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UNIVERSITY OF CALIFORNIA SAN DIEGO

**Coordinated Drosophila Class II PI3-kinase Variants Interact with Myotubularin
Phosphatase to Regulate Autophagy and Longevity**

A thesis submitted in partial satisfaction of the requirements for the degree
Master of Science

in

Biology

by

Chenlu Bao

Committee in charge:

Professor Amy Kiger, Chair
Professor Enfu Hui, Co-Chair
Professor Steve Wasserman

2019

The Thesis of Chenlu Bao is approved and it is accepted in quality and form of publication on microfilm and electronically:

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University of California San Diego

2019

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Figure 7 and figure 11 are coauthored with Chenlu Bao and Jean-Francois Groulx.

ABSTRACT OF THE THESIS

Coordinated *Drosophila* Class II PI3-kinase Variants Interact with Myotubularin Phosphatase to Regulate Autophagy and Longevity

by

Chenlu Bao

Master of Science in Biology

University of California San Diego, 2019

Professor Amy Kiger, Chair

Professor Enfu Hui, Co-Chair

Autophagy is the degradation of cytoplasm in lysosomes to enable cellular homeostasis. It is also the adaptation strategy to deal with cellular damage and nutrient shortages. Autophagy is critical to cell health and relevant to diseases, such as Huntington's disease and cancers. Class I and III PI3-kinases are well-studied and have been shown to regulate autophagy, but roles for class II PI3-Kinase (PI3KC2) have not been fully investigated. We utilized a well-built system in fat body of *Drosophila* larvae to study functions of PI3KC2 in autophagy. In flies, we found two alternatively spliced PI3KC2 variants: PI3KC2 kinase and a truncated, non-catalytic form

(PI3KC2-short). Using targeted deletions, we showed that wild-type PI3KC2 is required to restrict basal autophagy levels, while PI3KC2-short is needed to derepress this inhibition to induce autophagy. Our past data uncovered indirect protein interactions between PI3KC2 and Mtm (myotubularin) PI3-phosphatase, raising questions on the significance of PI3KC2-short in protein complex with Mtm and enzyme regulation of autophagy. This thesis presents strong evidence of protein-protein interactions between PI3KC2-short with both PI3KC2 and Mtm, and further genetically supports that PI3KC2-short acts upstream of both enzymes to promote autolysosomal maturation. Although deletion of PI3KC2-short causes a block in autolysosomal maturation with a lack of cathepsin B activity, another cysteine cathepsin protein L was processed to normal mature forms. The two PI3KC2 variants were both found to be broadly co-expressed and co-required for normal adult fly longevity, motivating initial studies to explore PI3KC2 requirements for protein aggregate clearance with fly aging. In contrast, mutants lacking only PI3KC2 resulted in extended survival in response to adult fly starvation, suggesting that the enhanced autophagy levels in this condition may provide a stress response advantage. In conclusion, PI3KC2 is a key regulator of autophagy levels. Future study of PI3KC2 in flies allows better understanding of its functions which can be applied to solve pathological issues in humans.

Introduction

Autophagy is a cellular recycling pathway that maintains homeostasis through lysosomal degradation of damaged organelles and misfolded proteins (Glick *et al.*, 2010). Thus, autophagy is considered as a possible target of treatments for many neurodegenerative diseases, such as Huntington's disease (Mizushima, 2005) and Parkinson's disease (Rivero-Ríos *et al.*, 2016). Autophagic clearance of protein aggregates also contributes to counter cellular aging (Mizushima, 2005). Furthermore, autophagy is found to be involved in tumor suppression and elimination of bacteria (Mizushima, 2005). Importantly, autophagy is a survival strategy that cells use to balance nutrient levels under stress, such as starvation (Glick *et al.*, 2010) and apoptosis (cell death) (Maiuri *et al.*, 2007). However, extensive autophagy could also induce cell death (Maiuri *et al.*, 2007). Therefore, regulation of autophagy levels can be critical to determine cell fate.

As one of the most prevalent types of autophagy, macroautophagy (referred here as autophagy) occurs in all eukaryotic cells (Levine & Klionsky, 2004). In this pathway, cytoplasm is sequestered and delivered in the form of autophagosomes (double membrane vesicles) to lysosomes for degradation (Ravikumar *et al.*, 2009). Delivery of autophagic cargo requires intersections with the endosome-lysosome (endolysosome) membrane pathways through either a one- or two-step membrane fusion process for autolysosome formation. Membrane fusion can occur directly between autophagosomes and lysosomes (Jahreiss *et al.*, 2008) (Figure 1). The alternative two-step pathway happens first between autophagosomes and late endosomes, forming non-degradative amphisomes that then subsequently fuse with acidified lysosomes to

mature into degradative autolysosomes (Berg *et al.*, 1998). Coordination between autophagosome biogenesis (initiation), membrane trafficking (fusion) and autolysosome activity (degradation) all must occur in order to adapt autophagy levels to meet the changing cellular demands. For example, the degradation of autophagic cargo is a key final step for successful autophagy. This has been revealed by a number of human disease pathologies that derive from conditions where although membrane fusion properly delivers autophagic cargo to autolysosomes, the late autophagic compartments are arrested with defective degradation due to impaired lysosomal clearance mechanisms (Platt *et al.*, 2012; Rivero-Ríos *et al.*, 2016).

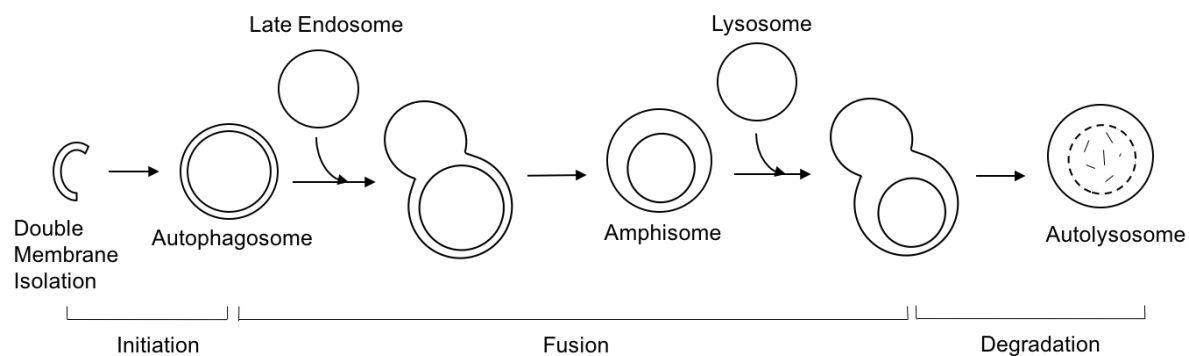


Figure 1. Autophagy: Membrane pathway for lysosomal degradation of cytoplasmic cargo.

Autophagy can proceed at the level of “baseline autophagy” or “induced autophagy” (Mizushima, 2005). At basal level, autophagy is hardly detectable due to rapid flux, but it is critical for clearance of damaged organelles and misfolded proteins as a mediator of cell damage (Mizushima, 2005). Upon starvation – a strong inducer of autophagy – autophagic degradation is significantly elevated to increase nutrient turnover (Mizushima, 2005). Small building blocks, such as amino acids, lipids, sugars, and nucleosides, are produced at the end of autophagy and released into the cytoplasm for reutilization (Rabinowitz and White, 2010). Changes in the levels

of autophagy flux and rates of degradation, therefore, require the ability to regulate autophagosome formation and autolysosome activity (Scott *et al.*, 2004).

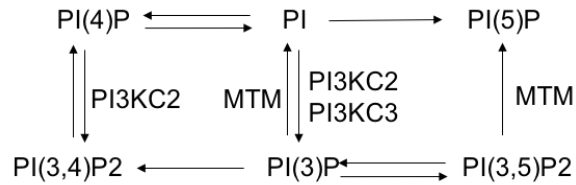


Figure 2. Interconversion of the phosphoinositides selectively targeted by PI3KC2 PI3-kinase and Mtm PI3-phosphatase.

The levels of autophagy are highly regulated to meet changing functional demands by a number of proteins. The family of phosphoinositide 3-kinases (PI3Ks) are key regulators of autophagy. There are three classes of PI3K, classes I, II and III, which have distinct but overlapping catalytic activities (Jean and Kiger, 2014). Overall, PI3Ks function by phosphorylating the 3' hydroxyl groups of the inositol ring of phosphatidylinositols (PtdIns). Products of PI3-kinases are signaling molecules that recruit effector proteins to mediate downstream pathways (Jean and Kiger, 2014, Figure 2). Thus far, two classes of PI3Ks have been ascribed with distinct positive or negative roles in the control of autophagy (Jean and Kiger, 2014). Class I PI3K preferentially phosphorylates PI(4,5)P₂ to PI(3,4,5)P₃ at the plasma membrane in signaling pathways that regulate cell fate, including suppression of autophagy levels (Yu *et al.*, 2015). In contrast, class III PI3K phosphorylates PI to PI(3)P at intracellular membranes for well-established roles in both endosomal membrane trafficking and to promote autophagy at the level of autophagosomes biogenesis (Simonsen and Tooze, 2009). Finally, class II PI3K (PI3KC2) can produce PI(3)P and PI(3,4)P₂ from PI and PI(4)P, respectively, that have

been attributed functions in both cell signaling and membrane trafficking (Jean and Kiger, 2014), and only a preliminary understanding of potential functions significant in autophagy.

Table 1. Class II phosphoinositide PI3-kinases (PI3KC2)

Kinases			Products	Function
PI3KC2	Human	PI3KC2 α	PI(3)P, PI(3,4)P ₂	Endosomal trafficking; Regulation on autophagy: ??
		PI3KC2 β		
		PI3KC2 γ		
	Drosophila	Pi3k68D		
	Yeast	None	--	--

In recent years, better understandings of PI3KC2 functions have begun to emerge.

