The autophagy-related gene Atg101 in Drosophila regulates both neuron and midgut homeostasis

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Running title: Atg101 regulates both neuron and midgut homeostasis

Abstract

Atg101 is an autophagy-related gene identified in worms, flies, mice, and mammals and encodes a protein that functions in autophagosome formation by associating with the ULK1–Atg13–Fip200 complex. In the last few years, the critical role of Atg101 in autophagy has been well established through biochemical studies and the determination of its protein structure. However, Atg101’s physiological role both during development and in adulthood remains less understood. Here, we describe the generation and characterization of an Atg101 loss-of-function mutant in Drosophila and report on the roles of Atg101 in maintaining tissue homeostasis in both adult brains and midguts. We observed that homozygous or hemizygous Atg101 mutants were semi-lethal, with only some of them surviving as adults. Both developmental and starvation-induced autophagy processes were defective in the Atg101 mutant animals, and Atg101 mutant adult flies had a significantly shorter lifespan and displayed a mobility defect. Moreover, we observed accumulation of ubiquitin-positive aggregates in Atg101 mutant brains, indicating a neuronal defect. Interestingly, Atg101 mutant adult midguts were shorter and thicker and exhibited abnormal morphology with enlarged enterocytes. Detailed analysis also revealed that the differentiation from intestinal stem cells to enterocytes was impaired in these midguts. Cell type-specific
rescue experiments disclosed that Atg101 had a function in enterocytes and limited their growth. In summary, the results of our study indicate that *Drosophila* Atg101 is essential for tissue homeostasis in both adult brains and midguts. We propose that Atg101 may have a role in age-related processes.

**Introduction**

Autophagy (Macroautophagy) is a process in which cytoplasmic materials, including organelles and macromolecules, are delivered to and degraded in the lysosome [1-4]. As a major intracellular degradation system, autophagy plays important roles in development, tissue homeostasis and aging [5-7]. Defects in the autophagy pathway can cause various human diseases, such as cancer and neurodegenerative diseases [8-10].

Genetic studies from budding yeast have identified more than thirty Atg genes, which function at various steps during autophagy [2, 3, 11]. Most of these genes are highly conserved from yeast to mammals [3, 11]. Among them, the Atg1 complex acts at the initiation stage of autophagy and functions as a scaffold for the recruitment of downstream Atg proteins to the pre-autophagosomal structure [4, 11-13]. The yeast Atg1 complex consists of Atg1, Atg13, Atg17, Atg29 and Atg31, while its mammalian counterpart is composed of ULK1 (or ULK2), Atg13, FIP200 (also known as RB1CC1) and Atg101 [4, 11-13]. Mammalian ULK1 and ULK2 are homologs of Atg1 [13]. FIP200 is generally considered as a homologue of yeast Atg11 and Atg17 [13]. Homologs of Atg29 and Atg31 are not found in higher eukaryotes [13]. In contrast, Atg101 is present in most eukaryotes, with the exception of budding yeast [13]. It has been proposed that the regulatory mode of the Atg1 complex and the regulatory mechanism for controlling autophagy initiation might have evolved from yeast to animal cells [13]. Due to its absence in budding yeast, Atg101 has not been studied extensively as other components of the ULK1 complex until the recent determination of the crystal structure of Atg13-Atg101 complex [14-20]. Atg101 contains a single HORMA domain and forms a heterodimer with Atg13 via this domain [14, 15, 18-23]. The interaction between Atg101 and Atg13 helps to stabilize Atg13 and ULK1 in the complex [14, 15, 18-23]. Although the functional requirement of Atg101 in autophagy has been established in mammalian cells, *C elegans* and *Drosophila*, the physiological role of Atg101 remains largely unexplored [14-17].

*Drosophila* has increasingly become an attractive model system to study autophagy, especially the physiological functions of autophagy in tissue homeostasis and neurogenesis [24-26]. In this study, we generate an *Atg101* loss-of-function mutant using the CRISPR/Cas9 approach in *Drosophila*, and report on the roles of Atg101 in regulating neuron and midgut homeostasis.

**Results**
**Generation and characterization of Atg101 loss-of-function mutant alleles**

*Drosophila* Atg101 is located in an intron of the S6KL gene, which encodes a S6 kinase-like protein (Figure 1A). To explore the physiological function of Atg101, we generated an Atg101 loss-of-function mutant allele, *Atg101*^6h^, using the recently developed CRISPR/Cas9 system (Figure 1A). *Atg101*^6h^ contains a thirteen-nucleotide deletion in the coding region which causes a frameshift mutation (Figure 1A). To examine whether this *Atg101* mutation affects the expression of its host gene S6KL, we extracted total RNA from both wild-type and *Atg101*^6h^ mutant animals and performed quantitative PCR analysis using a pair of exon specific primers for S6KL. Our qPCR results showed that the mature S6KL level was approximately 1.2 times of wild type level in *Atg101*^6h^ mutants (Figure S1A). Previous studies have reported that overexpression and mutation of S6KL leads to decreased and increased bouton number at larval neuromuscular junctions (NMJs), respectively [27, 28]. To further determine whether loss of Atg101 affects S6KL function, we performed immunostaining analysis and examined larval NMJ development in *Atg101*^6h^ mutants. For this analysis, anti-CSP antibody was used to label presynaptic components at larval NMJs. Our results revealed that there was no significant changes in the number of boutons for the muscle 4 NMJ in *Atg101*^6h^ mutant larvae as compared to the control (Figure S1B and C). This indicated that *Atg101*^6h^ mutants exhibit no detectable defects during larval NMJ development and further confirmed that loss of *Atg101* doesn’t affect S6KL function.

