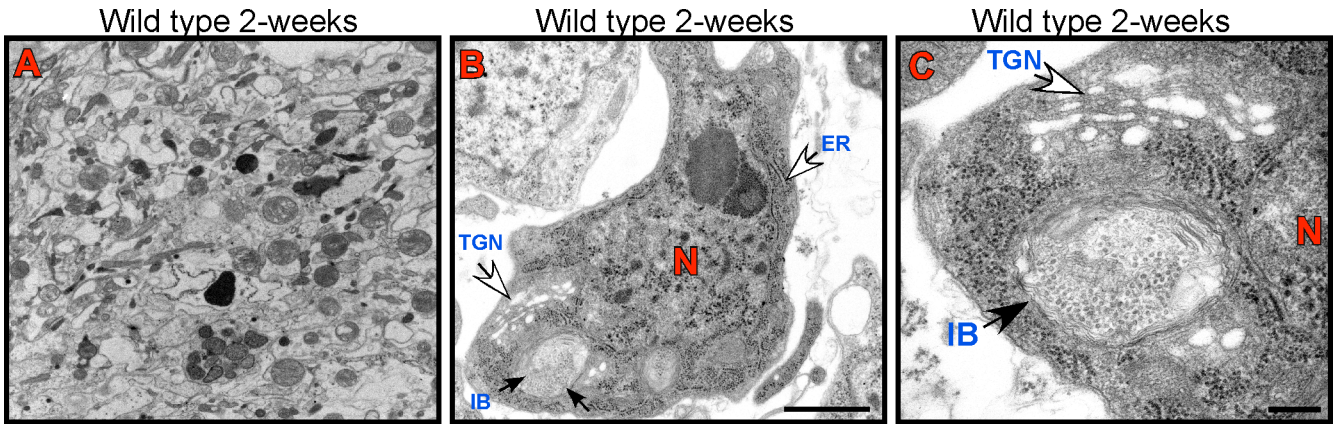
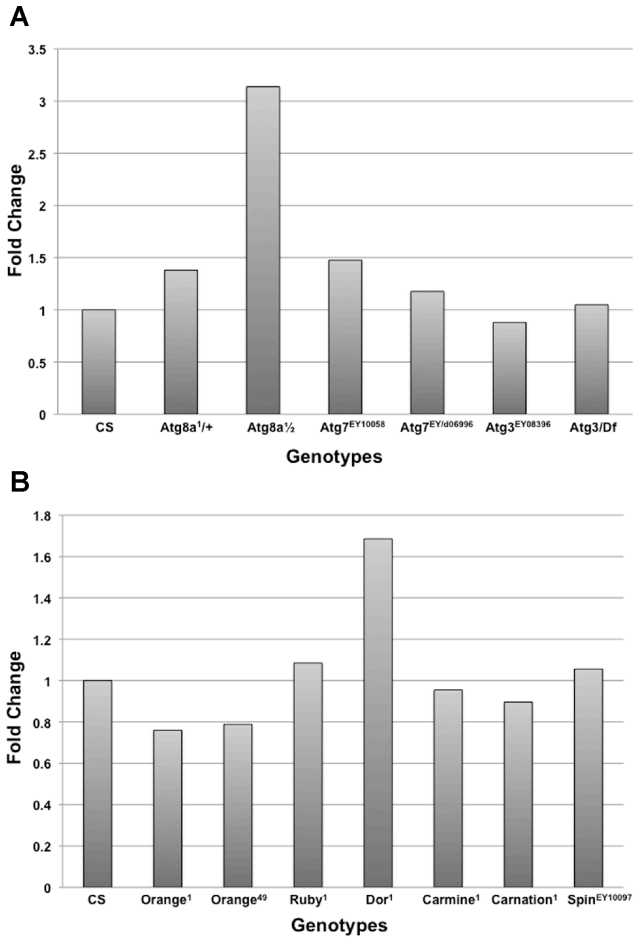
Supplemental Fig. 1.