Humans are found to encode three paralogous genes, PI3KC2 α , PI3KC2 β , and PI3KC2 γ (Jean and Kiger, 2014, Table 1) (Table 1). The three isoforms share overlapping cellular functions in regulating trafficking in recycling pathways via PI(3)P generation, but only PI3KC2 α and PI3KC2 β have been shown to generate PI(3,4)P₂ (Jean and Kiger, 2014). More specifically, PI3KC2 α , via PI(3)P pools, regulates delivery of recycling cargo from endosomes to endocytic recycling compartments (Campa *et al.*, 2018). In addition, PI3KC2 α phosphorylates PI(4)P to PI(3,4)P₂ that is enriched at plasma membrane, and is involved in clathrin-mediated endocytosis (Posor *et al.*, 2013). PI3KC2 β also generates PI(3)P that has been ascribed roles at the plasma membrane and endosomes, as well as was found to generate PI(3,4)P₂ at lysosomes and late endosomes to negatively regulate mTOR1 and subsequently cell growth (Marat *et al.*, 2017). Compared with PI3KC2 α and PI3KC2 β , PI3KC2 γ appears to have more restricted tissue expression with PI(3)P-mediated endosomal and signaling functions specifically in the liver

(Gulluni *et al.*, 2019). Both functions of PI3KC2 α and PI3KC2 β have been associated with requirements in autophagy (Merrill *et al.*, 2017; Marat *et al.*, 2017), although their specific functions in this process are still not well described or understood.

Whereas mammals encode for three different PI3KC2 genes and yeast encodes none (Jean and Kiger, 2014), *Drosophila* encodes for the single Pi3K68D gene (called here PI3KC2) (MacDougall *et al.*, 1995). The fruit fly presents an ideal system to address PI3KC2 functions in autophagy for the following reasons.

First, the single gene encoded in flies avoids potential masked functions by genetic redundancy found in human, yet provides metazoan context not possible to study in yeast that does not encode for PI3KC2.

Second, larval fat body is a well-established system to interrogate the functional and cellular requirements in autophagy. Fat body serves functions analogous to both mammalian liver and adipose tissue. It develops as a monolayer of cells that lines along the body cavity and is easily accessed by dissection in 3rd instar larvae (Lorincz *et al.*, 2017). This tissue is well studied and shown to undergo a robust autophagy induction in response to animal starvation (Lorincz *et al.*, 2017): starvation for amino acids leads to a striking increase of autophagy marked by an increased number of autophagosomes and increased number and size of autolysosomes (Scott *et al.*, 2004). A large number of conserved genes in the autophagy signaling regulation, hierarchical autophagosome biogenesis and flux have been well-established in the fat body system.

Third, previous work in *Drosophila* demonstrated that Pi3K68D shares overlapping cellular functions and structural similarities with human homologs, PI3KC2 α and PI3KC2 β (Gulluni *et al.*, 2019). Specifically, Pi3K68D is responsible for generation of significant endosomal pools of PI(3)P, with functions in endosomal trafficking and lysosomal homeostasis to control size of acidified lysosomes (Velichkova *et al.*, 2010). Opposite from Pi3K68D, the *Drosophila* Mtm PI3-phosphatase (orthologue of mammalian MTM1 and MTMR2 PI3-phosphatases) dephosphorylates the 3' phosphate position to regulate turnover of PI(3)P (Taylor *et al.*, 2000). Interestingly, Pi3K68D was found to interact (Jean *et al.*, 2012) and share important functional roles with the Mtm for membrane-trafficking in hemocytes (*Drosophila* macrophages) and muscles (Velichkova *et al.*, 2010; Ribeiro *et al.*, 2011; Jean *et al.*, 2012). The *mtm* function involved in lysosomal size in hemocytes exhibited features of being autophagy-dependent, including characteristics of autolysosomes (with cytoplasmic content) and a dependence on Atg1, a regulatory kinase of autophagy initiation (Velichkova *et al.*, 2010). Given the relationship between Mtm and PI3KC2 in endosomal recycling (Velichkova *et al.*, 2010), our interest next was to study whether Mtm also shares important functions with PI3KC2 on autophagy.



Figure 3. Two spliced variants of PI3KC2 in flies.

Our laboratory found that two strikingly distinct alternative splice variants were encoded for catalytic and non-catalytic PI3KC2 alleles (Groulx and Kiger, personal communication). One

variant spans the known full-length, or “long”, protein (referred to as PI3KC2 kinase or just PI3KC2). In contrast, a second major splicing variant results in a truncated protein (referred to as PI3KC2-short or PI3KC2-S) that only contains the most N-terminus region and four unique amino acids (Groulx and Kiger, personal communication) (Figure 3). The PI3KC2/PI3KC2-S N-terminus regions that contain a clathrin-binding domain is common to both variants, but PI3KC2-S does not have the other PI3KC2 lipid- and protein-binding domains nor the kinase domain. To explore functions of each variant, we generated variant-specific deletions. We used CRISPR Cas9 technique to specifically delete PI3KC2-S to generate $\Delta short$ or delete PI3KC2 to generate $\Delta long$. We also targeted the exon common for both variants to delete them simultaneously to generate $\Delta exon$.

Strikingly, we found that the two major PI3KC2 splice variants play opposite roles in a shared pathway to regulate levels of basal autophagy (Groulx and Kiger, personal communication). Under well-fed conditions, the $\Delta short$ mutant resulted in a decrease in levels of autophagosome formation and a block in autolysosomal maturation (Groulx and Kiger, personal communication). On the contrary, $\Delta long$ resulted in enhancement of the two processes, with increased levels of autophagosome biogenesis and hyperactive autolysosomes (Groulx and Kiger, personal communication). Interestingly, similar to $\Delta long$, knockout of both variants in a $\Delta exon$ null allele resulted in hyperactive autophagy, indicating a bypass of the autophagy in contrast to the block caused by $\Delta short$ (Groulx and Kiger, personal communication). Altogether, we propose that PI3KC2 normally inhibits autophagy while PI3KC2-S promotes autophagy, and specifically, both work together in a common pathway: PI3KC2-S acts upstream to negatively

regulate PI3KC2 and derepress autophagy (Groulx and Kiger personal communication).

In addition to PI3KC2, Mtm was found as necessary and sufficient to repress autophagy. The effects of Mtm on autophagy were also shared with PI3KC2 variants: *mtm* RNAi depletion led to enhanced autolysosomal activity (as also seen in $\Delta long$ mutants), while Mtm-WT overexpression blocked autophagy (as was seen with $\Delta short$) (Groulx and Kiger, personal communication). Double mutant combinations then revealed that Mtm acts downstream of PI3KC2-S and PI3KC2: *mtm* depletion reverted $\Delta short$ from blocked to enhanced autophagy, while overexpressed Mtm reverted $\Delta long$ mutants from enhanced to blocked autophagy (Groulx and Kiger, personal communication). Based on our results, we propose a model pathway with the two variants of PI3K-C2 and Mtm (Figure 4). We hypothesize that PI3KC2 activates Mtm to restrict autophagy, while PI3KC2-S inhibits PI3KC2 and Mtm in order to derepress autophagy to induced levels (Groulx and Kiger, personal communication).

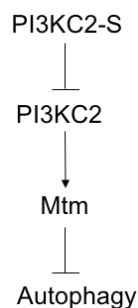


Figure 4. Hypothesized model.

My project included two major goals. First, I sought to better understand the protein-protein interactions between PI3KC2-S, PI3KC2 and Mtm, and how protein complex formations and other potential modifications may regulate autophagy in flies. Second, I explored the

physiological functions of PI3KC2-S and PI3KC2 variants *in vivo*, including the effects on viability, adaption to stress responses, and aging.

Materials and Methods

Drosophila strains

w[1118]; *P*{*w*[+*mC*]=*Cg*-*GAL4.A*}2 fly was used to induce UAS-driven gene expression in larval fat body. Genotypes included in this thesis: (1) *w*[1118]; *P*{*w*[+*mC*]=*UAS-lacZ.B*}*Bg4-1-2*. (2) *w*; *Sp*/*CyO*; *Pi3K68D*[*Δshort-009*]/*TM6C Sb Tb*; CRISPR/Cas9 deletion at 3' UTR of *PI3KC2-S*. (3) *w*; *Pi3K68D*[*Δlong-128*]; CRISPR/Cas9 deletion at exon unique to *PI3KC2*. (4) *w*; (*Sp*/*CyO*); *Pi3K68D*[*Δexon-058*]/*TM6C*; CRISPR/Cas9 deletion at N-terminus common for both *PI3KC2-S* and *PI3KC2-L*. (5) *y*[1] *w*[67c23]; *mtmΔ210*/*CyO*. (6) *y* *sc v*; *gRNAs*(*v*+)
Pi3k68d[*Δexon-1*]>*attP40*/*CyO*. (7) *w*[1118]; *P*{*w*+=*UAST-3XFLAG:Pi3K68Dshort*}2/(*CyO*). (8) *w*; *CgGal4*, *UAS-mCherry:mtm*/*CyO*. (9) *w*[1118]; *P*{*w*+=*UAST-3XFLAG:mtm*}3/*CyO*. (10) *w*[1118]; *Cg-Gal4*, *P*{*w*+, *UAS-mCherry:Pi3K68D*[2]}*Line2*/*CyO*. (11) *w*; *UAS-FLAG:Pi3K68D*/*CyO*.

Preparation of lysates and starvation

Flies were raised at 25°C unless stated otherwise. Fat body was dissected in 1x PBS (pH 7.4) at room temperature from 3rd stage instar larvae that were put in fresh food one day before extraction. Larvae directly taken from food were classified as “fed,” while larvae put in 20% sucrose solution for 4h in 25°C were classified as “starved.”

Different tissues were extracted from larval and adult flies in 1x PBS, and directly transferred to 2x SDS-loading buffer. Lysates were sonicated for 20sec-1min, and heated at 98°C for 5 min, and stored in -80°C or loaded onto SDS-PAGE gels. Protein concentrations were

quantified by BCA Protein Assay Kit.

Co-immunoprecipitation

Fat body was dissected from larvae (10-20) in 1x PBS and homogenized in 600 μ L lysis buffer (25 mM Tris-HCl, 1 mM EDTA, 0.1 mM EGTA, 5 mM MgCl₂, 150 mM NaCl, 1 mM DTT, 10% glycerol, 1% Triton X-100, and 1x protease inhibitor (Sigma)) on ice for 30min. Samples were then centrifuged at 13,200 RPM for 10 min at 4°C for 2 times, and clear supernatant was separated from white layers of lipids. Anti-FLAG M2 agarose beads (Sigma) or RFP Trap agarose beads (Chromo Tek) were washed with lysis buffer (without Triton X-100) for 2 times, 0.1 M Glycine pH 3.0, lysis buffer (without Triton X-100) for another 3 times. 200 mL of collected supernatant was transferred to pre-cleaned beads and immunoprecipitated for 2 h at 4°C. The remaining supernatant was used as “total lysate.” Beads were then washed with lysis buffer for 5 min for 5 times, followed by 1 wash with lysis buffer (without Triton X-100), and eventually dried. Samples were heated for 5 min in 100 μ L 2x SDS-loading buffer and stored in -80°C or loaded onto SDS-PAGE gels.