Homozygous or hemizygous *Atg101*^6h^ mutants were semi-lethal (Table S1). To determine whether the lethality we observed in *Atg101*^6h^ mutants was due to the specific loss of *Atg101* function, we made an UAS-*Atg101* rescue transgene. Overexpression of UAS-*Atg101* with a ubiquitously expressed Daughterless-Gal4 (Da-Gal4) in *Atg101*^6h^ mutants restored the viability of mutant animals, indicating that the lethality is a likely consequence of loss of *Atg101* (Table S1).

To analyze the lethality phenotype in more detail, we first examined the viability of wild-type and *Atg101*^6h^ mutants at different developmental stages under growth control conditions. Reduced viability was observed in both embryonic and post-embryonic stages in *Atg101*^6h^ mutants compared to the control, although the reduction of viability during larval and pupal stages was subtle (Figure S1B). *Atg101* resulted lethality before or during eclosion, similar to previously reported *Atg17* mutants (Figure S1C and D) [29]. *Atg101*^6h^ mutant adult flies also displayed an abnormal wing posture, which has been described in *Atg17* mutants (Figure S1E and F) [29]. This wing posture defect was rescued by overexpressing Atg101 with Da-Gal4 (Figure S1G). Some of the newly enclosed *Atg101*^6h^ mutant flies fell down easily into the food at the bottom of the vial (Data not shown). In addition, we found that *Atg101*^6h^
mutant animals developed relatively slower compared to the control and the time needed from the newly hatched first instar larvae to become pupae or adults was increased by 12 to 24 hours on average (Figure S1H and I).

We also determined the expression level of Atg101 at different developmental stages by performing RT-PCR analysis on RNA prepared from embryos, larvae, pupae and adults. Atg101 mRNA was expressed at all developmental stages, with the highest level in 0-12 hour embryos (Figure S1J). In addition, the expression level of Atg101 was comparable with the levels of Atg1 and Atg8a, but higher than the levels of Atg3, Atg4a and Atg7 in 0-12 hour embryos.

**Atg101 mutants exhibit defects in starvation-induced and developmental autophagy**

Atg101 is an important component of ULK1/Atg1 kinase complex in higher eukaryotes, and plays essential roles in the initiation of autophagy by interacting with Atg13 [13-15]. To demonstrate the role of Atg101 during the process of autophagy in vivo, we analyzed the starvation-induced and developmentally triggered autophagy in Atg101<sup>6h</sup> mutant and wild-type control larval fat bodies. The dual-tagged GFP-RFP-Atg8a reporter was commonly used to follow autophagic flux and monitor autophagic activities [30]. In this system, GFP fluorescence is normally quenched in the acidic environment of the autolysosome, but RFP fluorescence is pH independent. Thus, autophagosomes can be labeled by both GFP and RFP signals and appear as yellow. Autolysosomes are only positive for RFP and appear as red. We took this approach and examined the effects of Atg101 on autophagic activities. Loss of Atg101 prevented accumulation of both yellow and red punctate structures in fat body cells of both starved third instar larvae and late wandering third instar larvae, consistent with a previous report in which knockdown of Atg101 by RNAi blocked both starvation-induced and developmental autophagy (Figure 1B-I) [17]. It has been shown that Atg8a positive structures are larger in size upon Atg101 knockdown. However, we found that the size of Atg8a punctate structures was smaller in Atg101 mutant fat body cells than that in the control (Figure 1B-I). It is possible that some remaining Atg101 activities might affect the size of Atg8a positive structures.

Previous studies have shown that inhibition of autophagy leads to a delay in larval midgut cell death during Drosophila metamorphosis. Consistent with this, morphological analysis of Atg101<sup>6h</sup> mutant midguts revealed a delay in midgut cell death, as the gastric caeca still persisted at 4 h RPF (After Puparium Formation) in Atg101<sup>6h</sup> mutant animals (Figure S2A and B). Together, these data confirmed that animals lacking Atg101 function are impaired in their abilities to induce autophagy during normal development and in response to starvation.

**Atg101 mutant flies show decreased lifespans and impaired mobility**
Mutations in several Drosophila autophagy-related genes, including Atg7, Atg8 and Atg17, lead to reduced lifespans in adult flies [29, 31-33]. We therefore examined the effect on Drosophila lifespan when Atg101 was deleted. 50-70% of Atg101\(^6\)h mutants were able to develop to adulthood, and these flies were used for lifespan measurement. Compared to the control, Atg101\(^6\)h mutant flies exhibited a reduced adult lifespan and most of mutant animals died at three weeks of age (Figure 2A and B). In addition, we noticed that Atg101\(^6\)h mutant animals also showed a movement disorder and most of them were found at the bottom of vials (Data not shown). To confirm this, we performed a negative geotaxis assay with wild-type and mutant flies. When tapped to the bottom of a vial, wild-type flies respond by climbing to the top (Figure 2C). However, most Atg101\(^6\)h mutant flies failed to do so (Figure 3C). At 2 day of age, 70-80% of Atg101\(^6\)h mutant flies have impaired climbing ability in a climbing assay (Figure 2D). Their performance was much worse at day 10, with 100% of flies impaired (Figure 2D). These mobility defects were restored in the rescued flies (Figure 2C and D). Collectively, these data indicate that loss of Atg101 leads to reduced lifespan and impaired mobility in adult flies.