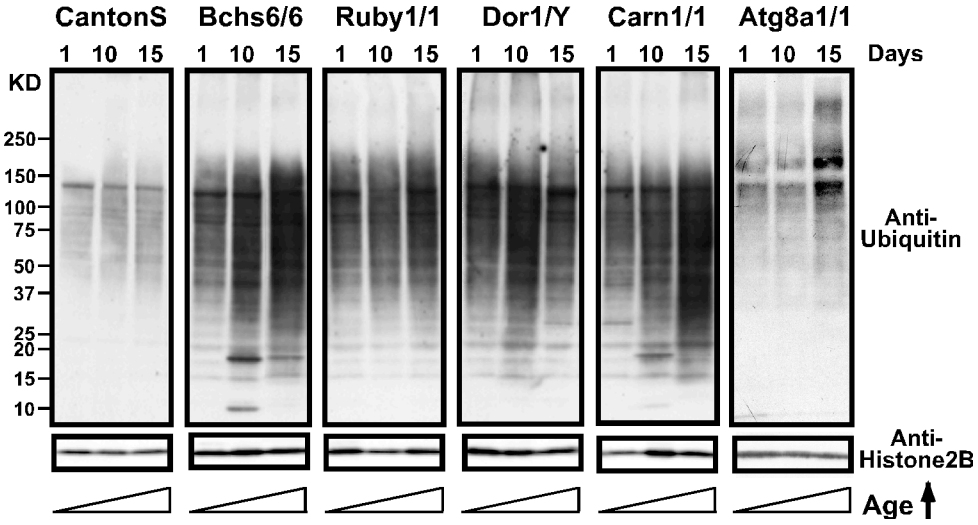
Neuronal TEM Images from control Drosophila. **A)** Electron microscopy of 2-week old wild type flies (Canton-S) shows a representative overview of normal brain tissue from neuropil regions, consisting primarily of axons. **B-C)** At this age wild type neurons also show the occasional formation of inclusion body or aggresome-like structures (IB, black arrow). However, these cytoplasmic structures are infrequent and the morphology of other sub-cellular organelles including the ER and Trans-golgi network (white arrows) appear normal even in affected neurons.[1]

Supplemental Fig. 2.



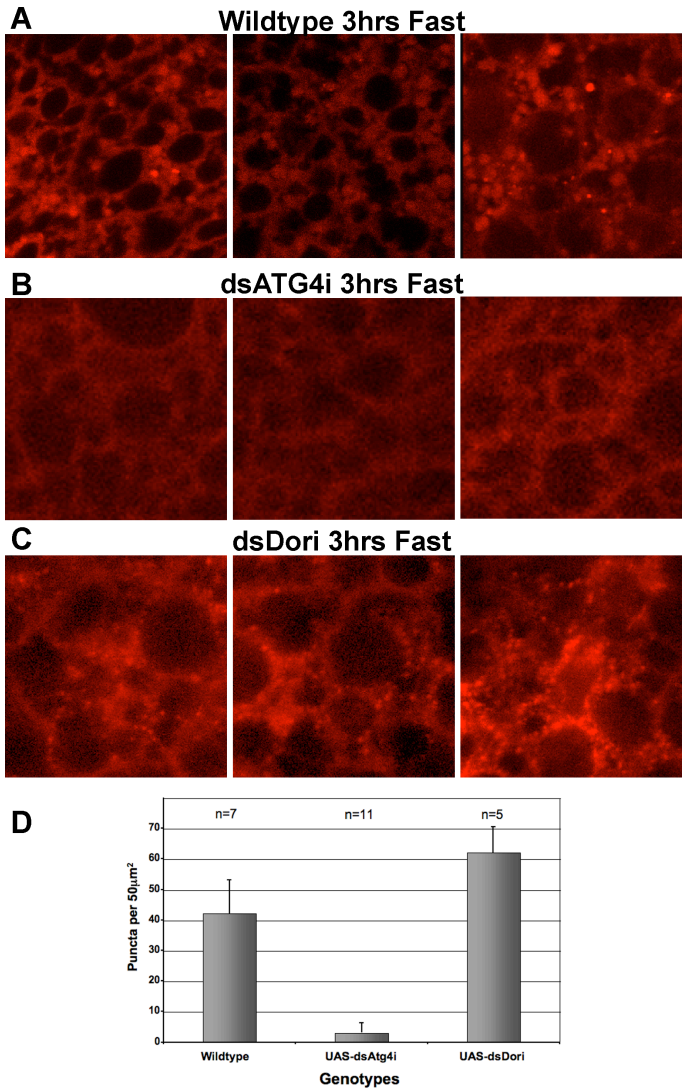
Quantified Ref(2)P protein profiles from Figure 3. **A)** Actin and Ref(2)P protein levels from Westerns in **Fig. 3A** were quantified from autophagy mutants using ImageJ software. Ref(2)P levels were corrected for loading using actin and wildtype values (CS) set at 1.0. **B)** Similarly corrected Ref(2)P levels are illustrated for lysosomal trafficking mutants in **Fig. 3B**.[1, 2]

