

Drosophila Gyf/GRB10 interacting GYF protein is an autophagy regulator that controls neuron and muscle homeostasis

Myungjin Kim,^{1,*} Ian Semple,¹ Boyoung Kim,¹ Alexandra Kiers,¹ Samuel Nam,¹ Hwan-Woo Park,^{1,2} Haeli Park,¹ Seung-Hyun Ro,¹ Jeong-Sig Kim,^{1,3} Gábor Juhász,⁴ and Jun Hee Lee^{1,*}

¹Department of Molecular and Integrative Physiology; University of Michigan; Ann Arbor, MI USA; ²Department of Cell Biology; College of Medicine; Konyang University; Daejeon, Korea; ³Department of Obstetrics and Gynecology; Soonchunhyang University Seoul Hospital; Seoul, Korea; ⁴Department of Anatomy, Cell and Developmental Biology; Eötvös Loránd University; Budapest, Hungary

Keywords: aging, autophagy, *Drosophila*, growth, neurodegeneration

Abbreviations: Atg, autophagy-related; dsRNA, double-stranded RNA; 4EHP, eukaryotic translation initiation factor 4E-homologous protein; GMR, glass multiple reporter; GRB10, growth factor receptor-bound protein 10; MiMIC, Minos-mediated integration cassette; PD, Parkinson disease; PtdIns3P, phosphatidylinositol 3-phosphate; ROS, reactive oxygen species; s.p.m., single pair mating; TORC1, TOR complex 1; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling; UAS, upstream activator sequence.

Autophagy is an essential process for eliminating ubiquitinated protein aggregates and dysfunctional organelles. Defective autophagy is associated with various degenerative diseases such as Parkinson disease. Through a genetic screening in *Drosophila*, we identified *CG11148*, whose product is orthologous to GIGYF1 (GRB10-interacting GYF protein 1) and GIGYF2 in mammals, as a new autophagy regulator; we hereafter refer to this gene as *Gyf*. Silencing of *Gyf* completely suppressed the effect of Atg1-Atg13 activation in stimulating autophagic flux and inducing autophagic eye degeneration. Although *Gyf* silencing did not affect Atg1-induced Atg13 phosphorylation or Atg6-Pi3K59F (class III PtdIns3K)-dependent Fyve puncta formation, it inhibited formation of Atg13 puncta, suggesting that *Gyf* controls autophagy through regulating subcellular localization of the Atg1-Atg13 complex. *Gyf* silencing also inhibited Atg1-Atg13-induced formation of Atg9 puncta, which is accumulated upon active membrane trafficking into autophagosomes. *Gyf*-null mutants also exhibited substantial defects in developmental or starvation-induced accumulation of autophagosomes and autolysosomes in the larval fat body. Furthermore, heads and thoraxes from *Gyf*-null adults exhibited strongly reduced expression of autophagosome-associated Atg8a-II compared to wild-type (WT) tissues. The decrease in Atg8a-II was directly correlated with an increased accumulation of ubiquitinated proteins and dysfunctional mitochondria in neuron and muscle, which together led to severe locomotor defects and early mortality. These results suggest that *Gyf*-mediated autophagy regulation is important for maintaining neuromuscular homeostasis and preventing degenerative pathologies of the tissues. Since human mutations in the *GIGYF2* locus were reported to be associated with a type of familial Parkinson disease, the homeostatic role of *Gyf*-family proteins is likely to be evolutionarily conserved.

Introduction

Autophagy is a highly conserved homeostatic process essential for bulk degradation of cytoplasmic contents. It is critical for elimination of toxic cytoplasmic inclusions, dysfunctional organelles such as damaged mitochondria,¹ excessive nutrient deposits such as lipid droplets,² and invading microorganisms.³ Defective regulation of autophagy in animal models can result in diverse pathological phenomena including neurodegeneration⁴ and muscular dystrophy⁵ that are associated with accumulation of protein aggregates and reactive oxygen species (ROS)-producing

dysfunctional mitochondria. In humans, genetic mutations leading to autophagy defects have been reported to be associated with hereditary neurodegenerative diseases such as several types of familial Parkinson disease (PD).⁶

The molecular basis for autophagy has been explored most thoroughly in yeast, which offers a simple and straightforward genetic model. Genetic screening in yeast has identified many components constituting the core autophagy-controlling machinery named autophagy-related (*ATG*) genes. Particularly, it has been well established that *ATG* genes play a central role in autophagy processes as they conjugate ATG12 and ATG8 or

*Correspondence to: Myungjin Kim; Email: myungjin@umich.edu, Jun Hee Lee; Email: leeju@umich.edu

Submitted: 10/03/2014; Revised: 06/03/2015; Accepted: 06/12/2015

<http://dx.doi.org/10.1080/15548627.2015.1063766>

MAP1LC3/LC3 onto target proteins and lipids in a mechanism analogous to the ubiquitin conjugating system.⁷ The conjugation of ATG8 or LC3 into target lipid membranes induces the formation of an autophagosome that will fuse with the lysosome, in which the cargo will become degraded.⁸ Subsequent biochemical and genetic studies in mammalian cells and animal models revealed that this core machinery is surprisingly well conserved in higher animals as well, including both vertebrates and invertebrates.⁷

However, unlike unicellular yeast, multicellular animals have developed more sophisticated molecular mechanisms for controlling autophagy, as autophagy needs to respond to a greater variety of physiological inputs such as hormonal regulation and developmental signaling. Furthermore, tissues that are quiescent and have a low cell turnover rate such as neurons and muscle cells will need to maintain their intracellular homeostasis by removing unnecessary and toxic cell constituents through autophagy. Indeed, a recent genetic screening in *C. elegans* and *Drosophila* identified several autophagy components that are not present in yeast cells, demonstrating the existence of metazoan-specific autophagy components.^{9,10} Still, the metazoan-specific autophagy pathway awaits more rigorous investigation.

To discover additional autophagy-controlling genetic components, we conducted our own genetic screening in *Drosophila*, from which we isolated *Gyff/CG11148*, whose human ortholog is mutated in a type of familial PD, as a new autophagy-regulating gene. We showed that Gyf is essential for starvation-induced and developmental autophagy, as well as for physiological autophagy which is critical for eliminating protein aggregates and dysfunctional organelles. Correspondingly, null mutants of *Gyff* exhibited a dramatically reduced life span and early impairment of mobility, which is associated with neurodegeneration, muscle degeneration, and accumulation of ubiquitinated proteins and dysfunctional mitochondria in the tissues. Taken together, our results establish Gyf as a new mediator of autophagy that protects tissue homeostasis and further prevents development of diverse degenerative pathologies in *Drosophila*.

Results

Identification of *Gyff/CG11148* as a new autophagy-regulating gene

In order to discover new autophagy regulators, we constructed a *Drosophila* line that stably expresses both Atg1 and Atg13 specifically in the eye using the *upstream activator sequence (UAS)-Atg1* and *UAS-Atg13* transgenes and a *glass multiple reporter (GMR)-Gal4* driver. Simultaneous transgenic expression of Atg1 and Atg13 provoked excessive autophagy in the developing eye, resulting in easily noticeable eye degeneration (Fig. 1A), as formerly reported.¹¹ We conducted a genetic screening by crossing the stable *GMR>Atg1+Atg13* line (the fly line with *UAS-Atg1* and *UAS-Atg13* transgenes whose expression is driven by *GMR-Gal4*) with double-stranded RNA (dsRNA) transgenic *Drosophila* lines obtained from the established libraries of dsRNA transgenic flies^{12,13} and searching for dsRNA lines that can suppress the

phenotype of the *GMR>Atg1+Atg13* transgene. A dsRNA line that targets an unnamed gene temporarily annotated as *CG11148* was identified to completely suppress the eye-degeneration phenotype of the *GMR>Atg1+Atg13* flies (Fig. 1A). The level of suppression by *CG11148* silencing was even comparable to the level conferred by silencing of *Atg1* itself (Fig. 1A). Silencing of *CG11148* alone did not affect eye development and morphology, similar to other autophagy regulators such as *Atg1* (Fig. 1B).

A sequence database search identified the protein product of *CG11148* as an ortholog of mammalian proteins named GIGYF1 (GRB10 interacting GYF protein 1) and GIGYF2. GIGYF2 is also known as KIAA0642, PERQ2, PERQ3, TNRC15, and PARK11.¹⁴ Considering that the protein product of *CG11148* is orthologous to both GIGYF1 and GIGYF2, we named this newly discovered gene *Drosophila Gyff* (hereafter *Gyff*). Strong primary sequence homology (approximately 30% similarity) was detected at both terminal regions of the protein, while the central region did not show any significant similarity between the mammalian and *Drosophila* orthologs (Fig. 1C). We subsequently designated the 2 homologous terminal regions as the N-terminal and C-terminal domain, respectively. The N-terminal domain of mammalian GIGYF2 contains 2 important protein-protein interaction motifs, namely EIF4E2/4EHP (eukaryotic translation initiation factor 4E-homologous protein)-binding motif¹⁵ and GRB10 (growth factor receptor-bound protein 10)-binding GYF motif.¹⁶ GIGYF2 can regulate protein translation and insulin signal transduction through these 2 motifs.^{15,16} Such motifs are conserved in *Drosophila* Gyf as well (Fig. 1C). Gyf-family proteins are found in most metazoan organisms (Fig. 1D), but no specific orthologs exist in fungi and plants. Expression of *Drosophila Gyff* mRNA and Gyf protein is detected throughout all developmental stages, although the expression was relatively low during larval development (Fig. 1E). Unlike *Gyff* whose silencing (Fig. 1F and G) can strongly suppress the eye phenotype of Atg1-Atg13 overexpression (Fig. 1A), silencing of *4EHP* (Fig. 1H) or *pico/Grb10* (Fig. 1I) did not suppress the Atg1-Atg13 effect (Fig. 1J), suggesting that Gyf may control autophagy independently of these formerly known interacting partners. In addition, although mammalian GIGYF2 was shown to modulate insulin signaling,^{16,17} the *Gyff* silencing construct did not produce any genetic interaction with insulin-target of rapamycin (TOR) signaling components in the eye system (Fig. S1).

Gyff silencing does not inhibit Atg1-Atg13 expression and activity

We further characterized the phenotypic interaction between Atg1-Atg13 activation and *Gyff* silencing through biochemical and histological methods. First, we checked the molecular status of overexpressed Atg1 and Atg13 in eye protein lysates. *Gyff* silencing did not reduce transgenic Atg1 expression; rather it strongly increased the Atg1 level by more than 2-fold in comparison to the control (Fig. 2A). It is formerly reported that, under the conditions of autophagy inhibition, there is compensatory upregulation of Atg1 protein

