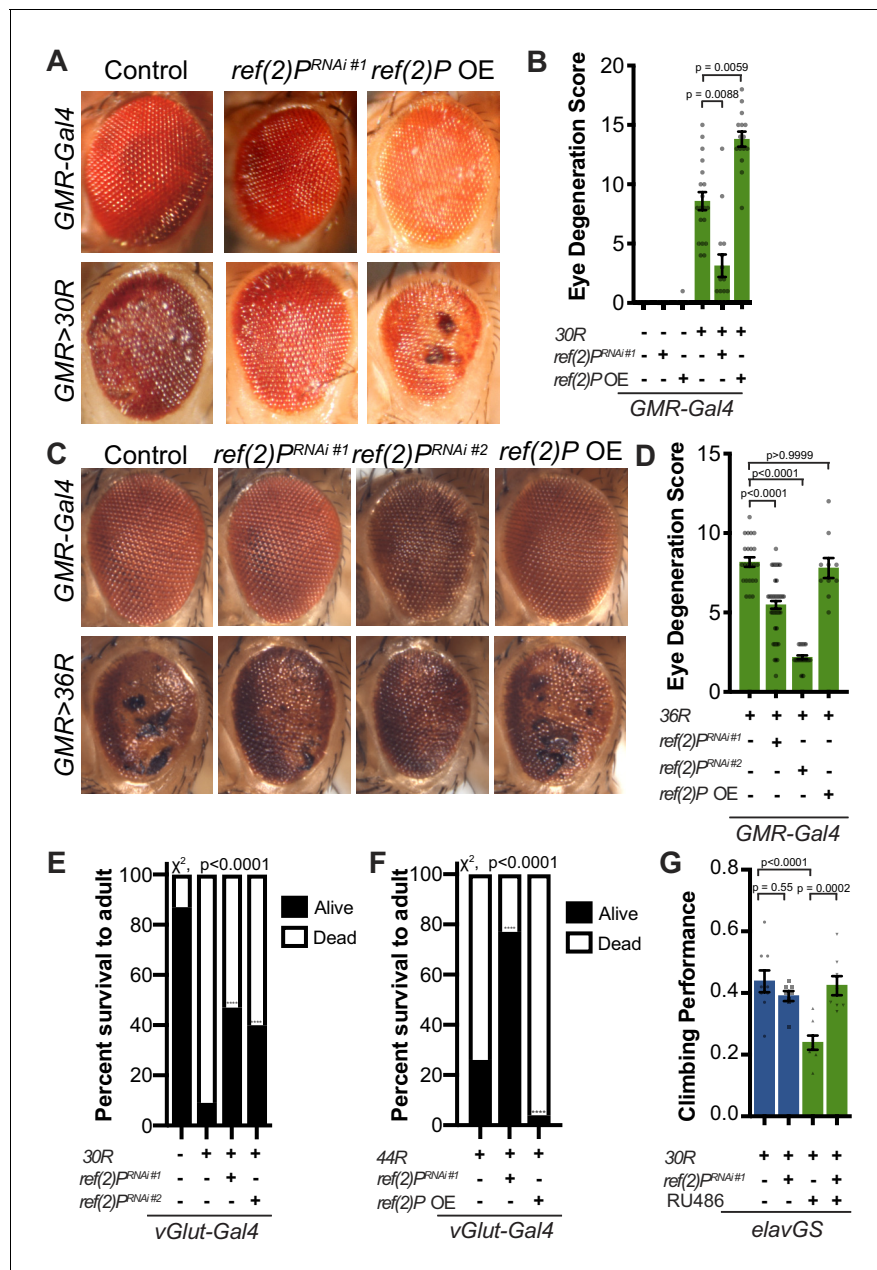


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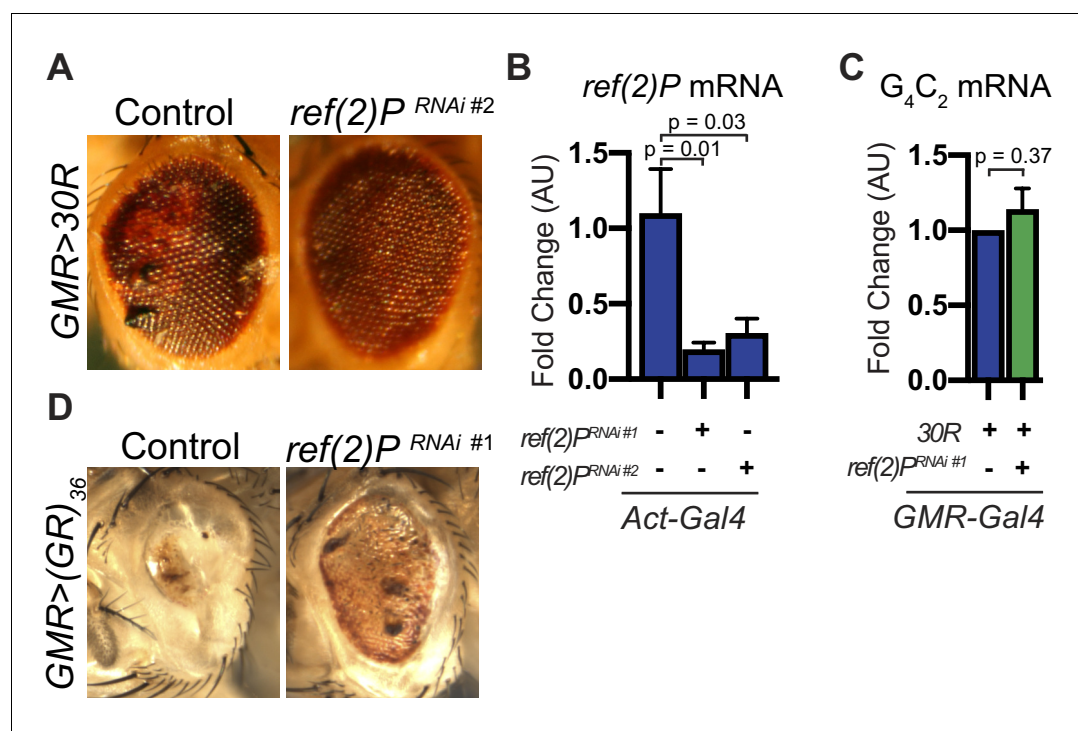
## Figures and figure supplements

TFEB/Mitf links impaired nuclear import to autophagolysosomal dysfunction in C9-ALS

