Atg7-dependent autophagy promotes neuronal health, stress tolerance, and longevity but is dispensable for metamorphosis in Drosophila

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Abstract

Autophagy, a cellular process of cytoplasmic degradation and recycling, is induced in Drosophila larval tissues during metamorphosis, potentially contributing to their destruction or reorganization. Unexpectedly, we find that flies lacking the core autophagy regulator Atg7 are viable, despite severe defects in autophagy. Although metamorphic cell death is perturbed in Atg7 mutants, the larval–adult midgut transition proceeds normally, with extended pupal development compensating for reduced autophagy. Atg7−/− adults are short-lived, hypersensitive to nutrient and oxidative stress, and accumulate ubiquitin-positive aggregates in degenerating neurons. Thus, normal levels of autophagy are crucial for stress survival and continuous cellular renewal, but not metamorphosis.

Results and Discussion

Generation of Atg7 deletions

Several P-element insertions are available in the genomic region of Atg7, located in the intron between exons 6 and 7, and upstream of or downstream from the gene (Fig. 1A; data not shown). All of these lines yielded viable adults when crossed to the deletion-bearing line Df(2R)P66, and none of them showed a noticeable effect on starvation-induced autophagy [data not shown].

As previously identified mutations in Drosophila Atg genes with a strong autophagy-defective phenotype were lethal [Scott et al. 2004], we initially screened for lethal lines after remobilization of the P-element Atg7EY10058, located 73 bases upstream of the predicted Atg7 transcription start site. Of the lethal lines recovered, two groups of deletions could be identified. The first contained deletions extending from the original P-element insertion site into a neighboring gene, Sec6 (data not shown). Lines in the second group had deletions extending into both Atg7 and Sec6, and none of them showed a noticeable effect on starvation-induced autophagy [data not shown].

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lethal deletions suggested that Atg7 might not be an essential gene. This was confirmed by remobilization of a second P-element located in the intron between exons 6 and 7 of Atg7. None of the recovered 54 lines that lost this element were lethal. We identified three groups of deletions based on sequence data. The first group, represented by Atg7d4, removed the last three exons of Atg7; the second group included deletions that removed most of CG5335, a gene located in the intron of Atg7 as in the case of CG5335d30; the third group consisted of deletions that completely removed CG5335 and part of Atg7, as in the case of Atg7d77, which lacks exons 5 and 6 and most of exon 4 [Fig. 1A]. As these exons code for the E1-like domain of Atg7 that is required for its enzymatic activity, we concluded that Atg7d77 is a likely null allele. As expected, the Atg7d77 and Atg7d4 deletions very strongly interfered with Atg7 expression. No product could be detected by RT–PCR for the sequences removed by these deletions, respectively, and mRNA levels for the remaining portions were also very strongly reduced in both cases [Fig. 1B].

In the experiments below, we refer to Atg7d77/Atg7d14 animals as Atg7d77 or simply Atg7 mutants [homozygous mutant for Atg7, heterozygous for Sec6 and CG5335], and we use flies of the genotype CG5335d30/Atg7d14 as control [heterozygous for Atg7, Sec6, and CG5335]. Most tests were repeated using flies of the genotypes Atg7d77/Df(2R)Pu66 and CG5335d30/Df(2R)Pu66, with similar results [Supplementary Table 1].