### Atg101 mutants show neurodegeneration defects

The age-related decline of mobility could be a reflection of neurodegeneration in the adult brain. Previous studies have shown that an accumulation of ubiquitinated proteins is associated with progressive neurodegeneration in Atg5 and Atg7 mutant mice as well as Atg7, Atg8 and Atg17 mutant flies [29, 31-35]. To ask whether the mobility defects in Atg101\(^6\)h mutant were due to neurodegeneration, we used an antibody against ubiquitin to examine Atg101\(^6\)h mutants. Ubiquitinated proteins were accumulated in one week age of Atg101\(^6\)h mutant fly head extracts as compared to the wild-type control (Figure 3A). To confirm the disruption of autophagy in Atg101 mutant fly head, we also examined the protein level of Ref(2)p in both wild-type and mutant fly heads. Consistently, Atg101\(^6\)h mutants had an obvious increase in Ref(2)p level as compared to the control (Figure 3B). Furthermore, our immunofluorescence staining also revealed that the ubiquitin and Ref(2)p punctate structures were accumulated in the mutant brain, suggesting the formation of protein aggregates in the central nervous system of Atg101\(^6\)h mutants (Figure 3C-D’ and G-H’, quantified in F and J). Partial co-localization between Ref(2)p and ubiquitin was also observed in Atg101\(^6\)h mutants (Figure S3A-B’). These protein aggregates defects were rescued by the overexpression of Atg101 (Figure 3E, E’, I and I’, quantified in F and J). To further determine whether Ref(2)p accumulation occurs in neurons or glial cells, we double-stained Atg101\(^6\)h mutant adult brains with anti-Ref(2)p and antibodies against Elav or Repo, which mark neuron and glia, respectively. Colocalization of Ref(2)p positive cells with Elav and Repo in
**Atg101** 6h mutant brains showed Ref(2)p accumulation in both neurons and glial cells (Figure 3K-L').

Taken together, these data indicate that Atg101 function is important for the elimination of protein aggregates and neuron homeostasis in the adult brain.

**Atg101 maintains midgut homeostasis**

Defects in the maintenance of intestinal stem cell homeostasis could result in short lifespan in adult flies [36-39]. During the course of our study, we noticed that the Atg101 6h mutant abdomen was enlarged as compared to the wild-type animals, which indicates a possible defect in the midgut tissue (Figure S4A and B). This defect was also rescued by overexpressing Atg101 (Figure S4C). We therefore extended our phenotypical analysis to the adult midgut tissue in Atg101 6h mutants. Interestingly, the midgut in Atg101 6h mutants was significantly shorter and thicker than that in wild-type controls (Figure 4A and quantified in 4B). This defect was suppressed in the rescued flies, confirming that the midgut phenotype was specifically due to the loss of Atg101 (Figure 4A and quantified in 4B). To begin understanding the basis for these defects, we then focused on the posterior midgut and stained the tissue with Phalloidin and DAPI to examine the posterior midgut morphology. Our Phalloidin staining revealed that the regular organization of the visceral muscles was disrupted in Atg101 6h mutant midguts, which suggests that the peristalsis was less efficient in the mutants (Figure 4C-D').

Cells with different-sized nuclei are present in the posterior midgut. The number of polyploid enterocytes with large nuclei was reduced, while the number of diploid cells with small nuclei was increased (Figure 4C-D', quantified in 4E). We also noticed that the nuclear size of enterocyte cells was larger in the mutant midguts compared to the control (Figure 4C' and D', quantified in 4F). Consistently, the enterocyte cell size was enlarged in Atg101 6h mutant midguts (Figure 4G and H). In addition, the midgut epithelium was thickened and the lumen size was increased in the Atg101 mutants (Figure 4I and J). Overexpression of Atg101 largely rescued all these posterior midgut defects (Figure 4C-M). It is likely that food digestion and nutrient absorption were less efficient in the mutant midgut due to the irregular organization of the visceral muscles and reduced number of polyploid enterocytes. Together, these data demonstrate that Atg101 is required for maintaining adult midgut homeostasis.

**Atg101 functions to promote intestinal stem cell differentiation**

To examine the posterior midgut defect in more detail, we next performed immunofluorescence staining with antibodies against various markers for different types of posterior midgut cells. Escargot (esg)-GFP is specifically expressed in intestinal stem cells (ISCs) and enteroblasts (EBs), which are collectively referred to as midgut precursor cells [40]. In Atg101 6h mutant midguts, the number of esg-
GFP-expressing cells was increased and cells often clustered (Figure 5A-B', quantified in C and D). Increase number of esg-GFP positive cells could be due to overproliferation of intestinal stem cell or a blockage of stem cell differentiation, or both. To further discriminate these possibilities, we used anti-phospho-Ser10-Histone H3 (pH3) antibodies to label cells in mitosis. In Atg101<sup>6h</sup> mutant midguts, the number of cells labeling for pH3 was comparable with the controls, indicating the expansion of esg-GFP positive cells was unlikely due to the increase of ISC proliferation (Figure 5E-F', quantified in G). We then considered the possibility that cell differentiation was blocked in Atg101<sup>6h</sup> mutants. To test this, we used anti-pros and anti-Pdm1 antibodies to stain for enteroendocrine cells (EEs) and ECs, respectively, and found a significant reduction of the number of cells for EC cell types in Atg101<sup>6h</sup> mutant flies, but not for EE cell types (Figure 5H-I' and K-L', quantified in J and M) [40-42]. Altogether, these analysis reveals that Atg101 is required for intestinal stem cell differentiation, especially the differentiation of EC cell lineage in adult midguts.