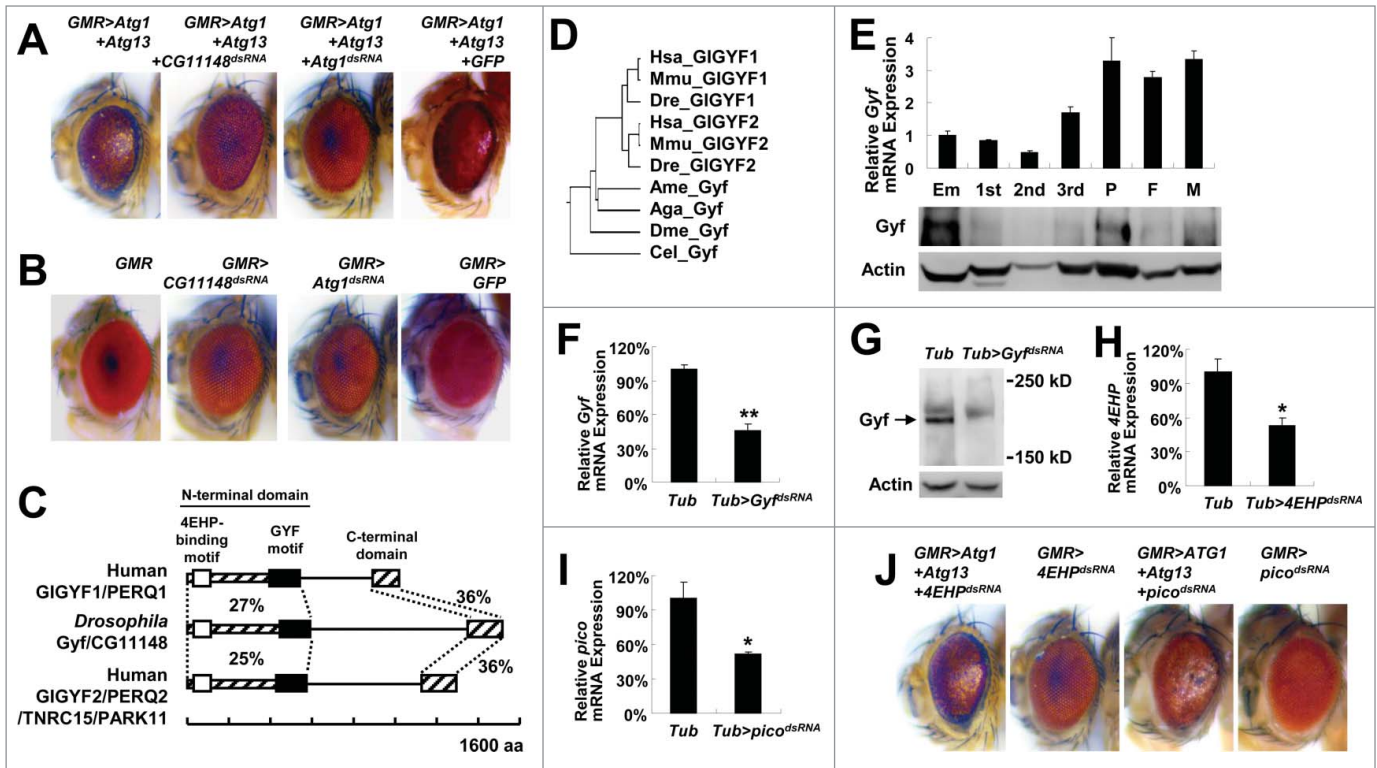


Figure 1. Isolation of *Gyf/CG11148* as a genetic modifier of the Atg1-Atg13-gain-of-function eye phenotype. **(A and B)** Eyes from the flies expressing indicated transgenes were imaged using light dissection microscopy. **(C)** Schematic representation of the comparison among *Drosophila* and human GYF orthologs. Amino acid sequence similarity is displayed as a percentage. **(D)** Phylogenetic tree analysis of GYF family proteins from *Drosophila melanogaster* (*Dme*), *Caenorhabditis elegans* (*Cel*), *Anopheles gambiae* (*Aga*), *Apis mellifera* (*Ame*), *Danio rerio* (*Dre*), *Mus musculus* (*Mmu*), and *Homo sapiens* (*Hsa*), constructed by the neighbor-joining algorithm. **(E)** Expression of GYF at different developmental stages of *Drosophila*. Em, embryo; 1st, first instar larva; 2nd, second instar larva; 3rd, wandering-stage third instar larva; P, pupa; F, adult female; M, adult male. Quantitative reverse transcriptase (RT)-PCR was performed, and GYF mRNA expression was normalized to ribosomal protein 49 (*rp49*) expression (upper panel, $n = 3$). Quantification data are represented as means \pm standard error. Immunoblot analysis was performed to monitor GYF (180 kDa) and Actin (40 kDa) expression. **(F-I)** Expression of GYF in indicated 1-wk-old flies was examined through quantitative RT-PCR **(F)** and immunoblotting **(G)**. Expression of 4EHP and *pico* in indicated 1-wk-old adult flies **(H)** or wandering-stage third instar larvae **(I)** was examined through quantitative RT-PCR. *Tub>pico^{dsRNA}* flies cannot develop into adulthood. Quantification data are represented as means \pm standard error ($n = 3$). *P* values were calculated using the Student *t* test. *, $P < 0.05$; **, $P < 0.01$; NS, not significant. Immunoblot analysis was performed to monitor GYF (180 kDa) and Actin (40 kDa) expression. **(J)** Eyes from the flies expressing the indicated transgenes were imaged using light dissection microscopy.

expression.^{11,18} Thus, it is possible that *Gyf* silencing inhibited autophagy. In contrast, *Atg1* silencing (positive control) strongly reduced the Atg1 expression level as expected (Fig. 2A). The amount of Atg13 expression was not altered by silencing of either *Atg1* or *Gyf*. However, the electromobility retardation (gel shift) of Atg13 was diminished by *Atg1* silencing (Fig. 2A) since the gel shift reflects the Atg1-mediated phosphorylation of Atg13.¹¹ The Atg13 gel shift is maintained after *Gyf* silencing (Fig. 2A), suggesting that *Gyf* silencing does not inhibit the catalytic activity of the Atg1 kinase.

Gyf silencing does not completely suppress morphogenetic abnormalities of ommatidia caused by Atg1-Atg13 activation

We then examined the histological morphology of Atg1-Atg13-overexpressing and *Gyf*-silenced adult eyes. Overexpression of Atg1-Atg13 induced pronounced degeneration of

retinal cells such as photoreceptors and cone cells, in addition to a complete disarray of the ommatidia structure and accumulation of membrane-dense vacuoles (Fig. 2B). After 4 wk, the retinal structure was no longer detectable inside the eye; the ommatidial structure was completely degenerated except for the corneal lens exocuticle (Fig. 2B). The silencing of *Atg1* completely prevented eye degeneration and restored ommatidial morphology at either one or 4 wk (Fig. 2B). *Gyf* silencing also dramatically suppressed an Atg1-Atg13-induced progressive eye degeneration phenotype; however, it failed to fully restore the photoreceptor morphology (Fig. 2B). It has been formerly shown that Atg1 regulates neuronal development independent of its autophagy-regulating activities.¹⁸⁻²¹ Therefore, it is possible that, although *Gyf* is required for Atg1-Atg13-induced autophagic eye degeneration, it does not play a critical role in regulating Atg1's neuronal morphogenesis-regulating activities.

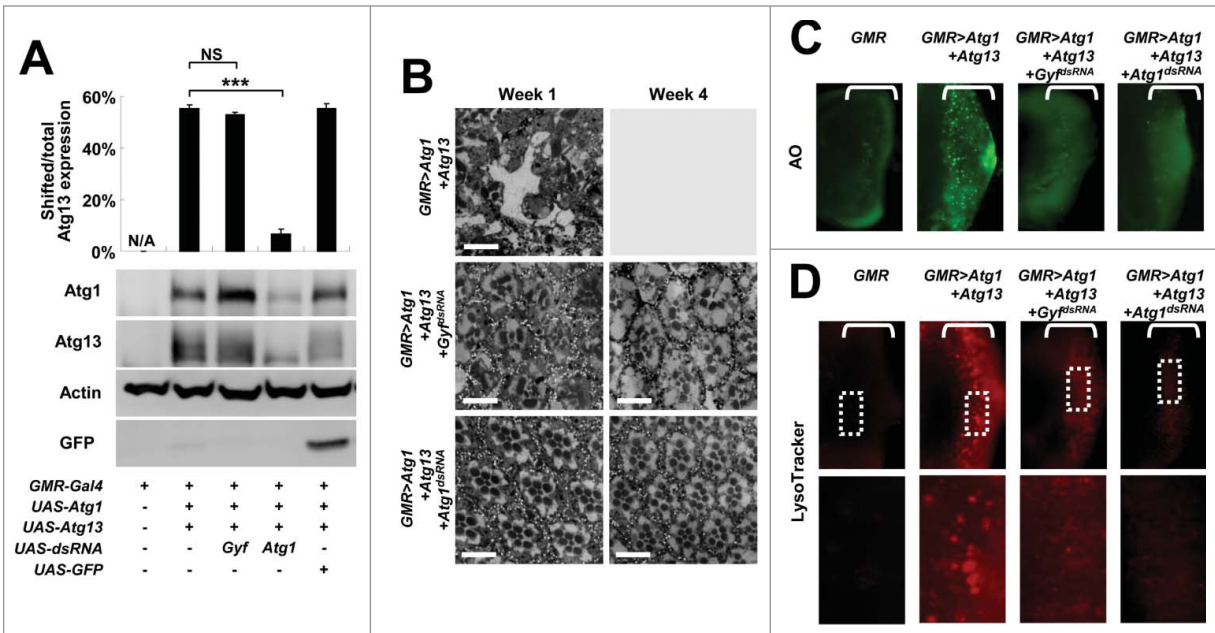


Figure 2. *Gyf* silencing suppresses Atg1-Atg13-induced eye degeneration and ectopic cell death. (A) Fly heads expressing indicated transgenic elements were subjected to immunoblotting to monitor the level of myc-Atg1 (Atg1, 120 kDa), Atg13-GFP (Atg13, 80 kDa), Actin (40 kDa), and GFP (25 kDa) expression. The level of Atg13 shift was quantified by densitometry of total and shifted Atg13 expression. Quantification data are represented as means \pm standard error of the shifted/total Atg13 protein ratios ($n=10$). P values were calculated using the Student t test. *** $P < 0.001$; NS, not significant. (B) Eyes from the flies expressing indicated transgenes were analyzed by electron microscopy to visualize photoreceptor structures. Scale bars: 10 μ m. (C and D) Developing eye discs from wandering-stage third instar larvae expressing indicated transgenes were analyzed by acridine orange (AO) staining (C) and LysoTracker Red staining (D) to visualize dying cells and autolysosomes, respectively. Brackets indicate the areas of differentiated ommatidia that express *GMR-Gal4*. Boxed regions in the top panel of (D) are magnified in the bottom panel to visualize the size of LysoTracker Red-positive vesicles.

Atg1-Atg13-induced ectopic cell death is suppressed by *Gyf* silencing

To decipher the underlying basis of Atg1-Atg13-induced eye degeneration and *Gyf*-silencing-mediated suppression of the degenerative phenotype, we examined the cell biological effects of Atg1-Atg13 and *Gyf* in developing eye discs. Because the *GMR-Gal4* driver is only expressed in differentiated ommatidia, Atg1-Atg13 overexpression and *Gyf* silencing occurred only posterior to the morphogenetic furrow (brackets in Fig. 2C). As formerly reported, Atg1-Atg13 overexpression induced prominent cell death in differentiated ommatidia (Fig. 2C), monitored by acridine orange (AO) staining. However, the ectopic cell death was completely suppressed by silencing of *Gyf* (Fig. 2C), demonstrating that *Gyf* is necessary for the Atg1-Atg13 signaling module to induce ectopic cell death. Atg1-Atg13-induced expansion of acidic compartments, which was visualized by the LysoTracker Red reagent to represent active autophagy, was also substantially reduced in size by silencing of *Gyf* (Fig. 2D), supporting the idea that *Gyf* is an autophagy controller downstream of, or in parallel with, Atg1-Atg13.

Atg1-Atg13-induced autophagic flux is inhibited by *Gyf* silencing

We directly measured the autophagic activity of developing eye disc using an autophagic flux marker GFP-mCherry-Atg8a (Fig. 3A).²² Due to the intrinsic nature of fluorescence proteins, mCherry matures, fluoresces, and fades slower than GFP

(Fig. 3B).^{23,24} Because mCherry-GFP is conjugated with Atg8a which becomes integrated into autophagosomes, autophagy can induce specific loss of GFP fluorescence; upon fusion with lysosomes, exposure to an acidic environment denatures GFP and quickly diminishes its fluorescence, while mCherry fluorescence can be maintained even in the acidic condition. Therefore, in conditions of active autophagy, GFP fluorescence is dramatically suppressed while mCherry fluorescence persists (Fig. 3B and C).

We expressed GFP-mCherry-Atg8a marker in differentiated ommatidia of the developing eye disc using a *GMR-Gal4* driver. In the disc, ommatidia differentiation is initiated at the morphogenetic furrow advancing in the anterior direction at the rate of approximately 2 h per column.²⁵ Therefore, by measuring the distance between ommatidia and the morphogenetic furrow, we are able to estimate how long *GMR-Gal4* has been expressed in the ommatidia.²⁵ GFP signal was strong in the control eye disc as expected because there is minimal autophagic activity in ommatidia during eye development.²⁶ (Fig. 3D and E). When overexpressed, expression of Atg1-Atg13 in the eye disc strongly interfered with GFP fluorescence emission while it did not affect mCherry fluorescence substantially (Fig. 3D and F), implying that Atg1-Atg13-induced autophagic flux reduced GFP fluorescence by degrading it in autolysosomes. However, silencing of *Gyf* completely restored GFP fluorescence (Fig. 3D, G and H), demonstrating that *Gyf* is required for Atg1-Atg13-induced stimulation of autophagic flux.