**Atg7 is required for starvation-induced autophagy**

To assess the role of fly Atg7 in autophagy, we stained larval fat bodies with Lysotracker Red, a vital dye used for the detection of acidic organelles including autolysosomes. Figure 1. Atg7 is required for starvation-induced autophagy in the fat body. [A] The genomic region of Atg7. The two P-element insertions used to generate deletions (blue arrowheads), and four deletions are shown. [B,B'] Atg7 mRNA expression was analyzed by RT–PCR in control and mutant third instar larvae. [B'] Thirty cycles reveal the expression of C-terminal Atg7 sequences in Atg7d77 and N-terminal sequences in Atg7d4 larvae. As expected, deleted parts are not expressed in the mutants. See A for Atg7 primer locations. [C-D'] Starvation-induced autophagy is severely impaired in Atg7d4 mutant fat body clones, as shown by GFP-Atg8a labeling [C'] and Lysotracker Red staining [D']. Mutant cells are marked by lack of myRFP (red, C) and GFP [green, D] expression, respectively. [E–J] Atg7d77 larval fat bodies show a strongly reduced autophagy in response to starvation. Very little Lysotracker staining is seen in Atg7d77 mutant fat bodies [F], while controls accumulate numerous Lysotracker-positive dots [E]. Transmission EM reveals many autophagosomes [arrowheads] and autolysosomes [arrows] in control [G,H], but not in Atg7d77 fat body cells following a 3-h starvation [I]. J shows a morphometric evaluation of EM images. [AP] Autophagosome; [AL] autolysosome. Error bars represent standard deviation. Bars: C–F, 10 µm; G–I, 1 µm. [B,E,G,H] Control: CG5335d30/Atg7d14. [B] Atg7d4: Atg7d4/Atg7d14. [B,F,I,J] Atg7d77: Atg7d77/Atg7d14. [C] FRT42D Atg7d4/CgGAL4 UAS-GFP-Atg8a FRT42D UAS-myrRFP. [D] FRT42D Atg7d4/UAS-2XeGFP FRT42D fb-GAL4.

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somes. We also monitored the intracellular localization of GFP-Atg8a, a widely used marker of autophagosomes. In control animals, starvation leads to accumulation of both Lysotracker Red-positive vesicles and GFP-Atg8a-positive punctate structures (Scott et al. 2004). The number of such structures was severely reduced in Atg7mutants. Similarly, starvation failed to induce punctate Lysotracker staining in fat bodies of Atg7mutant larvae (Fig. 1C,D).

We used transmission electron microscopy (EM) to quantify the autophagy defect seen in Atg7mutants. Following 3 h of starvation, many autophagosomes and autolysosomes were observed in control larval fat body cells (Fig. 1G,H). Atg7 mutants exhibited a strong reduction of these autophagic structures (Fig. 1I). Morphometric analysis revealed that autophagosomes occupied 0.53% and autolysosomes 1.79% of the cytoplasm in controls, whereas the volume ratio was 0.08% and 0.04% in Atg7 mutant fat body cells, respectively (Fig. 1I). Together, these results indicate that cells lacking Atg7 function are severely impaired in their ability to induce autophagy in response to starvation.

Atg7 is required for developmental autophagy in the larval midgut

At the onset of metamorphosis, autophagy is induced to high levels in larval tissues including the fat body, midgut, and salivary glands, and has been suggested to participate in the elimination of these tissues by programmed cell death events that take place during metamorphosis (Butterworth and Forrest 1984; Neufeld 2004; Penaloza et al. 2006). Accordingly, we observed numerous GFP-Atg8-positive punctate structures in the midgut gastric caeca of control larvae at the wandering stage, just prior to the onset of pupariation (Fig. 2A). Atg7 mutant gastric caeca showed a strong decrease of GFP-Atg8 dots (Fig. 2B), confirming that Atg7 is required for developmental as well as starvation-induced autophagy. Our EM studies also showed numerous autophagosomes and autolysosomes in control larval midguts, and the number and size of these structures were strongly reduced in mutant cells (Fig. 2C,D). Morphometric analysis revealed that autophagosomes occupied 0.95% and autolysosomes 3.22% of the cytoplasm in controls, whereas the volume ratio was 0.17% and 0.42% in Atg7 mutant midgut cells, respectively (Fig. 2E).