**Atg101 acts in ECs to limit cell growth**

Having shown that loss of Atg101 causes defects in adult midgut homeostasis, including the enlarged EC cell size and reduced intestinal stem cell differentiation, we next wanted to identify the cell types in which Atg101 might function. For this purpose, we performed the rescue experiment with the UAS-Atg101 transgene using several cell type-specific Gal4 drivers in adult midguts. First, esg-Gal4 combined with a temperature-sensitive GAL80 was used to overexpress Atg101 specifically in the adult ISC and EB population of Atg101<sup>6h</sup> mutant flies. In order to activate transgene expression, adult flies were shifted to the nonpermissive temperature. The results showed that there was no rescue effect on the enterocyte size when expressing UAS-Atg101 by esg-Gal4, as compared to Atg101<sup>6h</sup> mutant alone (Figure 6A-C). We then used the EC-specific Myo1A-Gal4 combined with Gal80<sup>ts</sup> to restrict the expression of UAS-Atg101 to ECs in the adult midgut. Interestingly, Myo1A-Gal4 driven Atg101 expression in Atg101<sup>6h</sup> mutant flies displayed a rescue of the enlarged enterocyte size normally seen in Atg101<sup>6h</sup> mutants (Figure 6D-F). Furthermore, no obvious rescue was observed when an EE-specific pros-Gal4 was used to drive Atg101 expression in Atg101<sup>6h</sup> mutant midguts (Figure G-I). Thus, we conclude that Atg101 has a cell autonomous effect in ECs to limit cell growth.

**Discussion**

Here we generated and characterized an Atg101 loss-of-function mutant in Drosophila. Atg101 mutant shows reduced viability in embryonic, larval and pupal stages. Most mutant animals can survive to adult stages, but have a short life-span. Our study also provides genetic evidence that Atg101 has a key role in maintaining neuron and midgut homeostasis.
Atg101 is a core subunit of the Atg1 complex, which is essential for autophagosome formation [13, 23]. Studies in mammalian cells have identified the role of Atg101 in autophagy initiation [14, 15]. Knockdown of Atg101 by RNAi in Drosophila also leads to autophagy defects [17]. Consistent with this, lacking of Atg101 function causes defects in both starvation-induced and developmental autophagy in Drosophila third instar larval fat body tissues. In addition, we observe a variety of phenotypes in adult flies, such as reduced lifespan, impaired locomotion, accumulation of ubiquitinated proteins and blockage of intestinal stem cell differentiation.

Autophagy has been implicated in the process of aging [43]. Suppression of autophagy disrupts age-dependent tissue homeostasis in various organs [43]. In flies, loss of Atg7, Atg8a or Atg17 in the entire organism leads to reduced lifespan as well as the accumulation of ubiquitinated protein aggregates in the brains [29, 31-33]. It has been proposed that the basal level of autophagy in the nervous system is required for the clearance of toxic proteins or damaged organelles [43, 44]. Similar to other Atg mutants, Atg1016h mutant flies also have a shorter lifespan. Protein aggregates were evident in Atg1016h mutant fly brains. The decline of locomotion ability in Atg1016h mutants during aging further demonstrates a neurondegeneration defects in the absence of autophagy function.

Recently, several reports indicate that autophagy-related genes also regulate intestine homeostasis. A core autophagy gene Atg16L1 has been shown to be associated with Crohn disease [45]. Later studies in mice showed that Paneth cells are abnormal in Atg16L1 or Atg5 knock-out mutant animals [46]. In flies, Atg9 has been shown to be required for JNK-mediated intestinal stem cell proliferation [47]. Interestingly, induction of autophagy can block stress-induced ISC proliferation [47]. In addition, Atg9 is also required for midgut homeostasis under normal physiological condition, and it specifically acts in the enterocytes to control cell growth by limiting TOR signaling [48]. Analysis of various Atg genes in Drosophila midguts have demonstrated that the Atg1 complex components, including Atg1, Atg13 and Atg17, play crucial roles in controlling enterocytes cell growth [48]. Furthermore, it appears that the Atg1 complex and Atg9 regulate TOR signaling via different mechanisms during enterocytes cell growth [48]. Interestingly, other core autophagy-related genes, such as Atg7, Atg12, Atg16, Atg18 and Vps32, are not required for enterocytes cell growth in Drosophila adult midguts, indicating that the role of the Atg1 complex and Atg9 in maintaining midgut homeostasis might be specific [48]. Consistent with the reported phenotype upon knocking down Atg1, Atg13 and Atg17, we found that loss of Atg101 causes defects in adult midgut homeostasis and results in abnormal midgut morphology with enlarged enterocytes. Interestingly, the number of intestinal progenitor cells was increased in Atg101 mutant midguts.
However, the number of dividing intestinal stem cells when stained with anti-pH3 remains the same as the control. In contrast, we observe reduced number of differentiating enterocyte cells. These data strongly indicate that Atg101 plays an important role in intestinal stem cell differentiation, but not proliferation. In addition, our cell type specific rescue experiments reveal that Atg101 functions in ECs to limit cell growth autonomously. It has been reported that apoptotic enterocytes promote intestinal stem cell division nonautonomously [49]. It remains unclear whether the overgrowth of EC has an effect on intestinal stem cell function. In summary, these findings indicate that Atg101 plays essential roles in maintaining neuron and midgut homeostasis, both of which may affect the aging process. A recent study also reveals that activation of autophagy in the adult brain by either expression of AMPK or Atg1 is able to induce autophagy in the intestine and leads to increased lifespan [50]. Further studies on the connection between the brain and midgut homeostasis in Drosophila Atg101 mutant animals likely provides novel insights into the cross-talk between the brain and the midgut.

**Experimental procedures**

**Drosophila stocks**

We used the following fly stocks: w1118, Daughterless-Gal4, esg-GFP^{P01986}/CyO, Cg-Gal4 UAS-GFP-RFP-Atg8a/CyO. Esg-Gal4 UAS-GFP/Cyo; Gal80^{ts}/TM6B, Myo1A-Gal4; Gal80^{ts}, pros-Gal4.

For experiments using Gal80^{ts}, crosses were set up and cultured at 18 °C to limit Ga4 activities. To inactivate Gal80^{ts}, 2-4 days old of F1 adult flies were shifted to 29 °C for 7 days before dissection.