Kinase activity assay

To test kinase activity of overexpressed protein, ~20 larvae were used for each condition. Procedures for lysate and anti-FLAG M2 agarose bead (Sigma) preparation were the same as co-immunoprecipitation. The amount of proteins in each sample was measured from supernatant using BCA Protein Assay Kit. Supernatant with the same amount of protein was transferred to

beads and immunoprecipitated for 1 h at 4°C.

To test endogenous kinase activity, ~40 larvae were used for each condition and processed in the same way as co-immunoprecipitation. The amount of proteins in each sample was measured from supernatant using BCA Protein Assay Kit. About 10 μ L of protein A agarose beads (Cell Signaling Technology) were washed for 1 time with lysis buffer (without Triton X-100). The co-immunoprecipitant was pre-cleaned with Protein A agarose bead (Cell Signaling Technology) for 1 h at 4°C. Supernatant was collected and incubated with antibody overnight at 4°C: 20 μ L anti-IgG (Santa Cruz Biotechnology; sc-2027) antibody for negative control; 20 μ L anti-Pi3K68D antibody (1:1,000; generated in (Jean *et al.*, 2012)) for others. On the following day, protein A agarose beads (Cell Signaling Technology) were washed for 2 times with lysis buffer (without Triton X-100). Lysates were transferred to beads and immunoprecipitated for 1 h at 4°C.

The following steps were the same for the two types of kinase assay. Beads were washed for 5 min with lysis buffer for 5 times, 1 time with lysis buffer (without Triton X-100), and 1 time with 1x kinase buffer (without ATP). Kinase activity was measured using V6930 ADP-Glo Kinase Assay (Promega). Beads were dried, mixed with 40 μ L 1x kinase buffer (with 2% ATP), and equally split into three tubes (one for PI; another for PI(4)P; one for Western Blot). In tubes for PI and PI(4)P, 2 μ L of PI or PI(4)P substrates were added and mixed with beads. Samples were then incubated at 37°C for 1h for reactions, mixed with 22 μ L ADP-Glo Reagents at room temperature for 45 min and 44 μ L Kinase Detection Reagents for another 45 min. Luminescence was read from supernatant (Analyst HT 96-384). Samples for Western Blot was re-suspended

with 2x SDS-Loading buffer and stored in -80°C or loaded onto SDS-PAGE gels.

Staining and microscopy

Fat body was extracted from 3rd stage instar larvae to detect mCherry:PI3KC2-L or stained with LysoTracker red DND-99 (1:5,000; Thermo Fisher Scientific, Waltham, MA) for 5 min or with Magic Red Cathepsin B Assay (1:260; ImmunoChemistry Technologies, Bloomington, MN) following manufacturer's instructions. Fat body was then mounted on slide glass for fluorescence microscopy at room temperature within 30 min.

Live images were taken by a Zeiss LSM 700 microscope with 40x oil/1.3 NA Plan Apochromatic objective, using Zen software (Carl Zeiss, Inc.). Images were then processed and cropped, using Adobe Photoshop CS5. The area of fat body was also measured by Adobe Photoshop CS5, while the LysoTracker staining was quantified by CellProfiler software. The histogram (Figure 2) was created with Prism6 (GraphPad).

Measurement of lifespan

The protocol was modified based on (Linford *et al.*, 2013). Flies were collected 2 days after eclosion. For each genotype, male and female flies were separated, and each vial only contained 10 flies or less. Under fed condition, flies were fed with regular food. Under starved condition, 2% agar gel base was boiled, split into empty vials, and used in place of food. Flies in both conditions were raised at 25°C, and flipped to fresh food or new agar base every 2-3 days. Numbers of deaths were counted every 1 or 2 days. Data was analyzed using Prism6 (GraphPad).

Detection of Ref(2)P (p62) from *Drosophila* heads

The protocol was modified based on (Bartlett *et al.*, 2011). About 40 flies were collected for each genotype and different ages. Heads were separated from body by vortexing after flash freezing in liquid nitrogen. Heads were then crashed by electronic homogenizers in 1% Triton buffer (1% Triton X-100, 1x PBS, 1x protease inhibitor (Sigma)). Samples were centrifuged at 13,200 RPM for 10 min at 4°C. Supernatant was collected as “Triton-soluble.” Debris of heads were washed with Triton buffer, and suspended with SDS buffer (50mM Tris pH 7.5, 2% SDS). Lysates were sonicated for 20-30sec, heated at 98°C for 5 min, and centrifuged at 13,200 RPM for 10 min at 4°C, and supernatant was collected as “Triton-insoluble.” The amount of proteins was quantified by BCA protein assay kit to ensure equal protein levels were loaded for Western Blot analysis.

Western Blot analysis

The amount of proteins in each sample was quantified by BCA Protein Assay kit, loaded on SDS-PAGE gels, and then transferred to polyvinylidene difluoride membranes. Membranes were blocked with 5% milk in PBST (1x PBS and 0.1% Tween 20) for 1 h at room temperature, and subsequently incubated with primary antibodies: rabbit anti-Pi3K68D (1:1,000; generated in (Jean *et al.*, 2012)), rabbit anti-Ref(2)P (1:2,500; AB_569199), rabbit anti-FLAG (1:1,000; AB_439687), rabbit anti-mCherry (1:1,000; AB_1975001), mouse anti-tubulin (1:5,000; AB_477593), mouse anti-ubiquitin (1:1,000; AB_331292), and mouse anti-cathepsinL (1:5,000, MAB22591) for overnight at 4°C. Membranes were washed for 3 times with PBST for 10 min,

followed by incubation of secondary anti-rabbit HRP or anti-mouse HRP antibodies (1:3,000) for 1 h at room temperature. Membranes were then washed for 3 times with PBST for 10 min and incubated by SuperSignal West Pico PLUS Chemiluminescent Substrate (Fisher Scientific) for 5 min and detected by ELC plus (GE Healthcare Life Sciences) and X-ray films. Intensities of bands were measured using ImageJ and normalized to that of LacZ (Figure 4). The histogram was created with Prism6 (GraphPad).

Results

PI3KC2-S interacts with both PI3KC2 and Mtm *in vivo*

In order to test whether shared functions and genetic interactions between PI3KC2-S, PI3KC2 and Mtm could be mediated by protein interactions *in vivo*, I performed co-immunoprecipitate experiments from fat body lysates of fed larvae. I found pairwise interactions between all combinations of the three proteins in lysates from well-fed larval conditions (Figure 5A, PI3KC2-S--Mtm, PI3KC2--Mtm, PI3KC2--PI3KC2-S). PI3KC2 and PI3KC2-S shared protein interactions, and both variant proteins also co-IPed Mtm. The combinations of protein interactions suggested that autophagy regulation may involve formation of a tri-protein complex, yet to be tested *in vivo* but was pulled down from triple co-expression in cell lines.

Regulation of PI3KC2 kinase activity

Our genetic results indicated that PI3KC2-S inhibits the repressive function of PI3KC2 in autophagy. However, it is unclear how or what aspect of PI3KC2 function is inhibited. We wondered if PI3KC2-S could regulate catalytic activity of PI3KC2. Our cell-based studies with overexpressed proteins suggested that PI3KC2 kinase activity against PI and PI(4)P could be inhibited by PI3KC2-S. To test this hypothesis *in vivo* with endogenous levels of PI3KC2, I immunoprecipitated endogenous PI3KC2 from fat body of well-fed wild-type versus $\Delta short$ mutant conditions lacking PI3KC2-S. I then used ADP-Glo kinase assays to test catalytic activity against PI and PI(4)P. The results showed that although we could IP endogenous PI3KC2, the detectable kinase activity was insignificant above the background in this assay; hence, we were

unable to determine whether or not the presence of PI3KC2-S influences PI3KC2 catalytic activity (Figure 5B). However, when PI3KC2 was overexpressed in flies, I could differentiate the kinase activity above the negative controls (Figure 5C). While there was a trend of increased kinase activity in starved versus fed larvae, we still did not get statistically significant differences. Overall, how PI3KC2 kinase activity is regulated is still to be determined.

Co-regulation of autolysosomal maturation by PI3KC2-S and Mtm

Our past results showed that autolysosomal maturation was impaired in fat body cells from *Δshort* mutants, while *mtm* knockdown resulted in enhanced autophagy that could bypass the blocked autolysosome maturation with *Δshort*. To validate the genetic relationship between *mtm* and PI3KC2-S, I tested autophagy levels in *Δmtm* genomic null mutants and double mutants with *Δshort* by monitoring LysoTracker Red staining of acidic compartments using confocal microscopy. As predicted, I found hyperactive autophagy with more Lysotracker-positive acidic compartments in *Δmtm* single homozygous mutants, as seen with *mtm* knockdown in fat body (Figure 6A-B). In the double mutants of *Δshort* and *Δmtm*, abnormally high autolysosomal activity was also observed, indicating bypass of the *Δshort* block and that Mtm acts downstream of PI3KC2-S to co-regulate autolysosomal maturation.

Interestingly, I also observed that *Δmtm/+* heterozygotes resulted in enhanced autophagy levels compared to *Δshort*, but at lower levels compared to *Δmtm* homozygotes. This result suggested that autophagy regulation may be dosage-sensitive to *mtm* expression levels.

Cathepsin L was processed in *Δshort*

We showed impaired autolysosomal activity in *Δshort* indicated by low LysoTracker Red as well as Magic Red staining, a read-out of cathepsin B protease activity. Cathepsin B is a cysteine lysosomal protease that, once activated in the acidic environment of endo-lysosomes, will cleave Magic Red substrate and exhibit fluorescence as an indicator of functional cathepsin activity. Using this technique, my colleague, Dr. Groulx, showed there is a lack of cathepsin B activity, indicating impaired autophagy in *Δshort* larval fat body (Figure 7A) (personal communication). This result indicated a block of autolysosomal maturation, suggesting possible underlying defects in either cathepsin trafficking and/or processing. To test cathepsin processing, we monitored another cysteine lysosomal protease, cathepsin L, which normally remains in the inactive pre-cathepsin L form until processed to the mature form by cleavage in acidic compartments. In our results, mature cathepsin L was detected in wild-type and all PI3KC2 mutant conditions under both fed and starved conditions (Figure 7B), demonstrating successful processing of cathepsin L even in *Δshort*. The future plan is to test if trafficking defects in protease delivery to lysosomes could account for the block of autophagy in *Δshort*.

