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Characterization of the *Autophagy related gene-8a (Atg8a)* promoter in *Drosophila melanogaster*

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ABSTRACT Autophagy is an evolutionarily conserved process which is upregulated under various stress conditions, including nutrient stress and oxidative stress. Amongst autophagy related genes (Atgs), *Atg8a* (*LC3* in mammals) is induced several-fold during nutrient limitation in *Drosophila*. The minimal *Atg8a* cis-regulatory module (CRM) which mediates transcriptional upregulation under various stress conditions is not known. Here, we describe the generation and analyses of a series of *Atg8a* promoter deletions which drive the expression of an *mCherry-Atg8a* fusion cassette. Expression studies revealed that a 200 bp region of *Atg8a* is sufficient to drive expression of Atg8a in nutrient rich conditions in fat body and ovaries, as well as under nutrient deficient conditions in the fat body. Furthermore, this 200 bp region can mediate *Atg8a* upregulation during developmental histolysis of the larval fat body and under oxidative stress conditions induced by H_2O_2. Finally, the expression levels of Atg8a from this promoter are sufficient to rescue the lethality of the *Atg8a* mutant. The 200 bp promoter-fusion reporter provides a valuable tool which can be used in genetic screens to identify transcriptional and post-transcriptional regulators of Atg8a.

KEY WORDS: *Autophagy, Atg8a, promoter, mCherry-Atg8a*

Macroautophagy (termed as autophagy henceforth) is a cytoplasmic degradative process that is induced within the cells in response to external as well as internal stress stimuli. It proceeds by formation of a double membrane structure called autophagosome that encompasses cytoplasmic contents including damaged organelles that are marked for destruction in the lysosomal compartment. The degraded products are exported to the cytosol for building macromolecules necessary to preserve cell structure and function in stress conditions (Mizushima, 2007). This process is vital for many physiological processes including development, differentiation, and maintenance of cellular homeostasis (Mizushima and Levine, 2010). It is dysregulated in several disease conditions that include cancer, infectious diseases, heart diseases, liver diseases, and neurological disorders (Choi *et al.*, 2013; Murrow and Debnath, 2012). Although autophagy is believed to be a non-selective process, several reports suggest that selective autophagy occurs under specific cellular contexts (Boya *et al.*, 2013).

Basal autophagy and induced autophagy employ multiple autophagy-related (Atg) proteins that take part in building the double membrane autophagosome. Autophagy is initiated by the formation of a cup-shaped double membrane structure referred to as phagophore/pre-autophagosomal structure (PAS) which further elongates to form the isolation membrane. Several Atgs are essential for the elongation of the isolation membrane which eventually completes forming the cargo laden vesicle called the autophagosome. Autophagosome can either mature into an amphisome through fusion with endosome or into an autolysosome through fusion with the lysosome. The lysosomal acid hydrolases then degrade the components of the autophagosome. The entire process of autophagosome formation and maturation is highly regulated (Mizushima *et al.*, 2011). Amongst several Atg proteins, the Atg8/MAP1LC3 (microtubule associated protein 1 light-chain 3, LC3, in mammals), a ubiquitin-like protein, has been shown to be essential for elongation and maturation of the autophagosome (Mizushima *et al.*, 1998; Nakatogawa *et al.*, 2007). Atg8 is processed by the Atg4 cysteine-protease to expose a C-terminal glycine residue. Cleaved Atg8 is conjugated to phosphatidylethanolamine (PE) by series of E1, E2 and E3-like enzymatic reactions catalyzed by Atg7 (E1-like enzyme), Atg3 (E2-like enzyme), and the Atg12-5-16 complex, to generate Atg8-PE (a membrane-bound form of Atg8). This lipidation event can be monitored using several cell

Abbreviations used in this paper: Atg, autophagy-related; CRM, cis-regulatory module.