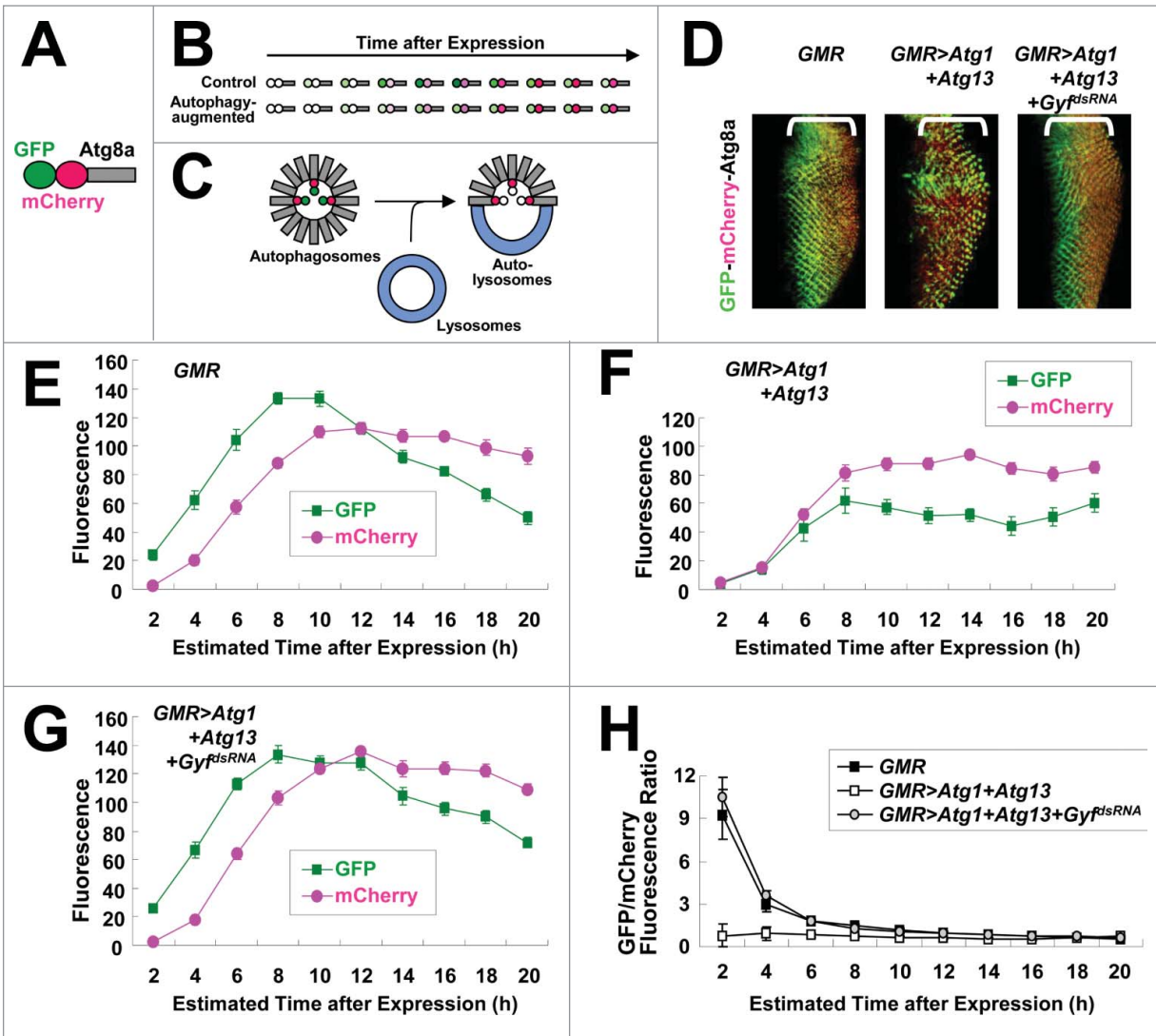


Figure 3. *Gyf* silencing interferes with Atg1-Atg13-induced autophagic flux. (A) Schematic representation of the GFP-mCherry-Atg8a autophagic flux reporter. (B) Due to the intrinsic nature of fluorescence proteins, GFP matures, fluoresces, and fades faster than mCherry. (C) Incorporation of GFP-mCherry-Atg8a into autolysosomes diminishes the fluorescence of GFP, but not that of mCherry. (D) Developing eye discs from wandering-stage third instar larvae expressing indicated transgenes and the GFP-mCherry-Atg8a reporter were observed using laser confocal microscopy. Brackets indicate the areas of differentiated ommatidia. (E–H) GFP and mCherry fluorescence intensities were measured from individual ommatidia expressing indicated transgenes and plotted into a graph against estimated time (h) after expression ($n \geq 15$ in each time period) (E–G). GFP/mCherry fluorescence ratio at each time period was calculated (H). Data are represented as means \pm standard error.

Gyf silencing does not interfere with PtdIns3P accumulation

It has been formerly shown that GIGYF2, the mammalian ortholog of *Gyf*, is present in endosomal compartments.²⁷ Because the activity of Atg6-Pi3K59F (class III PtdIns3K) is important for both endosomal trafficking and autophagosome formation, we tested if *Gyf* is necessary for the proper functionality of Atg6-Pi3K59F, which produces phosphatidylinositol 3-phosphate (PtdIns3P). The PtdIns3P level inside the tissue was monitored by Fyve-GFP, which has a PtdIns3P-binding domain conjugated with a GFP fluorophore.²⁸ *Gyf* silencing in the eye disc does not reduce the level of Fyve-GFP puncta (Fig. 4A, B and Fig. S2A), suggesting that *Gyf*

is not essential for the gross activity of Atg6-Pi3K59F in *Drosophila* eyes.

Gyf silencing inhibits Atg1-Atg13-induced Atg9 trafficking

During active autophagy in mammalian cells, endosomally localized Atg1-Atg13 induces trafficking of Atg9-labeled endosomal membranes to autophagosomes.^{29,30} Indeed, overexpression of Atg1-Atg13 in the developing eye disc induced formation of Atg9-positive puncta (Fig. 4C and D), which are known to be accumulated during active autophagy.^{31,32} We tested the requirement of *Gyf* in this process. Silencing of *Gyf* strongly abrogated the Atg9 puncta formation (Fig. 4E and Fig. S2B), suggesting

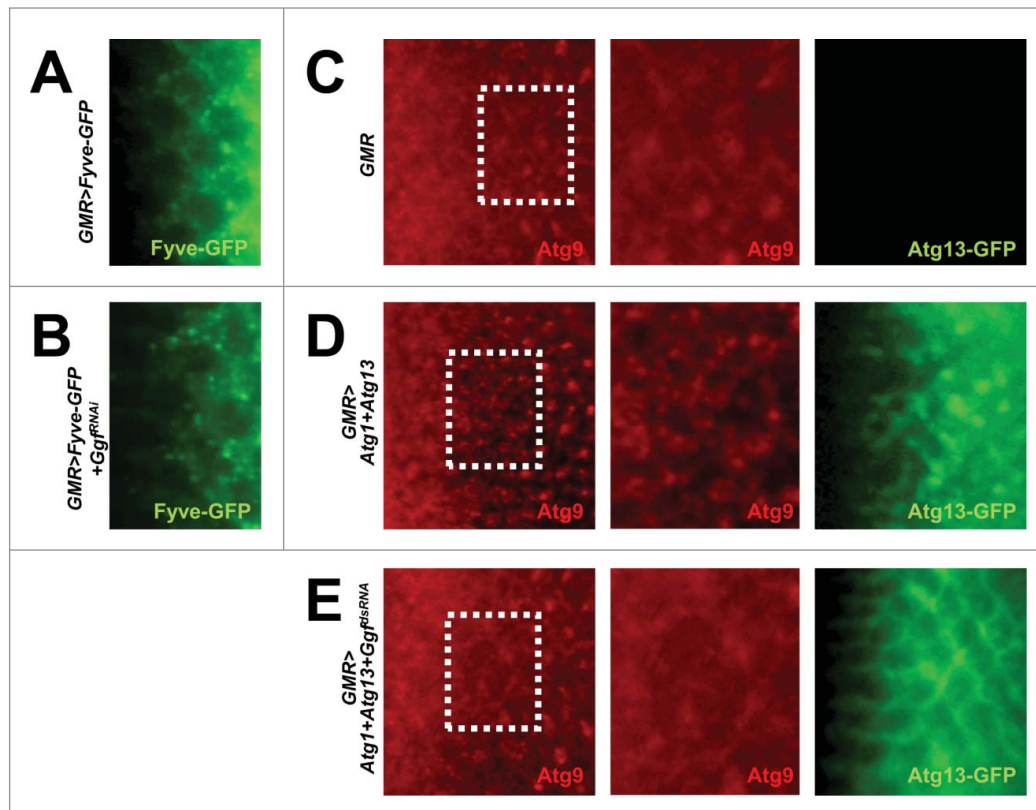


Figure 4. *Gyf* silencing does not interfere with the Atg6-Pi3K59F activity but inhibits Atg1-Atg13-induced Atg9 trafficking. **(A and B)** Developing eye discs from wandering-stage third instar larvae expressing indicated transgenes were observed under a fluorescence microscope to visualize Fyve-conjugated GFP (green) that monitors PtdIns3P. **(C–E)** Developing eye discs from wandering-stage third instar larvae expressing indicated transgenes were analyzed by anti-Atg9 (red) staining, which monitors trafficking of endosomes to autophagosomes. Boxed areas in the left-most panels are magnified in the middle panels. The right-most panels display fluorescence of Atg13-conjugated GFP (green) in the region corresponding to the left-most panel.

that *Gyf* is required for Atg1-Atg13-induced trafficking of the endosomal membrane and Atg9. Interestingly, Atg13 puncta formation, which is observed in Atg1-Atg13-overexpressing eye disc, was also completely prevented by silencing of *Gyf* (Fig. 4D, E and Fig. S2C). This result suggests that *Gyf* is required for proper subcellular localization of the Atg1-Atg13 complex, which is required for instigation of active Atg9 trafficking and autophagosome formation.

Gyf silencing does not interfere with classical apoptosis

Because *Gyf* silencing inhibited Atg1-Atg13-induced cell death and eye degeneration, we speculated on whether *Gyf* can also serve as a general inhibitor of cell death such as apoptosis. Rpr/Reaper and Hid proteins in *Drosophila* antagonize the inhibitor of apoptosis (Thread/dIAP1) proteins, thereby inducing activation of caspase cascades that lead to apoptotic cell death.³³ Eye-specific overexpression of either of these proteins induced significant cell death, as monitored by acridine orange (AO) or terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining, in the developing eye disc (Fig. 5A and B), and reduced eye size in adults (Fig. 5C). These apoptotic phenotypes were not suppressed at all by *Gyf* silencing (Fig. 5A–C), suggesting that *Gyf* specifically regulates the autophagic signaling pathway.

Identification and characterization of *Gyf*-null mutant flies

To further study the physiological function of the *Gyf* gene, we isolated *Gyf*-null mutant flies. A FlyBase search identified 2 *Minos* transposon insertions, *Gyf*^{MB01094} and *Gyf*^{MI00455} (designated as *Gyf*^{MB} and *Gyf*^{MI}), in the fourth intron and fifth exon of the *Gyf* gene respectively (Fig. 6A). These mutants were formerly generated by the large-scale *Drosophila* gene disruption projects.^{34,35} Although the *Gyf*^{MB} mutation did not affect the expression of *Gyf* mRNA (data not shown), the *Gyf*^{MI} mutation, which disrupts the coding sequence of the *Gyf* gene, completely prevented *Gyf* mRNA (Fig. 6B) and *Gyf* protein (Fig. 6C) expression. Thus, we characterized *Gyf*^{MI} as the null mutant of *Gyf* gene.

Gyf-null mutants are short lived

Gyf^{MI} mutants developed into adulthood well without any noticeable developmental problems. Because many *Drosophila* mutants with autophagy defects are short-lived,^{18,36} we measured longevity of WT control and *Gyf*^{MI} mutants under the standard laboratory condition. Interestingly, *Gyf*^{MI} mutants exhibited a high mortality rate in the early ages of their life, making their life span drastically shorter than WT counterparts (Fig. 6D). We then confirmed whether the early onset of mortality was due to the mutation of the *Gyf* gene. The genomic sequence including

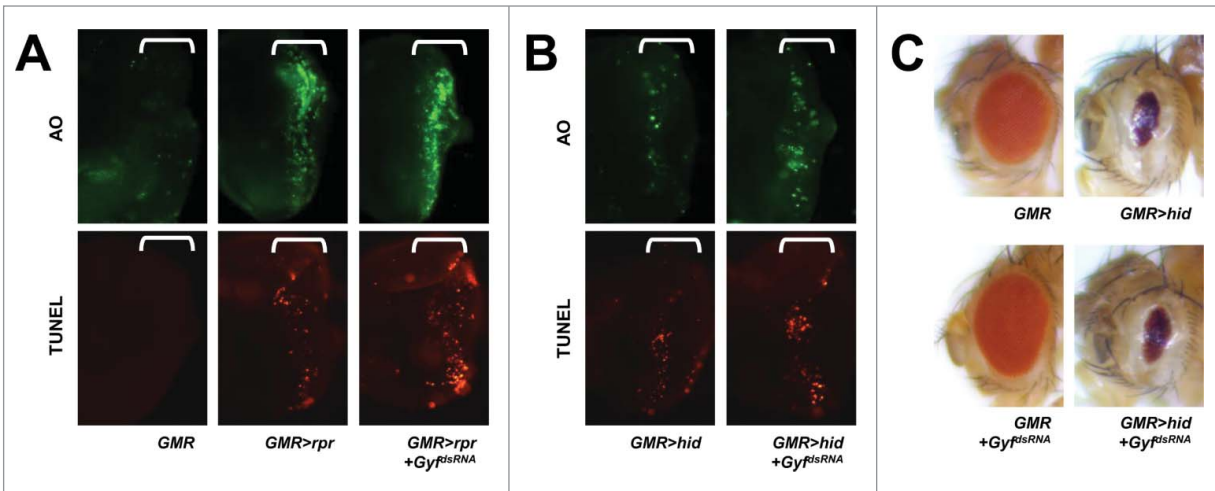


Figure 5. *Gyf* silencing does not interfere with apoptotic cell death. **(A and B)** Developing eye discs from wandering-stage third instar larvae expressing indicated transgenes were analyzed by acridine orange (AO, upper panels) and TUNEL (lower panels) staining to visualize apoptotic cells. Brackets indicate the areas of differentiated ommatidia. **(C)** Eyes from the flies expressing indicated transgenes were imaged using light dissection microscopy. *GMR>rpr* flies cannot develop into adulthood.

