon carcinoma cells, Class I PtdIns 3-kinase and Akt inhibit autophagy whereas PTEN stimulates autophagy. Thus, signals that extend life span in *C. elegans* are autophagy-stimulatory in mammalian cells whereas signals that shorten life span in *C. elegans* are autophagy-inhibitory (Arico et al., 2001; Petiot et al., 2000). There is also direct evidence that this insulin-like/PtdIns 3-kinase signaling pathway controls autophagy in *C. elegans*, since increased autophagy is observed in nematodes with a loss-of-function mutation in the insulin-like tyrosine kinase receptor, *daf-2* (Melendez et al., 2003). Notably, at least certain components of the autophagic machinery are required for the life span extension in *daf-2* mutant animals; siRNA-mediated interference of the *C. elegans* ortholog of *ATG6* (*bec-1*) blocks the life span extension of *daf-2* mutant animals (Melendez et al., 2003). While further studies with other *atg* gene mutations in *C. elegans* and in other animal model systems will be important to confirm these findings, these data provide preliminary genetic evidence for a role of the autophagic machinery in longevity.

The identification of autophagy as a potential mechanism of life span extension provides a framework for the conceptual integration of the oxidative damage theory of aging and the known stimuli that influence aging in different organisms, including caloric restriction and signaling through the insulin-like signaling pathway in *C. elegans* and *Drosophila*. Although the molecular determinants of cargo recognition have not been defined, autophagy is thought to selectively remove damaged mitochondria (Elmore et al., 2001), a function that is expected to decrease levels of intracellular reactive oxygen species and protect cells against oxidative damage. Many of the long-lived mutants in *C. elegans*, *Drosophila*, and mice are resistant to oxidative stress; mice and rats whose life spans are increased by caloric restriction are resistant to oxidative stress; and overexpression of antioxidant enzymes in *Drosophila* extends life span (Johnson et al., 2000; Lin et al., 1998; reviewed in Guarente and Kenyon, 2000). Conversely, mutations that increase oxidative damage shorten life span in *C. elegans*. As noted above, caloric restriction and loss-of-function mutations in the insulin-like signaling pathway positively regulate autophagy. Thus, the activation of autophagy, leading to increased mitochondrial turnover and decreased oxidative cellular damage, may represent a unifying mechanism by which life span is extended in diverse animals.

Autophagy and Cell Growth Control

An understudied question of fundamental importance for developmental biology is the role of autophagy in cell growth control. In the 1970s, it was first proposed that protein catabolism through autophagy is a determinant of the rate of cell growth (Amenta et al., 1978; Gunn et al., 1977). According to this model, both cell mass and the rate of cell growth is a balance between the amount of protein synthesized and the amount of autophagic protein degradation. Although this model has received little attention in recent years, interest in the role of autophagy in cell growth control has reemerged in light of new biochemical and genetic links between autophagy and the negative regulation of tumorigenesis.

Several different oncogenic signaling molecules, including members of the insulin signaling pathway (e.g., Class I PtdIns 3-kinase, Akt) and members of the Rho and Ras families of GTPases, negatively regulate autophagy in mammalian cells, and the PTEN tumor suppressor positively regulates autophagy (reviewed in Furuya et al., 2004). Furthermore, the autophagy inhibitor, Tor, is an important positive regulator of cell growth in diverse organisms, ranging from *Drosophila* to mice and humans (reviewed in Schmelzle and Hall, 2000). In *Drosophila*, inactivation of Tor signaling, either genetically or by rapamycin treatment, dramatically inhibits cell growth (Neufeld, 2004). In mice, rapamycin reduces neoplasia in animals with upstream mutations that result in aberrant Tor activation (Neshat et al., 2001; Podsypanina et al., 2001). In humans, rapamycin has promising anticancer activity in early phases of clinical trials (reviewed in Huang and Houghton, 2003). Suppression of autophagy has been suggested to be the predominant means by which Tor promotes cell growth in *Drosophila* (Neufeld, 2004), but this is speculative in view of the pleiotropic effects of Tor, including transcriptional and translational regulation. Moreover, it is not known whether autophagy induction contributes to the antitumor effects of rapamycin in mice and humans. Thus, further studies are needed to dissect the role of autophagy regulation in the cell growth control effects of Tor and other oncogenic and tumor suppressor signaling molecules.

The identification of a component of the autophagic machinery, Beclin 1, as a tumor suppressor provides more direct genetic evidence for a role of autophagy in

cell growth control. *Beclin 1* is monoallelically deleted in a high percentage of human breast, ovarian, and prostate cancers (Aita et al., 1999); *beclin 1* gene transfer inhibits cell growth and tumorigenicity of human breast cancer cells (Liang et al., 1999); and *beclin 1* heterozygous disruption in mice results in increased spontaneous tumorigenesis (Yue et al., 2003; Qu et al., 2003). Furthermore, the mammary glands of autophagy-deficient *beclin 1* heterozygous-deficient mice have abnormally high levels of mammary epithelial cell proliferation and abnormal ductal morphogenesis (Qu et al., 2003). It will be important to determine whether similar defects in growth control are observed in other autophagy gene knockout animals.

Together, these observations suggest that autophagic protein degradation may not only function in recycling nutrients and structural remodeling, but may also function as a critical "brake" in cell growth control during development. This theory is consistent with the observations that growth-suppressive signals such as contact inhibition and substrate detachment induce autophagy whereas treatments that stimulate growth (e.g., growth factor addition, partial hepatectomy, refeeding after starvation) inhibit autophagy (reviewed in Neufeld, 2004). An important, testable hypothesis is that the proper temporal and spatial control of autophagy in the developing organism is a fundamental mechanism for orchestrating the growth of specific tissues. At the molecular level, it remains to be determined how autophagy regulates cell growth. Is it related in a general manner to the bulk degradation of cytoplasmic contents and/or altered cellular metabolism or is it related more specifically to the degradation of particular cellular components that, in turn, play a role in cell growth control?

Conclusion

Research in the last decade has revolutionized our understanding of the molecular mechanisms involved in autophagy. As a consequence of the identification of evolutionarily conserved components of the autophagic machinery, it has become possible to use reverse genetics to unravel the functions of autophagy in eukaryotic development. Through such approaches, we have learned that autophagy genes are essential for sporulation in yeast, multicellular development in *Dictyostelium discoideum*, the development of multicellular organisms (including *Drosophila*, *Arabidopsis*, *C. elegans*, and mice), and life span extension in *C. elegans*. This most likely reflects a common need for an efficient catabolic pathway that can rapidly recycle intracellular components in diverse aspects of development across a wide range of taxa. Depending on the specific context, the recycling of cargo through autophagic delivery to the lysosome may be necessary for adequate cellular nutrition, for architectural remodeling, for growth control, or for self-protection against dangerous cytoplasmic contents. However, in contrast to the considerable genetic and molecular genetic advances that have recently been made in autophagy research, our understanding of the biology of autophagy in development remains quite primitive. The major challenge for future research will be to determine the precise functions of autophagy that make this destructive process such an essential part of life.

Acknowledgments

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