Atg7 is required for developmental autophagy and apoptotic DNA fragmentation in the midgut

Developmental autophagy results in punctate GFP-Atg8a localization in control midguts (Fig. 2A). Atg7mutant midguts show a more uniform GFP-Atg8a signal with very few dots. Ultrastructural analysis reveals numerous autophagosomes and autolysosomes in control (C), but not in mutant midguts (D). E shows a morphometric evaluation of EM images; labels as in Figure 1. (F–M) Adult epithelium is formed normally in Atg7mutant midguts during metamorphosis. (F, G) The larval midgut is composed mostly of large, polypliod cells, with intercalated small, diploid imaginal cells in control and mutant animals at the time of puparium formation [white prepupal stage). (H–K) The adult epithelium is formed from proliferating imaginal cells by 5 h RPE, accompanied by shortening and thickening of the entire midgut (note increased diameter in cross-section between F-H or G-I), and shedding of the larval cell layer into the lumen [dark cell masses in H, I]). (L) Although appearing less condensed at this time, larval cells of Atg7 mutants are also shed into the lumen. (J, K) Formation of the adult midgut epithelium [ae) proceeds normally in both control and mutant animals. Aberrant structures reminiscent of protein aggregates were seen in the apical area of mutant midgut cells at 5 h RPE [M], in the region where control cells accumulated numerous large autolysosomes [L]. TUNEL staining of white prepupal midguts reveals defects in DNA fragmentation in dying Atg7mutant larval midgut cells (O), when compared with the robust nuclear staining seen in controls (N). Bars: A, B, F–I, 10 µm; C, D, J–M, 1 µm; N, O, 200 µm. [A, C, E, F, H, J, L, N] Control: CG5335/Atg7+1-2. [R, P, E, G, J, K, M, O] Atg7+1-2. Atg7ad14. 10 µm; C, D, J–M, 1 µm; N, O, 200 µm. [A, C, E, F, H, J, L, N] Control: CG5335/Atg7+1-2. [R, P, E, G, J, K, M, O] Atg7+1-2. Atg7ad14.
Atg7 mutants are hypersensitive to nutrient and oxidative stress and have a reduced life span

As Atg7 mutants were fully viable [Supplementary Fig. 2A], we were able to investigate the role of autophagy in adult functions such as stress responses and aging. Under conditions of complete starvation or a sugar-only diet, Atg7 mutants displayed an accelerated lethality compared with control flies [Fig. 3B,C], consistent with a conserved role for autophagy in promoting starvation survival by recycling cytoplasmic constituents. Previous studies in cultured mammalian cells have suggested that autophagy may protect against oxidative damage by targeting damaged macromolecules and organelles for elimination (Cuervo et al. 2005). To test whether autophagy contributes to oxidative stress resistance in an organismal context, we measured the survival of age-matched Atg7 mutant and control flies treated with agents that induce oxidative stress. Atg7 mutants were hypersensitive to paraquat treatments, dying at a two-fold faster rate than controls [Fig. 3D]. Similar results were observed using hydrogen peroxide [Supplementary Fig. 2B]. In addition, loss of Atg7 resulted in a reduced life span under normal conditions [Fig. 3E; see Supplementary Table 2 for detailed statistical analysis of survival curves]. Atg7 mutant flies also displayed a significant age-dependent decline in climbing performance tests [Fig. 3F], an established measure of nervous system function [Martinez et al. 2007]. Together, these results indicate the importance of autophagy in stress survival and longevity, potentially through elimination and recycling of damaged cellular components generated in response to induced oxidative stress or during normal aging, thus promoting constant cellular renewal.