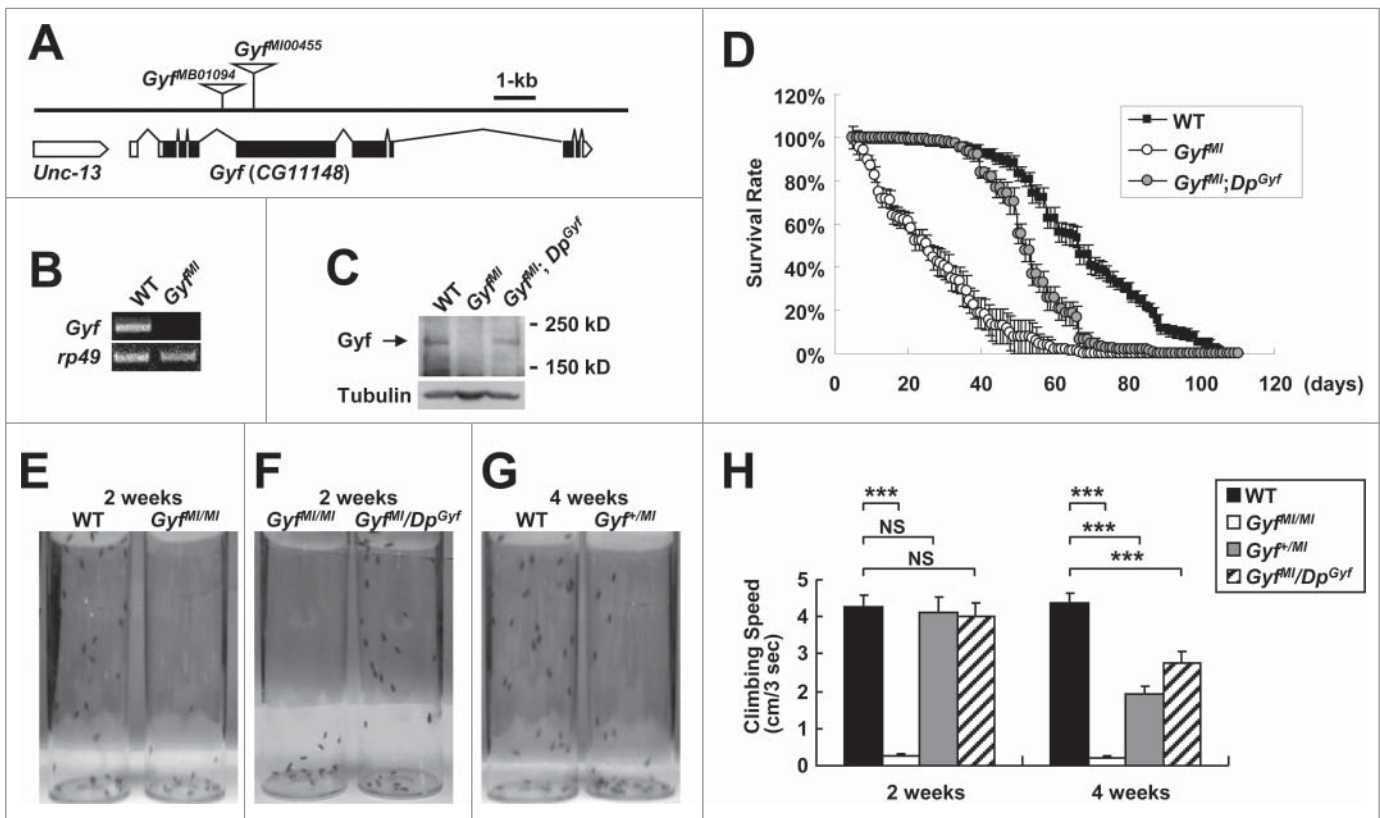


Figure 6. *Gyf* mutant flies exhibit shortened life span and mobility defects. **(A)** Schematic genomic organization of the *Gyf* (*CG11148*) locus and *Gyf* mutants. Triangles, transposon insertions; open boxes, untranslated exons; closed boxes, protein-coding exons. Scale bar, relative length of 1-kb genomic span. **(B and C)** Absence of *Gyf* expression in *Gyf*-null flies (*Gyf^{MI}*). RT-PCR of *Gyf* and *rp49* **(B)** and immunoblotting of *Gyf* (180 kDa) and Tubulin (50 kDa) **(C)** were conducted from the flies of indicated genotypes. **(D)** Survivorship of WT (*w¹¹¹⁸* control), *Gyf*-null mutant (*Gyf^{MI}*) and *Gyf*-null mutant with *Gyf* genomic rescue (*Gyf^{MI}*, *Dp^{Gyf}*) male flies ($n \geq 180$). Survival of 4 independent cohorts is presented as mean \pm standard error. **(E-G)** Photographs of the vials containing 2- or 4-wk-old adult male flies of indicated genotypes taken at 3 sec after negative geotaxis induction. **(H)** Quantification of the climbing speeds of indicated 2- or 4-wk-old adult male flies ($n \geq 80$). Climbing speed is presented as mean \pm standard error. *P* values were calculated using the Student *t* test. *** $P < 0.001$; NS, not significant.

and surrounding *Gyff* gene on the fourth chromosome was cloned into a bacterial artificial chromosome (BAC) vector and transgenically inserted into an independent genomic locus on the third chromosome by phiC31 integrase-mediated recombination. This allele, which was formerly generated by Duplication Consortium,³⁷ was designated as the *Dp^{Gyf}* duplication. Using the *Dp^{Gyf}* strain, we tested if transgenic expression of *Gyf* can suppress the longevity defects of *Gyff^{MI}* mutants. *Dp^{Gyf}* was able to partially restore *Gyf* protein expression in *Gyff^{MI}* mutant (Fig. 6C). This expression was enough to substantially prevent an early rise in the mortality rate of *Gyff^{MI}* mutant (Fig. 6D), suggesting that the absence of *Gyf* protein is indeed responsible for the observed life-span phenotype.

Gyf-null mutants exhibit mobility defects

We observed that the early mortality rate of *Gyff^{MI}* mutants is associated with a dramatic decline in locomotor ability, as most of the flies were found at the bottom of vials with a largely impaired ability to fly or climb the walls. A negative geotaxis assay confirmed the loss of mobility in *Gyff^{MI}* mutants, which was already prominent at 2 wk of age (Fig. 6E). The mobility defect was again substantially restored by *Dp^{Gyf}* genomic rescue (Fig. 6F). Interestingly, the heterozygotic *Gyff^{MI/+}* flies and rescued flies showed a slight decrease in climbing ability at 4 wk of age (Fig. 6G and H), suggesting that the reduced expression of *Gyf* in these flies may have caused a motor deficit in later ages. These results indicate that *Gyf* is important for preservation of mobility in adult flies.

Gyf-null mutants exhibit autophagy defects

We then returned to the question of whether *Gyf* is a genuine regulator of autophagy. Because Atg1-Atg13, which showed strong genetic interaction with the *Gyf*-silencing construct, is essential for developmental and starvation-induced autophagy, we questioned the contribution of *Gyf* to these processes. As previously reported,^{36,38} late wandering-stage third instar WT larvae exhibited prominent autophagic activities, such as accumulation of autophagosomes (monitored by Atg8a staining), and acidic vesicles such as autolysosomes (monitored by LysoTracker Red) in their fat bodies (Fig. 7A–D). However, these autophagic activities were strongly abrogated by the *Gyff*-null mutation (Fig. 7A–D). *Gyff*-null mutant larvae were also defective in starvation-induced autophagy (Fig. 7E–H). Because the *Gyff*-null mutant showed degenerative phenotypes at the adult stage, we were also curious if *Gyf* controls autophagy at the adult stage. To biochemically monitor the autophagic activity in adult tissues, we have utilized an anti-Atg8 antibody, which can detect an endogenous level of Atg8a protein expression in *Drosophila* (Fig. S3). Head and thorax tissues of WT adults exhibited considerable Atg8a-II expression (Fig. 7I–L) which correlates with the basal level of physiological autophagy that is necessary for maintaining tissue homeostasis. However, *Gyff*-null mutants showed substantial decreases in Atg8a-II expression in both head and thorax tissues (Fig. 7I–L). These results collectively indicate that *Gyf* is a gene that is critical for developmental, starvation-induced and physiological autophagy processes.

Gyff loss downregulates TOR signaling in adult tissues

As briefly mentioned above, GIGYF2, the mammalian ortholog of *Gyf*, was formerly shown to regulate the insulin-TOR signaling pathway.^{16,17} Because TOR complex 1 (TORC1) signaling antagonizes autophagy and inhibits Atg8-II processing through downregulation of Atg1/ULK1,³⁹ we were curious whether the organism-level *Gyf* deficiency affects autophagy through activation of TORC1. Phosphorylation of the TORC1 substrate S6k (RPS6-p70-protein kinase) was, however, dramatically downregulated in *Gyff*-null mutant tissues (Fig. 7M and N), suggesting that TORC1 signaling was inhibited, not activated, by the *Gyff* loss. Phosphorylation of a TORC2 substrate Akt1 was also downregulated, but to a less extent when compared to the S6k phosphorylation (Fig. 7M and N). TORC1-induced inhibitory phosphorylation of Atg1, which induces a gel shift of Atg1 in *Drosophila*,¹¹ was also substantially reduced by *Gyf* loss (Fig. 7M and N). This TORC1 downregulation could be as an unsuccessful compensatory response to restore autophagy in *Gyff*-null mutant tissues.

Gyf-null mutants accumulate large amounts of ubiquitinated proteins and dysfunctional mitochondria

Autophagy is important for clearance of ubiquitinated proteins and maintenance of protein homeostasis. Mice and flies with autophagy defects accumulate a large amount of ubiquitinated proteins inside their body, provoking neurodegeneration and muscular dysfunction^{4,5} which can together interfere with mobility. As the data indicate that *Gyf* is important for physiological autophagy in neuron and skeletal muscle tissues (Fig. 7I–L), we inferred that *Gyff*-null mutants may have defects in eliminating ubiquitinated proteins in these tissues. Indeed, 2-wk-old *Gyff*-null mutants accumulated a highly elevated amount of ubiquitinated proteins inside the body, which was suppressed by *Dp^{Gyf}* genomic rescue (Fig. 8A and B).

In addition to ubiquitinated proteins, dysfunctional organelles such as damaged mitochondria are physiologically important substrates of autophagy.⁴⁰ Defective clearance of ROS-producing mitochondria can also facilitate neurodegeneration and muscle degeneration in autophagy-defective flies and mice.^{5,41–44} Brain (Fig. 8C and D, optic lobe medulla) and skeletal muscle tissues (Fig. 8E and F) of *Gyff*-null mutants exhibited a number of damaged mitochondria, characterized by vacuolization and disorganized cristae structure which are rarely observed in WT control flies. The mitochondrial dysfunctions were also associated with extensive apoptotic cell death in both tissues (Fig. 9). In muscle, the Z band in the sarcomere structure was frequently broadened (Fig. 8E and G), indicative of muscle dysfunction and degeneration.^{45–47} These results highlight the critical role of *Gyf* in maintenance of tissue homeostasis that prevents degeneration of neuron and muscle tissues.

Discussion

Through screening for mediators of Atg1-Atg13-induced autophagy, we have isolated *Gyf* as a new autophagy-regulating