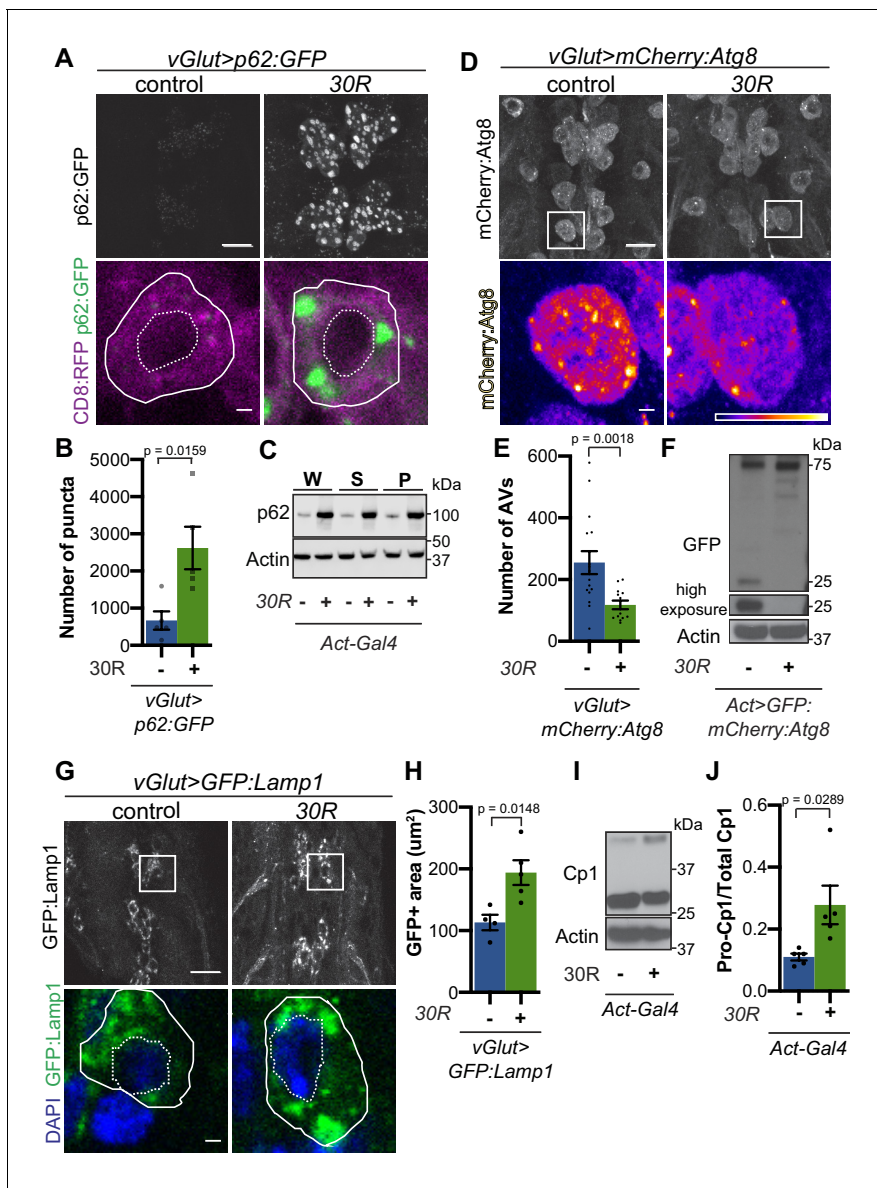
**Kathleen M Cunningham *et al***



**Figure 1.** Autophagy receptor Ref(2) P/p62 genetically suppresses G4C2-HRE-mediated degeneration. (A) 15-day-old *Drosophila* eyes expressing GMR-Gal4 +/- UAS-30R (GMR>30R) with RNAi background (control), *ref(2)<sup>P</sup> RNAi #1* or overexpression (OE) of *ref(2)<sup>P</sup>*. (B) Quantification of external eye degeneration in A by semi-quantitative scoring system. Data are reported as mean  $\pm$  SEM. Kruskal-Wallis test,  $p < 0.0001$ , followed by Dunn's multiple comparisons,  $n \geq 15$  adults. (C) 15-day-old *Drosophila* eyes expressing GMR-Gal4 +/- UAS-36R (GMR >36R) along with UAS-luciferase<sup>RNAi</sup> (control), UAS-*ref(2)<sup>P</sup> RNAi #1*, UAS-*ref(2)<sup>P</sup> RNAi #2*, or UAS-*ref(2)<sup>P</sup> OE*. (D) Quantification of external eye degeneration in C by semi-quantitative scoring system. Data are reported as mean  $\pm$  SEM. Kruskal-Wallis test,  $p < 0.0001$ , followed by Dunn's multiple comparisons,  $n = 23, 62, 28, 10$  adults respectively. (E) Percent of pupal eclosion of adult flies expressing the motor neuron driver vGlut-Gal4 +/- UAS-30R and RNAi background control or UAS-*ref(2)<sup>P</sup> RNAi #1*. Fisher's exact test,  $n \geq 100$  pupa. (F) Percent of pupal eclosion of adult flies expressing the motor neuron driver vGlut-Gal4 +/- UAS-44R along with UAS-luciferase RNAi, UAS-*ref(2)<sup>P</sup> RNAi #1*, or UAS-*ref(2)<sup>P</sup> OE*. Fisher's exact test,  $n \geq 55$  pupa. (G) Adult *Drosophila* expressing UAS-30R under the control of the inducible, pan-neuronal elavGS induced with 200  $\mu$ M RU486 or vehicle alone and co-expressing control or UAS-*ref(2)<sup>P</sup> RNAi #1*. Data are reported as mean  $\pm$  SEM. One-way ANOVA, \*\*\*\* $p < 0.0001$ , with Sidak's multiple comparisons test,  $n = 9, 8, 8, 8$  groups of 10 flies.

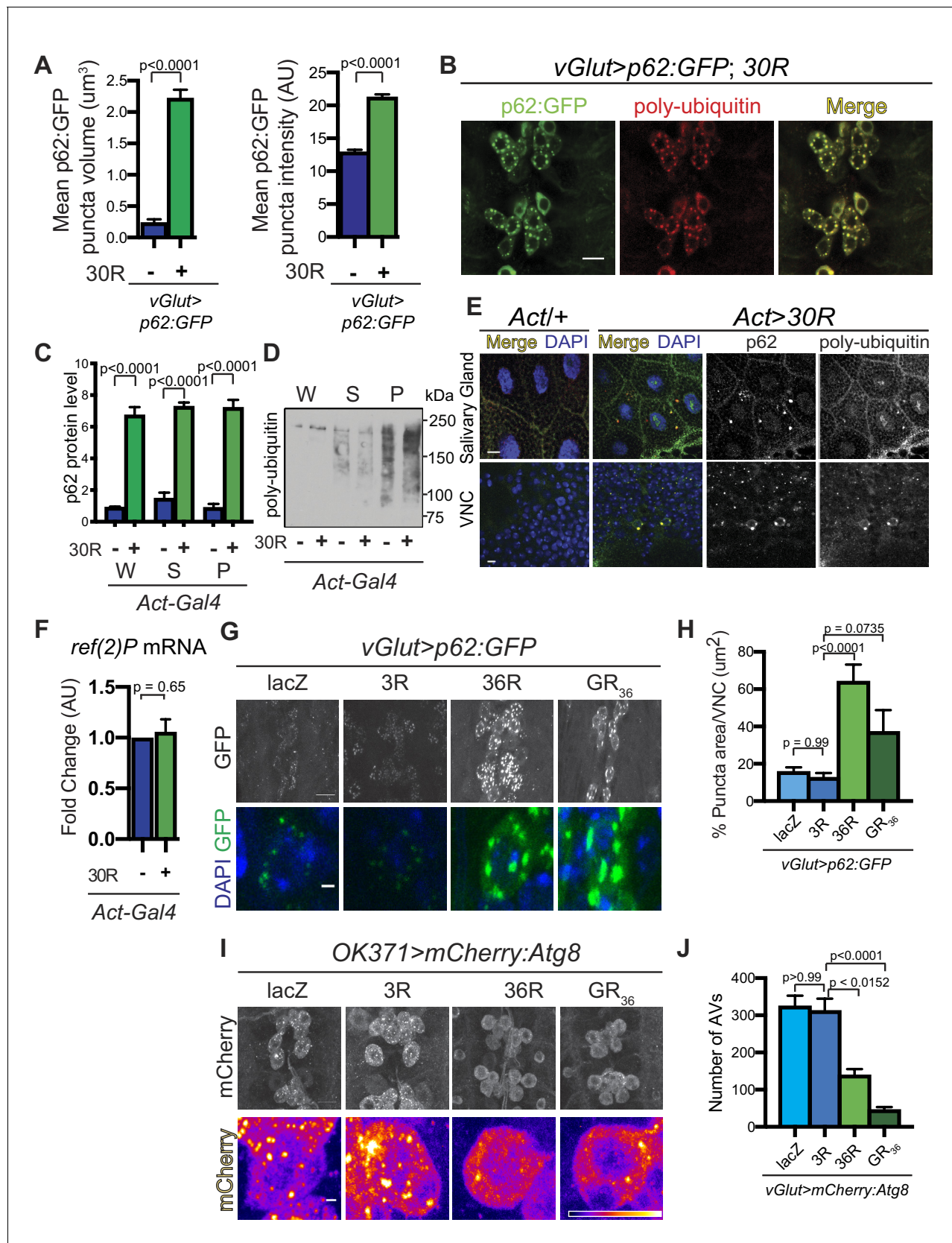


**Figure 1—figure supplement 1.** Ref(2)P/p62 genetically modifies G4C2-HRE. (A) *GMR > 30R* expressing fly eyes at 15 days of age crossed to control (*Canton S*) or *UAS-ref(2)P*<sup>RNAi #2</sup>, demonstrating suppression of 30R-mediated eye degeneration with *ref(2)P* knockdown. (B) Levels of *ref(2)P* mRNA measured by quantitative RT-PCR in *Drosophila* expressing *UAS-30R* under the control of *Act-Gal4* with *UAS-ref(2)P*<sup>RNAi #1</sup> or *UAS-ref(2)P*<sup>RNAi #2</sup>. Data are presented as mean ± SEM. One-way ANOVA, *p*=0.0105 followed by Sidak's multiple comparisons, *n* = 5 per genotype. (C) Levels of G4C2 mRNA measured by quantitative RT-PCR in flies expressing *UAS-30R* under the control of *GMR-Gal4* either with or without *UAS-ref(2)P*<sup>RNAi</sup>. Data are presented as mean ± SEM. Student's *t*-test, *n* = 3 per genotype. (D) *GMR-Gal4* expressing an alternate codon *UAS-poly(GR)*<sub>36</sub> alone (control) or co-expressed with *UAS-ref(2)P*<sup>RNAi #1</sup> at 15 days of age.



**Figure 2.** G4C2 repeat expression impairs autophagic flux. (A) *Drosophila* motor neurons expressing *UAS-p62:GFP* +/- *UAS-30R*, showing multiple motor neuron cell bodies (top) or a representative cell co-expressing the membrane marker CD8:RFP (bottom). Plasma membrane outlined with solid white line; nucleus outlined with dotted line. Scale bar = 10  $\mu$ m (top), 1  $\mu$ m (bottom). (B) Quantification of number of p62:GFP puncta in *Drosophila* motor neuron cell bodies. Data are reported as mean  $\pm$  SEM. Mann-Whitney test,  $n = 5$  larvae per genotype. (C) Western blot of anti-p62 and anti-beta-actin showing the whole (W), supernatant (S) and pellet (P) fractions of lysates from *Drosophila* larvae ubiquitously expressing +/- *UAS-30R* under the control of *Act-Gal4*. (D) *Drosophila* motor neurons expressing *UAS-mCherry:Atg8* +/- *UAS-30R* showing cell bodies (top) with an example single cell highlighting mCherry:Atg8-positive puncta (bottom). Scale bar = 10  $\mu$ m (top), 1  $\mu$ m (bottom). (E) Quantification of mCherry:Atg8-positive autophagic vesicles (AVs) in the ventral nerve cord of *vGlut-Gal4/+* or *vGlut >30R* expressing flies. Data are reported as mean  $\pm$  SEM. Mann-Whitney test,  $n = 16$  and 13 larvae, respectively. (F) Western blot of anti-GFP and anti-beta-actin of lysates from whole *Drosophila* larvae ubiquitously expressing *UAS-GFP:mCherry:Atg8* +/- *UAS-30R* under the control of *Act-Gal4* showing full length GFP:mCherry:Atg8 at 75 kDa and cleaved GFP at 25 kDa. (G) *Drosophila* motor neurons expressing *UAS-GFP:Lamp1* (with N-terminal [luminal] GFP) +/- *UAS-30R* under the control of *vGlut-Gal4* in multiple cell bodies (top) or in a representative cell (bottom). Scale bar = 10  $\mu$ m (top), 1  $\mu$ m (bottom). (H) Quantification of GFP:Lamp1 positive area in G. Data are reported as mean  $\pm$  SEM. Student's t-test,  $n = 5$  larvae. (I) Western of whole *Act-Gal4* *Drosophila* larvae +/- *UAS-30R* blotted for the lysosomal protease Cp1, showing pro- (inactive, upper band) and cleaved (active, lower band) Cp1. (J) Quantification of the ratio of pro-Cp1 to total Cp1 in I. Data are reported as mean  $\pm$  SEM. Student's t-test,  $n = 5$  biological replicates.