Progressive neuronal degeneration in Atg7 mutants

The age-related decline of nervous system function in Atg7 mutants suggested that the loss of autophagy in these flies leads to a neurodegenerative phenotype. Previous studies of Atg5 and Atg7 mutant mice reported an accumulation of ubiquitinated proteins and progressive neurodegeneration in these animals [Hara et al. 2006; Komatsu et al. 2006]. We found that ubiquitinated proteins also accumulated in aged Atg7 mutant fly head extracts, suggesting that protein aggregates form in the CNS [Fig. 4A]. Ultrastructural analysis confirmed the presence of inclusion bodies in neurons of mutant, but not control fly brains [Fig. 4B,C]. Immunoelectron microscopy further revealed that these aberrant structures are ubiquitin positive [Fig. 4D]. The number of inclusion bodies increased over time in neurons of Atg7 mutant brains: Larval neurons never contained such structures, whereas they were readily observed in 3-d-old adults, with the highest numbers in 30-d-old adults [Fig. 4E]. Accumulation of these ubiquitin-positive aggregates was associated with a progressive neurodegeneration, as dead neurons were readily identified in Atg7 mutant brains [Fig. 4F]. Also, most brain cells of aged Atg7 mutants showed extensive DNA fragmentation [Fig. 4H,I], whereas we observed no TUNEL labeling in controls [Fig. 4G,I]. Neurodegeneration was also revealed by moderate vacuolization of 30-d-old mutant brains, compared with controls [Fig. 4K,L]. As expected, we detected no DNA fragmentation in control or Atg7 mutant brains at 3 d after eclosion [data not shown], but the presence of inclusion bodies in mutants may account for the slight decrease in climbing performance at this time. Interestingly, other tissues like indirect flight muscles were largely unaffected by the mutation, and overall ommatidial morphology was also similar to controls [Supplementary Fig. 3].

In conclusion, our findings indicate that normal levels...
we recently showed that the lethality of Atg1 mutants can be partially rescued by expression of a kinase-defective form of the Atg1 protein that inhibits starvation-induced autophagy [Scott et al. 2007], suggesting that one or more essential functions of Atg1 may be independent of autophagy. The identification of viable mutations in Drosophila Atg8a also supports this conclusion [Scott et al. 2007]. Alternatively, the viability of Atg7 mutants may reflect a less severe disruption or a qualitative difference in autophagy compared with other Atg mutants. Analysis of null mutations in additional Atg genes will further clarify this issue.

The role of autophagy in cell death has not been fully resolved. Although autophagy is generally considered a prosurvival cellular defense pathway, elevated levels of autophagy have been shown to cause cell death, at least under certain experimental conditions [Kroemer and Jaattela 2005; Pattingre et al. 2005; Scott et al. 2007]. Our results provide in vivo evidence supporting a dual role for autophagy in cell death. During metamorphosis, extremely high autophagic activity in larval tissues such as the midgut epithelium precedes DNA fragmentation and cell death. Mutation of Atg7 results in a marked decrease of autophagy and an inhibition of DNA fragmentation, suggesting that autophagy contributes to the normal cell death process in this case. As reported earlier, this cell death defect does not interfere with adult midgut epithelial development, which drives morphogenetic events during midgut reconstruction in Drosophila [Lee et al. 2002]. In contrast to the case in these larval tissues, loss of Atg7 activity in the adult brain leads to DNA fragmentation and neuronal cell death, suggesting that autophagy acts to maintain neuronal health and prevent cell death in these cells. Our findings thus suggest that the net effect of autophagy on cell survival in vivo is dependent on both cell type and level of autophagic induction. Better understanding of the regulation of autophagy and identification of relevant substrates may provide new insight into neurodegenerative diseases and aging.

Materials and methods

P-element excisions and mutant fat body cell clones were generated as described earlier [Scott et al. 2007]. Three- to five-day-old males were treated as follows: For complete starvation and the sugar-only diet, a paper towel soaked in water or 20% sucrose solution was placed in vials. Additional liquid was added as needed, and dead flies were counted daily. Paraquat or H2O2 was stirred into the food at a final concentration of 30 mM or 1%, respectively, and dead flies were counted daily. For life-span analysis, newly eclosed males were transferred to fresh food every 2–3 d, and dead flies were counted. Climbing assays were performed as described [Martinez et al. 2007]. See the Supplemental Material for additional procedures.

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