Supplemental Fig. 3.



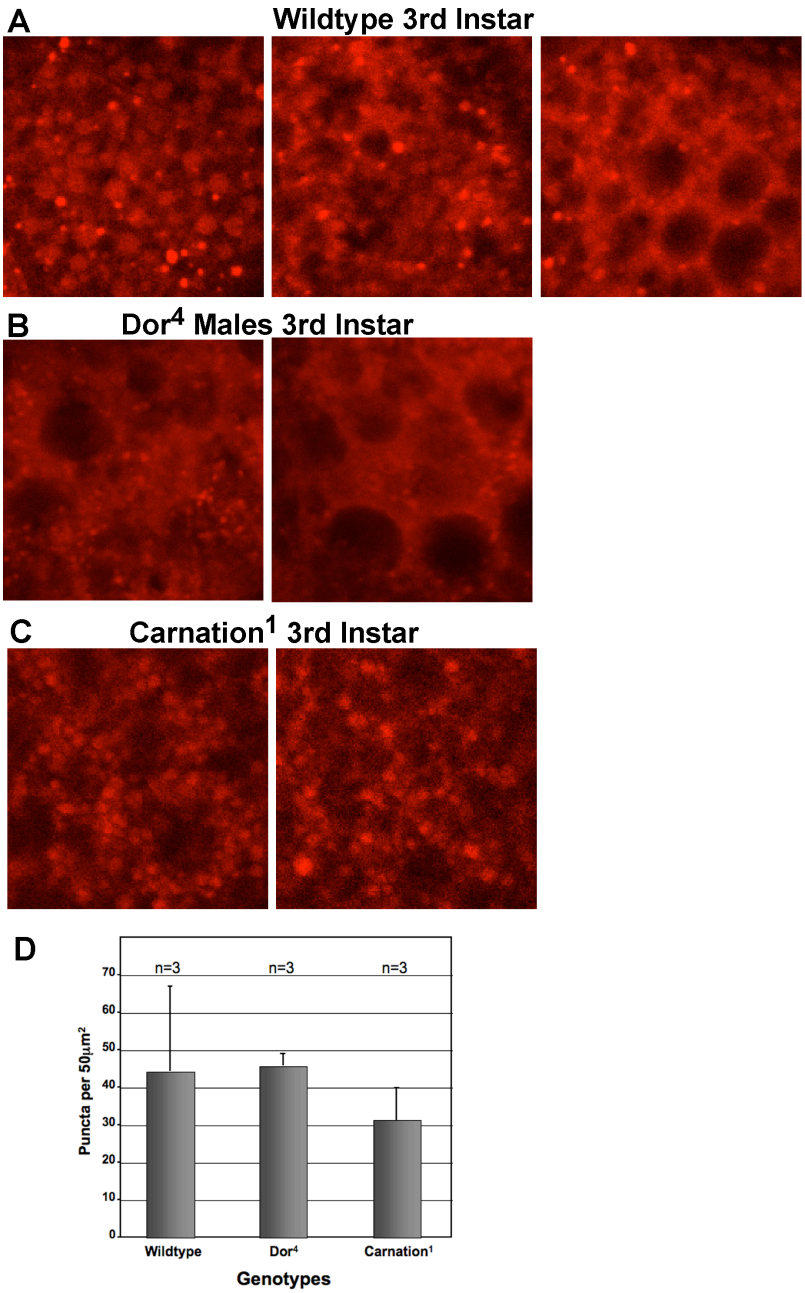
IUP profiles of autophagy and lysosomal mutants. Neural extracts were prepared from control flies (Canton-S), autophagy (*bcsh*$^{6/6}$ and *Atg8a*$^{1/1}$) and lysosomal trafficking mutant genotypes (*ruby*$^{1/1}$, *deep orange*$^{1/1}$, *carnation*$^{1/1}$) at 1-, 10- or 15-days of age. Western blots were prepared from the SDS protein fraction and probed with anti-ubiquitin and anti-Histone2B antibodies.[2, 3] When compared to age-matched controls the IUP profiles in both lysosomal and autophagy trafficking mutants were significantly altered suggesting defects at multiple points of vesicle trafficking results in the abnormal accumulation of ubiquitinated proteins.[3]