Changes of protein levels of PI3KC2 variants

We investigated interactions between PI3KC2 variants and their regulations in autophagy, but we're also interested in how cell change protein behaviors for better adaptation to stress. I explored changes in endogenous protein levels of PI3KC2-S and PI3KC2 under fed and starved conditions, as well as in different mutants. Comparison of protein levels between fed and

starved conditions showed that starvation did not lead to changes in PI3KC2 protein levels, but in two experiments, did lead to decreased PI3KC2-S levels significantly (Figure 8A-B). In addition, with starvation in *Δlong* mutants, I found decreased expression of PI3KC2-S levels compared to control, although this difference was insignificant. Thus, with induced autophagy, PI3KC2-S protein levels may in turn be limited, resulting in a negative-feedback loop. I made this observation with only two independent experiments, so more data are needed to make conclusions.

Localization of PI3KC2

Besides protein levels, cells might also change other protein behaviors of PI3KC2 in response to different cellular demands. We would like to find out changes of localization of PI3KC2 under different autophagy levels. We first examined the localization and dynamics of overexpressed PI3KC2 in fat body from fed larvae raised at different temperatures in order to address any changes due to levels of temperature-sensitive GAL4-induced expression. My results in limited studies found that the localization of PI3KC2 was partially altered with temperature (Figure 9A-B). At 18°C, most PI3KC2 was distributed to puncta throughout cell, while only a minority was localized at the cell periphery. As temperature increased (coinciding with increased levels of GAL4-induced PI3KC2 levels), PI3KC2 tended to shift to puncta along the cell periphery. Given that PI3KC2 shifted from a peripherally to centrally localized puncta under starved conditions (Groulx and Kiger, personal communication), we propose that PI3KC2 localization may shift in response to environmental changes and functional needs.

Physiological effects of PI3KC2 variants

Other than effects on autophagy, we were also interested in the physiological roles for the two PI3KC2 variants *in vivo*; hence, we investigated longevity of adult aged flies with different PI3KC2 mutants. When flies were well-fed, the function of just one variant or the other ($\Delta short$ or $\Delta long$) or half the genetic amount of both PI3KC2 variants ($\Delta exon/+$) were able to maintain survival with aging of female and male flies as in controls (LacZ), although statistically significant differences were only found in female flies (Figure 10A; Table 2). On the contrary, deletion of both variants in the null allele ($\Delta exon$) significantly shortened the longevity of flies. We could find the similar trend that flies with $\Delta exon$ mutant tended to die earlier than controls under starvation (Figure 10B). Furthermore, $\Delta exon/+$ and $\Delta long$ mutants not only maintained normal longevity of female flies, but enabled improved survival of flies under starvation. $\Delta exon/+$ and $\Delta short$ mutants had the similar effects on male flies. Cumulatively, these data suggested that PI3KC2 contributes to maintaining longevity of flies and is significant for better survival under stress.

PI3KC2 expressions and functions in other tissues

Variants of PI3KC2 were expressed in fat body and critical for autophagy and nutrient turnover. Other than fat body, I found that PI3KC2 variants proteins were broadly detected in other tissues: brains and gonads of adult flies, and the central nervous system of larvae (Figure 11A). The expression in brains inspired us to study if PI3KC2 could regulate autophagic aggregate clearance in brain. The cytoplasmic scaffold protein, p62, delivers ubiquitinated cargo

for autophagic degradation, and levels of p62 have been used as an indicator of autophagic activity. I used solubility of Ref(2)P, the *Drosophila* p62 homolog, as a measurement of autophagic activity to detect if PI3KC2 regulates aggregate clearance at different ages of flies. I found that from both 1-week and 4-week old wild-type flies, more insoluble aggregates were detected than soluble proteins, but more soluble proteins were found in 3-week old flies (Figure 11B). Different PI3KC2 mutants did not result in significant differences compared to controls when flies were 1 week old. However, from 3-week-old flies, both $\Delta short$ and $\Delta long$ mutants resulted in more insoluble aggregates compared to controls, but less insoluble aggregates in $\Delta exon$ mutant. When flies were 4 weeks old, more soluble aggregates were found in $\Delta exon$ mutant compared to controls. Overall, however, this experiment was inconclusive because of inconsistent and incomplete results.

Discussion

Conserved interactions between PI3KC2 and Mtm *in vivo*

My thesis research contributes to understanding the coordination required between lipid regulators, PI3KC2 and Mtm, to adapt autophagy levels to the cellular demands. My data demonstrates that both PI3KC2 protein variants can physically interact with each other and with Mtm, and that Mtm acts downstream of PI3KC2-S to co-regulate autolysosomal maturation. Previously, Mtm was found to work together with PI3KC2 kinase to regulate hemocyte (macrophage) dynamics and hemocyte-dependent animal viability (Velichkova *et al.*, 2010). The strong interactions between PI3KC2 and Mtm discovered in flies predict a similar pairing present in mammals. Pi3K68D (PI3KC2) loss of function was shown to suppress *mtm*-associated defects in fly muscle organization (Ribeiro *et al.*, 2011), and subsequently, it was similarly found that removal of PI3KC2 β was able to prevent and rescue features of myotubular myopathy in *MTM1*-deficient mouse models (Sabha *et al.*, 2016). In addition, both PI3KC2 β and MTMR6 were found to regulate calcium-activated K⁺ channels via PI(3)P mediated regulation (Srivastava *et al.*, 2005, 2009). In conclusion, interactions between PI3KC2 and MTM family members are conserved and may be critical to understand PI(3)P-mediated pathways and functions. PI3KC2 may also be studied as the treatment of Mtm-dependent diseases.

Regulation of kinase activity

We demonstrated that PI3KC2-S promotes autophagy by inhibiting PI3KC2 function that restricts basal autophagy levels, and then questioned how PI3KC2-S regulates PI3KC2. Studies

have shown that PI3KC2 α protein can be autoregulated to mediate endocytosis. On the one hand, structural studies revealed that protein folding of PI3KC2 α C-terminal PX-C2 protein domains interferes with catalytic activity of the kinase domain (Wang *et al.*, 2018). Likewise, PI3KC2 α kinase activity was elevated when the N-terminal end (the protein region that shares sequence homology with both fly PI3KC2 and PI3KC2-S variants) was bound with clathrin or deleted (Gaidarov *et al.*, 2001), suggesting potential derepression of another inhibitory intramolecular interaction. It is also plausible that such inhibition was resulted by intermolecular interactions by two PI3KC2 molecules. Similar to human PI3KC2, the *Drosophila* PI3KC2 contains a RBD (Ras-binding domain), the PI3K core which includes kinase domain, and lipid-binding PX-C2 domains at the C-terminus (Gulluni *et al.*, 2019), while PI3KC2-S only contains the most N-terminus clathrin-binding domain and four unique amino acids. I explored whether levels of endogenous PI3KC2 kinase activity was affected by loss of PI3KC2-S function. We predicted to see elevated PI3KC2 kinase activity in *Δshort*. However, the endogenous kinase activity signals were not significantly above background in my assay. The question remained to be answered potentially through optimization of this kinase assay. The future direction would be to detect at which domain PI3KC2-S interacts with PI3KC2 to better understand how this inhibition is done.

Regulation of PI3KC2-S

Although we know that PI3KC2-S inhibits PI3KC2, it is still unclear how PI3KC2-S itself is regulated. Both PI3KC2 and PI3KC2-S encode for an N-terminus clathrin-binding

domain, suggesting that clathrin could be involved in their regulation. PI3KC2 α was found to co-localize with endocytic vesicles (clathrin coated pits), as well as autophagy markers, and suggested to function as a bridge between endocytosis and autophagy (Merrill *et al.*, 2017). In other words, it is possible that PI3KC2 α receives signals from endocytosis, clathrin in particular, and subsequently regulates autophagy. The clathrin-binding motif of PI3KC2-S might allow it to function in such a way. Hence, one possible regulator of PI3KC2-S could be clathrin or other endocytic signals.

Cathepsin proteins processing in $\Delta short$

We have shown impaired autolysosomal maturation in $\Delta short$ with a defect in appropriate levels of cathepsin B activity. Previously, the cysteine cathepsins B and L were demonstrated to be critical for maintaining lysosomal degradation (Cermak *et al.*, 2016). Their processed forms were also decreased when lysosomal functions were defective (Yogalingam and Pendergast, 2008). Our results indicated that activity of cathepsin B fit expectation, but processing of cathepsin L was not impacted in $\Delta short$. Alternatively, the non-degradative lysosomes discovered in $\Delta short$ could be caused by a defect in trafficking of cathepsin cargoes. To test this, we plan to detect colocalization of cathepsin L and markers for different compartments along endomembrane trafficking pathways to identify any defects in cathepsin delivery to the autolysosomes.

Effects of PI3KC2 on adult fly longevity

We have shown that PI3KC2 is crucial for autophagy at cellular levels, but found that it also has significant physiological importance. Along with our previous findings that PI3KC2-S and PI3KC2 derepresses and inhibits autophagy, respectively, we expected the opposite trends on longevity previously shown to be sensitive to autophagy ability (Nakamura and Yoshimori, 2018). However, the result showed that having either variant was sufficient enough to maintain relatively normal adult fly lifespans under normal (non-stressed) conditions. Mountford and colleagues showed that the effect of PI3KC2 α on survival of mouse model was dependent on its expression. In this study, homozygous PI3KC2 α -deficient mice died at embryo stages, and yet while heterozygous mice survived past embryogenesis, they also exhibited phenotypes (Mountford *et al.*, 2015). We found that $\Delta exon$ flies die much earlier, while the $\Delta exon/+$ flies have normal lifespans, suggesting that PI3KC2 is required for survival. This result showed that only half the genetic amount of PI3KC2 – implying that possibly half the amount of PI3KC2 proteins – was sufficient to rescue the shorter lifespan phenotype. Importantly, these $\Delta exon/+$ flies and $\Delta long$ mutant flies could live even longer than wild-type under starvation, suggesting that enhanced autophagy might help animals to survive in response to stress. However, the $\Delta short$ mutant, which resulted in block of autophagy in both fed and starved conditions, did not improve survival of flies. Therefore, PI3KC2 may function in other pathways to regulate longevity and survival. Other than starvation, other stresses, such as heat shock, were also shown to regulate autophagy pathways (Dokladny *et al.*, 2015). Our future plan is to stress flies with different methods to test the importance of PI3KC2 and its regulation on autophagy in response to these stresses.