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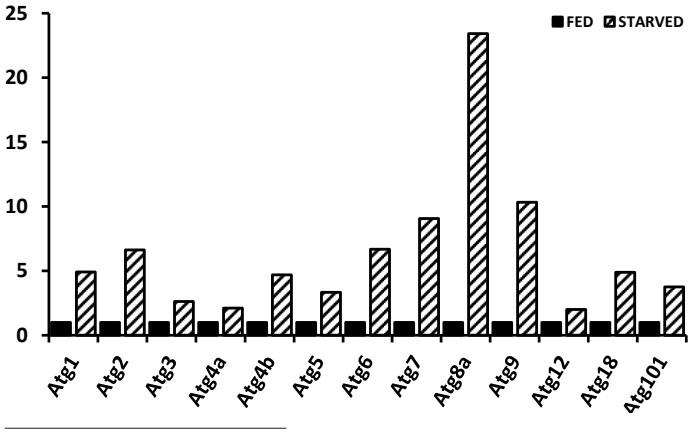

Fig. 1 (Left). Autophagy specific genes are upregulated upon nutrient limitation in *Drosophila*. *Expression analysis of Atg genes in fat body from third instar larvae in fed and starved conditions by qPCR. Transcript levels were normalized against the internal control gene tubulin and represented as relative expression. Note the massive upregulation of* Atg8a *expression upon starvation.*

biological and biochemical techniques to understand the process of autophagy. The processing and conjugation of Atg8 are crucial for formation of autophagosomes, the size of autophagosomes and the number of autophagosomes. For instance, when Atg8 levels are reduced during starvation, significantly smaller sized autophagosomes are formed, suggesting that the amount of this protein regulates autophagosome size (Weidberg *et al.*, 2010; Xie *et al.*, 2008). Thus, the expression of both *Atg8* mRNA and Atg8 protein appear to be crucial to understanding the kinetics of autophagy. *Drosophila* possesses two *Atg8/LC3* homologs, *Atg8a* and *Atg8b*, of which *Atg8a* has been routinely used as a marker to monitor autophagy. Previous reports have demonstrated that ~2kb upstream region of Atg8a was sufficient for the reporter gene expression in larval tissues including fat body, midgut and salivary glands of *Drosophila* (Denton *et al.*, 2009; Nelson *et al.*, 2014). In this study, we have characterized Atg8a promoter that mediates *Atg8a* expression in *Drosophila melanogaster* under distinct physiological conditions. Experiments with transgenic *Drosophila* revealed that a 200bp region of *Atg8a/CG32672* is sufficient for the wild type-like expression of *mCherry-Atg8a* fusion reporter in the fat body as well as in the germline cells of the ovary under unstressed conditions. Also, this promoter fragment is sufficient to drive upregulation of *mCherry-Atg8a* under nutrient stress as well as oxidative stress. Finally, this promoter-protein fusion reporter can rescue the lethality of *Atg8a*^{KG07569} mutants.

Results and Discussion

Autophagy is stimulated in response to nutrient limitation and as a consequence transcription of several autophagy genes is upregulated several folds (Füllgrabe *et al.*, 2016; Scott *et al.*,

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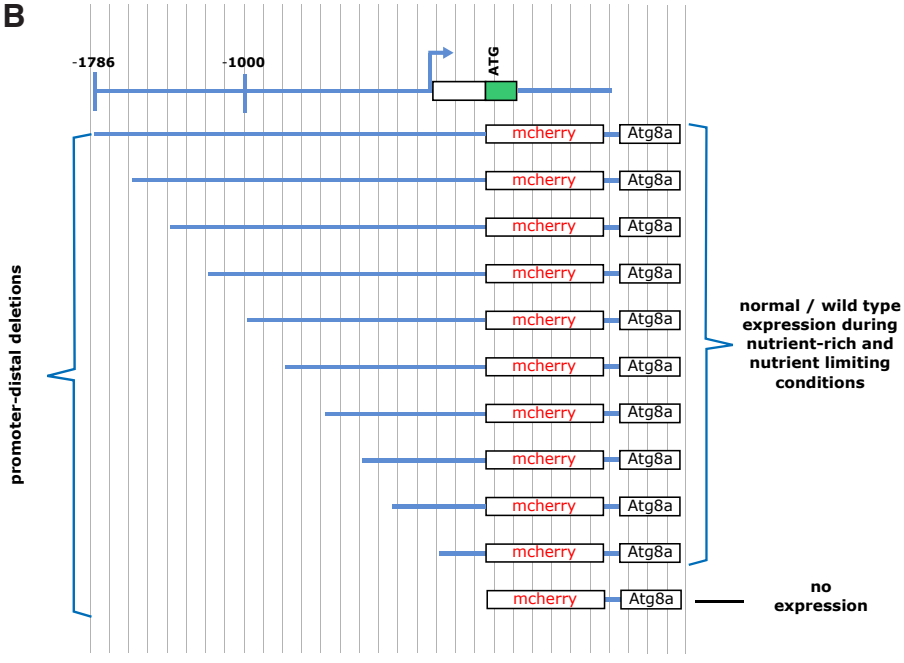