Supplemental Fig. 4.



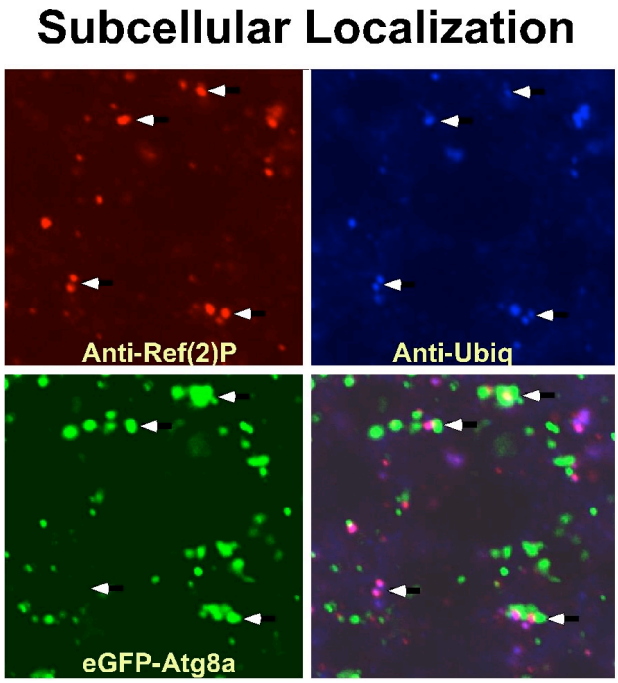
Formation of LysoTracker positive vesicles in larval fat body tissues following a fast. **A-C)** 2nd instar larvae from wildtype controls, and larvae expressing dsAtg4 and dsDor constructs (Vienna stock collection) in the larval fat body tissues (Cg-Gal4) were collected and fasted for 3 hours.[4-7] Tissues were collected and stained with Lysotracker Red (1: 1,000 dilution in PBS, Invitrogen) for 10min, rinsed and lightly fixed in 3.5% paraformaldehyde.[6, 7] Tissues were mounted and imaged using a Lieca confocal microscope and processed using Photoshop CS3 and Canvas X imaging software. The dark unstained regions are lipid droplets.[4] **A)** Images from wildtype control tissues shows a marked increase in LysoTracker positive vesicles. **B)** Tissues from larvae expressing a dsAtg4-RNAi construct have few positive puncta following a fast.[8-10] Previously we had shown the Dor protein has a dual function in late endosomal trafficking and mediating autophagosome-lysosome interactions.[7] **C)** Here we find that dsRNAi suppression of the *dor* message also changes the formation and morphology of LysoTracker positive puncta.[7] **D)** The graph quantifies the number of positive puncta formed within 50µm^2 areas of fat body tissues from multiple fasted larvae.[7] This work indicates that even particle suppression of autophagy can be detected using complementary imaging and Western analysis techniques.

Supplemental Fig. 5.