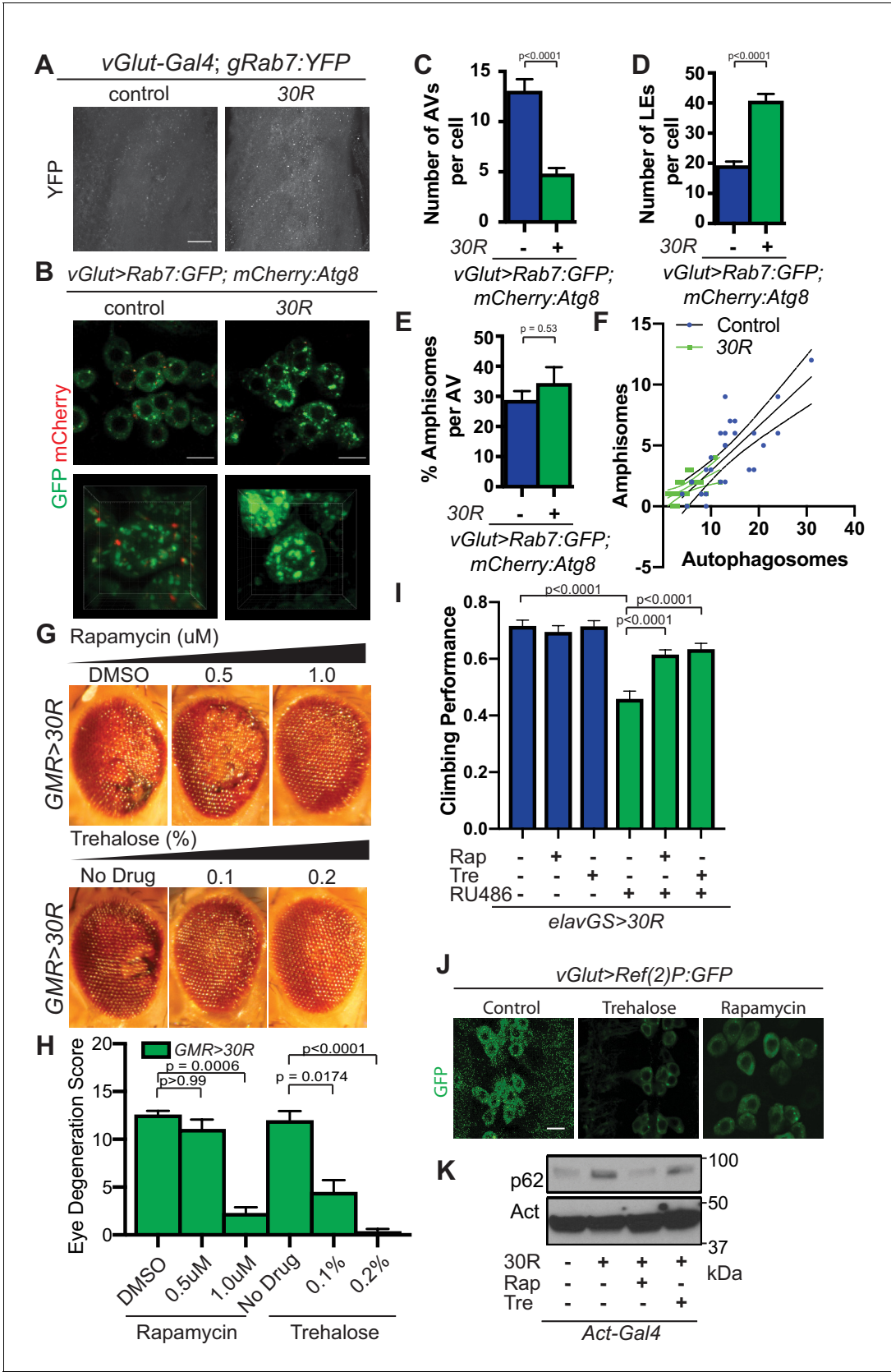




**Figure 2—figure supplement 1.** p62:GFP accumulates in C9-ALS fly models and co-localizes with poly-ubiquitin. (A) Quantification of mean volume and intensity of p62:GFP puncta in *vGlut*/+ or *vGlut* >30R motor neuron cell bodies. Data are presented as mean ± SEM. Student's t-test with Welch's correction.  $p < 0.0001$ . Figure 2—figure supplement 1 continued on next page

## Figure 2—figure supplement 1 continued

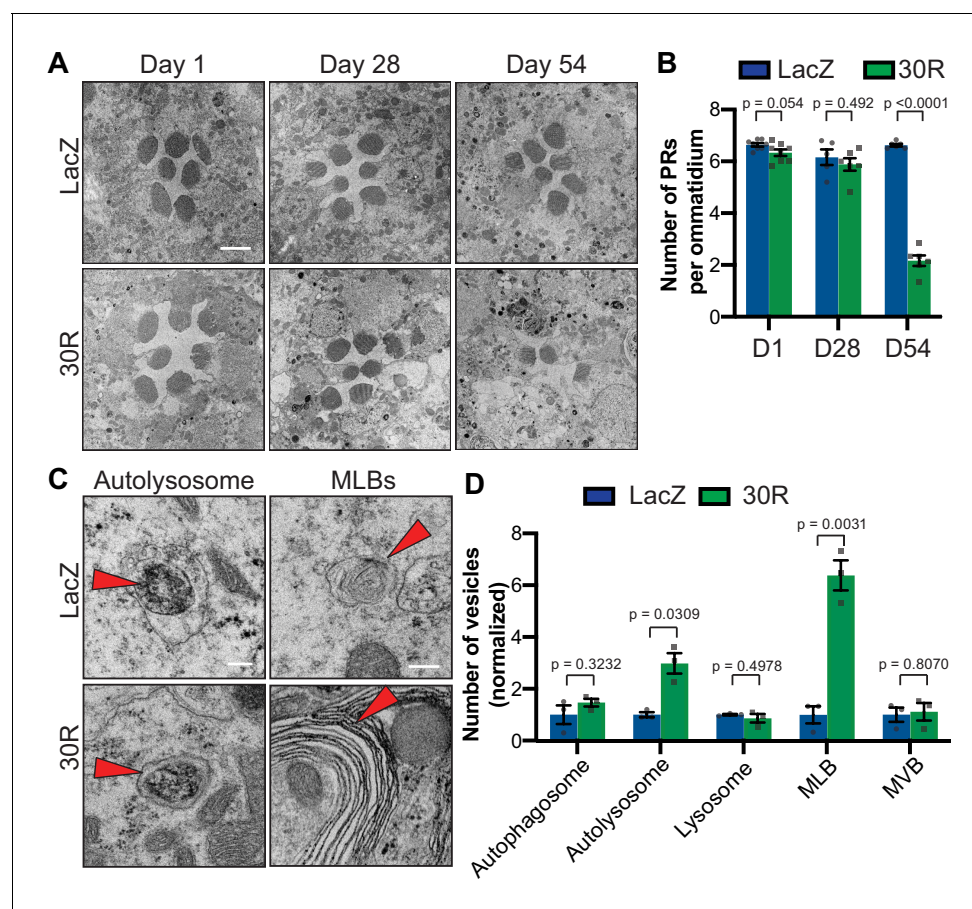
correction,  $n = 5$  per genotype. (B) *Drosophila* *vGlut-Gal4* motor neurons expressing *UAS-30R* and *UAS-p62:GFP* (green) co-stained with anti-poly-ubiquitin (red). Scale bar = 10  $\mu\text{m}$ . (C) Quantification of p62 levels in the whole (W), supernatant (S) and pellet (P) fractions of lysates from *Drosophila* L3 larvae ubiquitously expressing *UAS-30R* under the control of *Act-Gal4* shown in western blot in **Figure 2C**. One-way ANOVA,  $p < 0.0001$ , followed by Sidak's multiple comparisons,  $n = 3$  per genotype. (D) Western blot of anti-poly-ubiquitin showing the whole (W), supernatant (S) and pellet (P) fractions of *Drosophila* L3 larvae ubiquitously expressing *30R* under the control of *Act-Gal4*. (E) *Drosophila* L3 *Act-Gal4/+* or *Act > 30R* larvae ubiquitously expressing G4C2 repeats showing anti-p62 (red) and anti-ubiquitin (green) staining in the larval salivary gland (top) and ventral nerve cord (VNC) (bottom). Scale bar = 10  $\mu\text{m}$ . (F) Levels of *ref(2)P* RNA measured by quantitative RT-PCR in lysates from *Drosophila* larva expressing *UAS-30R* ubiquitously under control of *Actin-Gal4*. Data are presented as mean  $\pm$  SEM. Student's t-test,  $n = 5$  per genotype. (G) *vGlut > p62:GFP* showing motor neurons of the VNC (top) or a representative cell body (bottom) with two control (*UAS-LacZ* or *UAS-3R*) and two alternate C9-ALS model lines, one expressing a G4C2 repeat expansion (*UAS-36R*) and another expressing the dipeptide-repeat protein polyGR (*UAS-poly(GR)<sub>36</sub>*) using alternate codons. Top panels, scale bar = 10  $\mu\text{m}$ ; bottom panels, scale bar = 1  $\mu\text{m}$ . (H) Quantification of the total GFP+ area of p62:GFP positive puncta in G. Data are presented as mean  $\pm$  SEM. One-way ANOVA,  $p < 0.0001$ , with Sidak's multiple comparisons,  $n = 13$ –18 larva per genotype. (I) *vGlut > mCherry:Atg8* showing the VNC (top) or a representative cell body (bottom) with two control overexpression (*UAS-LacZ* or *UAS-3R*) and two alternate C9-ALS model lines, one expressing a G4C2 repeat expansion (*UAS-36R*) and another expressing the alternate codon arginine dipeptide GR (*UAS-poly(GR)<sub>36</sub>*). Top panels, scale bar represents 10  $\mu\text{m}$ ; bottom panels, scale bar = 1  $\mu\text{m}$ . (J) Quantification of mCherry:Atg8 puncta in the motor neuron cell bodies in I. Kruskal-Wallis test,  $p < 0.0001$ , followed by Dunn's multiple comparisons,  $n = 13$  larva per genotype. Data are presented as mean  $\pm$  SEM.