Functions of PI3KC2 in other tissues

PI3KC2 variants were found in larval fat body, but they were also found to be broadly expressed in many other fly tissues. Based on our results in fat body that two PI3KC2 variants regulate autophagy in opposite ways, it raises the questions whether there are similar functions in other tissues. In mouse and human brains with Huntington's disease, autophagy has been shown to be defective, resulting in empty autophagosomes and failure of cargo recognition (Matrinez-Vicente *et al.*, 2010). Moreover, more insoluble aggregates were detected from older and autophagy defective (Atg8s mutant) fly brains (Barlett *et al.*, 2011). We predicted that one PI3KC2 role in autophagy regulation may have consequences in brains in order to meet the demand of clearance of neural protein aggregates with aging. However, we were unable to see more aggregates nor ubiquitinated proteins in aged wild-type flies as expected, suggesting that our experiments may not be optimized or reflect differences due to genetic backgrounds. Our next plan is to optimize this experiment, or try different assays to monitor the accumulation of aggregates. For example, fluorescent-tagged membrane proteins in *Drosophila* eyes could quantitatively report Htt protein degradation (Burr *et al.*, 2014). This powerful technique might be tested in other tissues, such as guts and muscles, to monitor degradation in future directions.

My thesis research presents both physical and functional connections between PI3KC2 and Mtm, and their regulations on autophagy, as well as new insights on the physiological importance of PI3KC2. Importantly, it uncovers the significance of PI3KC2-S on autophagy and interactions with other proteins, although it is still unclear if human PI3KC2 has a similar

alternatively spliced variant. Future experiments will address unsolved questions for better understanding of regulation of PI3KC2 on autophagy and relevance of human diseases.

Figures and Tables

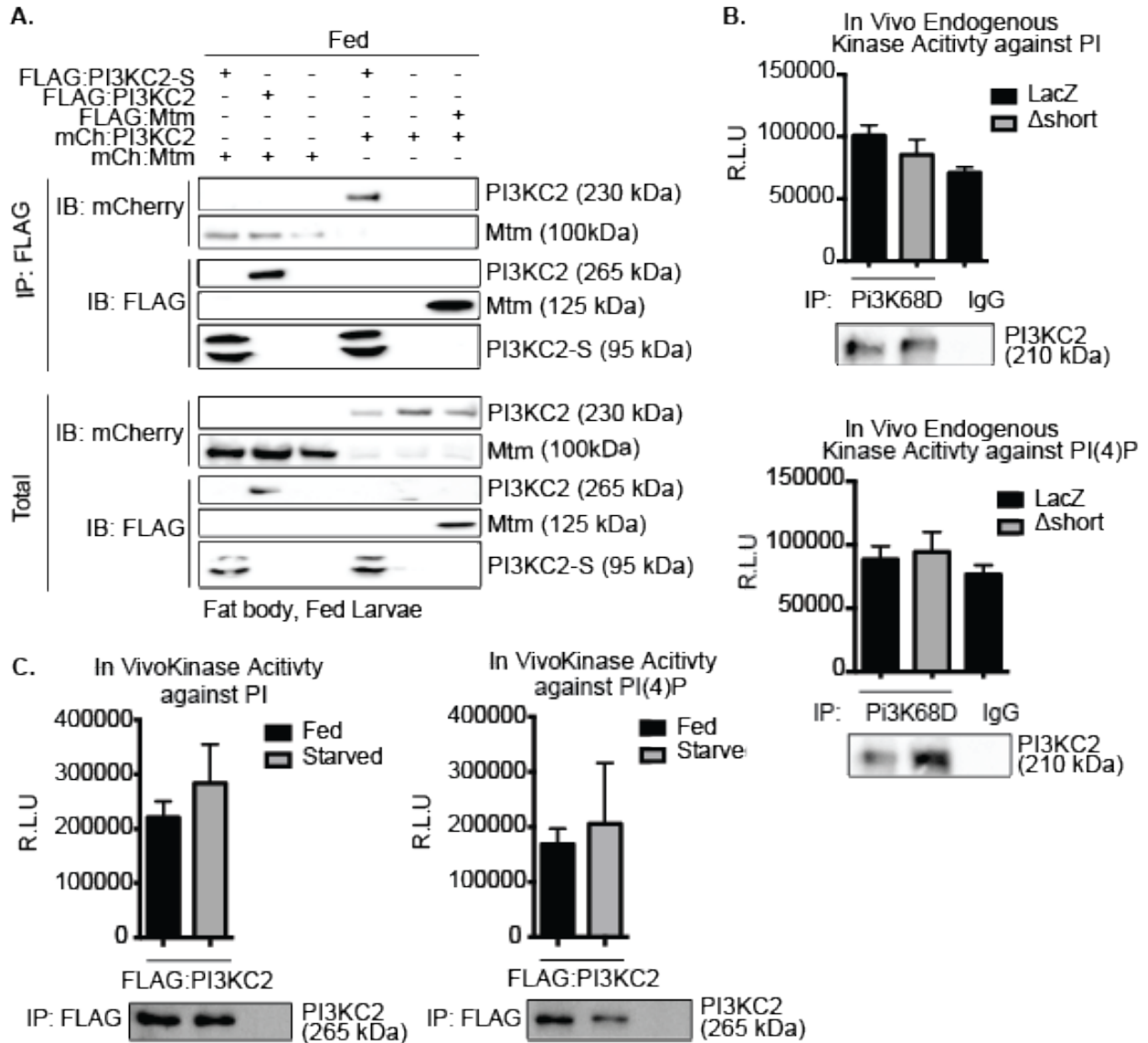


Figure 5. Protein-protein interactions between PI3KC2-S, PI3KC2 and Mtm. **A.** Co-IP of PI3KC2-S, PI3KC2, and Mtm. FLAG and mCherry Western Blot of IP-FLAG protein or total protein lysates from fat body of fed larvae. **B.** The effect of PI3KC2-S on endogenous PI3KC2 kinase activity against PI and PI(4)P was measured by ADP-Glo Kinase Assay (R.L.U: relative luminescence units). IP-Pi3K68D or -IgG from fat body of fed larvae, shown by Western Blot. 3 independent experiments; $P \geq 0.05$, using one-way ANOVA test. **C.** The effect of starvation on 3xFLAG PI3KC2 kinase activity was measured by ADP-Glo Kinase Assay (R.L.U, relative luminescence units), and normalized to fat body of fed LacZ. IP-FLAG from fat body of fed or starved larvae, shown by Western Blot. Error bars indicate 1 standard error above and below means. 3 independent experiments; $P \geq 0.05$, using one-way ANOVA test.

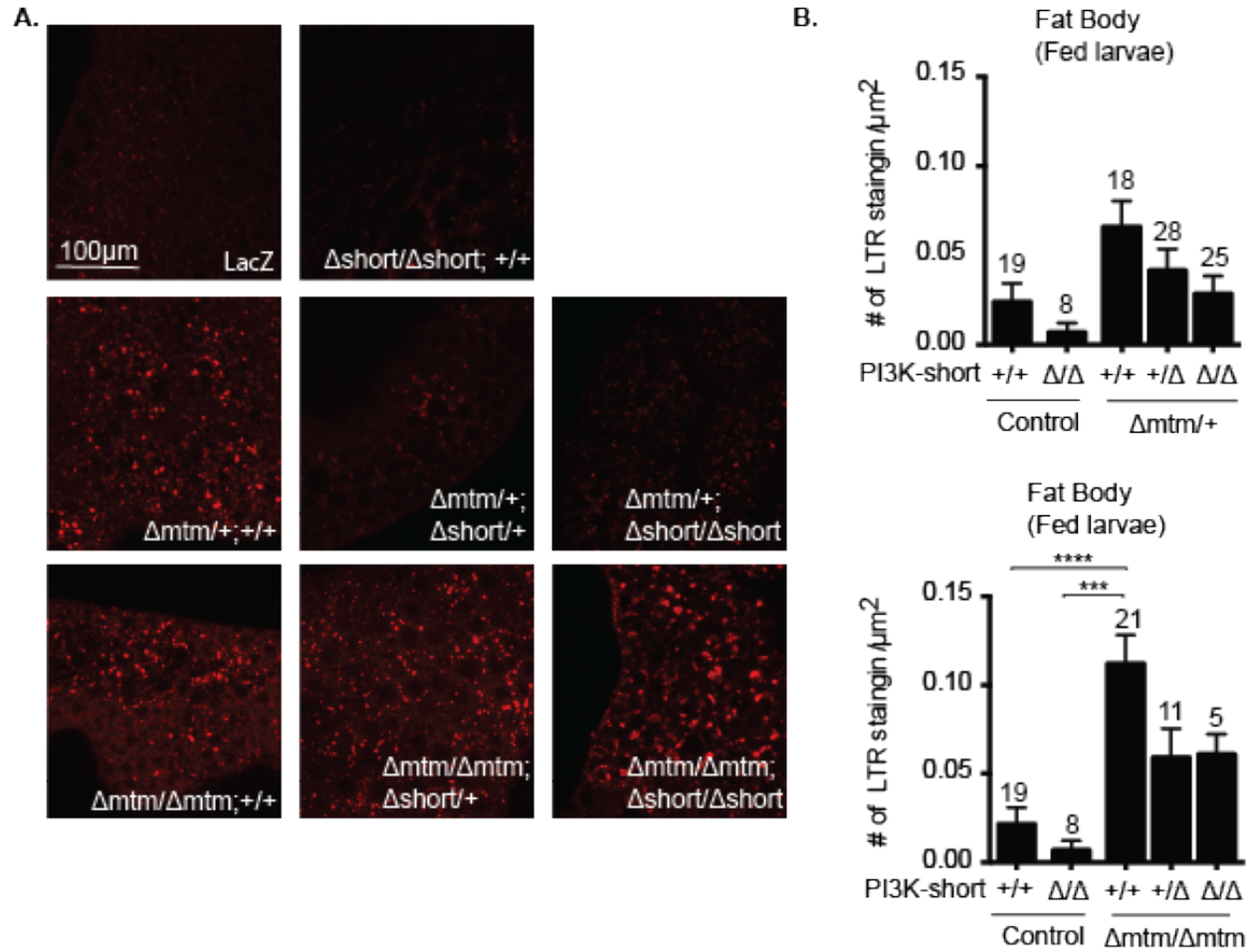


Figure 6. PI3KC2-S and Mtm are dosage sensitive co-regulators of autolysosomal activity.

A. LysoTracker (LTR) staining is normally low with basal autophagy levels in fat body of fed wild-type larvae. While LTR signals remained low with blocked autophagy in $\Delta short$ single mutant, in contrast, high levels of LTR acidification appeared with abnormally induced autophagy in Δmtm single mutant. Double mutants lead to high LTR-positive autolysosomal activity, indicating Δmtm can induce autolysosomes that bypass the block in $\Delta short$. **B.** Quantifications of LysoTracker positive puncta. Error bars indicate 1 standard error above and below means. *** $P \leq 0.001$; **** $P \leq 0.0001$, from one-way ANOVA test.