**Generation of Atg101 mutant and transgenic fly lines**

CRISPR-mediated mutagenesis was performed according to a previous report [51]. Briefly, Atg101 target sequence and PAM site were determined using http://tools.flycrispr.molbio.wisc.edu/targetFinder/. After identifying the target region, two primers (TAATACGACTCACTATAGGAGGTGTGGACGGTGCACCGTTTTAGAGCTAGAAATA GC and AAAAAAGCACGACTCGGTGCCAC) were used to amplify the DNA fragment from the pMD19-T gRNA scaffold vector. The PCR products were used for gRNA in vitro transcription with the RibomAX Large Scale RNA Production Systems T7 Kit. To synthesis Cas9 mRNA, pSP6-2sNLS-spCas9 plasmid was first cut by XbaI and then purified. Transcription was performed using the Sp6 mMESSAGE mMACHINE Kit (Ambion, USA). The poly(A) tails were added to the 3'-end of Cas9 mRNAs using E. coli Poly(A) polymerase Kit (New England BioLabs, USA). Cas9 mRNAs and Atg101 gRNAs were then mixed and injected into w1118 fly embryos. Genomic DNA from dead embryos were used for PCR amplification and sequencing to determine the efficiency and usefulness of gRNA. The primers used for amplifying the target region were TTTACACCGTCCTTTCCAC and
ATGATGGGAGGATTTGCGTTC.

Detection of a string of “double peaks” in the sequencing chromatogram indicates the mismatched region and the usefulness of gRNA. Single flies were selected and balanced over FM6B. Exact deletions were determined by PCR screening and sequencing for individual flies. Atg101 \textsuperscript{6h} mutants have a 13 bp deletion (From ChX: 18792261 to ChX: 18792273).

For the generation of UAS-Atg101-HA transgenic flies, an Atg101 cDNA fragment was amplified using the primers: CGGCCGCCATGAACGCAGGTTTGCA GGT and CGCTCGAGCATTGCGAGCGTTTCCTTGA, and cloned into a modified pUAST vector with a 3HA tag at the C-terminal. The pUAST-Atg101-HA construct was then injected into the ZF-25C landing site on Chromosome II using standard methods.

**Quantitative PCR and RT-PCR**

Total RNA was extracted using the Trizol reagent (Ambion, 15596-026). cDNA was synthesized with PrimeScript RTase (TaKaRa, PrimeScript™ II 1st Strand cDNA Synthesis Kit, Code No. 621A). Quantitative PCR was performed on the ABI 7900HT Fast Real-Time PCR system using the following primers: S6KL: 5’- GTCAGATGCTGACGCAG -3’ and 5’- GCCATCACACTGCGGATAC -3’; rp49: 5’- GCTAAGCTGTGCAGCACAAA -3’ and 5’- TCCGGTGCCGACGATGTGG -3’. RT-PCR was performed using the following primers: Rp49: 5’- GCTAAGCTGCTGACGACCAA -3’ and 5’- TCCGGTGGCCGACGATGTGG -3’; Atg101: 5’- GAGGTGTGGACGGTGACC -3’ and 5’- GATGTGTCGAAGATCAG -3’; Atg1: 5’- GAAAATTGTTGTTTTGCGGATC -3’ and 5’- CAGAGATCCGCTTTGGAATC -3’; Atg3: 5’- CGCCCGGTGTTTTGAGGATC -3’ and 5’- TGCTTTGGTCTCTCATGACCG -3’; Atg4a: 5’- TACTGCGCTTCGATGACTGG -3’ and 5’- TATGACAGCAGTGGCCTGCC -3’; Atg7: 5’- GATGTGTCGAGCGGAAAG -3’ and 5’- GCCAGCTCCTTACGAGGAT -3’; Atg8a: 5’- TCATTGCGACGACGCAATCCA -3’ and 5’- AGTCCTCTCCGATGTCCTCC -3’.

**Western blotting**

Adult fly heads were collected and lysed in modified RIPA lysis buffer (50mM Tris-\(\text{HCl}\), pH 8.0, 150mM NaCl, 1% (v/v) (\(\text{v}\)/v) IGRPA CA-630, 0.5% (w/v) sodium deoxycholate) with protease inhibitor cocktail (Roche, Cat. No. 04693132001) and PhosStop phosphatase inhibitor cocktail (Roche, Cat. No. 4906845001). Samples were then subjected to SDS-PAGE and transferred to polyvinylidene fluoride membrane. Membranes were immunoblotted with Mouse anti-Ubiquitin (1:1000, P4D1, Cell Signaling Technology Cat# 3936S), Rabbit anti-Ref(2)p (1:500, ab178440, Abcam), Mouse anti-GAPDH (1:1000, Goodhere, AB-M-M001) and Mouse anti-tubulin (1:1000, Beyotime, AT819-1). Detection of proteins was performed using the ChemiLucent™ ECL detection reagents (Millipore, WBKLS0500). Images were taken using the Chemiluminescence Imaging System (Clinx Science Instruments, Shanghai).