**Figure 2—figure supplement 2.** Rescuing G4C2-mediated lysosome defects reduces neurodegeneration. (A) *vGlut*<sup>+/+</sup> or *vGlut*<sup>>30R</sup> with genomically tagged *Rab7:YFP* (*gRab7:YFP*) in the L3 larval ventral nerve cord (VNC). Scale bar = 10 μm (B) Control *vGlut* motor neurons co-expressing *Rab7:GFP* Figure 2—figure supplement 2 continued on next page

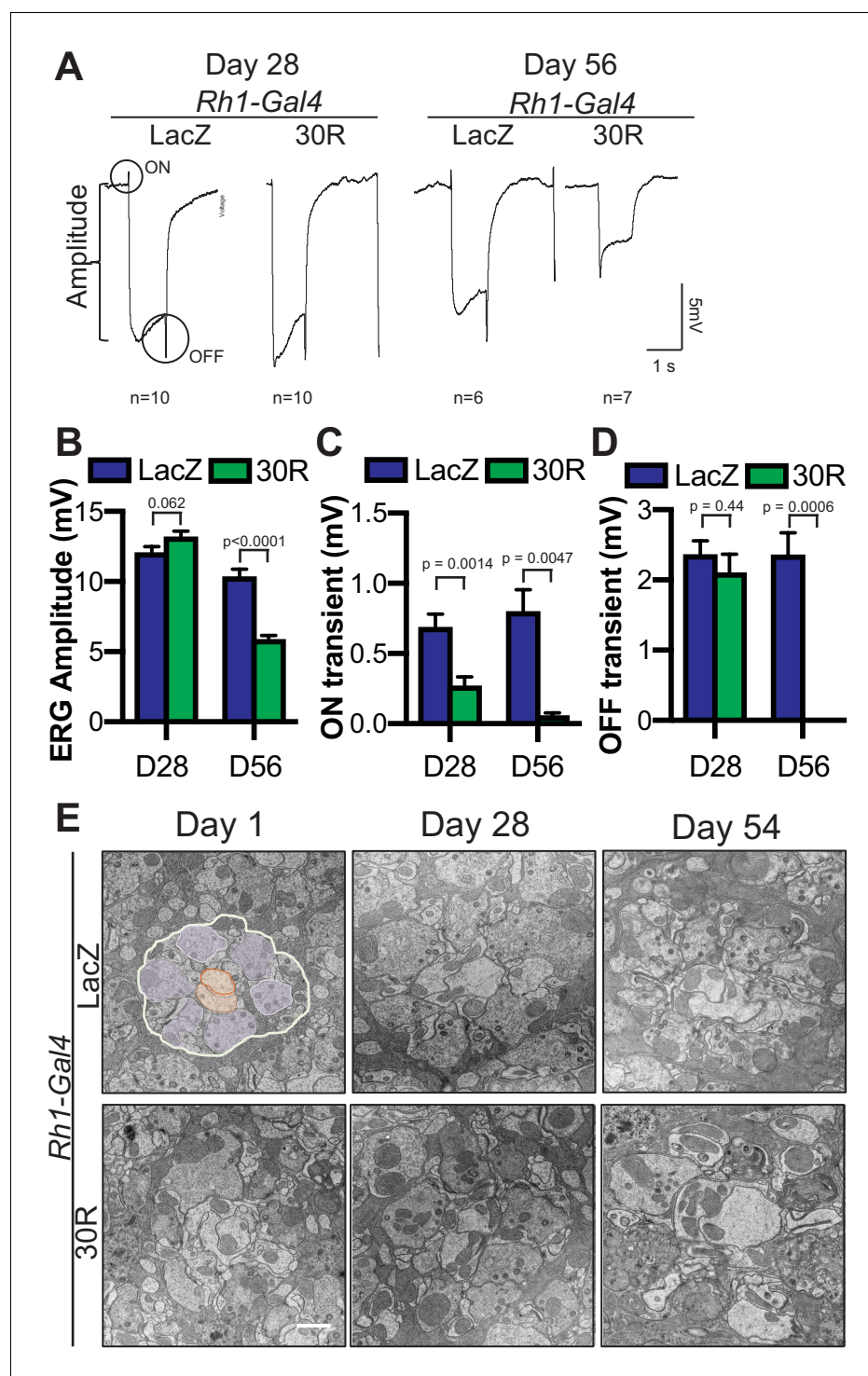
## Figure 2—figure supplement 2 continued

and -mCherry:Atg8 in the VNC (top) or a representative single cell (bottom). Scale bar = 10  $\mu$ m (C) Quantification of number of Atg8 positive autophagic vesicles (AV) per cell. Data are presented as mean  $\pm$  SEM. Mann-Whitney test, n = 29 and 24 cells, respectively. (D) Quantification of number of Rab7 positive late endo/lysosomes (LE) per cell. Data are presented as mean  $\pm$  SEM. Mann-Whitney test, n = 29 and 24 cells, respectively. (E) Number of amphisomes (co-localized mCherry:Atg8 and Rab7:GFP puncta) per cell normalized to the total number of autophagosomes. Data are presented as mean  $\pm$  SEM. Mann-Whitney test, n = 29 and 24 cells respectively. (F) Number of amphisomes (co-localized mCherry:Atg8 and Rab7:GFP puncta) plotted against number of autophagic vesicles for *vGlut/+* control and *vGlut >30R* motor neurons. Curves represent simple linear regression for each genotype. (G) Eyes from 15-day-old *Drosophila* expressing *UAS-30R* under the control of *GMR-Gal4* fed with increasing concentrations of rapamycin (DMSO, 0.5  $\mu$ M, or 1  $\mu$ M) and trehalose (no drug, 0.1%, or 0.2%). (H) Quantification of eye degeneration in G. Data are presented as mean  $\pm$  SEM. Kruskal-Wallis test,  $p < 0.0001$ , followed by Dunn's multiple comparisons, n = 10 per genotype. (I) Adult *Drosophila* expressing *UAS-30R* under the control of the inducible, pan-neuronal *elavGS* driver induced with 200  $\mu$ M RU486 have decreased climbing ability at 7 days compared to non-induced controls, which is rescued by supplementing food with rapamycin or trehalose. One-way ANOVA,  $p < 0.0001$ , with Sidak's multiple comparisons test, n = 10 groups of 10 flies per genotype. (J) *Drosophila* motor neurons expressing *UAS-p62:GFP* and *UAS-30R* from 3<sup>rd</sup> instar larvae fed DMSO (control), 1.0  $\mu$ M rapamycin, or 0.2% trehalose. (K) Western of lysates from whole *Drosophila* larvae expressing no repeats or *UAS-30R* driven by *Act-Gal4* and fed DMSO, 1.0  $\mu$ M rapamycin, or 0.2% trehalose and blotted for p62.