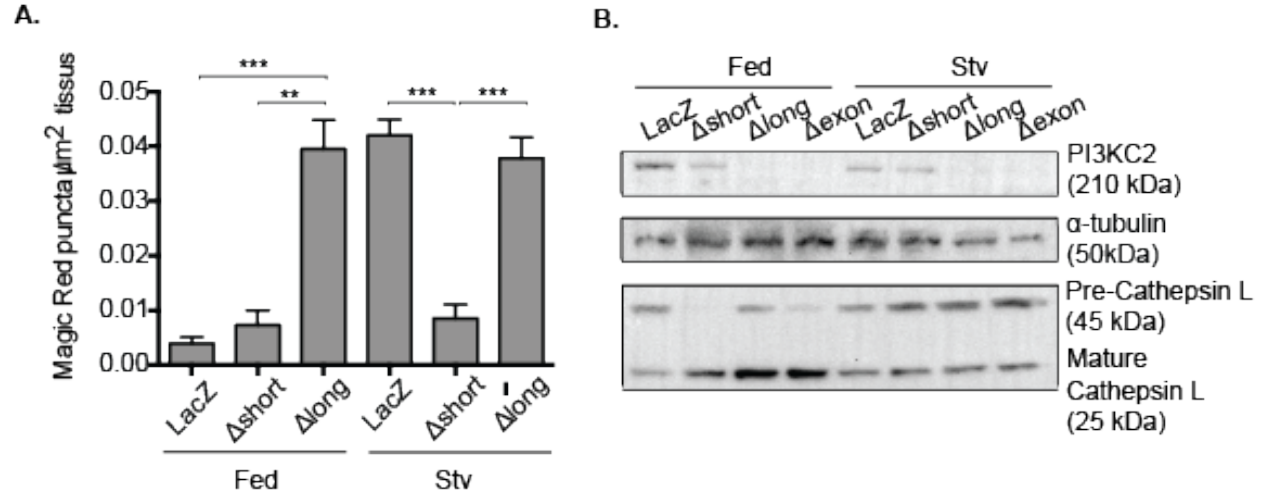


Figure 7. PI3KC2 affect regulation of cathepsin B activity, but not cathepsin L processing.

A. Magic Red (Cathepsin B) activity in fat body from fed or starved larvae. Cathepsin B activity was abnormally induced in fed $\Delta long$ larvae and abnormally blocked in starved $\Delta short$ larvae (puncta number per area; *** $P \leq 0.001$; **** $P \leq 0.0001$) (Groulx, personal communication). **B.** Western Blot analysis of cathepsin L processing from pre-cathepsin L to mature cathepsin L. Mature Cathepsin L was present in all PI3KC2 mutant conditions under fed or starvation condition. Figure 7 is coauthored with Chenlu Bao and Jean-Francois Groulx.

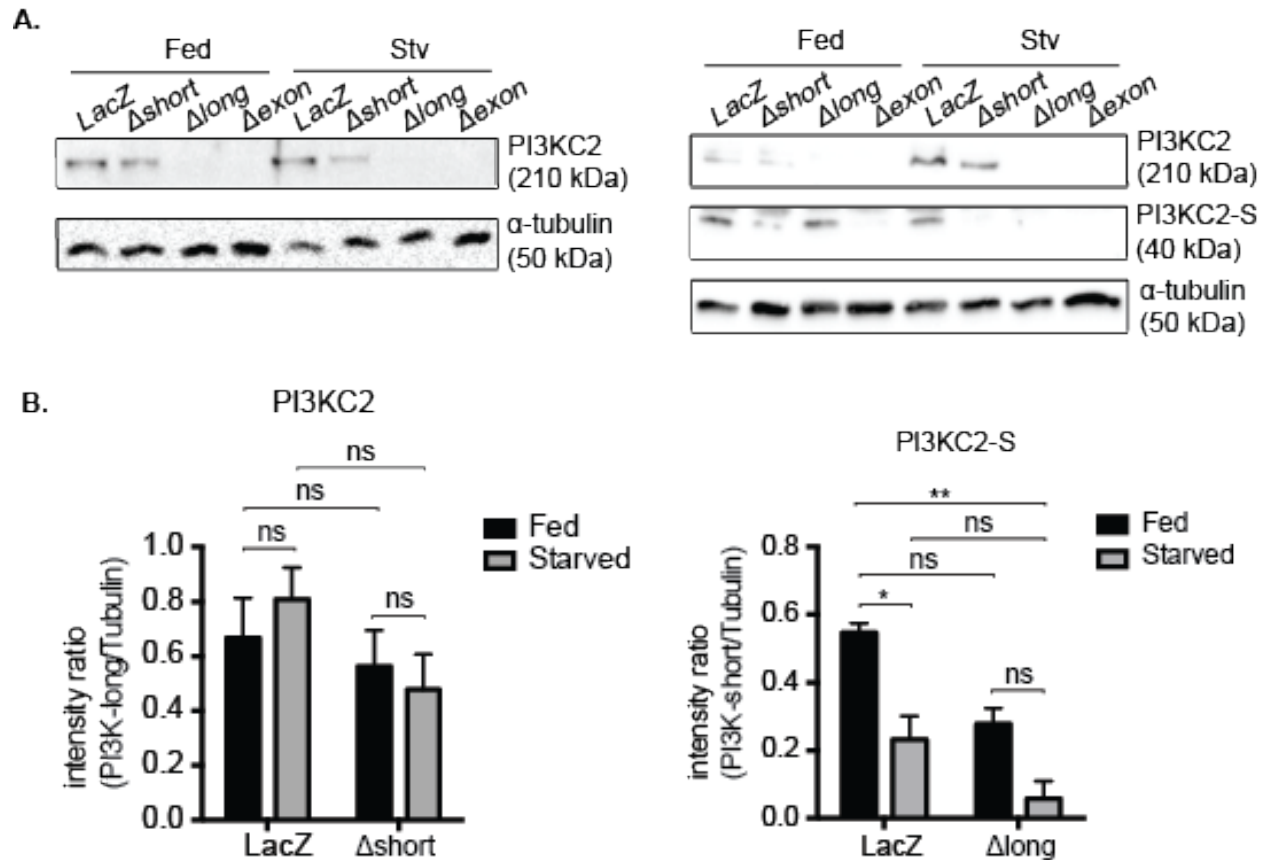


Figure 8. Expression of PI3KC2 and -S in mutants under fed and starved conditions. A. Two representative Western Blot analyses. **B.** Intensity ratios of PI3KC2 and -S protein levels, normalized to α -tubulin levels. Protein levels of PI3KC2 mildly decreased in $\Delta short$ mutant under fed and starved conditions. 3 independent experiments; $P \geq 0.05$, using one-way ANOVA test. Starvation did not significantly alter PI3KC2 levels. Protein levels of PI3KC2-S were decreased in $\Delta long$ mutant and by starvation. 2 independent experiments; $*P \leq 0.05$; $**P \leq 0.01$, using one-way ANOVA test.

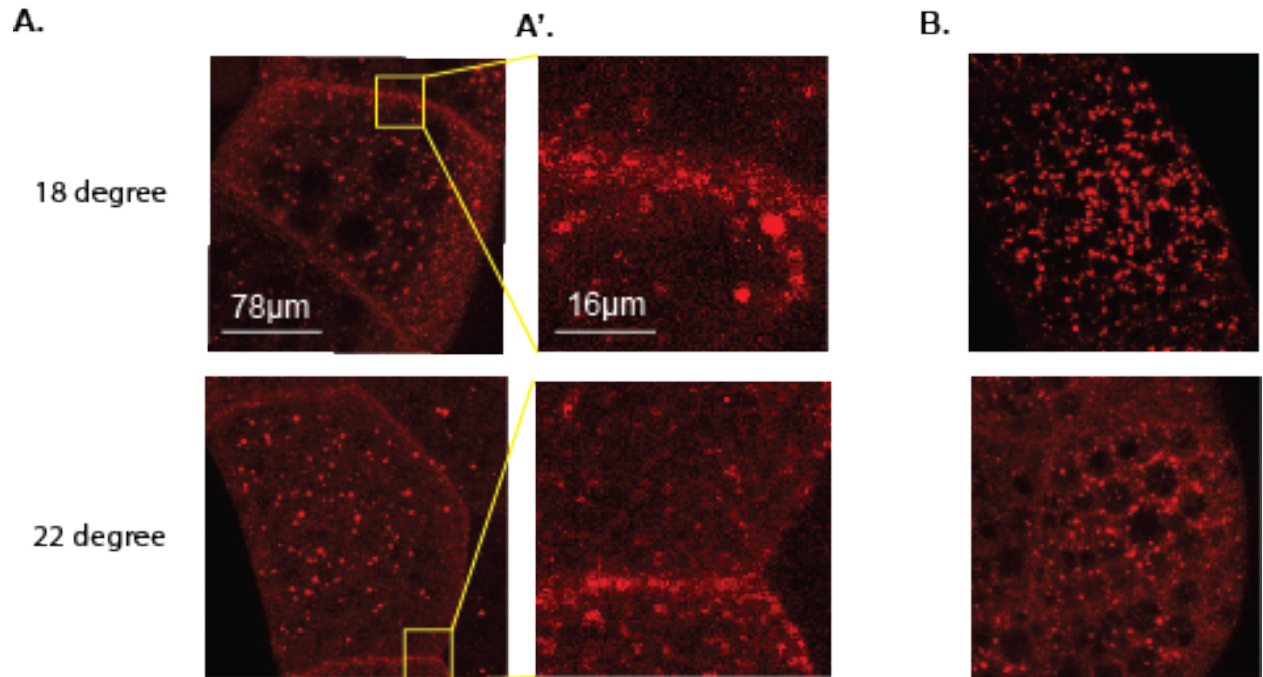


Figure 9. Localization of GAL4-induced mCherry:PI3KC2 raised at different temperatures. A&B. LysoTracker staining on fat body of fed larvae raised in 18°C and 22°C. **A-A'.** PI3KC2 localized to periphery. **B.** Another observed pattern of localization of PI3KC2. At 18°C, most PI3KC2 localized to central puncta. At 22°C, most PI3KC2 localized peripherally, either on the plasma membrane or to adjacent foci (endosomes) and to some centrally localized puncta.