**Immunostaining and microscopy**

Drosophila adult midguts were dissected in SD medium and fixed with 4% paraformaldehyde in PBS for 40
min with rocking at room temperature. Midguts were washed three times in PBT (0.1% Triton X-100 in PBS) before blocking for 1 hour in PBT plus 3% BSA buffer at room temperature. Next, midguts were incubated with the primary antibodies overnight at 4 °C. After four washes in PBT, midguts were incubated with secondary antibodies for 2 h at room temperature with rocking. DAPI was added for the last 20 min. After four further washes with PBT, midguts were mounted in Vectashield mounting medium. 7-day old adult males were used in all the analysis. The posterior region of the midgut was chosen for imaging. Drosophila adult brains and larval NMJs were dissected in SD medium. Samples were then fixed with 4% paraformaldehyde in PBS for 20 min and stained as above. The following primary antibodies were used: Chicken anti-GFP (1:2000; ab13970, Abcam), Mouse anti-CSP (1:50, 6D6, DSHB), Mouse anti-Ubiquitin (1:400, P4D1, Cell Signaling Technology Cat# 3936S), Rabbit anti-Ref(2)p (1:500, ab178440, Abcam), Rat anti-Elav (1:50, 7E8A10, DSHB), Mouse anti-Repo (1:50, 8D12, DSHB), Rabbit anti-pH3 (1:500, Millipore), Mouse anti-prospero (1:100), Rabbit anti-Pdm1 (1:500), Mouse anti-Dlg (1:500, DSHB, 4F3). Fluorescent secondary antibodies (Alexa Fluor 488 or 555 - conjugated, anti-rabbit, anti-mouse, anti-chicken) were obtained from Molecular Probes (1:500). Phalloidin (Phalloidin 568, Invitrogen A12380, 1033926) was used in 1:1000 dilution. DNA was labeled with DAPI (1ug/ml, Sigma). To induce starvation, middle L3 stage larvae were collected and transferred to a 20% sucrose solution for 4 hours. For live imaging, larval fat body tissues were dissected in SD medium. Imaging were performed on an Olympus FV1000 confocal microscope and images were processed using Image J and Adobe Photoshop.

Quantification and statistical analysis

In Figure 1D, E, H and I, the number of RFP-Atg8a spots were counted manually, and the size of RFP-Atg8a spots were measured with NIS-elements. In Figure 3F and J, the number of ubiquitin positive spots and the area of Ref(2)p positive spots were measured with ImageJ. In Figure 4B, midgut length and width were measured with NIS-elements from images of whole midguts acquired with a Nikon Eclipse 80i microscope. In Figure 4F and G, the number of nuclei was counted manually, and the size of nuclei was measured with NIS-elements. Posterior midgut regions R4a and R4b were chosen. In Figure 5C, J and M, the number of esg-positive, pros-positive and pdm1-positive cells were counted manually. Posterior midgut regions R4a and R4b were chosen. In Figure 5D, the number of esg-positive cell clusters was counted manually for the entire frame. In Figure 5G, the number of pH3 positive cells was counted manually for each midgut. In Figure S2B, the gastric caeca size was measured with NIS-elements. The sample size for the quantification analysis was indicated in the figure legend. Statistical analysis was performed using GraphPad Prims 5.
Hatching rate, pupation rate, eclosion rate
3-4 days old flies were collected and put in the cages. Embryos were collected every two hours. To measure hatching rate, 200 embryos were transferred to a fresh plate, and the number of hatched first instar larvae were counted. To monitor the rate and timing of pupation and eclosion, 50 first instar larvae were collected and cultured in a vial. The number of pupae and adults were counted every 12 hours. Pupation rate was calculated as the percentage of the number of pupae versus the number of first instar larvae contained in each vial. Eclosion rate was calculated as the percentage of the number of eclosed adults versus the total number of pupae contained in each vial.

Climbing assay
For negative geotaxis assay, aged flies were separated by gender and grouped in cohorts of 20 animals. Before testing, flies were transferred to a tube which was made by two vertically joined empty vials, and allowed to rest for 1 hour before the assay. After tapping the flies down to the bottom of the vial, we measured the number of flies that can climb above the 15-cm mark by 15 seconds. A climbing index was calculated as the percentage of the number of flies above the mark versus the total number of flies in the tube. Six replicate sets of experiments were performed for each genotype.

Lifespan assay
For lifespan analysis, groups of 20 newly eclosed males or females were collected and transferred to vials with fresh food every 2 to 3 days. The number of dead flies were counted. The survival rates were calculated as the percentage of the number of surviving flies versus the total number of flies. Three replicate sets of experiments were performed for each genotype.

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Conflict of Interest
The authors declare no conflict of interest.
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Abbreviations and nomenclature

Atg, Autophagy-related gene; ULK1/2, UNC-51 like autophagy activating kinase 1/2; Fip200, Focal adhesion kinase family interacting protein of 200 kDa; CRISPR, Clustered regularly interspaced short palindromic repeats; PAM, Protospeacer adjacent motif; RFP, red fluorescent protein; GFP, green fluorescent protein; Ref(2)p, refractory to sigma P; DAPI, 4’,6-diamidino-2-phenylindole; esg, escargot; ISC, intestinal stem cell; EB, enteroblast; EC, enterocyte; EE, enteroendocrine cell; pH3, phospho-Ser10-Histone H3; JNK, Jun N-terminal kinases; TOR, target of rapamycin; PCR, polymerase chain reaction

Figure legends

**Figure 1 Loss of Atg101 causes defects in starvation-induced and developmental autophagy**

(A) Schematic diagram for the Atg101 gene locus. Coding exons are in black, and non-coding exons are in white. Sequence of guide RNA target is indicated in red. PAM sequence is indicated in purple. Hyphens indicates the deleted sequence. Atg101<sup>6h</sup> has a 13 bp deletion around the guide RNA target site.

(B-B’’) Accumulation of GFP-Atg8a and RFP-Atg8a punctate structures in fat body cells of middle stage wild-type third instar larvae in response to starvation.

(C-C’’) Absence of GFP-Atg8a and RFP-Atg8a punctate structures in Atg101<sup>6h</sup> mutant animals under the same growth condition as (B-B’’).

(D) Quantification of the number of RFP-Atg8a punctate spots from B’ and C’. 145 cells from 16 wild-type fat body samples and 114 cells from 15 Atg101<sup>6h</sup> mutant fat body samples were counted.