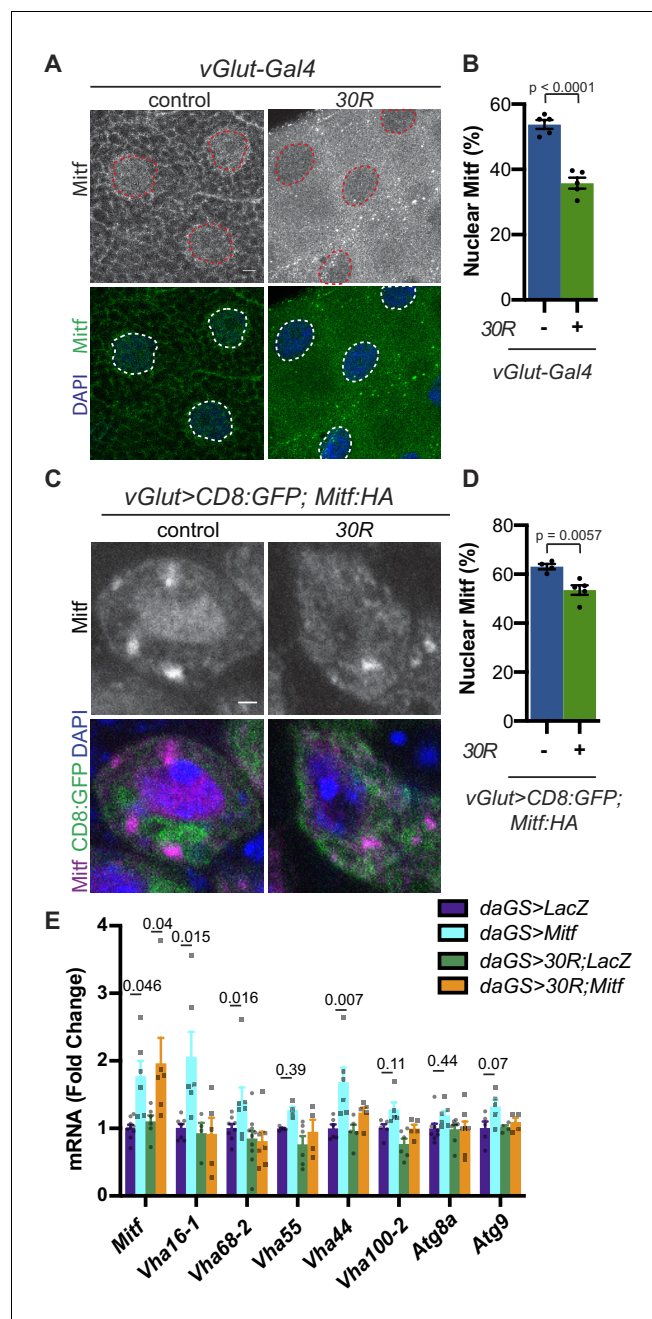
**Figure 3.** Autophagolysosomal defects precede neurodegeneration in photoreceptor neurons. (A) Transmission electron microscopy (TEM) of rhabdomeres (cell bodies) in *Rhodopsin1-Gal4* (*Rh1-Gal4*) driving *UAS-LacZ* (control) or *UAS-30R* at Day 1, Day 28, and Day 54 after eclosion. Scale bar = 2  $\mu$ m. (B) Quantification of number of healthy (not split) photoreceptors (PRs) per ommatidium in A. Data are reported as mean  $\pm$  SEM. Student's t-test,  $n = 8, 8, 6, 6, 6$ , and 6 flies, respectively. (C) TEM images at 28 days of *Drosophila* eyes (rhabdomeres) +/- 30R repeats expressed by *Rh1-Gal4* showing representative autolysosomes and multilamellar bodies (MLBs), marked with red arrows. Scale bar = 200 nm. (D) Quantification of different vesicle types (autophagosomes, autolysosomes, lysosomes, MLBs, and multivesicular bodies (MVBs)) shown in TEM of rhabdomeres with *Rh1-Gal4* driving *UAS-LacZ* or *UAS-30R* (as in C) normalized to LacZ (control). Data are reported as mean  $\pm$  SEM. Student's t-test,  $n = 3$  adults per genotype.



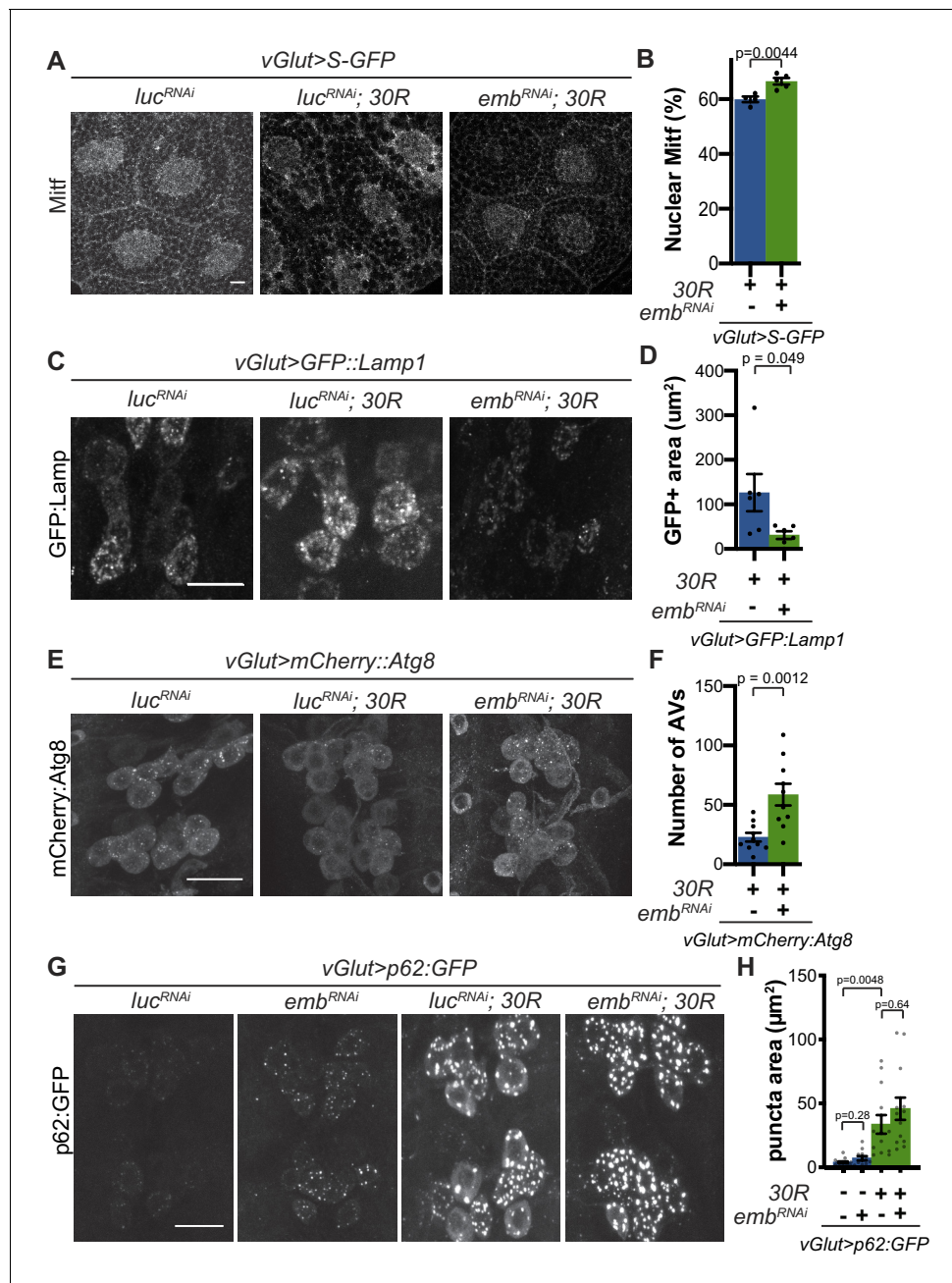


**Figure 3—figure supplement 1.** Progressive synapse degeneration in G4C2-expressing photoreceptor neurons. (A) Representative electroretinogram (ERG) of *Rhodopsin1-Gal4* driving *UAS-LacZ* (control) or *UAS-30R* in adult eyes at Day 28 or Day 56 after eclosion. (B) Quantification of mean ERG amplitude in A. Data are presented as mean  $\pm$  SEM. Student's t-test, n = 10, 10, 6, 7. (C) Quantification of mean ON transient amplitude in (A). (D) Quantification of mean OFF transient amplitude in (A). (E) Transmission electron microscopy (TEM) of terminals (synapses) in *Rhodopsin1-Gal4* driving *UAS-LacZ* (control) or *UAS-30R* adult eyes at Day 1, Day 28, and Day 54 after eclosion. For the Day 1 control image, presynaptic terminals are pseudocolored in purple and post-synaptic terminals are pseudocolored in orange. Scale bar = 1  $\mu$ m.