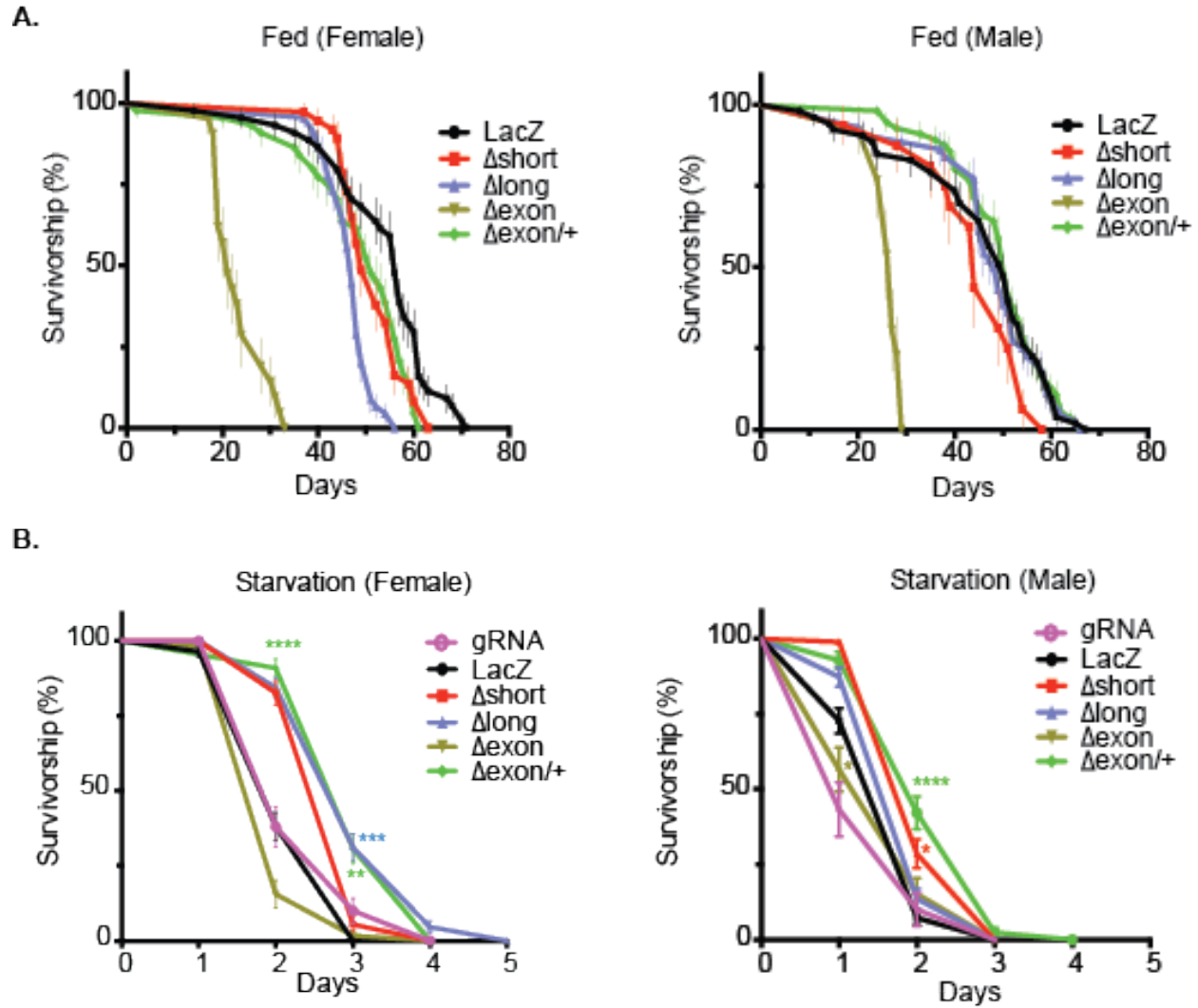


Figure 10. PI3KC2 is required for regulating longevity of adult flies (females and males). Survival rates of adult flies control (LacZ, gRNA) and PI3KC2 homozygous ($\Delta short$, $\Delta long$, $\Delta exon$) or heterozygous ($\Delta exon/+$) mutant flies over time (days) when raised under (A) fed or (B) starved conditions. Error bars indicate one standard error above and below means. * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, **** $P \leq 0.0001$, using one-way ANOVA test comparing with LacZ flies on the same day.

Table 2. Lifespan of fed female and male flies when fed. Log-Rank test; comparison between mutants and LacZ (control).

Female/ Genotype	Sample size	Mean lifespan (Days)	Median lifespan (Days)	Mean lifespan difference	Log-Rank Prob.
LacZ	44	53.18	56	/	/
$\Delta short$	37	51.03	49	4.04%	0.0161
$\Delta long$	45	46.42	47	12.71%	< 0.0001
$\Delta exon$	21	23.14	21	56.48%	< 0.0001
$\Delta exon/+$	44	48.05	51	9.65%	0.0029

Male/ Genotype	Sample size	Mean lifespan (Days)	Median lifespan (Days)	Mean lifespan difference	Log-Rank Prob.
LacZ	54	45.89	50	/	/
$\Delta short$	16	43.81	44	4.53%	ns (> 0.05)
$\Delta long$	44	47.45	48.5	3.40%	ns (> 0.05)
$\Delta exon$	13	26.31	27	42.67%	< 0.0001
$\Delta exon/+$	56	49.21	50	7.23%	ns (> 0.05)

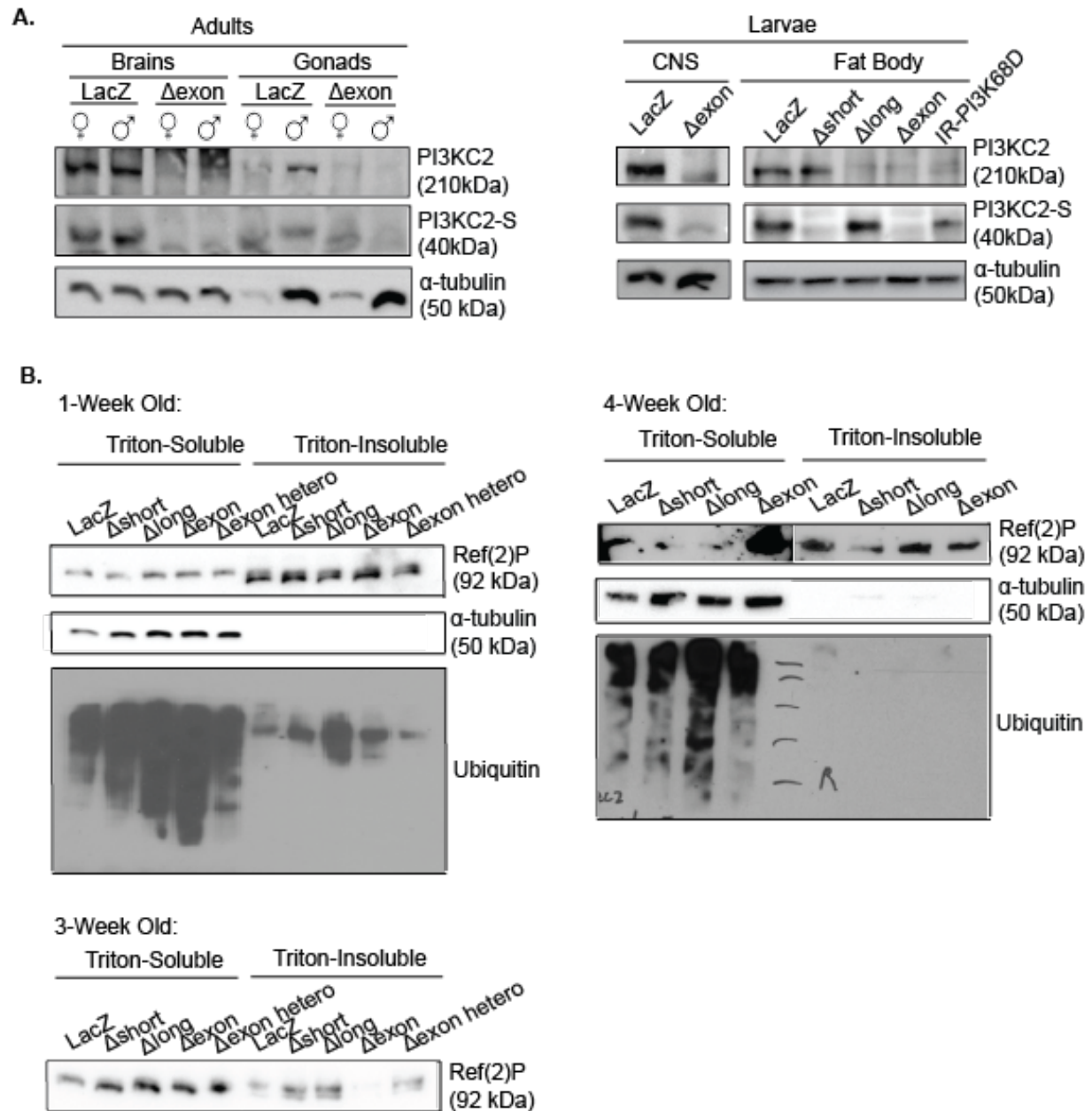


Figure 11. PI3KC2 variant proteins are both broadly expressed in different tissues. A.

Expressions of PI3KC2 and -S protein from adult brains or gonads, and larval central nervous system (CNS) or fat body (Western Blot analysis of fat body was performed by Jean-Francois Groulx).

B. Detection of insoluble proteins as an indicator of accumulated aggregates in brains of adult flies at different ages in different PI3KC2 mutant conditions, marked by Ref(2)P. Figure 11 is coauthored with Chenlu Bao and Jean-Francois Groulx.