Fig. 2 (Left). Promoter-protein reporter constructs identify the minimal cis- regulatory region driving normal mCherry-Atg8a expression. (A) *The* Atg8a *promoter from -1786 to + 269. 5′UTR is shown in orange. +1 depicts transcription initiation site. TATA boxes are depicted in blue.* **(B)** *The schematic drawing at the top represents the sequence region at the transcription start site of the* Atg8a *locus, extending -1786 bp upstream and +269 bp downstream. Chromosomal orientation is inverted. The cloned reporter constructs are depicted below. The black lines mark the extent of the promoter fragments driving the expression of mCherry-Atg8a. The effects are indicated on the right.*

2004 and Fig.1A). In particular, *Atg8a* is expressed atleast 20-fold higher in amino acid starved *Drosophila* larvae as compared in nutrient rich conditions (Fig.1).Previously, we had described the generation of *GFP-Atg8a* fusion reporter for monitoring autophagy driven by an 2kb *Atg8a* upstream promoter (Denton *et al.*, 2009). This ~ 2 kb fragment contains 1786bp of the cis-regulatory region upstream of the transcription start site and 269bp of the 5'UTR region of *Atg8a* (Fig. 2A; http://flybase.org). This transgene can rescue the lethality associated with $Atg8a^{KG07569}$ transposon insertion.

To identify the minimal cis-regulatory region of *Atg8a* essential for wild-type expression upon starvation, we generated a series of *Atg8a* promoter deletion *mCherry-Atg8a* reporter constructs (called *pCAtg8a* henceforth). In the deletion series, each of the subsequent constructs possesses a 200bp deletion in the *Atg8a* promoter (Fig.2B). These distal deletions are shown in Fig. 2B. The entire set of *Atg8a* promoter distal deletions were subjected to starvation-induced autophagy using fat body of *Drosophila* as a model (Scott *et al.*, 2004). Fig.3 shows expression of mCherry-Atg8a for all promoter-distal deletions under both fed and starved conditions. Our analyses of mCherry-Atg8a expression suggest that all promoter fragments including the smallest promoter fragment (200bp) can drive uniform expression of the mCherry-Atg8a in the fat body in nutrient rich conditions. mCherry-Atg8a was found to be predominantly cytoplasmic in these conditions. This suggests that the minimal *Atg8a* promoter is 200bp or less, and this fragment possesses binding sites for Transcription Factors (TFs) that are necessary for basal expression of *Atg8a*. We subjected these transgenic lines to starvation-induced autophagy assay and monitored the expression and localization of mCherry-Atg8a (Scott *et al.,* 2004). Atg8a displays diffuse cytoplasmic staining in nutrient rich conditions, and upon starvation, it undergoes a translocation to the autophagosome membrane and could be observed as punctate (mCherry-Atg8a; red puncta) structures in the fat body cells (Scott *et al.*, 2004). mCherry-Atg8a puncta were detected in all fat body cells dissected from *2kb-0.2kb-pCAtg8a* transgenic lines. Our data suggest that all promoter fragments carry binding sites/ cis-regulatory modules (CRMs) for transcription factors that induce *mCherry-Atg8a* transcriptional upregulation upon starvation.