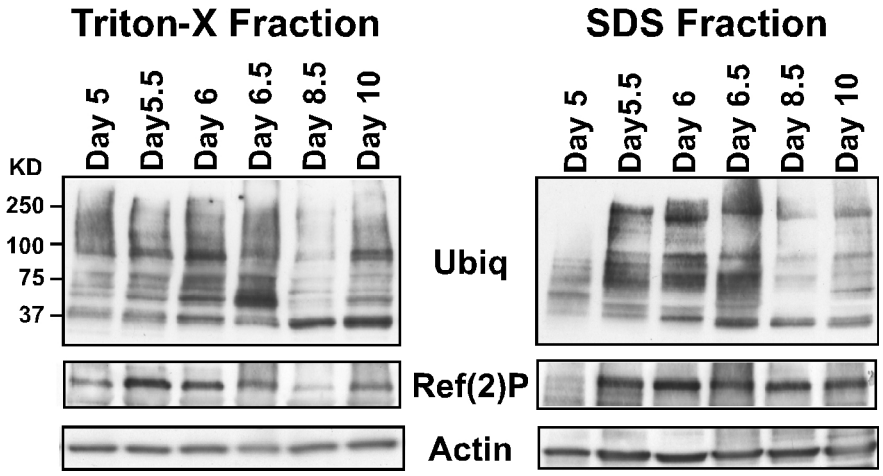
LysoTracker positive vesicles in fat body cells following ecdysone induction of autophagy. Following a pulse of the regulatory hormone 20-hydroxyecdysone (ecdysone), fat body cells from late 3rd instar larvae undergo extensive autophagy.[4, 5] **A)** As a result tissues from wildtype larvae are replete with LysoTracker™ positive puncta and vesicle morphology depends on the cellular depth where the image is taken. Unstained regions are lipid droplets. **B)** *Deep orange* mutants (*dor*^{4}, Vps18) show altered puncta and lipid droplet morphology, while **C)** *carnation*^{1} mutants (Vps33, sec1 protein) morphology patterns that are similar to controls.[3] Carnation/Vps33 is involved with late endosomal-lysosomal trafficking.[3] The pronounce IUP accumulation seen for *carnation*^{1} mutants in **Supplementary Fig. 3** and the lack of Ref(2)P accumulation seen in **Fig. 3B**, suggests this protein has little or no effect on autophagosome trafficking. As a result the build up of neuronal IUP may be attributed to defects in endosomal or other trafficking pathways that target ubiquitinated substrates to the lysosome.

Supplemental Fig. 6.