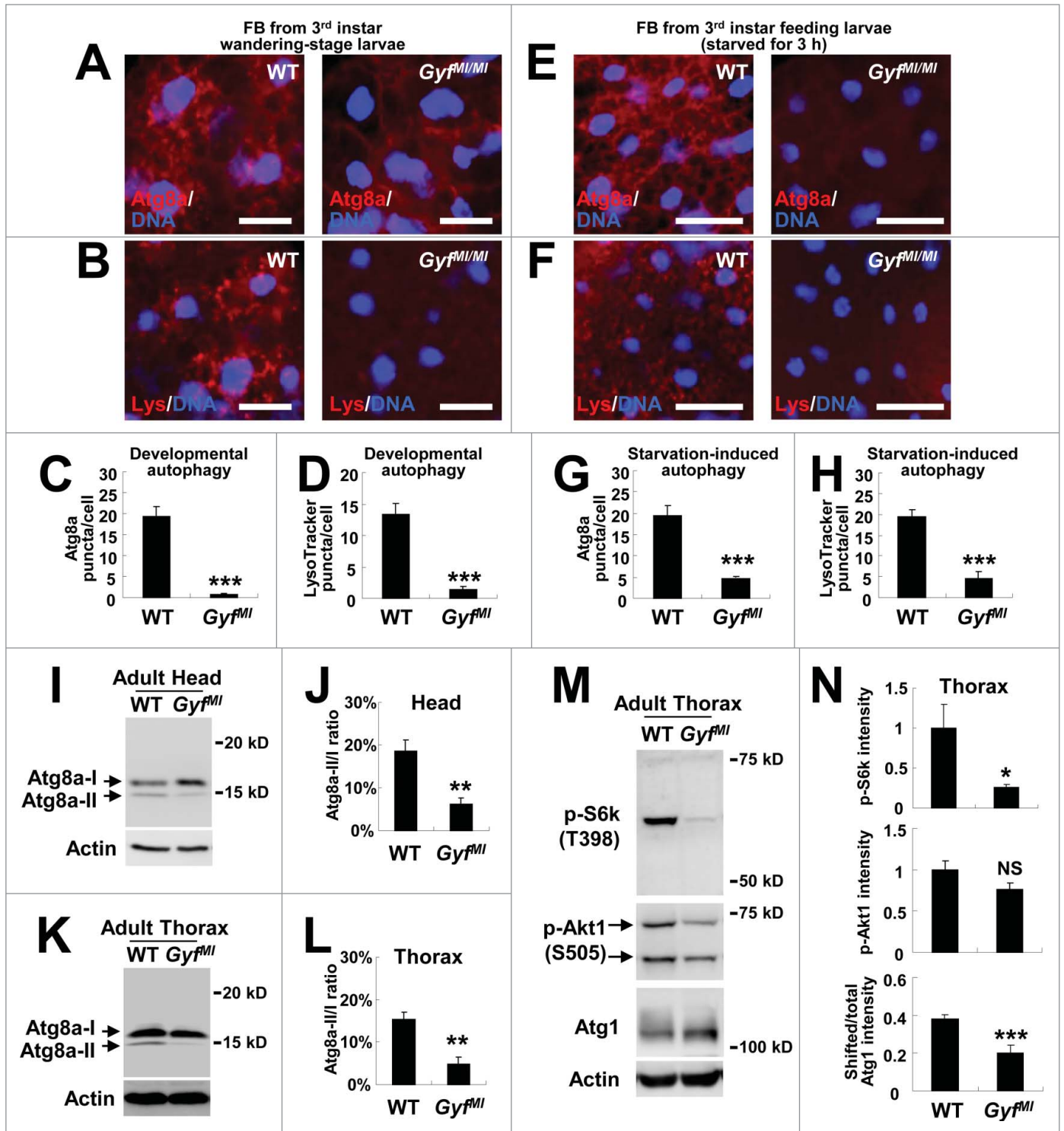


Figure 7. *Gyf* is essential for developmental, starvation-induced and physiological autophagy. (A–H) Fat bodies (FB) of wandering-stage third instar larvae (A–D) or feeding-stage third instar larvae that were placed on 20% sucrose solution for 3 h (E–H) of WT and *Gyf^{MI/MI}* flies were subjected to anti-Atg8a immunostaining (A, C, E and G) or LysoTracker Red (Lys) staining (B, D, F and H). Hoechst 33258 (DNA) was used to visualize nuclei. Scale bars: 50 μ m. Number of Atg8a or LysoTracker Red puncta per cell in fat bodies are presented as means \pm standard error ($n \geq 40$) (C, D, G and H). (I–L) Heads (I and J) and thoraxes (K and L) of 1-wk-old WT and *Gyf^{MI/MI}* flies were analyzed through immunoblotting of Atg8a-I (16 kDa), Atg8a-II (14 kDa) and Actin (40 kDa). The ratios of Atg8a-II to Atg8a-I are presented as means \pm standard error ($n = 7$) (J and L). (M and N) Thoraxes of 1-wk-old WT and *Gyf^{MI/MI}* flies were analyzed through immunoblotting of phospho-Thr398 S6k (70 kDa), phospho-Ser505 Akt1 (60 and 70 kDa), Atg1 (120 kDa) and Actin (40 kDa). The immunoblot results were quantified and presented as means \pm standard error ($n = 4$). *P* values were calculated using the Student *t*-test. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; NS, not significant.

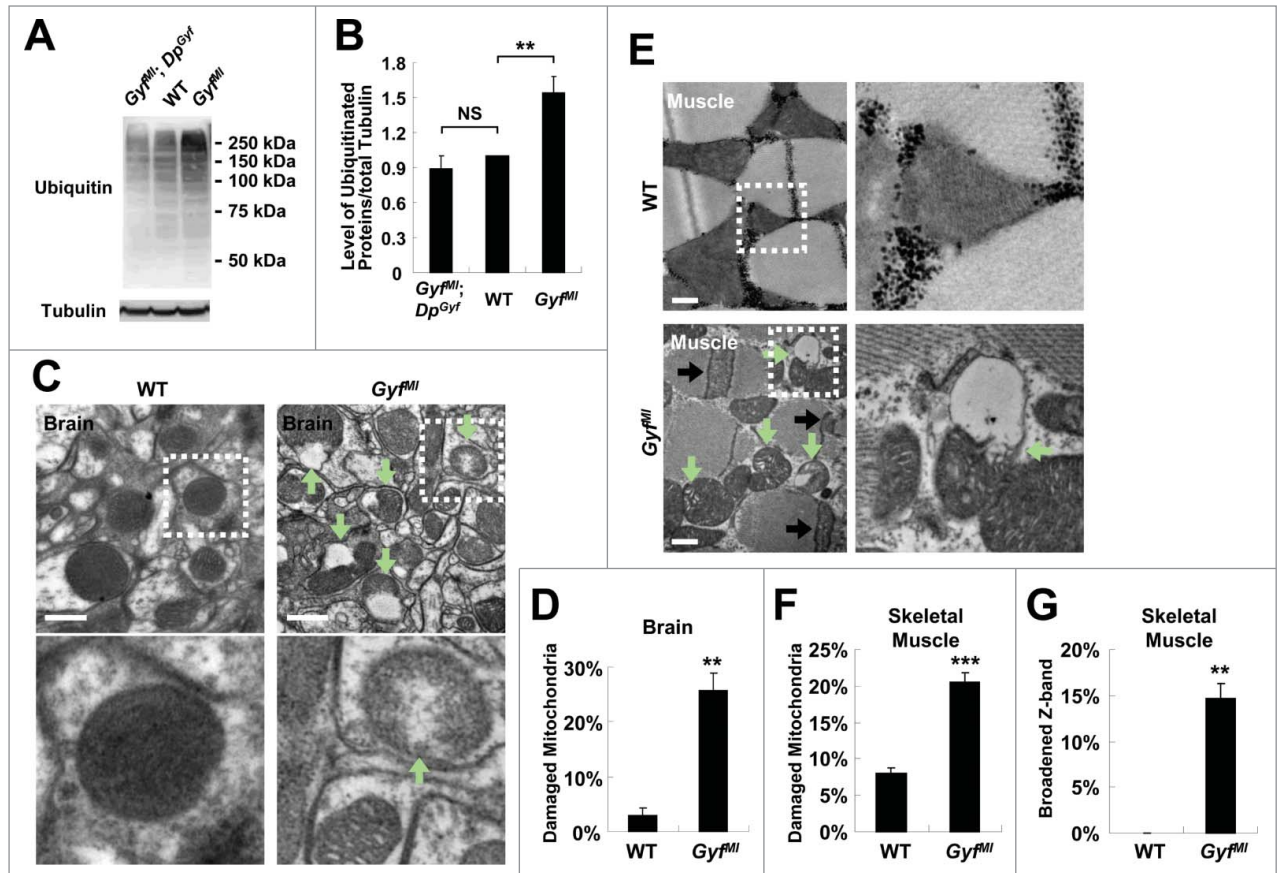


Figure 8. Accumulation of ubiquitinated proteins and damaged mitochondria in *Gyf* mutant flies. (**A and B**) 2-wk-old adult male flies of indicated genotypes were subjected to immunoblot analyses with ubiquitin and tubulin (50 kDa) antibodies (**A**). Relative protein level was quantified and presented as mean \pm standard error (**B**). (**C and E**) Electron micrographs reveal the presence of dysfunctional mitochondria (green arrows) in the brain (**C**, optic lobe medulla) and the skeletal muscle (**E**) of 4-wk-old *Gyf* mutant flies. Boxed areas with normal or damaged mitochondria are magnified in lower (**C**) or right (**E**) panels. In the skeletal muscle of *Gyf* mutant flies, broadened Z-bands (black arrows) were frequently observed. Scale bars: 500 nm. (**D, F and G**) Quantification data are represented as means \pm standard error ($n \geq 3$). *P* values were calculated using the Student *t* test. **, $P < 0.01$; ***, $P < 0.001$; NS, not significant.

gene. *Gyf* is required for the Atg1-Atg13 complex to stimulate autophagic flux, autolysosome formation and cell death in the case of excessive autophagy. It is also physiologically essential for developmental and starvation-induced autophagy as well as autophagic elimination of ubiquitinated proteins and dysfunctional mitochondria from neuron and skeletal muscle tissues. This physiological role of *Gyf* is critical for preventing neurodegeneration and myopathy as well as for preserving life span and mobility.

Although *Gyf* is required for Atg1-Atg13-induced autophagy, *Gyf*-null mutant phenotypes are milder than those of *Atg1*-, *Atg13*-, or *Atg17/Fip200*-null mutants, which all fail to develop into viable adult flies.^{11,18,48,49} Thus, it is likely that *Gyf* does not mediate all biological functions of the Atg1-Atg13 complex. Consistently, we observed that *Gyf* silencing did not restore some phenotypic outputs of the Atg1-Atg13 overexpression, such as interference with photoreceptor morphogenesis. Nevertheless, accumulation of autophagy substrates in *Gyf*-null mutants, such as ubiquitinated proteins and dysfunctional mitochondria, shows that *Gyf* is indeed required for the physiological autophagy that

is dependent on Atg1-Atg13. Accumulation of ubiquitinated proteins was also formerly observed in flies deficient in several autophagy-regulating gene products, such as *Atg7*,³⁶ *Atg8a*,⁵⁰ and *Bchs/ALFY*.⁵¹ However, mitochondrial dysfunction or muscle degeneration was not observed in some of these flies,³⁶ and it has been determined that these components are not necessarily essential for mitochondrial autophagy (mitophagy) that functions to eliminate damaged mitochondria.⁵²⁻⁵⁵ On the contrary, the Atg1-Atg13-Atg17 system is essential for mitophagy,⁵²⁻⁵⁶ and hypomorphic mutations of *Atg1* or *Atg17* provoke accumulation of damaged mitochondria,^{18,44} similar to the currently described *Gyf*-null mutant phenotypes. Thus, *Gyf* is a genetic component that is essential for Atg1-Atg13-dependent autophagy of both ubiquitinated proteins and dysfunctional mitochondria in the physiological context.

Although our current study uncovered the genetic function of *Gyf* in regulating autophagy and neuromuscular homeostasis, the exact biochemical role of *Gyf* in autophagy process still awaits further investigation. As there is no known catalytic activity or

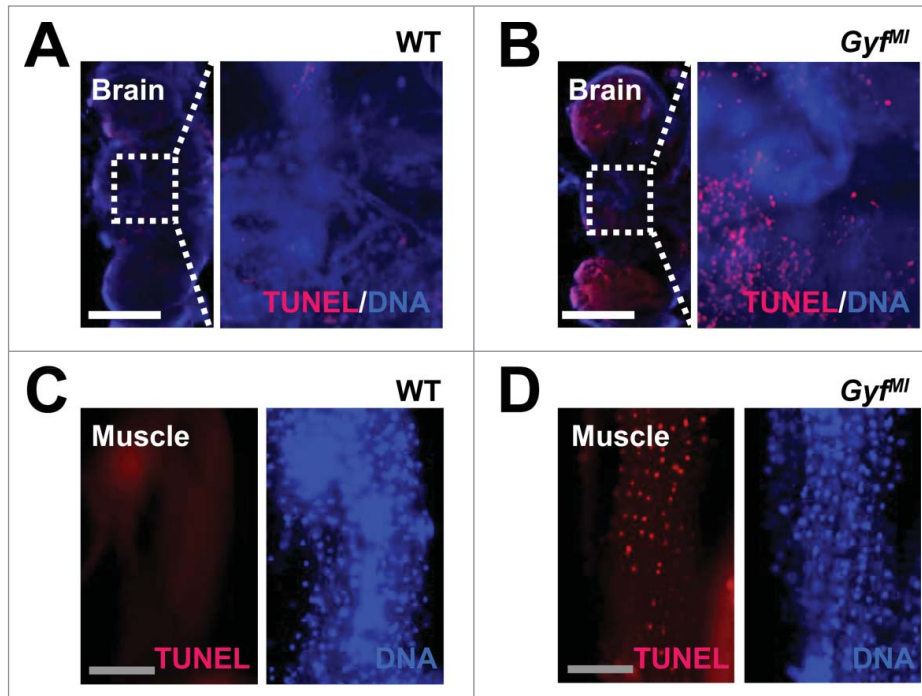


Figure 9. Degenerative phenotypes of brain and muscle tissues of *Gyf* mutant flies. (A–D) TUNEL (red) and DAPI (blue, DNA) staining reveals apoptotic cells in the brain and skeletal muscle of 4-wk-old *Gyf* mutant flies. Right panel images are corresponding to the boxed areas in the left panel images (A and B). Scale bars: 200 μm (white), 50 μm (gray).

motif in *Gyf* or its related mammalian orthologs, it is likely that *Gyf* works with other proteins to regulate autophagy. For example, formerly known interacting partners of *Gyf*, such as 4EHP and Pico/GRB10,^{15,16} may play some roles in *Gyf*-mediated autophagy. However, as these molecules are largely dispensable for Atg1-Atg13-induced autophagy, additional molecules should be also involved in *Gyf*-regulated autophagy process. One report shows that GIGYF2 in human cells is localized in endosomal compartments.²⁷ As Atg1/ULK1 protein kinase is known to localize into endosomes and endosomes contribute to the growth of the autophagosomal membrane,³⁰ *Gyf*/GIGYF2 may promote autophagosome formation by regulating Atg1-Atg13-mediated trafficking of endosomal membrane constituents into autophagosomes. Supporting this idea, we have found that *Gyf* is required for Atg1-Atg13-induced Atg9 puncta formation, which reflects active membrane trafficking to autophagosomes.^{31,32} In addition, although *Gyf* does not control Atg1-induced Atg13 phosphorylation, it was necessary for Atg1-induced Atg13 puncta formation in developing eye discs, suggesting that *Gyf* may control autophagy by regulating subcellular localization of the Atg1-Atg13 protein complex.