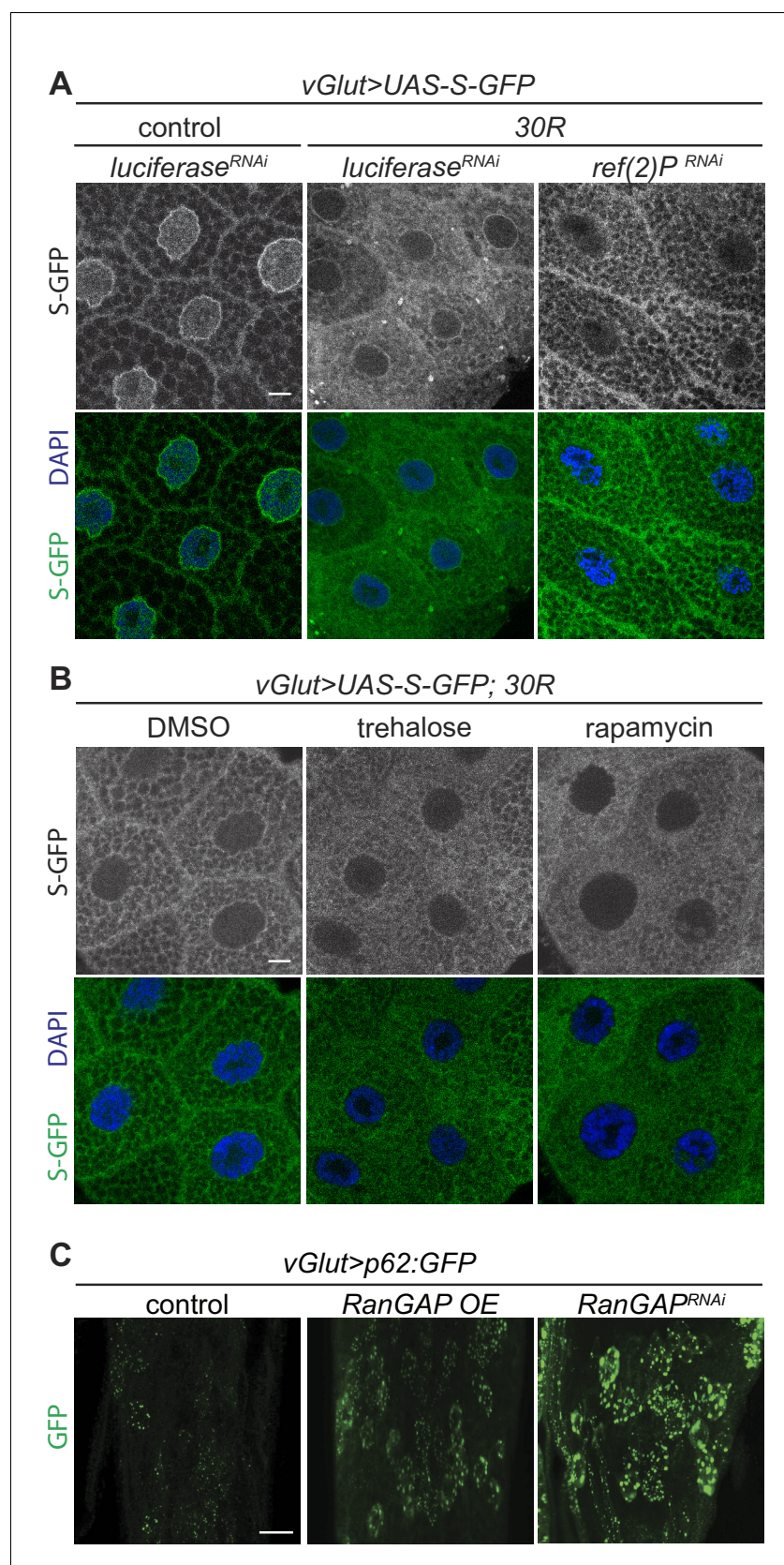




**Figure 4.** Mitf/TFEB is mislocalized from the nucleus and inactivated. (A) *Drosophila* larval salivary glands  $-/+$  UAS-30R under the control of *vGlut-Gal4* stained with anti-Mitf and DAPI. Dotted lines outline nuclei. Scale bar = 10  $\mu$ m. (B) Quantification of percent (%) nuclear Mitf (nuclear Mitf fluorescence/total fluorescence) in A. Data are reported as mean  $\pm$  SEM. Student's t-test,  $n = 5$  larvae per genotype. (C) *Drosophila* motor neurons (MNs) expressing UAS-Mitf-HA and UAS-CD8:GFP  $-/+$  UAS-30R under the control of *vGlut-Gal4* stained with anti-HA, anti-GFP (membrane), and DAPI to show nuclear localization. Scale bar = 1  $\mu$ m. (D) Quantification of percent (%) nuclear Mitf in C. Data are reported as mean  $\pm$  SEM. Student's t-test,  $n = 4$  and 5 larvae, respectively, with at least 10 motor neurons per larva. (E) Quantitative RT-PCR to assess transcript levels of *Mitf* and seven target genes from lysates of *Drosophila* heads expressing control (UAS-LacZ) or UAS-30R driven by *daGS* in control conditions or with overexpression of *Mitf*. Data are reported as mean  $\pm$  SEM. One-way ANOVA,  $p < 0.0001$ , with Sidak's multiple comparisons test,  $n \geq 4$  biological replicates of 30 heads per genotype.



**Figure 5.** Modulation of nucleocytoplasmic transport rescues autophagolysosome dysfunction. (A) *Drosophila* larval salivary glands stained with anti-Mitf and DAPI expressing +/- UAS-30R, UAS-shuttle-GFP (S-GFP, not shown), and either control RNAi (UAS-*luc<sup>RNAi</sup>*) or exportin RNAi (UAS-*emb<sup>RNAi</sup>*) under the control of *vGlut-Gal4*. Scale bar = 10  $\mu\text{m}$ . (B) Quantification of percent (%) nuclear Mitf in A. Data are reported as mean  $\pm$  SEM. Student's t-test,  $n = 4$  and 5 larvae, respectively. (C) *Drosophila* motor neurons expressing UAS-GFP::Lamp1 (N-terminal, luminal GFP) +/- UAS-30R and UAS-*luc<sup>RNAi</sup>* or exportin RNAi (UAS-*emb<sup>RNAi</sup>*). Scale bar = 10  $\mu\text{m}$ . (D) Quantification of C. Student's t-test,  $n = 6$  larvae. (E) *Drosophila* motor neurons expressing UAS-mCherry::Atg8 +/- UAS-30R and either control RNAi (UAS-*luc<sup>RNAi</sup>*) or exportin RNAi (UAS-*emb<sup>RNAi</sup>*). Scale bar = 10  $\mu\text{m}$ . (F) Quantification of E. Data are reported as mean  $\pm$  SEM. Mann-Whitney test,  $n = 10$  larvae. (G) *Drosophila* motor neurons expressing UAS-p62::GFP +/- UAS-30R and either control RNAi (*luc<sup>RNAi</sup>*) or exportin RNAi (*emb<sup>RNAi</sup>*) under the control of *vGlut-Gal4*. Scale bar = 10  $\mu\text{m}$ . (H) Quantification of G. Data are reported as mean  $\pm$  SEM. Brown-Forsythe and Welch ANOVA test,  $p < 0.0001$ , followed by Dunnett's T3 multiple comparisons,  $n = 12$ –14 larvae per genotype.