Developmental autophagy is induced in the larval fat body when the late 3rd instar larvae transition to the pupal stage. During this transition autolysosomes generated in the fat body contain glycogen and mitochondria in various stages of degeneration (Butterworth *et al.*, 1988; Butterworth and Forrest, 1984). This developmental autophagy is characterized by rather large sized autophagosomes and autolysosomes and can range from 2-5 μm in size. *2kb-0.2kb-pCAtg8a* transgenic lines were subjected to developmental autophagy assay in the fat body. As expected in entire series of *2kb-0.2kb-pCAtg8a* transgenic lines fat body cells exhibited 2-5 μm sized autolysosomes (Fig.4 A,B).

Autophagy has been shown to be essential for proper progression of oogenesis. *Atg8a* is induced in germline cells as well as follicle cells in response to starvation during *Drosophila* oogenesis (Barth *et al.*, 2011). To determine if mCherry-Atg8a is expressed at a basal level, we dissected ovaries from *2kb-0.2kbpCAtg8a* transgenic flies and imaged for mCherry-Atg8a. Few red puncta were detected in nurse cells at stages 6 to stage 14 egg chambers in all promoter deletions tested indicating that the 200bp fragment includes cis-regulatory modules driving the expression of mCherry-Atg8a during oogenesis (Fig.4 C,D).

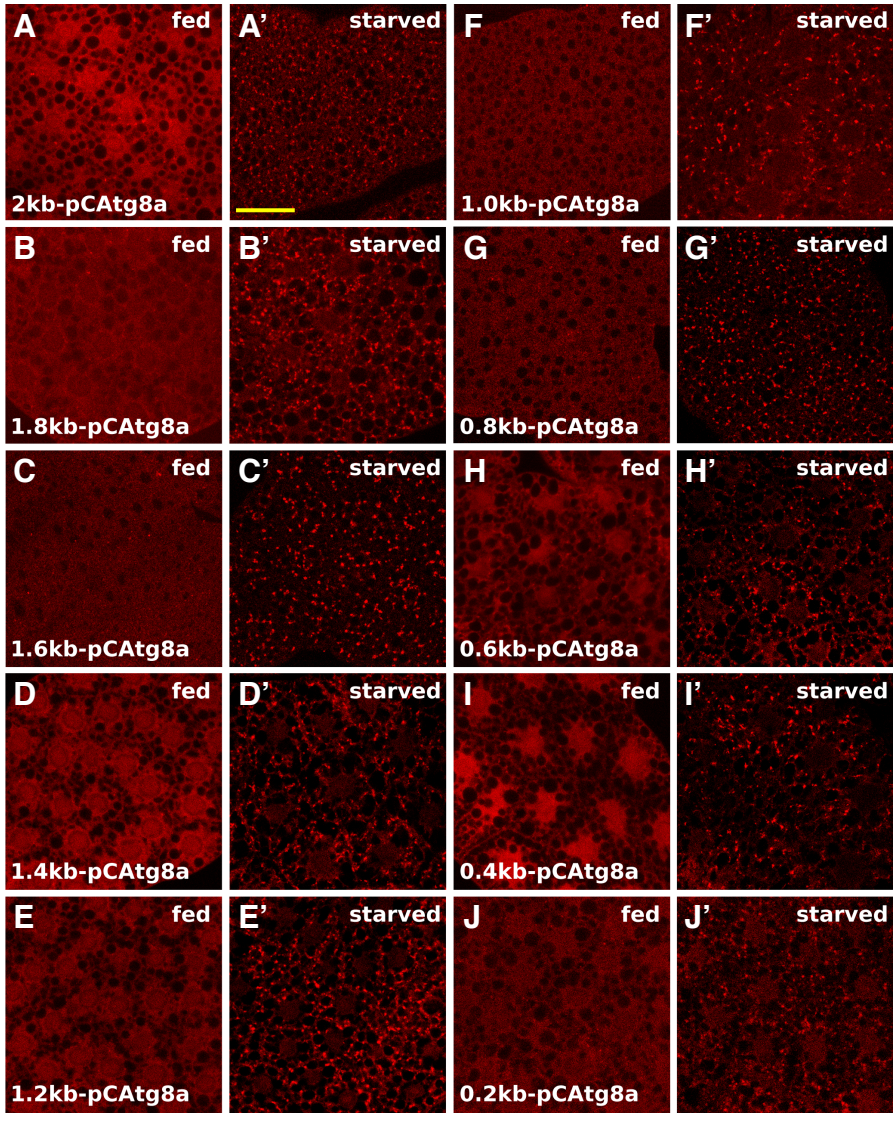
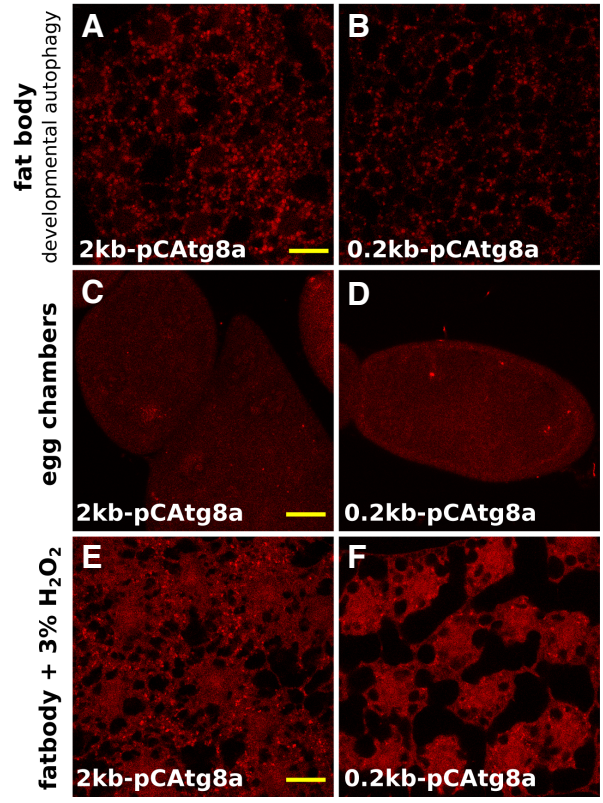