It should be noted that mutations in *GIGYF2* gene, one of the 2 human orthologs of *Gyf*, are associated with several cases of familial PD.¹⁴ Although *GIGYF2*/*PARK11* mutations are actually excluded from the frequent causes of PD in the general human population,^{57–60} *GIGYF2* may still play important roles in maintaining neuronal homeostasis and preventing neurodegeneration. *Gigylf2*^{-/-} mice are reported to exhibit perinatal

mortality and *Gigylf2*^{+/-} mice display early onset of age-associated neurodegenerative phenotypes that include neuronal protein inclusion and a significant decline in mobility.¹⁷ Our results of *Gyf*^{+/-} flies exhibiting a slight motor deficit and *Gyf*^{-/-} flies displaying an early mortality rate and neurodegeneration-associated mobility defects are highly reminiscent of the mouse *Gigylf2* and human PD phenotypes.^{14,17,61} The prominent cell death observed in neuron and muscle tissues of *Gyf*^{-/-} flies (Fig. 9) is most likely due to excessive accumulation of dysfunctional mitochondria and protein aggregates (Fig. 8) as a consequence of chronic autophagy downregulation (Fig. 7). Thus, it should be investigated in the future whether mammalian GIGYF1 and GIGYF2 proteins play roles in neuronal autophagy as *Drosophila* *Gyf* does. In addition, since many autophagy-defective mouse mutants are lethal at early neonatal stage due to their defects in responding to neonatal starvation,^{62,63} it would be interesting to investigate later whether perinatal lethality of *Gigylf2*^{-/-} mice¹⁷ is associated with autophagic defects. Based on the current results, it is highly likely that *Gyf*-family proteins are evolutionarily conserved regulators of neuronal autophagy that is critical for prevention of neurodegenerative phenotypes.

Human mutations in several genes have been implicated as playing a causal role in familial PD. These genes include *SNCA*/*PARK1*, *PARK2*/*Parkin*, *UCHL1*/*PARK5*, *PINK1*/*PARK6*, *PARK7*/*DJ-1*, *LRRK2*/*PARK8* and *ATP13A2*/*PARK9*. It is interesting to note that all of these genes play some roles in autophagic elimination of ubiquitinated proteins or damaged mitochondria.^{6,53,64,65} Because our current report suggests *GIGYF2* as

another potential regulator of the autophagy process, autophagy defects that impede cellular protein and oxidative metabolism homeostasis seem to be a general underlying pathogenetic cause of the familial PD syndrome. The genetic role of *PARK* genes also seems to be evolutionarily conserved because *Drosophila* strains bearing mutations of *SNCA*,⁶⁶ *park/Parkin*,^{67,68} *Pink1*,^{41,42} *Dj-1*⁶⁹⁻⁷¹ and *Lrrk*⁷²⁻⁷⁴ genes show neurodegenerative phenotypes similar to those of PD patients, and *Gyf*-null mutants also exhibited such phenotypes. Therefore, *Gyf* is among the family of neuroprotective proteins that are important for preventing PD-like degenerative pathologies.

Materials and Methods

Fly strains and culture

Atg8a^{d4}, *UAS-myc-Atg1* (*UAS-Atg1*) and *UAS-Atg13-GFP* (*UAS-Atg13*) were gifts from Dr. Thomas Neufeld (University of Minnesota). *GMR-Gal4*, *UAS-Atg1^{dsRNA}*, *UAS-Gyf^{dsRNA}*, *UAS-4EHP^{dsRNA}*, *Gyf^{MB01094}* (*Gyf^{MB}*), *Gyf^{MI00455}* (*Gyf^{MI}*) and *PBac* [*y⁺ w⁺ Dp(4;3)RC049*]/*VK00033* (*Dp^{Gyf}*), *UAS-GFP*, as well as balancer and WT strains, were obtained from Bloomington *Drosophila* Stock Center (BDSC). *UAS-pico^{dsRNA}* was obtained from Vienna *Drosophila* RNAi Center (VDRC, 16369). The flies were reared on standard cornmeal-agar medium with humidity (70%), temperature (25°C or as indicated) and light (12/12 h light/dark cycle) control. For negative geotaxis assays, pictures were taken 3 sec after negative geotaxis induction, and climbing speed was calculated from the pictures.¹⁸

Construction of the *GMR>Atg1+Atg13* line

UAS-Atg1 and *UAS-Atg13* were interbred to generate *UAS-Atg1/UAS-Atg13* females, which were then crossed with *TM3Sb/TM6B* balancer male flies. The male progenies from this cross were mated with *GMR-Gal4*; *TM2/TM6B* virgins (50 single pair mating (s.p.m.)). *GMR-Gal4/+*; *UAS-Atg1 UAS-Atg13/TM6B* flies with noticeable eye degeneration were selected from the s.p.m crosses and interbred. Finally, a stable *GMR-Gal4*; *UAS-Atg1 UAS-Atg13/TM6B* line (*GMR>Atg1+Atg13*) was constructed, and expression of *Atg1* and *Atg13* was confirmed through immunoblotting. However, we noticed that *GMR>Atg1+Atg13* flies exhibited reduced viability and fecundity. Raising the flies at 18°C restored fertility while maintaining the eye phenotypes that allowed us to distinguish the flies; therefore, all genetic experiments using the *GMR>Atg1+Atg13* transgene were conducted at 18°C.

Gyf mutants

The *Gyf* gene locus is located on the fourth chromosome. *Gyf^{MB}* and *Gyf^{MI}* mutant alleles were generated by an insertion of genetically engineered *Minos* transposons called *MiET1*³⁴ and *MiMIC*,³⁵ respectively. The original *Gyf^{MB}* stock was maintained as homozygotes while the *Gyf^{MI}* stock was balanced with *In(4)ci^D* inversion. Because the fourth chromosome does not undergo recombination, this genetic combination is enough to maintain *Gyf^{MI}* allele whose homozygosity produces early mortality and extreme sickness. Due to these defects, we were unable to

establish a *Gyf^{MI}* homozygotic fly line. Therefore, all *Gyf^{MI}* homozygotes were collected from the progenies of *Gyf^{MI}/In(4)ci^D* intercrosses by absence of *ci^D* marker.

Dp^{Gyf} allele was generated by the BDSC Duplication Consortium using a formerly described method.^{37,75} A duplication of 165,448 bp-long sequence from the fourth chromosome (coordinates 4:798,603..964,051, *Drosophila melanogaster* genome release 5; encompassing *Gyf* (4:874,342..887,651) and several neighboring genes) derived from the BACR11A04 BAC clone was inserted at the *PBac*[*y⁺-attP-3B*]/*VK00033* docking site on the third chromosome using *phiC31*-mediated recombination.

Dp^{Gyf} was incorporated into *Gyf^{MI}* mutants by means of the following genetic scheme. *Dp^{Gyf}* contains *y⁺* and *w⁺* genetic markers. *Gyf^{MI}* contains *y⁺* genetic marker only. *w⁻/Y*; *Dp^{Gyf}/TM6Sb* males were crossed with *y⁻ w⁻*; *Gyf^{MI}/In(4)ci^D* females. The resulting *y⁻ w⁻/Y*; *Dp^{Gyf}/+*; *Gyf^{MI}/+* males were backcrossed with *y⁻ w⁻*; *Gyf^{MI}/In(4)ci^D* females. Males with *y⁺ w⁺ ci⁺* phenotypes, whose genotype should be either *y⁻ w⁻/Y*; *Dp^{Gyf}/+*; *Gyf^{MI}/Gyf^{MI}* or *y⁻ w⁻/Y*; *Dp^{Gyf}/+*; *+/Gyf^{MI}*, were collected and bred again with *y⁻ w⁻*; *Gyf^{MI}/In(4)ci^D* females in s.p.m. crosses. The males that produce progenies with *y⁻ w⁻ ci^D* phenotypes in s.p.m. crosses are judged to have genotype of *y⁻ w⁻/Y*; *Dp^{Gyf}/+*; *Gyf^{MI}/+*, therefore removed from the study. *y⁺ w⁺ ci⁺* male progenies from all the other s.p.m. crosses will be *y⁻ w⁻/Y*; *Dp^{Gyf}/+*; *Gyf^{MI}/Gyf^{MI}* flies (designated as *Gyf^{MI}*; *Dp^{Gyf}*) that were found to be fully viable and fertile.

Antibodies

Guinea pig anti-*Gyf* antibodies were made as follows: a cDNA fragment encoding a.a. 500 to 1000 of *Gyf* was cloned into pGEX and transformed into *E. coli* BL21. Insoluble GST-fusion proteins were purified from SDS-PAGE gel bands and injected into guinea pigs (Pocono farms, Inc.). Sera were subjected to affinity purification using PVDF-immobilized proteins. Anti-*Atg1* and anti-*Atg9* antibodies were formerly described.^{18,32} *Atg13-GFP* was detected using anti-GFP antibodies (Santa Cruz Biotechnology, sc-9996; 1:50). Anti-TUBA/ α -tubulin (Sigma, T5168; 1:1000), anti-ACTA1/actin (DSHB, JLA20; 1:100), anti-ubiquitin (Santa Cruz Biotechnology, sc-8017; 1:100), anti-phospho Thr398 *Drosophila* S6k (Cell Signaling Technology, 9209; 1:1000) and anti-phospho-Ser505 *Drosophila* Akt1 (Cell Signaling Technology, 4054; 1:1000) antibodies were used for immunoblot analyses. Anti-GABARAP (anti-*Atg8*) antibody (Abcam, ab109364) was used to detect endogenous levels of *Drosophila* *Atg8a* in both immunostaining (1:200) and immunoblotting (1:1000) experiments. According to Abcam, this rabbit monoclonal antibody was raised against the evolutionarily conserved region of GABARAP, and therefore can cross-react with *Atg8*-family proteins from a variety of species including *Drosophila* and silkworm. In *Drosophila*, this antibody detected endogenous *Atg8a* in immunoblotting and immunostaining experiments (Fig. S3).

Histology

Immunostaining,⁴⁴ LysoTracker Red staining,⁷⁶ acridine orange staining,⁷⁷ Terminal deoxynucleotidyl transferase dUTP

nick end labeling (TUNEL) staining,⁷⁸ transmission electron microscopy⁴⁴ and observation of the GFP-mCherry-Atg8a autophagy flux indicator²² were done as previously described. Fluorescence images and electron micrographs were quantified using NIH ImageJ software or manually in a blinded manner. To simultaneously conduct autophagy phenotyping and PCR genotyping of *Gyjf* from a single larva, we thoroughly rinsed the larvae in phosphate-buffered saline (PBS; Life Technologies, 14190-144) and dissected them individually in a drop of PBS, using fresh disposable slides and an 18-gauge needle every time. Fat bodies were transferred to a well in a 96-well plate filled with Brower Fix⁴⁴ (for immunostaining)⁷⁹ or to a well in a 9-well glass plate filled with PBS plus 100 nM LysoTracker Red DND-99 (Invitrogen, L7528) and 20 µg/ml Hoechst 33258 (Sigma, 23491-45-4) (for LysoTracker Red staining),⁷⁶ and the remaining carcass was transferred to a microcentrifuge tube. For PCR genotyping of larval carcasses, an individual carcass was digested in 100 µL mouse tail digestion buffer (100 mM Tris-HCl, pH 8.5, 200 mM NaCl, 5 mM EDTA, 0.2% SDS [IBI Scientific, IB07060], 0.2 mg/mL proteinase K [Roche, 3115879]) at 65°C overnight, and diluted 2 fold with water. After centrifugation at 15,000 g for 10 min, 1 µL of the supernatant fraction was used for a PCR reaction with relevant primers as described below.

Biochemical analyses

Total RNA was isolated, reverse transcribed, and analyzed using quantitative RT-PCR method using relevant primers as previously described.⁴⁴ Protein lysate preparation and immunoblotting were performed using a standard cell lysis buffer or the indicated buffers as previously described.^{44,80} Predicted protein molecular weights were calculated using the ExPASy molecular weight computation tool. Observed protein size was estimated by comparison of the protein band to protein size marker bands (Bio-Rad, 161-0374).

Primer sequences

For genotyping of the *Gyjf* gene, 5'-GACCGAAAAGTAAA-GACCTGTC-3' (forward) and 5'-AGTGCAGCTG-TAAACCCGT-3' (reverse) primers were used. Homozygosity of *Gyjf^{MI}* insertion abolishes PCR amplification from the *Gyjf* locus

with these primers. Primers detecting *rp49* locus, 5'-ACGTTGTGCACCAGGAACCTT-3' (forward) and 5'-CCAGTCGGATCGATATGCTAA-3' (reverse), were used as a positive control reaction. Quantitative RT-PCR of *Gyjf* and *rp49* (normalization control) was also done using the same primers described above. Quantitative RT-PCR of *4EHP* and *pico* was done using 5'-GCGAGTAGAGCGACCACC-3' (forward) and 5'-GAACAGCGCCACAGGAAC-3' (reverse), and 5'-AGTGC-CACGTCATCAGGTT-3' (forward) and 5'-CGTCGGC-TGTTTGTCAAGG-3' (reverse), respectively.