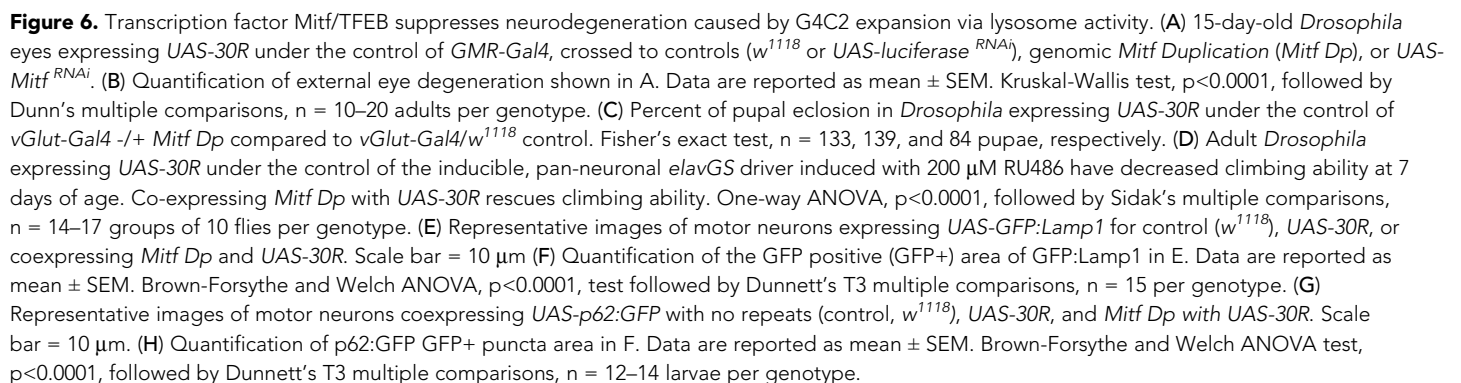


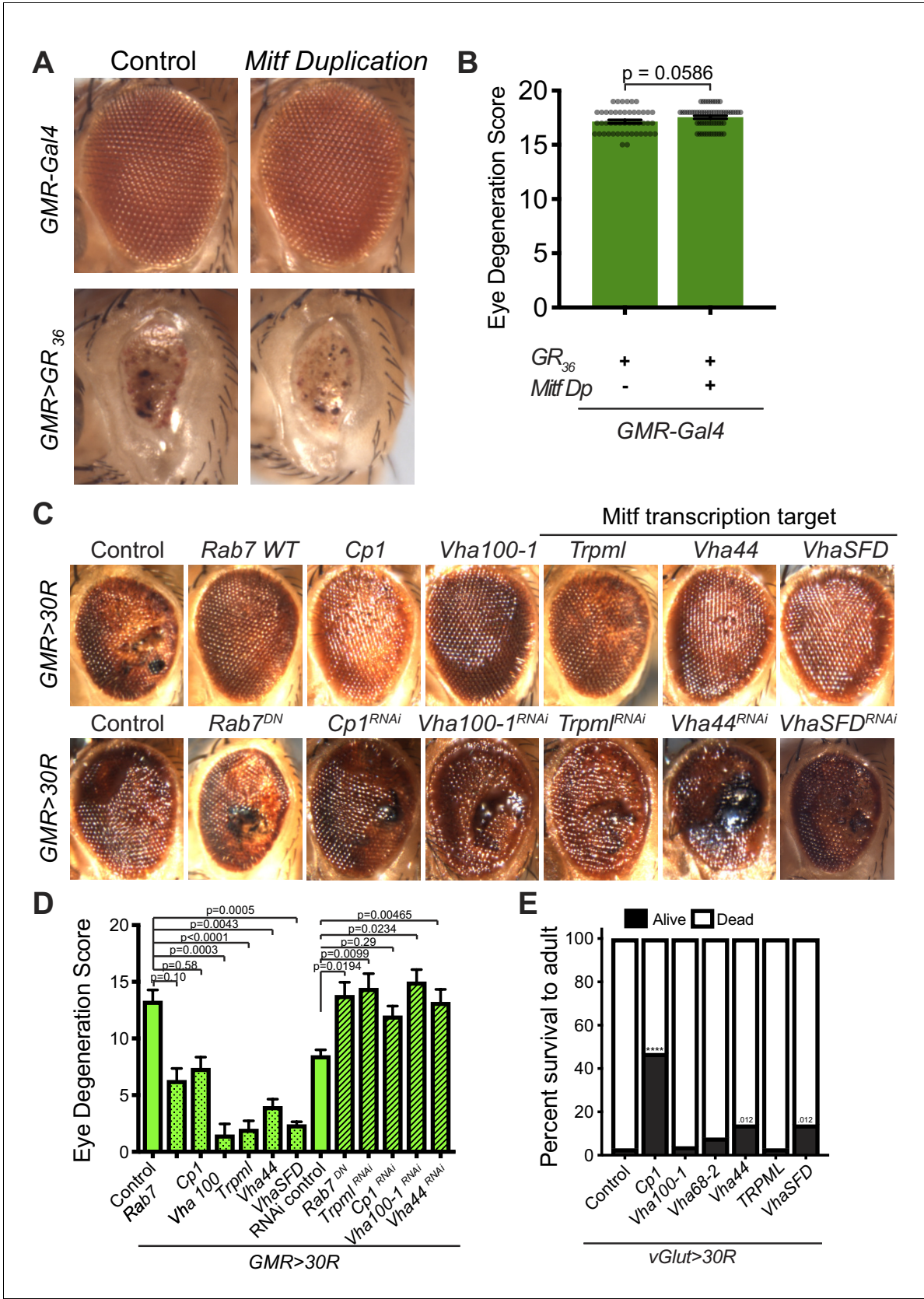
**Figure 5—figure supplement 1.** Nucleocytoplasmic transport disruption is upstream of autophagic defects. (A) Images of the nucleocytoplasmic transport marker shuttle-GFP (S-GFP) in control and *vGlut > 30R* with either UAS-  
Figure 5—figure supplement 1 continued on next page

*Figure 5—figure supplement 1 continued*

*luciferase* (control) or *UAS-Ref(2)P RNAi #1* expressed in *Drosophila* salivary glands. (B) Images of L3 salivary gland expressing *UAS-S-GFP* in *vGlut > 30R Drosophila* after feeding supplemented with control (DMSO), 0.2% trehalose, or 1.0  $\mu\text{m}$  rapamycin. (C) Representative images of ventral nerve cords expressing control, knockdown of *RanGAP* (*UAS-RanGAP<sup>RNAi</sup>*), or overexpression of *RanGAP* (*UAS-RanGAP*), along with *UAS-p62:GFP* in *vGlut* motor neurons. Scale bars = 10  $\mu\text{m}$ .





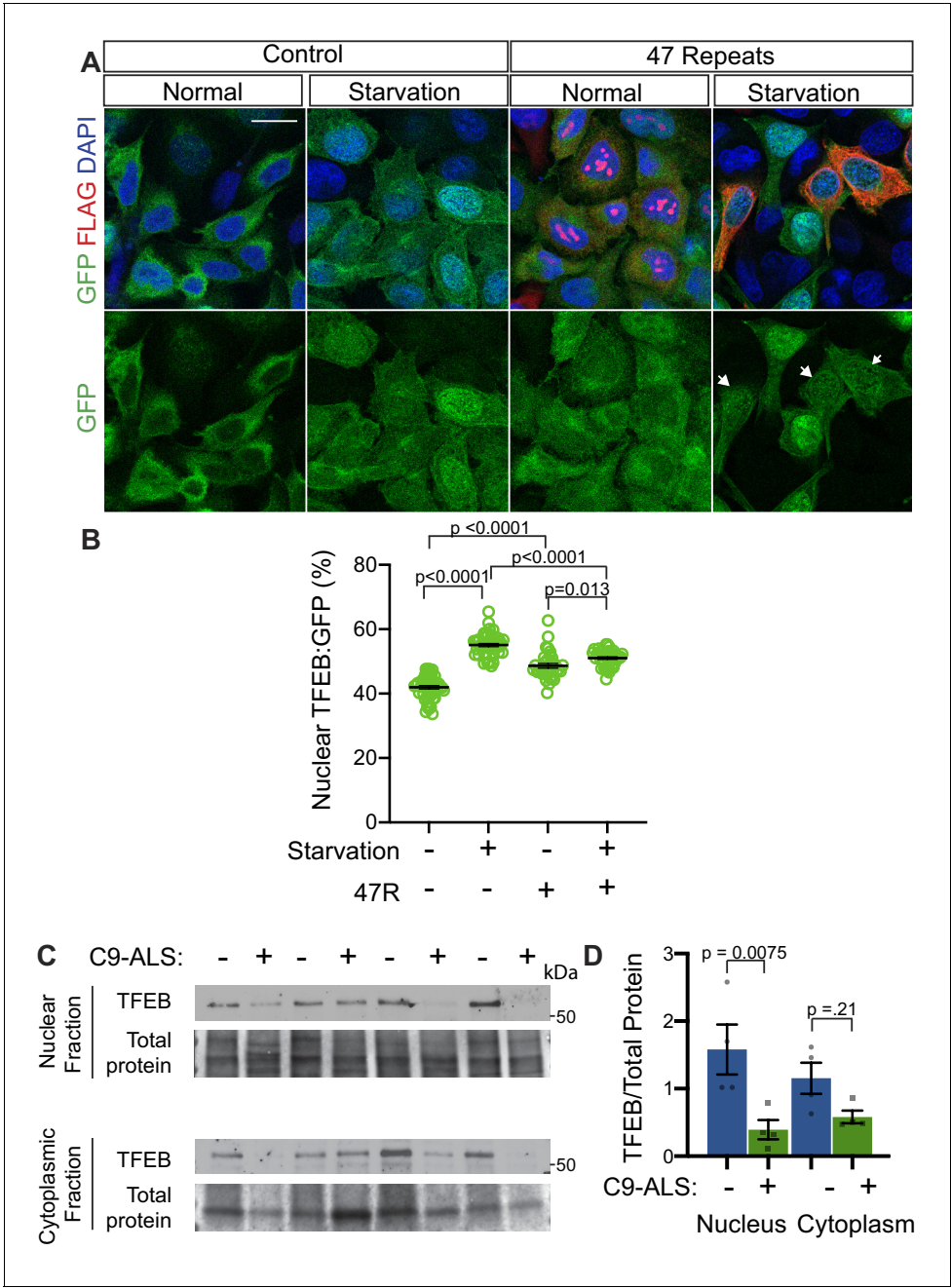


**Figure 6—figure supplement 1.** Genetic increase of lysosome function rescues degeneration caused by G4C2 expression but not by poly-GR. (A) 15-day-old *Drosophila* eyes expressing poly-GR<sub>36</sub> under the control of the *GMR-Gal4* with control (*w<sup>1118</sup>*) or *Mitf Dp*. (B) Quantification of external eye Figure 6—figure supplement 1 continued on next page