Fig. 3. Expression patterns of distal *Atg8a* promoter deletion fusion reporter constructs. (A-J') *Fat body showing the expression pattern of the mCherry-Atg8a from all* Atg8a *promoter fragments in both nutrient rich and nutrient deficient conditions. Atg8a fusion reporter is visualized by mCherry fluorescence (red) as diffused staining under fed conditions. Note the punctate pattern of mCherry-Atg8a expression in starved conditions.* **(A-A')** 2.0kb-pCAtg8a; **(B-B')** 1.8kb-pCAtg8a; **(C-C')** 1.6kb-pCAtg8a; **(D-D')** 1.4kb-pCAtg8a; **(E-E')** 1.2kb-pCAtg8a; **(F-F')** 1.0kb-pCAtg8a; **(G-G')** 0.8kb-pCAtg8a; **(H-H')** 0.6kb-pCAtg8a; **(I-I')** 0.4kb-pCAtg8a; **(J-J')** 0.2kb-pCAtg8a; **(K-K')**. *Scale bar, 40 μm.*



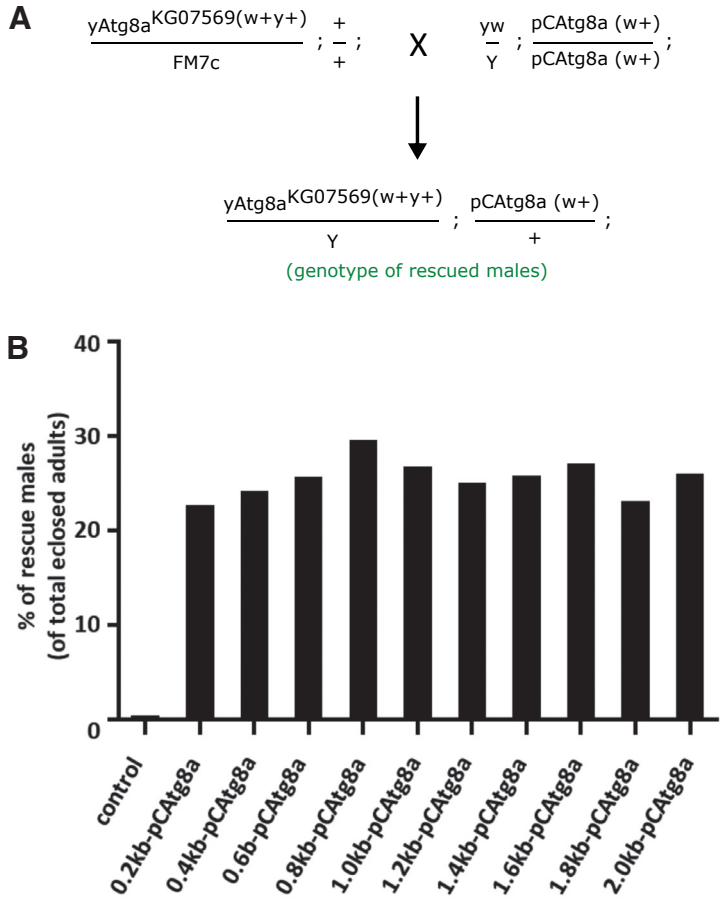


Fig. 4 (Top). Expression of mCherry-Atg8a driven by *Atg8a* promoter fragments during developmental autophagy, basal autophagy and in oxidative stress. (A,B) *mCherry-Atg8a expression during developmental autophagy in fat body cells;* **(A)** 2.0kb-pCAtg8a; **(B)** 0.2kb-pCAtg8a. **(C,D)** *Basal expression of mCherry-Atg8a in ovaries;* **(C)** 2.0kb-pCAtg8a; **(D)** 0.2kb-pCAtg8a. **(E,F)** *Upregulation of mCherry-Atg8a expression in fat body cells upon exposure to 3% H_2O_2 ;* **(E)** 2.0kb-pCAtg8a; *(F)* 0.2kb-pCAtg8a. *Scale bars: 40μm in (A,B,E,F); 10 μm in (C,D).*

Fig. 5 (Right). Complementation of *Atg8a*^{KG07569} mutants by different *pCAtg8a* transgenes. (A) *Crossing strategy for assessing rescue of lethality associated with* Atg8a ^{KG07569} *allele.* **(B)** *Graph showing the percentage of rescue genotype observed in the complementation experiment.*

It has been demonstrated that reactive oxygen species can stimulate autophagy (Filomeni *et al.*, 2015). To test further if *2-0.2kb-pCAtg8a* reporter transgenic lines respond to oxidative stress, we exposed larvae to 3% H_2O_2 for a period of 24 hours. Fat body cells were imaged for presence of mCherry-positive puncta. mCherry-Atg8a puncta were detected in fat body cells dissected from the entire series of transgenic lines in response to oxidative stress (Fig. 4 E,F). These observations suggest that CRMs mediating redox stress response are present in 200bp minimal promoter.

Next we tested if *2kb-0.2kb-pCAtg8a* transgenes can rescue lethality associated with *Atg8a*^{KG07569} transposon insertion. Atg8a protein cannot be detected on western blots performed using protein extracts prepared from homozygous *Atg8a*^{KG07569} insertion mutants (Chang *et al.*, 2013). This rescue experiment tests if the expression levels of *mCherry-Atg8a* from the different promoter fragments are sufficient for the complementing the deficiency of Atg8a in homozygous *Atg8a*^{KG07569} mutants. All transgenic lines harbouring promoter deletions *2-0.2kb-pCAtg8a* are able to rescue the lethality of *Atg8a*^{KG07569} mutants. The rescue genotypes displayed Mendelian ratios of inheritance Fig. 5A,B and Supplementary Table S2.