Statistical analysis

A 2-tailed Student *t* test was used to calculate statistical significance of differences between 2 groups. *P* values equal to or above 0.05 were judged to be not statistically significant (NS).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

We thank Drs. T. Neufeld (UMN), R.J. Wessells, S. Pletcher, R.A. Miller (UM) and G.C. Chen (National Taiwan University), and DSHB (Iowa), DGRC (Indiana), VDRC (Austria), Santa Cruz Biotech. Inc., TRiP (Harvard, GM084947) and Bloomington stock centers (Indiana) for cell lines, fly strains, reagents and access to lab equipment. We thank S. Meshinchi for imaging assistance.

Funding

Work was supported by grants from Ellison Medical Foundation (AG-NS-0932-12) and NIH (R21OD018265, P30AG024824 and P30AG013283).

Supplemental Material

Supplemental data for this article can be accessed on the publisher's website.

References

1. Yen WL, Klionsky DJ. How to live long and prosper: autophagy, mitochondria, and aging. *Physiology* 2008; 23:248-62; PMID:18927201; <http://dx.doi.org/10.1152/physiol.00013.2008>
2. Singh R, Cuervo AM. Autophagy in the cellular energetic balance. *Cell Metab* 2011; 13:495-504; PMID:21531332; <http://dx.doi.org/10.1016/j.cmet.2011.04.004>
3. Levine B, Mizushima N, Virgin HW. Autophagy in immunity and inflammation. *Nature* 2011; 469:323-35; PMID:21248839; <http://dx.doi.org/10.1038/nature09782>
4. Komatsu M, Waguri S, Chiba T, Murata S, Iwata J, Tanida I, Ueno T, Koike M, Uchiyama Y, Kominami E, et al. Loss of autophagy in the central nervous system causes neurodegeneration in mice. *Nature* 2006; 441:880-4; PMID:16625205; <http://dx.doi.org/10.1038/nature04723>
5. Masiero E, Agatea L, Mammucari C, Blaauw B, Loro E, Komatsu M, Metzger D, Reggiani C, Schiaffino S, Sandri M. Autophagy is required to maintain muscle mass. *Cell Metab* 2009; 10:507-15; PMID:19945408; <http://dx.doi.org/10.1016/j.cmet.2009.10.008>
6. Lynch-Day MA, Mao K, Wang K, Zhao M, Klionsky DJ. The role of autophagy in Parkinson's disease. *Cold Spring Harb Perspect Med* 2012; 2:a009357; PMID:22474616; <http://dx.doi.org/10.1101/cshperspect.a009357>
7. Feng Y, He D, Yao Z, Klionsky DJ. The machinery of macroautophagy. *Cell Res* 2014; 24:24-41; PMID:24366339; <http://dx.doi.org/10.1038/cr.2013.168>
8. Xie Z, Klionsky DJ. Autophagosome formation: core machinery and adaptations. *Nat Cell Biol* 2007; 9:1102-9; PMID:17909521; <http://dx.doi.org/10.1038/ncb1007-1102>
9. Tian Y, Li Z, Hu W, Ren H, Tian E, Zhao Y, Lu Q, Huang X, Yang P, Li X, et al. C. elegans screen identifies autophagy genes specific to multicellular organisms. *Cell* 2010; 141:1042-55; PMID:20550938; <http://dx.doi.org/10.1016/j.cell.2010.04.034>
10. Arsham AM, Neufeld TP. A genetic screen in *Drosophila* reveals novel cytoprotective functions of the autophagy-lysosome pathway. *PLoS One* 2009; 4:e6068; PMID:19562034; <http://dx.doi.org/10.1371/journal.pone.0006068>
11. Chang YY, Neufeld TP. An Atg1/Atg13 complex with multiple roles in TOR-mediated autophagy regulation. *Mol Biol Cell* 2009; 20:2004-14; PMID:19225150; <http://dx.doi.org/10.1091/mbc.E08-12-1250>
12. Dietzl G, Chen D, Schnorrer F, Su KC, Barinova Y, Fellner M, Gasser B, Kinsey K, Oettel S, Scheiblaue S, et al. A genome-wide transgenic RNAi library for conditional gene inactivation in *Drosophila*. *Nature* 2007; 448:151-6; PMID:17625558; <http://dx.doi.org/10.1038/nature05954>
13. Cook KR, Parks AL, Jacobus LM, Kaufman TC, Matthews KA. New research resources at the Bloomington *Drosophila* Stock Center. *Fly (Austin)* 2010; 4:88-91;

- PMID:20160480; <http://dx.doi.org/10.4161/fly.4.1.11230>
14. Lautier C, Goldwurm S, Durr A, Giovannone B, Tsiras WG, Pezzoli G, Brice A, Smith RJ. Mutations in the GIGYF2 (TNRC15) gene at the PARK11 locus in familial Parkinson disease. *Am J Hum Genet* 2008; 82:822-33; PMID:18358451; <http://dx.doi.org/10.1016/j.ajhg.2008.01.015>
 15. Morita M, Ler LW, Fabian MR, Siddiqui N, Mullin M, Henderson VC, Alain T, Fonseca BD, Karashchuk G, Bennett CF, et al. A novel 4EHP-GIGYF2 translational repressor complex is essential for mammalian development. *Mol Cell Biol* 2012; 32:3585-93; PMID:22751931; <http://dx.doi.org/10.1128/MCB.00455-12>
 16. Giovannone B, Lee E, Laviola L, Giorgino F, Cleveland KA, Smith RJ. Two novel proteins that are linked to insulin-like growth factor (IGF-I) receptors by the Grb10 adapter and modulate IGF-I signaling. *J Biol Chem* 2003; 278:31564-73; PMID:12771153; <http://dx.doi.org/10.1074/jbc.M211572200>
 17. Giovannone B, Tsiras WG, de la Monte S, Klysk J, Lautier C, Karashchuk G, Goldwurm S, Smith RJ. GIGYF2 gene disruption in mice results in neurodegeneration and altered insulin-like growth factor signaling. *Hum Mol Genet* 2009; 18:4629-39; PMID:19744960; <http://dx.doi.org/10.1093/hmg/ddp430>
 18. Kim M, Park HL, Park HW, Ro SH, Nam SG, Reed JM, Lee JH. *Drosophila* Fip200 is an essential regulator of autophagy that attenuates both growth and aging. *Autophagy* 2013; 9:1201-13; PMID:23819996; <http://dx.doi.org/10.4161/aut.24811>
 19. Mochizuki H, Toda H, Ando M, Kurusu M, Tomoda T, Furukubo-Tokunaga K. UNC-51/ATG1 controls axonal and dendritic development via kinesin-mediated vesicle transport in the *Drosophila* brain. *PLoS One* 2011; 6:e19632; PMID:21589871; <http://dx.doi.org/10.1371/journal.pone.0019632>
 20. Toda H, Mochizuki H, Flores R, 3rd, Josowitz R, Kravieva TB, Lamorte VJ, Suzuki E, Gindhart JG, Furukubo-Tokunaga K, Tomoda T. UNC-51/ATG1 kinase regulates axonal transport by mediating motor-cargo assembly. *Genes Dev* 2008; 22:3292-307; PMID:19056884; <http://dx.doi.org/10.1101/gad.1734608>
 21. Ahanitarig A, Chadwell LV, Terrazas IB, Garcia CT, Nazarian JJ, Lee HK, Lundell MJ, Cassill JA. Molecular characterization of Pegarn: a *Drosophila* homolog of UNC-51 kinase. *Mol Biol Rep* 2009; 36:1311-21; PMID:18636236; <http://dx.doi.org/10.1007/s11033-008-9314-4>
 22. Nezis IP, Shrivage BV, Sagona AP, Lamark T, Bjorkoy G, Johansen T, Rusten TE, Brech A, Bachrecke EH, Stenmark H. Autophagic degradation of dBruce controls DNA fragmentation in nurse cells during late *Drosophila melanogaster* oogenesis. *J Cell Biol* 2010; 190:523-31; PMID:20713604; <http://dx.doi.org/10.1083/jcb.201002035>
 23. Khmelinskii A, Keller PJ, Bartosik A, Meurer M, Barry JD, Mardin BR, Kaufmann A, Trautmann S, Wachsmuth M, Pereira G, et al. Tandem fluorescent protein timers for in vivo analysis of protein dynamics. *Nat Biotechnol* 2012; 30:708-14; PMID:22729030; <http://dx.doi.org/10.1038/nbt.2281>
 24. Hebisch E, Knebel J, Landsberg J, Frey E, Leisner M. High variation of fluorescence protein maturation times in closely related *Escherichia coli* strains. *PLoS One* 2013; 8:e75991; PMID:24155882; <http://dx.doi.org/10.1371/journal.pone.0075991>
 25. Pollock JA, Ellisman MH, Benzer S. Subcellular localization of transcripts in *Drosophila* photoreceptor neurons: chaotic mutants have an aberrant distribution. *Genes Dev* 1990; 4:806-21; PMID:2143163; <http://dx.doi.org/10.1101/gad.4.5.806>
 26. Klionsky DJ, Abdalla FC, Abeliovich H, Abraham RT, Acevedo-Arozena A, Adeli K, Agholme L, Agnello M, Agostinis P, Aguirre-Ghiso JA, et al. Guidelines for the use and interpretation of assays for monitoring autophagy. *Autophagy* 2012; 8:445-544; PMID:22966490; <http://dx.doi.org/10.4161/aut.19496>
 27. Higashi S, Iseki E, Minegishi M, Togo T, Kabuta T, Wada K. GIGYF2 is present in endosomal compartments in the mammalian brains and enhances IGF-1-induced ERK1/2 activation. *J Neurochem* 2010; 115:423-37; PMID:20670374; <http://dx.doi.org/10.1111/j.1471-4159.2010.06930.x>
 28. Wucherpfennig T, Wilsch-Brauninger M, Gonzalez-Gaitan M. Role of *Drosophila* Rab5 during endosomal trafficking at the synapse and evoked neurotransmitter release. *J Cell Biol* 2003; 161:609-24; PMID:12743108; <http://dx.doi.org/10.1083/jcb.200211087>
 29. Young AR, Chan EY, Hu XW, Kochl R, Crawshaw SG, High S, Hailey DW, Lippincott-Schwartz J, Tooze SA. Starvation and ULK1-dependent cycling of mammalian Atg9 between the TGN and endosomes. *J Cell Sci* 2006; 119:3888-900; PMID:16940348; <http://dx.doi.org/10.1242/jcs.03172>
 30. Longatti A, Lamb CA, Razi M, Yoshimura S, Barr FA, Tooze SA. TBC1D14 regulates autophagosome formation via Rab11- and ULK1-positive recycling endosomes. *J Cell Biol* 2012; 197:659-75; PMID:22613832; <http://dx.doi.org/10.1083/jcb.201111079>
 31. Tang HW, Liao HM, Peng WH, Lin HR, Chen CH, Chen GJ. Atg9 interacts with dTRAF2/TRAF6 to regulate oxidative stress-induced JNK activation and autophagy induction. *Dev Cell* 2013; 27:489-503; PMID:24268699; <http://dx.doi.org/10.1016/j.devcel.2013.10.017>
 32. Nagy P, Hegedus K, Piracs K, Varga A, Juhasz G. Different effects of Atg2 and Atg18 mutations on Atg8a and Atg9 trafficking during starvation in *Drosophila*. *FEBS Lett* 2014; 588:408-13; PMID:24374083; <http://dx.doi.org/10.1016/j.febslet.2013.12.012>
 33. Steller H. Regulation of apoptosis in *Drosophila*. *Cell Death Differ* 2008; 15:1132-8; PMID:18437164; <http://dx.doi.org/10.1038/cdd.2008.50>
 34. Metaxakis A, Oehler S, Klinakis A, Savakis C. Minos as a genetic and genomic tool in *Drosophila melanogaster*. *Genetics* 2005; 171:571-81; PMID:15972463; <http://dx.doi.org/10.1534/genetics.105.041848>
 35. Venken KJ, Schulze KL, Haelterman NA, Pan H, He Y, Evans-Holm M, Carlson JW, Levis RW, Spradling AC, Hoskins RA, et al. MIMIC: a highly versatile transposon insertion resource for engineering *Drosophila melanogaster* genes. *Nat Methods* 2011; 8:737-43; PMID:21985007; <http://dx.doi.org/10.1038/nmeth.1662>
 36. Juhasz G, Erdi B, Sass M, Neufeld TP. Atg7-dependent autophagy promotes neuronal health, stress tolerance, and longevity but is dispensable for metamorphosis in *Drosophila*. *Genes Dev* 2007; 21:3061-6; PMID:18056421; <http://dx.doi.org/10.1101/gad.1600707>
 37. Venken KJ, Popodi E, Holtzman SL, Schulze KL, Park S, Carlson JW, Hoskins RA, Bellen HJ, Kaufman TC. A molecularly defined duplication set for the X chromosome of *Drosophila melanogaster*. *Genetics* 2010; 186:1111-25; PMID:20876565; <http://dx.doi.org/10.1534/genetics.110.121285>
 38. Berry DL, Bachrecke EH. Growth arrest and autophagy are required for salivary gland cell degradation in *Drosophila*. *Cell* 2007; 131:1137-48; PMID:18083103; <http://dx.doi.org/10.1016/j.cell.2007.10.048>
 39. Cornu M, Albert V, Hall MN. mTOR in aging, metabolism, and cancer. *Curr Opin Genet Dev* 2013; 23:53-62; PMID:23317514; <http://dx.doi.org/10.1016/j.gde.2012.12.005>
 40. Kanki T, Klionsky DJ. The molecular mechanism of mitochondria autophagy in yeast. *Mol Microbiol* 2010; 75:795-800; PMID:20487284; <http://dx.doi.org/10.1111/j.1365-2958.2009.07035.x>
 41. Clark IE, Dodson MW, Jiang C, Cao JH, Huh JR, Seol JH, Huh JR, Seol JH, Yoo SJ, Hay BA, Guo M. *Drosophila* pink1 is required for mitochondrial function and interacts genetically with parkin. *Nature* 2006; 441:1162-6; PMID:16672981; <http://dx.doi.org/10.1038/nature04779>
 42. Park J, Lee SB, Lee S, Kim Y, Song S, Kim S, Bae E, Kim J, Shong M, Kim JM, et al. Mitochondrial dysfunction in *Drosophila* PINK1 mutants is complemented by parkin. *Nature* 2006; 441:1157-61; PMID:16672980; <http://dx.doi.org/10.1038/nature04788>
 43. Liang CC, Wang C, Peng X, Gan B, Guan JL. Neural-specific deletion of FIP200 leads to cerebellar degeneration caused by increased neuronal death and axon degeneration. *J Biol Chem* 2010; 285:3499-509; PMID:19940130; <http://dx.doi.org/10.1074/jbc.M109.072389>
 44. Lee JH, Budanov AV, Park EJ, Birse R, Kim TE, Perkins GA, Ocorr K, Ellisman MH, Bodmer R, Bier E, et al. Sestrin as a feedback inhibitor of TOR that prevents age-related pathologies. *Science* 2010; 327:1223-8; PMID:20203043; <http://dx.doi.org/10.1126/science.1182228>
 45. Price HM, Gordon GB, Pearson CM, Munsat TL, Blumberg JM. New evidence for excessive accumulation of Z-band material in nemaline myopathy. *Proc Natl Acad Sci U S A* 1965; 54:1398-406; PMID:5218258; <http://dx.doi.org/10.1073/pnas.54.5.1398>
 46. Maron BJ, Ferrans VJ, Roberts WC. Ultrastructural features of degenerated cardiac muscle cells in patients with cardiac hypertrophy. *Am J Pathol* 1975; 79:387-434; PMID:1245333
 47. Friden J, Sjoström M, Ekblom B. A morphological study of delayed muscle soreness. *Experientia* 1981; 37:506-7; PMID:7250326; <http://dx.doi.org/10.1007/BF01986165>
 48. Lee SB, Kim S, Lee J, Park J, Lee G, Kim Y, Kim JM, Chung J. ATG1, an autophagy regulator, inhibits cell growth by negatively regulating S6 kinase. *EMBO Rep* 2007; 8:360-5; PMID:17347671; <http://dx.doi.org/10.1038/sj.embor.7400917>
 49. Scott RC, Juhasz G, Neufeld TP. Direct induction of autophagy by Atg1 inhibits cell growth and induces apoptotic cell death. *Curr Biol* 2007; 17:1-11; PMID:17208179; <http://dx.doi.org/10.1016/j.cub.2006.10.053>
 50. Cumming RC, Simonsen A, Finley KD. Quantitative analysis of autophagic activity in *Drosophila* neural tissues by measuring the turnover rates of pathway substrates. *Methods Enzymol* 2008; 451:639-51; PMID:19185743
 51. Simonsen A, Cumming RC, Lindmo K, Galaviz V, Cheng S, Rusten TE, Finley KD. Genetic modifiers of the *Drosophila* blue cheese gene link defects in lysosomal transport with decreased life span and altered ubiquitinated-protein profiles. *Genetics* 2007; 176:1283-97; PMID:17435236; <http://dx.doi.org/10.1534/genetics.106.065011>
 52. Nishida Y, Arakawa S, Fujitani K, Yamaguchi H, Mizuta T, Kanaseki T, Komatsu M, Otsu K, Tsujimoto Y, Shimizu S. Discovery of Atg5/Atg7-independent alternative macroautophagy. *Nature* 2009; 461:654-8; PMID:19794493; <http://dx.doi.org/10.1038/nature08455>
 53. Winklhofer KF. Parkin and mitochondrial quality control: toward assembling the puzzle. *Trends Cell Biol* 2014; 24:332-41; PMID:24485851; <http://dx.doi.org/10.1016/j.tcb.2014.01.001>
 54. Itakura E, Kishi-Itakura C, Koyama-Honda I, Mizushima N. Structures containing Atg9A and the ULK1 complex independently target depolarized mitochondria at initial stages of Parkin-mediated mitophagy. *J Cell Sci* 2012; 125:1488-99; PMID:22275429; <http://dx.doi.org/10.1242/jcs.094110>
 55. Chang TK, Shrivage BV, Hayes SD, Powers CM, Simin RT, Wade Harper J, Bachrecke EH. Uba1 functions in ATG7- and Atg3-independent autophagy. *Nat*