(E) Quantification of the size of RFP-Atg8a punctate spots from B’ and C’. 1000 spots from 12 wild-type fat body samples and 198 spots from 16 Atg101<sup>6h</sup> mutant fat body samples were measured.

(F-F’’) Accumulation of GFP-Atg8a and RFP-Atg8a punctate structures in fat body cells of wandering-stage wild-type third instar larvae.
(G-G’’) Absence of GFP-Atg8a and RFP-Atg8a punctate structures in Atg101\textsuperscript{6h} mutant animals.

(H) Quantification of the number of RFP-Atg8a punctate spots from F’ and G’. 82 cells from 15 wild-type fat body samples and 162 cells from 29 Atg101\textsuperscript{6h} mutant fat body samples.

(I) Quantification of the size of RFP-Atg8a punctate spots from B’ and C’. 150 spots from 6 wild-type fat body samples and 410 spots from 4 Atg101\textsuperscript{6h} mutant fat body samples were measured.

Data are presented as mean ± SEM. Unpaired t-test was used for statistical analysis. *** indicates that p<0.001.

Scale bars: 50 um.

Figure 2 Loss of Atg101 leads to decreased adult lifespan and impaired locomotion

(A) Lifespan of Atg101\textsuperscript{6h} mutant females is reduced in comparison with control females. The survival rates were calculated as the percentage of the number of surviving females versus the total number of females at the indicated days. Data are presented as mean ± SD.

(B) Lifespan of Atg101\textsuperscript{6h} mutant males is reduced in comparison with control males. The survival rates were calculated as the percentage of the number of surviving males versus the total number of males at the indicated days. Data are presented as mean ± SD.

(C) A photograph of vials containing 7-day old male flies with the indicated genotypes taken 15 seconds after taping the vials.

(D) Groups of male flies with the indicated genotypes were tested for locomotor activity at Day 2 and Day 10. Data are presented as mean ± SEM. Paired t-test was used for statistical analysis. ** indicates that p<0.01.

Figure 3 Accumulation of ubiquitinated proteins and Ref(2) aggregates in Atg101 mutant brains
(A) Western blotting reveals that the level of ubiquitinated proteins is increased in Atg101\textsuperscript{6h} mutant fly heads. 7 day-old wild-type and Atg101\textsuperscript{6h} mutant flies were used. Tubulin as a loading control.

(B) Increased Ref(2) protein levels in Atg101\textsuperscript{6h} mutant fly heads. 7 day-old wild-type and Atg101\textsuperscript{6h} mutant flies were used. GAPDH as a loading control.

(C-E') Aggregates of ubiquitinated proteins accumulate in Atg101\textsuperscript{6h} mutant brains. Shown are confocal images of Drosophila brains of 7 day-old wild-type, Atg101\textsuperscript{6h} mutant and rescue flies, stained with anti-Ubiquitin antibody. Scale bars: 50 um.

(F) Quantification of the number of ubiquitin positive spots in wild-type, Atg101\textsuperscript{6h} mutant and rescue fly brains. n=12, 11, 15, respectively. * indicates that p<0.05.

(G-I') Aggregates of Ref(2)p proteins accumulate in Atg101\textsuperscript{6h} mutant brains. Shown are confocal images of Drosophila brains of 7 day-old wild-type, Atg101\textsuperscript{6h} mutant and rescue flies, stained with anti-Ref(2)p antibody. Scale bars: 50 um.

(J) Quantification of the total areas of Ref(2)p positive spots in wild-type, Atg101\textsuperscript{6h} mutant and rescue fly brains. n=6. * indicates that p<0.05.

(K-K'') Colocalization of Ref(2)p positive cells with Elav, a marker of neuronal nuclei. Phalloidin was used to label F-actin. Scale bars: 50 um.

(L-L'') Colocalization of Ref(2)p positive cells with Repo, a marker of glial nuclei. Phalloidin was used to label F-actin. Scale bars: 50 um.

**Figure 4 Loss of Atg101 results in adult midgut defects**

(A) Atg101\textsuperscript{6h} mutant adult midguts are shorter and thicker than the wild-type control. Shown are images of midguts from adult flies with the indicated genotypes.

(B) Quantification of the length of entire midguts and the width of posterior midguts from adult flies with the indicated genotypes. White lines indicate the location for measuring the width of posterior midguts. 10 midguts were used for each genotype. Data are presented as mean ± SEM. Unpaired t-test was used for statistical analysis. ** indicates that p<0.01.
(C–E') Z-projection confocal images of wild-type, *Atg101*<sup>6h</sup> mutant and rescue fly posterior midguts stained for DNA (using DAPI) and actin (using Phalloidin) as indicated. Top right panels in (C'), (D') and (E') show higher magnification of boxed area. Stars indicate the nuclei of EC cells.

(F) Quantification of the number of cells with large nuclei. Cells in 18 defined regions from 9 wild-type midguts, 18 defined regions from 10 *Atg101*<sup>6h</sup> mutant midguts and 10 defined regions from 5 rescue fly midguts were counted. Data are presented as mean ± SEM. Unpaired t-test was used for statistical analysis. ** indicates that p<0.01.

(G) Quantification of the size of large nuclei. Sizes of 60 nuclei from 6 wild-type midguts, 90 nuclei from 9 *Atg101*<sup>6h</sup> mutant midguts and 90 nuclei from 9 rescue fly midguts were measured. Data are presented as mean ± SEM. Unpaired t-test was used for statistical analysis. ** indicates that p<0.01.

(H–J) Enlarged enterocytes in *Atg101*<sup>6h</sup> mutant midguts. Single plane confocal images of wild-type, *Atg101*<sup>6h</sup> mutant and rescue fly posterior midguts stained for anti-Dlg, which labels the cell membrane. Scale bars: 10 µm.