The minimal-promoter-protein-reporter construct could be used to uncover post-transcriptional regulators in both forward and reverse genetic screens. The promoter-protein fusion reporter described here is driven by an endogenous promoter it will avoid potential problems associated with heterologous promoter driven reporters. Transgenes from heterologous promoter may be expressed at lower or higher levels in different tissues leading to increased incidences of uncovering false negative and false positive candidates. The 0.2kb-*pCAtg8a* transgenic line generated in this study could be a valuable tool for designing and conducting genetic screens to identify genes that regulate Atg8a both transcriptionally and post-transcriptionally.

Materials and Methods

Drosophila *maintenance*

Flies were reared at 25°C on standard cornmeal/molasses/agar media. The following *Drosophila melanogaster* stocks were used: *y,w1118*,

CantonS, *OregonR, Atg8a$^{KG07569\ BL-1}$4639* (Bloomington *Drosophila* Stock Center, USA)*, 2kb-pCAtg8a, 1.8kb-pCAtg8a, 1.6 kb-pCAtg8a, 1.4 kb-pCAtg8a, 1.2kb-pCAtg8a, 1kb-pCAtg8a, 0.8kb-pCAtg8a, 0.6kb-pCAtg8a, 0.4kb-pCAtg8a, 0.2kb-pCAtg8a, 0 kb-pCAtg8a* (this study).

Generation of promoter fusion reporter constructs

A 1786bp fragment upstream of the *Drosophila Atg8a* gene along with 269 bp of the 5′ *UTR* sequences (–1786 to +269bp, Fig.1) was PCR amplified from *Drosophila CantonS* genomic DNA using restriction enzyme sites NotI incorporated in the primers as shown in Supplementary Table 1. The P-element based transformation plasmid *pCasper4* was used to generate *pC4-2kbAtg8a* promoter (–1786 to +269). A *mcherry-atg8a* fragment was cut with XbaI from *pPGW-mcherry-Atg8a* (Nezis *et al*., 2009) and ligated in *pC4-2kbAtg8a* promoter to generate *pC4-2kbAtg8a-mcherry-Atg8a (2kb-pCAtg8a).* This construct was used to generate a series of mCherry-Atg8a fusion reporter constructs with 5′ deletions of *Atg8a* promoter. A series of 200bp deletions were generated using standard PCR and cloned upstream of *mCherry-Atg8a*. All recombinant plasmids were analysed and confirmed by PCR, restriction digestion and DNA sequencing. After confirming the sequence and orientation of the inserts, the plasmids were sent for microinjections.

Generation of transgenic lines

Each of the *2-0.2kb-pCAtg8a* constructs were sent to C-CAMP, Bangalore, India for generating transgenic lines. A total of 47 transgenic lines were screened for mcherry-Atg8a expression and rescue of *Atg8a^{KG07569}* mutant.

Starvation induced autophagy assay

Starvation induced autophagy was performed according to Scott et. al.,2004 (Scott *et al*., 2004). Briefly, 2nd instar larvae were fed on live yeast for 24 hours. Early 3rd instar larvae were then subjected to starvation by transferring them to Petri dish containing 20% sucrose in 1X PBS for 3 hours.

Dissection of fat body

Fat body dissection was carried according to Shravage *et al*., 2013 (Shravage *et al*., 2013).

Real Time PCR

RNA was isolated from larval fat bodies (n=25) using Trizol Reagent (Invitrogen, USA) and was treated with DNase I to remove any residual DNA. cDNA was generated from 1 μg of RNA, using Superscript III Reverse Transcriptase (Invitrogen, USA), following manufacturer's instructions. Real time PCR was performed according to (Denton *et al*., 2010). Primers were used for real time PCR reaction are listed in Supplementary Table S1.

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