- Cell Biol 2013; 15:1067-78; PMID:23873149; <http://dx.doi.org/10.1038/ncb2804>
56. Egan DF, Shackelford DB, Mihaylova MM, Gelino S, Kohnz RA, Mair W, Vasquez DS, Joshi A, Gwinn DM, Taylor R, et al. Phosphorylation of ULK1 (hATG1) by AMP-activated protein kinase connects energy sensing to mitophagy. *Science* 2011; 331:456-61; PMID:21205641; <http://dx.doi.org/10.1126/science.1196371>
 57. Di Fonzo A, Fabrizio E, Thomas A, Fincati E, Marconi R, Tinazzi M, Breedveld GJ, Simons EJ, Chien HF, Ferreira JJ, et al. GIGYF2 mutations are not a frequent cause of familial Parkinson's disease. *Parkinsonism Relat Disord* 2009; 15:703-5; PMID:19482505; <http://dx.doi.org/10.1016/j.parkreldis.2009.05.001>
 58. Zhang Y, Zheng L, Zhang T, Wang Y, Xiao Q, Fei QZ, Cui PJ, Cao L, Chen SD. GIGYF2 Asn56Ser mutation is rare in Chinese Parkinson's disease patients. *Neurosci Lett* 2009; 463:172-5; PMID:19638301; <http://dx.doi.org/10.1016/j.neulet.2009.07.067>
 59. Sutherland GT, Siebert GA, Newman JR, Silburn PA, Boyle RS, O'Sullivan JD, Mellick GD. Haplotype analysis of the PARK 11 gene, GIGYF2, in sporadic Parkinson's disease. *Mov Disord* 2009; 24:449-52; PMID:19117363
 60. Prestel J, Sharma M, Leitner P, Zimprich A, Vaughan JR, Durr A, Bonifati V, De Michele G, Hanagasi HA, Farrer M, et al. PARK11 is not linked with Parkinson's disease in European families. *Eur J Hum Genet* 2005; 13:193-7; PMID:15523496; <http://dx.doi.org/10.1038/sj.ejhg.5201317>
 61. Maraganore DM, de Andrade M, Lesnick TG, Strain KJ, Farrer MJ, Rocca WA, Pant PV, Frazer KA, Cox DR, Ballinger DG. High-resolution whole-genome association study of Parkinson disease. *Am J Hum Genet* 2005; 77:685-93; PMID:16252231; <http://dx.doi.org/10.1086/496902>
 62. Komatsu M, Waguri S, Ueno T, Iwata J, Murata S, Tanida I, Ezaki J, Mizushima N, Ohsumi Y, Uchiyama Y, et al. Impairment of starvation-induced and constitutive autophagy in Atg7-deficient mice. *J Cell Biol* 2005; 169:425-34; PMID:15866887; <http://dx.doi.org/10.1083/jcb.200412022>
 63. Kuma A, Hatano M, Matsui M, Yamamoto A, Nakaya H, Yoshimori T, Ohsumi Y, Tokuhisa T, Mizushima N. The role of autophagy during the early neonatal starvation period. *Nature* 2004; 432:1032-6; PMID:15525940; <http://dx.doi.org/10.1038/nature03029>
 64. Dehay B, Martinez-Vicente M, Caldwell GA, Caldwell KA, Yue Z, Cookson MR, Klein C, Vila M, Bezdard E. Lysosomal impairment in Parkinson's disease. *Mov Disord* 2013; 28:725-32; PMID:23580333; <http://dx.doi.org/10.1002/mds.25462>
 65. Kabuta T, Wada K. Insights into links between familial and sporadic Parkinson's disease: physical relationship between UCH-L1 variants and chaperone-mediated autophagy. *Autophagy* 2008; 4:827-9; PMID:18635949; <http://dx.doi.org/10.4161/auto.6560>
 66. Feany MB, Bender WW. A *Drosophila* model of Parkinson's disease. *Nature* 2000; 404:394-8; PMID:10746727; <http://dx.doi.org/10.1038/35006074>
 67. Greene JC, Whitworth AJ, Andrews LA, Parker TJ, Pallanck LJ. Genetic and genomic studies of *Drosophila* parkin mutants implicate oxidative stress and innate immune responses in pathogenesis. *Hum Mol Genet* 2005; 14:799-811; PMID:15689351; <http://dx.doi.org/10.1093/hmg/ddi074>
 68. Greene JC, Whitworth AJ, Kuo I, Andrews LA, Feany MB, Pallanck LJ. Mitochondrial pathology and apoptotic muscle degeneration in *Drosophila* parkin mutants. *Proc Natl Acad Sci U S A* 2003; 100:4078-83; PMID:12642658; <http://dx.doi.org/10.1073/pnas.0737556100>
 69. Meulener M, Whitworth AJ, Armstrong-Gold CE, Rizzu P, Heutink P, Wes PD, Pallanck LJ, Bonini NM. *Drosophila* DJ-1 mutants are selectively sensitive to environmental toxins associated with Parkinson's disease. *Curr Biol* 2005; 15:1572-7; PMID:16139213; <http://dx.doi.org/10.1016/j.cub.2005.07.064>
 70. Park J, Kim SY, Cha GH, Lee SB, Kim S, Chung J. *Drosophila* DJ-1 mutants show oxidative stress-sensitive locomotive dysfunction. *Gene* 2005; 361:133-9; PMID:16203113; <http://dx.doi.org/10.1016/j.gene.2005.06.040>
 71. Menzies FM, Yeniseti SC, Min KT. Roles of *Drosophila* DJ-1 in survival of dopaminergic neurons and oxidative stress. *Curr Biol* 2005; 15:1578-82; PMID:16139214; <http://dx.doi.org/10.1016/j.cub.2005.07.036>
 72. Lee SB, Kim W, Lee S, Chung J. Loss of LRRK2/PARK8 induces degeneration of dopaminergic neurons in *Drosophila*. *Biochem Biophys Res Commun* 2007; 358:534-9; PMID:17498648; <http://dx.doi.org/10.1016/j.bbrc.2007.04.156>
 73. Liu Z, Wang X, Yu Y, Li X, Wang T, Jiang H, Ren Q, Jiao Y, Sawa A, Moran T, et al. A *Drosophila* model for LRRK2-linked parkinsonism. *Proc Natl Acad Sci U S A* 2008; 105:2693-8; PMID:18258746; <http://dx.doi.org/10.1073/pnas.0708452105>
 74. Imai Y, Gehrke S, Wang HQ, Takahashi R, Hasegawa K, Oota E, Lu B. Phosphorylation of 4E-BP by LRRK2 affects the maintenance of dopaminergic neurons in *Drosophila*. *EMBO J* 2008; 27:2432-43; PMID:18701920; <http://dx.doi.org/10.1038/emboj.2008.163>
 75. Venken KJ, Carlson JW, Schulze KL, Pan H, He Y, Spokony R, Wan KH, Koriabine M, de Jong PJ, White KP, et al. Versatile [lacman] BAC libraries for transgenesis studies in *Drosophila melanogaster*. *Nat Methods* 2009; 6:431-4; PMID:19465919; <http://dx.doi.org/10.1038/nmeth.1331>
 76. Neufeld TP. Genetic manipulation and monitoring of autophagy in *Drosophila*. *Methods Enzymol* 2008; 451:653-67; PMID:19185744
 77. Lee JH, Lee E, Park J, Kim E, Kim J, Chung J. In vivo p53 function is indispensable for DNA damage-induced apoptotic signaling in *Drosophila*. *FEBS Lett* 2003; 550:5-10; PMID:12935877; [http://dx.doi.org/10.1016/S0014-5793\(03\)00771-3](http://dx.doi.org/10.1016/S0014-5793(03)00771-3)
 78. Lee JH, Koh H, Kim M, Park J, Lee SY, Lee S, Chung J. JNK pathway mediates apoptotic cell death induced by tumor suppressor LKB1 in *Drosophila*. *Cell Death Differ* 2006; 13:1110-22; PMID:16273080; <http://dx.doi.org/10.1038/sj.cdd.4401790>
 79. Hunt LC, Demontis F. Whole-mount immunostaining of *Drosophila* skeletal muscle. *Nat Protoc* 2013; 8:2496-501; PMID:24232251; <http://dx.doi.org/10.1038/nprot.2013.156>
 80. Park HW, Park H, Semple IA, Jang I, Ro SH, Kim M, Cazares VA, Stuenkel EL, Kim JJ, Kim JS, et al. Pharmacological correction of obesity-induced autophagy arrest using calcium channel blockers. *Nat Commun* 2014; 5:4834; PMID:25189398; <http://dx.doi.org/10.1038/ncomms5834>