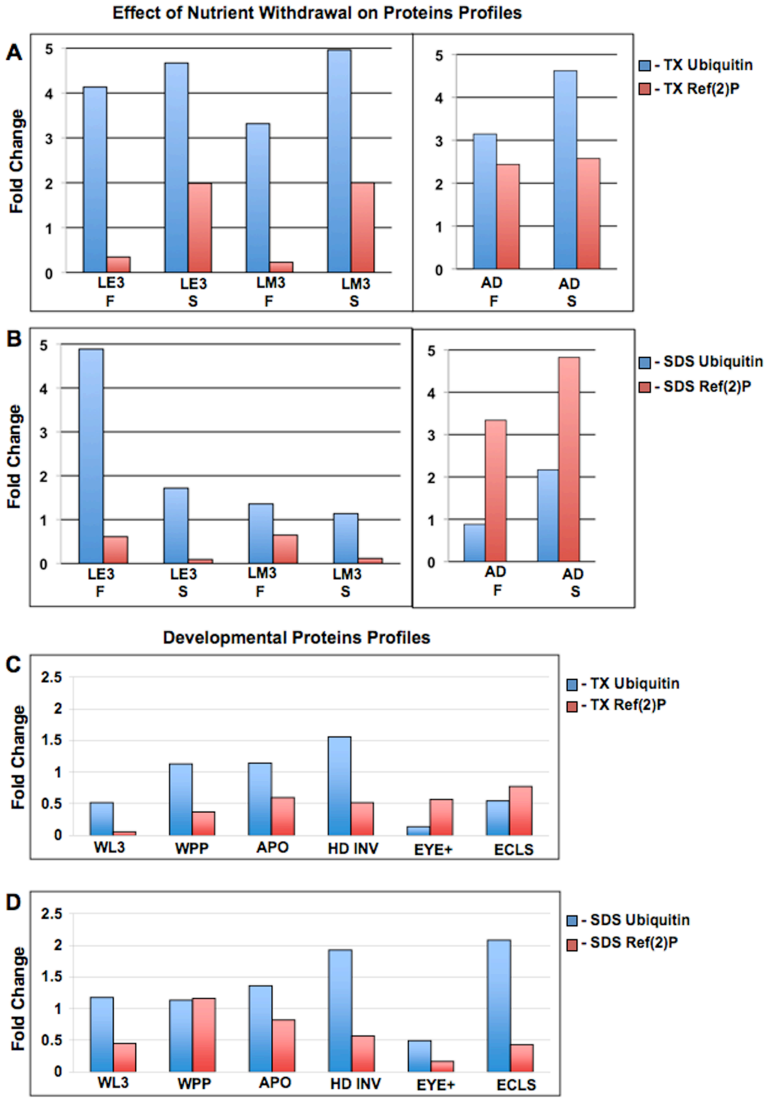
Co-localization of GFP-Atg8a, Ref(2)P, ubiquitinated proteins in Drosophila fat body tissues. Previous studies have shown that fat body tissue from wandering 3rd instar larvae show extensive autophagosome formation. Tissues from this stage, which expressed the UAS-eGFP-Atg8a marker (**green**, *Cg-Gal4,UAS-eGFP-Atg8a*), were collected and used for immunofluorescence analysis. Fixed tissues were incubated with anti-Ref(2)P (1:200 dilution, **red**) and anti-ubiquitin (1:200 diluted, Cell Signaling**,** **blue**) antibodies overnight at 4°C.[5] Rinsed tissues were incubated for 1-hour (RT) with anti-Rabbit Cy3-conjugated secondary and anti-mouse Cy2-conjugated secondary antibodies (1:200 dilution, Jackson ImmunoResearch Laboratories, Inc.) before being mounted and imaged using a Leica TCS SP2 AOBS confocal microscope. Arrows indicate the co-localization of Ref(2)P, ubiquitin and Atg8a positive structures. Ubiquitin and Ref(2)P positive structures closely associate, indicating the formation of pre-autophagic structures called sequestosomes occurs in this tissue. GFP-Atg8a labeled vesicles shows a more limited co-localization with ubiquitin and Ref(2)P positive structures, suggesting the formation of autophagosomes and the flux of substrates via autophagy is a highly dynamic process.

Supplemental Fig. 7.



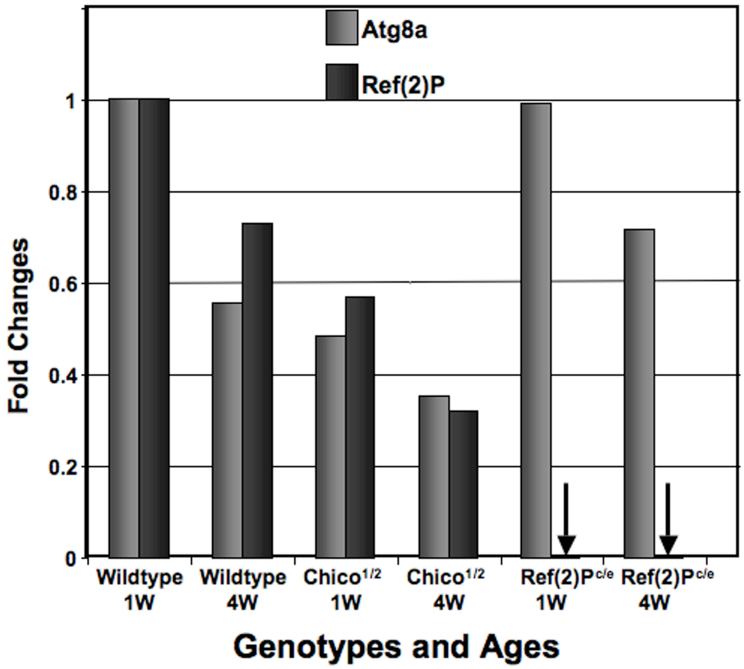
Developmental profiles of Ref(2)P and ubiquitinated proteins. Mated female Canton-S flies were allowed to lay eggs for 4-hours and used for timed egg-collections. Larvae and pupae representing different developmental stages were collected between Day 5 to Day 10 of development and used for serial detergent protein extractions. Western analysis was done for ubiquitin, Ref(2)P and actin proteins. There is an robust induction of autophagy that occurs in wandering 3rd instar larvae (~Day 5.5) following the initial pulse of the hormone ecdysone. Western analysis of both ubiquitinated proteins and Ref(2)P show a dynamic change in the overall levels as well as solubility profiles for both proteins. IUP profiles show a significant increase at the larval-pupal boundary, which is preceded slightly by Ref(2)P accumulation. At later stages (Day-8.5 and Day-10) soluble and IUP are reduced while Ref(2)P profiles remain elevated through the entire pupal phase of development. Most autophagy-linked cell death occurs earlier in metamorphosis and is followed by the rapid development and differentiation of cells into adult tissues and structures. Western analysis suggests that ubiquitin conjugated proteins are effectively cleared during this complex development period, likely by both autophagy and the ubiquitin-proteosome system (UPS). Elevated Ref(2)P levels at later developmental stages suggests key autophagy components may become depleted, thus preventing the effective clearance of substrates. Quantified values are shown in **Supplementary Fig. 8C**, **D**.