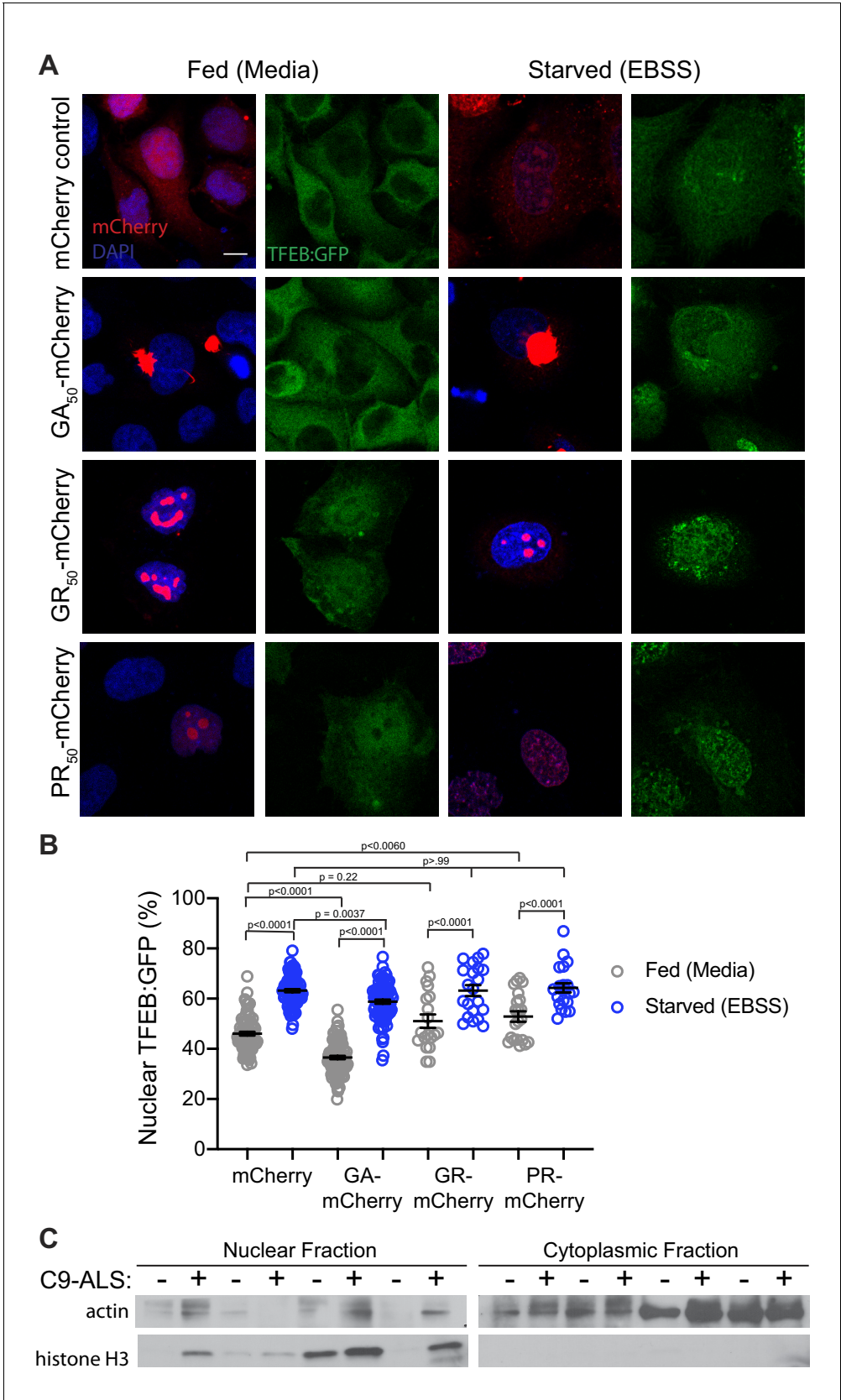


## Figure 6—figure supplement 1 continued

degeneration in A. Data are presented as mean  $\pm$  SEM. Mann-Whitney test,  $n = 48$  and  $59$  adult flies. (C) 15-day-old *Drosophila* eyes expressing UAS-30R under the control of the *GMR-Gal4* driver accompanying overexpression (top) or knockdown (bottom) of lysosomal genes. (D) Quantification of external eye degeneration in A. Data are presented as mean  $\pm$  SEM. Kruskal-Wallis test,  $p < 0.0001$  (left) and  $p = 0.0202$  (right) followed by Dunn's multiple comparisons,  $n = 10, 10, 10, 10, 8, 4, 5, 8, 6, 10, 9, 10, 4$ , and  $10$  adult flies, respectively. (E) Percent of pupal eclosion in *Drosophila* expressing UAS-30R under the control of the motor neuron *vGlut-Gal4* driver with UAS-lacZ (control) or overexpression of lysosomal genes. Fisher's exact test,  $n = 89, 120, 88, 146, 119, 123$ , and  $105$  pupa.



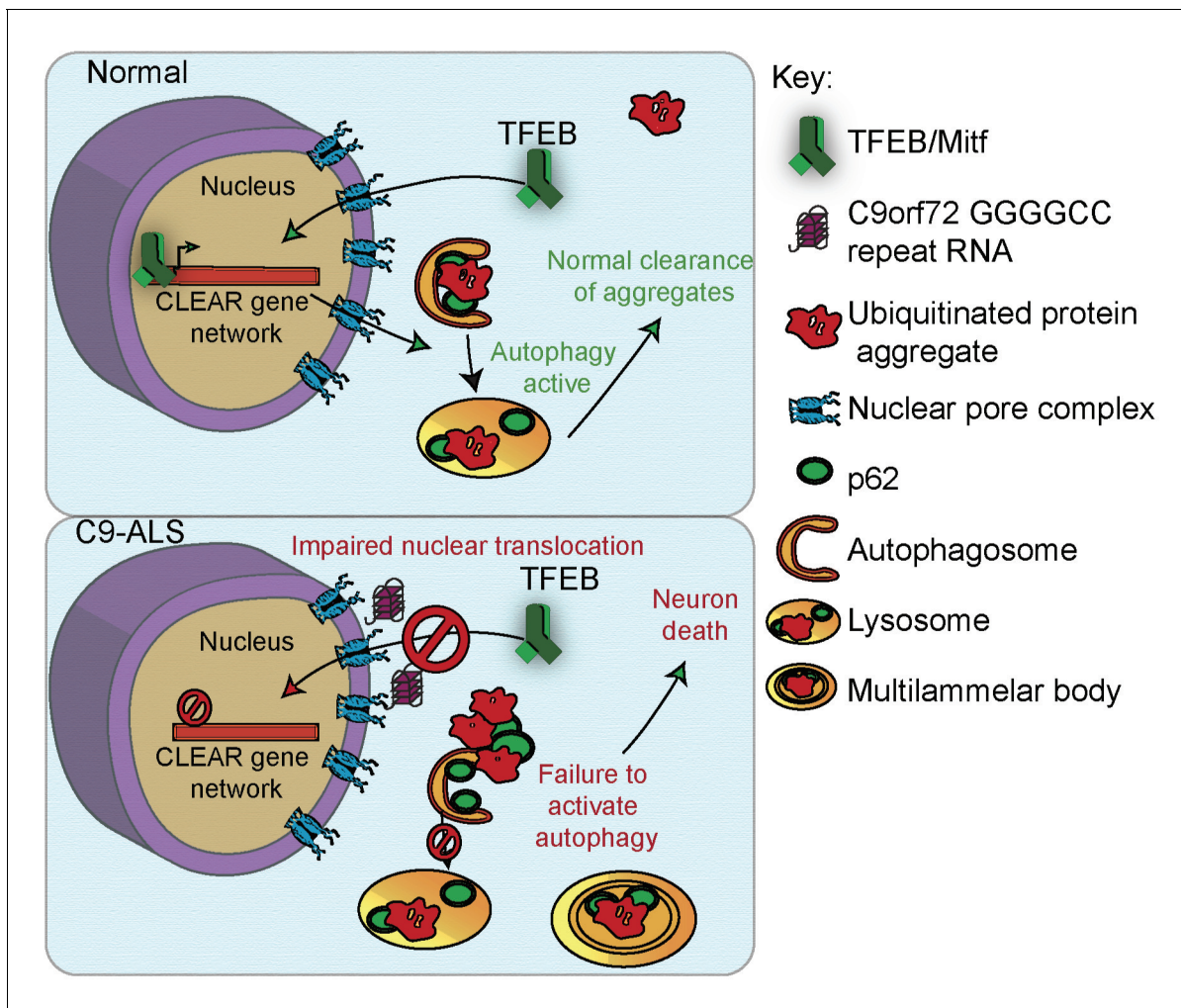
**Figure 7.** Nuclear TFEB is reduced in human cells expressing GGGGCC repeats and in C9-ALS human motor cortex. **(A)** HeLa cells stably expressing TFEB:GFP transfected with 0R (Control) or a 47R construct (Flag tag in frame with poly-GR) in normal media (DMEM) or starved (3 hr in EBSS) conditions. White arrowheads indicate transfected cells in the 47R starved group. **(B)** Quantification of cells from A showing the percent (%) nuclear TFEB:GFP (nuclear/total) for each group. Data are presented as mean  $\pm$  SEM. One-way ANOVA,  $p < 0.0001$ , with Sidak's multiple comparisons,  $n = 47, 47, 35$ , and  $38$  cells. **(C)** Western blot for TFEB of human motor cortex samples fractionated into cytoplasmic and nuclear samples from postmortem control and C9-ALS patient brains. **(D)** Quantification of TFEB levels against total protein loading (Faststain) in control and C9-ALS patients. Data reported are mean  $\pm$  SEM. One-way ANOVA,  $p = 0.0142$ , with Sidak's multiple comparisons,  $n = 4$ .



**Figure 7—figure supplement 1.** DPRs affect TFEB import in HeLa Cells. (A) Representative images of transfection of codon-optimized poly-GA-mCherry, poly-GR-mCherry, and poly-PR-mCherry into HeLa cells with stably incorporated TFEB:GFP. (B) Quantification of percent nuclear TFEB:GFP in Figure 7—figure supplement 1 continued on next page

*Figure 7—figure supplement 1 continued*

A. Data are presented as mean  $\pm$  SEM. One-way ANOVA,  $p < 0.0001$ , with Sidak's multiple comparisons  $n = 80, 90, 92, 84, 19, 21, 21$ , and  $21$  cells, respectively. Scale bars =  $20 \mu\text{m}$ . (C) Nuclear and cytoplasmic fractions of human motor cortex samples in **Figure 7C** blotted for cytoplasmic (beta-actin) and nuclear (histone H3) control proteins.



**Figure 8.** A proposed model of GGGGCC repeat expansion pathogenesis. G4C2 repeat expansion causes nucleocytoplasmic transport disruption through multiple proposed mechanisms including G4C2 RNA binding of RanGAP and stress granule recruitment of nucleocytoplasmic transport machinery. Transport disruption leads to a blockage in the translocation of autophagy-mediating transcription factors such as Mitf/TFEB to the nucleus in response to proteotoxic stress. Failure to induce autophagic flux leads to autophagy pathway disruption such as the accumulation of large, non-degradative lysosomes and MLBs. Loss of autophagic flux leads to accumulation of Ref(2)P/ p62 and ubiquitinated protein aggregates, leading to chronic protein stress signaling and eventually neuronal cell death.