References

- Glick, D., Barth, S., & Macleod, K. F. (2010). Autophagy: cellular and molecular mechanisms. *The Journal of Pathology*, 221(1), 3–12.
- Mizushima, N. (2005). The pleiotropic role of autophagy: from protein metabolism to bactericide. *Cell Death and Differentiation*, 12(S2), 1535–1541.
- Rivero-Ríos, P., Madero-Pérez, J., Fernández, B., & Hilfiker, S. (2016). Targeting the Autophagy/Lysosomal Degradation Pathway in Parkinson's Disease. *Current Neuropharmacology*, 14(3), 238–249.
- Maiuri, M. C., Zalckvar, E., Kimchi, A., & Kroemer, G. (2007). Self-eating and self-killing: crosstalk between autophagy and apoptosis. *Nature Reviews Molecular Cell Biology*, 8(9), 741–752.
- Levine, B., & Klionsky, D. J. (2004). Development by Self-Digestion: Molecular Mechanisms and Biological Functions of Autophagy. *Developmental Cell*, 6(4), 463–477.
- Ravikumar, B., Futter, M., Jahreiss, L., Korolchuk, V. I., Lichtenberg, M., Luo, S., Massey, D. C., Menzies, F. M., Narayanan, U., Renna, M., Jimenez-Sanchez, M., Sarkar, S., Underwood, B., Winslow, A., & Rubinsztein, D. C. (2009). Mammalian macroautophagy at a glance. *Journal of Cell Science*, 122(Pt 11), 1707–1711.
- Jahreiss, L., Menzies, F. M., & Rubinsztein, D. C. (2008). The Itinerary of Autophagosomes: From Peripheral Formation to Kiss-and-Run Fusion with Lysosomes. *Traffic*, 9(4), 574–587.
- Berg, T. O., Fengsrud, M., Strømhaug, P. E., Berg, T., & Seglen, P. O. (1998). Isolation and characterization of rat liver amphisomes. Evidence for fusion of autophagosomes with both early and late endosomes. *The Journal of Biological Chemistry*, 273(34), 21883–21892.
- Platt, F. M., Boland, B., & van der Spoel, A. C. (2012). The cell biology of disease: lysosomal storage disorders: the cellular impact of lysosomal dysfunction. *The Journal of Cell Biology*, 199(5), 723–734.
- Rabinowitz, J. D., & White, E. (2010). Autophagy and metabolism. *Science (New York, N.Y.)*, 330(6009), 1344–1348.
- Klionsky, D. J., Eskelinen, E.-L., & Deretic, V. (2014). Autophagosomes, phagosomes, autolysosomes, phagolysosomes, autophagolysosomes... wait, I'm confused. *Autophagy*, 10(4), 549–551.

- Scott, R. C., Schuldiner, O., & Neufeld, T. P. (2004). Role and Regulation of Starvation-Induced Autophagy in the *Drosophila* Fat Body. *Developmental Cell*, 7(2), 167–178.
- Jean, S., & Kiger, A. A. (2014). Classes of phosphoinositide 3-kinases at a glance. *Journal of Cell Science*, 127(Pt 5), 923–928.
- Yu, X., Long, Y. C., & Shen, H.-M. (2015). Differential regulatory functions of three classes of phosphatidylinositol and phosphoinositide 3-kinases in autophagy. *Autophagy*, 11(10), 1711–1728.
- Simonsen, A., & Tooze, S. A. (2009). Coordination of membrane events during autophagy by multiple class III PI3-kinase complexes. *The Journal of Cell Biology*, 186(6), 773–782.
- Campa, C. C., Margaria, J. P., Derle, A., Del Giudice, M., De Santis, M. C., Gozzelino, L., Copperi, F., Bosia, C., & Hirsch, E. (2018). Rab11 activity and PtdIns(3)P turnover removes recycling cargo from endosomes. *Nature Chemical Biology*, 14(8), 801–810.
- Posor, Y., Eichhorn-Gruenig, M., Puchkov, D., Schöneberg, J., Ullrich, A., Lampe, A., Müller, R., Zarbakhsh, S., Gulluni, F., Hirsch, E., Krauss, M., Schultz, C., Schmoranz, J., Noe, F., & Haucke, V. (2013). Spatiotemporal control of endocytosis by phosphatidylinositol-3,4-bisphosphate. *Nature*, 499(7457), 233–237.
- Marat, A. L., Wallroth, A., Lo, W.-T., Müller, R., Norata, G. D., Falasca, M., Schultz, C., & Haucke, V. (2017). mTORC1 activity repression by late endosomal phosphatidylinositol 3,4-bisphosphate. *Science (New York, N.Y.)*, 356(6341), 968–972.
- Gulluni, F., De Santis, M. C., Margaria, J. P., Martini, M., & Hirsch, E. (2019). Class II PI3K Functions in Cell Biology and Disease. *Trends in Cell Biology*.
- Merrill, N. M., Schipper, J. L., Karnes, J. B., Kauffman, A. L., Martin, K. R., & MacKeigan, J. P. (2017). PI3K-C2 α knockdown decreases autophagy and maturation of endocytic vesicles. *PloS One*, 12(9), e0184909.
- MacDougall, L. K., Domin, J., & Waterfield, M. D. (1995). A family of phosphoinositide 3-kinases in *Drosophila* identifies a new mediator of signal transduction. *Current Biology*, 5(12), 1404–1415.
- Lőrincz, P., Mauvezin, C., & Juhász, G. (2017). Exploring Autophagy in *Drosophila*. *Cells*, 6(3).
- Velichkova, M., Juan, J., Kadandale, P., Jean, S., Ribeiro, I., Raman, V., Stefan, C., & Kiger, A. A. (2010). *Drosophila* Mtm and class II PI3K coregulate a PI(3)P pool with cortical and endolysosomal functions. *Journal of Cell Biology*, 190(3), 407–425.

Taylor, G. S., Maehama, T., & Dixon, J. E. (2000). Myotubularin, a protein tyrosine phosphatase mutated in myotubular myopathy, dephosphorylates the lipid second messenger, phosphatidylinositol 3-phosphate. *Proceedings of the National Academy of Sciences of the United States of America*, 97(16), 8910–8915.

Jean, S., Cox, S., Schmidt, E. J., Robinson, F. L., & Kiger, A. (2012). Sbf/MTMR13 coordinates PI(3)P and Rab21 regulation in endocytic control of cellular remodeling. *Molecular Biology of the Cell*, 23(14), 2723–2740.

Ribeiro, I., Yuan, L., Tanentzapf, G., Dowling, J. J., & Kiger, A. (2011). Phosphoinositide Regulation of Integrin Trafficking Required for Muscle Attachment and Maintenance. *PLoS Genetics*, 7(2), e1001295.

Linford, N. J., Bilgir, C., Ro, J., & Pletcher, S. D. (2013). Measurement of lifespan in *Drosophila melanogaster*. *Journal of Visualized Experiments : JoVE*, (71).

Bartlett, B. J., Isakson, P., Lewerenz, J., Sanchez, H., Kotzebue, R. W., Cumming, R. C., Harris, G. L., Nezis, I. P., Schubert, D., Simonsen, A., & Finley, K. D. (2011). p62, Ref(2)P and ubiquitinated proteins are conserved markers of neuronal aging, aggregate formation and progressive autophagic defects. *Autophagy*, 7(6), 572–583.

Sabha, N., Volpatti, J. R., Gonorazky, H., Reifler, A., Davidson, A. E., Li, X., Eltayeb, N. M., Dall'Armi, C., Di Paolo, G., Brooks, S. V., Buj-Bello, A., Feldman, E. L., & Dowling, J. J. (2016). PIK3C2B inhibition improves function and prolongs survival in myotubular myopathy animal models. *The Journal of Clinical Investigation*, 126(9), 3613–3625.

Srivastava, S., Li, Z., Lin, L., Liu, G., Ko, K., Coetzee, W. A., & Skolnik, E. Y. (2005). The phosphatidylinositol 3-phosphate phosphatase myotubularin-related protein 6 (MTMR6) is a negative regulator of the Ca²⁺-activated K⁺ channel KCa3.1. *Molecular and Cellular Biology*, 25(9), 3630–3638.

Srivastava, S., Di, L., Zhdanova, O., Li, Z., Vardhana, S., Wan, Q., Yan, Y., Varma, R., Backer, J., Wulff, H., Dustin, M. L., & Skolnik, E. Y. (2009). The Class II Phosphatidylinositol 3 kinase C2β Is Required for the Activation of the K⁺ Channel KCa3.1 and CD4 T-Cells. *Molecular Biology of the Cell*, 20(17), 3783–3791.

Wang, H., Lo, W.-T., Vujičić Žagar, A., Gulluni, F., Lehmann, M., Scapozza, L., Haucke, V., & Vadas, O. (2018). Autoregulation of Class II Alpha PI3K Activity by Its Lipid-Binding PX-C2 Domain Module. *Molecular Cell*, 71(2), 343–351.e4.

Gaidarov, I., Smith, M. E., Domin, J., & Keen, J. H. (2001). The Class II Phosphoinositide 3-Kinase C2 α Is Activated by Clathrin and Regulates Clathrin-Mediated Membrane Trafficking. *Molecular Cell*, 7(2), 443–449.

Cermak, S., Kosicek, M., Mladenovic-Djordjevic, A., Smiljanic, K., Kanazir, S., & Hecimovic, S. (2016). Loss of Cathepsin B and L Leads to Lysosomal Dysfunction, NPC-Like Cholesterol Sequestration and Accumulation of the Key Alzheimer's Proteins. *PLOS ONE*, 11(11), e0167428.

Yogalingam, G., & Pendergast, A. M. (2008). Abl Kinases Regulate Autophagy by Promoting the Trafficking and Function of Lysosomal Components. *Journal of Biological Chemistry*, 283(51), 35941–35953.

Nakamura, S., & Yoshimori, T. (2018). Autophagy and Longevity. *Molecules and Cells*, 41(1), 65–72.

Mountford, J. K., Petitjean, C., Putra, H. W. K., McCafferty, J. A., Setiabakti, N. M., Lee, H., Tonnesen, L. L., McFadyen, J. D., Schoenwaelder, S. M., Eckly, A., Gachet, C., Ellis, S., Voss, A. K., Dickins, R. A., Hamilton, J. R., & Jackson, S. P. (2015). The class II PI 3-kinase, PI3KC2 α , links platelet internal membrane structure to shear-dependent adhesive function. *Nature Communications*, 6(1), 6535.

Dokladny, K., Myers, O. B., & Moseley, P. L. (2015). Heat shock response and autophagy—cooperation and control. *Autophagy*, 11(2), 200–213.

Martinez-Vicente, M., Talloczy, Z., Wong, E., Tang, G., Koga, H., Kaushik, S., de Vries, R., Arias, E., Harris, S., Sulzer, D., & Cuervo, A. M. (2010). Cargo recognition failure is responsible for inefficient autophagy in Huntington's disease. *Nature Neuroscience*, 13(5), 567–576.

Burr, A. A., Tsou, W.-L., Ristic, G., & Todi, S. V. (2014). Using Membrane-Targeted Green Fluorescent Protein To Monitor Neurotoxic Protein-Dependent Degeneration of Drosophila Eyes. *Journal of Neuroscience Research*, 92(9), 1100.