Supplemental Fig. 8.



Quantification of IUP and Ref(2)P levels. Fed=F; Fasted/Starved=S; LE3=early third instar larvae; LM3 =mid third instar larvae; A=adults (2-days). **A)** There is a moderate increase in soluble ubiquitinated proteins when larvae and adults (50%) are fasted (see **Fig. 4A**). However, only fasted larvae show a significant increase in soluble Ref(2)P levels, while the adult profiles were not altered. **B)** In the SDS protein fractions a significant change in IUP profiles is not detect, with the exception of a sharp decrease in fasted early third instar larvae (LE3 S) (see **Fig. 4A**). Larval adipose tissues show a consistent decrease in Ref(2)P following a fast, while the adult CNS levels shows a slight increase. **C-D)** WL3=wandering third instar larvae; WPP= white pre-pupae; APO=brown pupae; Hd Inv=pupal head involution; Eye+=pupal eye pigmentation; ECLS=eclosion. The developmental profiles of soluble and insoluble Ref(2)P and ubiquitinated proteins were highly variable, reflecting the dynamic physiology changes and remodeling that occurs throughout metamorphosis (see **Suppl. Fig. 7**). Westerns were digitally scanned and Ref(2)P, ubiquitin and actin values quantified using ImageJ software and corrected values graphed using Excel (NIH, http://rebweb.nih.gov/ij). Illustrations were generated using Microsoft PowerPoint and Adobe Photoshop CS3.

Supplemental Fig. 9.



Neuronal Atg8a and Ref(2)P expression profiles. Total neuronal mRNA was prepared from heads taken from 1- and 4-week old wildtype (CS) controls as well as from *chico*$^{1/2}$ and *Ref(2)P*$^{c/e}$ mutant flies (Trizol, Invitrogen). cDNAs were prepared from each mRNA sample (cDNA synthesis kit, #K1622, Fermentas) and used for qRT-PCR analysis of actin, *Atg8a* and *ref(2)P* messages using gene specific primers.[1] Actin values were used to correct for loading. 1-week wildtype values were set at 1.0 and other values correspondingly corrected. As seen previously, both *Atg8a* and *ref(2)P* expression levels are lower in the aged animals and *ref(2)P* mutants (*Ref(2)P*$^{c/e}$) a similar decline in Atg8a message profiles (arrows note *ref(2)P* values).[11] Flies with defects in the *chico* gene (*chico*$^{1/2}$, insulin receptor substrate homologue)[12] have profound developmental defects and at 1-week of age have lower levels of *Atg8a* and *ref(2)P* expression, which is further reduced over time (4-weeks). It has been suggested that insulin-signaling defects may alter metabolic rates thus directly reducing oxidative stress and rates of cellular aging. This also suggests that the reduction in aggrephagy markers and expression of key autophagy genes may reflect a decreased rate of metabolism.

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