(K–M) Cross-section of midgut epithelium from wild-type, *Atg101*<sup>6h</sup> mutant and rescue adult flies. The white double-headed arrows indicate the intestinal lumen and the white lines indicate the intestinal wall. Scale bars: 50 µm.

**Figure 5** *Atg101* is required for the differentiation of intestinal stem cell

(A–B') Z-projection confocal images of the posterior midgut of wild-type and *Atg101*<sup>6h</sup> mutant flies expressing *esg-GFP*, which labels both ISC and EB cells.

(C) Quantification of *esg-GFP* positive cells. For quantification, Z-projection confocal images of the posterior midgut were acquired, and then the number of *esg-GFP* positive cells and all the rest cells were counted in a defined region. For each defined region, at least 100 cells were present. Cells in 8 defined regions from 7 wild-type midguts and 15 defined regions from 9 *Atg101*<sup>6h</sup> mutant midguts were counted. Data are presented as mean ± SEM. Unpaired t-test was used for statistical analysis. ** indicates that p<0.01.
(D) Quantification of esg-GFP positive cell clusters. Quantification was performed as Figure 5C. Cell clusters (at least 3 cells) in 21 defined regions from wild-type midguts and 27 defined regions from Atg101<sup>6h</sup> mutant midguts were counted. Data are presented as mean ± SEM. Unpaired t-test was used for statistical analysis. ** indicates that p<0.01.

(E-F') Z-projection confocal images of the posterior midgut of wild-type and Atg101<sup>6h</sup> mutant flies stained for anti-phospho-H3 (pH3), which labels mitotic ISCs.

(G) Quantification of pH3 positive cells within the entire gut. 20 wild-type and 24 Atg101<sup>6h</sup> mutant midguts were used for the quantification. Data are presented as mean ± SEM. Unpaired t-test was used for statistical analysis. N.S. indicates it is not significant. DAPI was used to visualize DNA. Scale bars: 50 um.

(H-I') Z-projection confocal images of wild-type and Atg101<sup>6h</sup> mutant posterior midguts stained for anti-prospero (pros), which labels EB cells.

(J) Quantification of pros positive cells. Quantification was performed as Figure 5C. Cells in 27 defined regions from 25 wild-type midguts and 23 defined regions from 14 Atg101<sup>6h</sup> mutant midguts were counted. Data are presented as mean ± SEM. Unpaired t-test was used for statistical analysis. N.S. indicates it is not significant.

(K-L') Z-projection confocal images of wild-type and Atg101<sup>6h</sup> mutant posterior midguts stained for anti-Pdm1, which labels EC cells.

(M) Quantification of Pdm1 positive cells. Quantification was performed as Figure 5C. Cells in 10 defined regions from 10 wild-type midguts and 9 defined regions from 9 Atg101<sup>6h</sup> mutant midguts were counted. Data are presented as mean ± SEM. Unpaired t-test was used for statistical analysis. ** indicates that p<0.01.

**Figure 6 Atg101 functions in ECs to regulate cell growth**

(A-C) esg-Gal4 driven Atg101 expression in both ISC and EB population failed to rescue the enlarged enterocyte size defects in Atg101 mutants. Shown are confocal images of the esg-Gal4 control, Atg101<sup>6h</sup> mutant and rescued fly
posterior midguts stained for DNA and Dlg as indicated. For conditional induction of Atg101, esg-Gal4 UAS-GFP; Gal80\textsuperscript{ts} was used in the rescue experiment.

(D-F) Myo1A-Gal4 driven Atg101 expression in EC cells was able to rescue the enlarged enterocyte size defects in Atg101 mutants. Shown are confocal images of the Myo1A-Gal4 control, Atg101\textsuperscript{6h} mutant and rescued fly posterior midguts stained for DNA and Dlg as indicated. For conditional induction of Atg101, Myo1A-Gal4 UAS-GFP; Gal80\textsuperscript{ts} was used in the rescue experiment.

(G-I) pros-Gal4 driven Atg101 expression in EE cells failed to rescue the enlarged enterocyte size defects in Atg101 mutants. Shown are confocal images of the pros-Gal4 control, Atg101\textsuperscript{6h} mutant and rescued fly posterior midguts stained for DNA and Dlg as indicated. pros-Gal4 was used in the rescue experiment. Scale bars: 20 um.
Figure 2

A

B

C

D

Survival rate (%)

Days

Survival rate (%)

Days

Climbing index (%)

2 Days

10 Days

wt Atg101th Rescue

wt Atg101th Rescue

wt Atg101th Rescue

wt Atg101th Rescue

(Female)

(Male)

wt

AlgL01th

Rescue

wt

AlgL01th

Rescue

wt

AlgL01th

Rescue

wt

AlgL01th

Rescue
Figure 3

A

B

C

C'

G

G'

D

D'

H

H'

E

E'

I

I'

F

J

K

K'

K''

K'''

L

L'

L''

L'''

Numbers of Ub dots (x10^2)

Total areas of Ref(2)p dots (x1000 arbitrary units)
Figure 6

|   | Dlg+DAPI | Dlg+DAPI | Dlg+DAPI |
|---|---------|---------|---------|
| A | esg-Gal4 | Myo1A-Gal4 | pros-Gal4 |
| B | esg-Gal4; Atg101\textsuperscript{ts} | Myo1A-Gal4; Atg101\textsuperscript{ts} | pros-Gal4; Atg101\textsuperscript{ts} |
| C | esg-Gal4; UAS-Atg101; Atg101\textsuperscript{ts} | Myo1A-Gal4; UAS-Atg101; Atg101\textsuperscript{ts} | pros-Gal4; UAS-Atg101; Atg101\textsuperscript{ts} |
The autophagy-related gene Atg101 in Drosophila regulates both neuron and midgut homeostasis

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