Supplementary Procedures

High pressure freezing, freeze substitution, and electron microscopy

Selection of *Drosophila* larvae: Larvae were picked from growth medium and kept for a short time on a drop of PBS buffer prior to freezing. For correlative light electron microscopy, larvae were typically preselected to be genetic mosaics containing DsRed-labeled homozygous mutant cells using a Leica MZ12 dissecting scope equipped with epifluorescence illumination. Larvae were loaded into 100µm Type A specimen carriers (Cat. # 241; Technotrade, Manchester, NH) and the carrier was filled with *E. coli* paste, used as heat-transmitting medium and cryo-protectant. Loaded carriers were closed with the flat side of Type B specimen carriers (Technotrade, cat. # 242) and high pressure-frozen using a BAL-TEC HPM 010 freezer (BAL-TEC, Inc., Carlsbad, CA).

A limitation on all HPF protocols is the size of the specimen that can processed, since larger samples cannot fit into the chambers used for rapid freezing, and the rate of freezing of deep tissues is not fast enough for good preservation of morphology. We find that L1 and early L2 *Drosophila* larvae can be reliably frozen with excellent preservation of morphology. Late L2 larvae can also be used, though they require some manipulation to position them in the caps, and in these samples internal morphology is generally less well conserved. L3 larvae are too large for our procedure, though we have had some success in performing crude dissections and rapidly freezing a portion of the sample.

Freeze substitution procedure for epoxy embedding: Frozen larvae were quickly transferred for a freeze substitution to a -90°C pre-cooled mix of 1-2% osmium tetroxide (OsO_4) and 0.1% uranyl acetate in 97% acetone (McDonald and Müller-Reichert, 2002) in an automated freeze

substitution device (AFS 2; Leica Microsystems, Buffalo Grove, IL). To enhance membrane contrast we added 3% of water to the fixative (Walther and Ziegler, 2002). Specimens were freeze substituted for 72 hours at -90°C, then gradually warmed up at the rate of 5°C/hour to -20°C and maintained at this temperature for another 8-16 hrs. The temperature was then slowly raised to 20°C at the rate of 10°C/hour and processed immediately. Cryovials were removed from the AFS unit and transferred at room temperature. Once at a room temperature the specimens were rinsed immediately with pure acetone five times as follows: 2x15 minutes each, 1x30 minutes, and 2x1 hour each.

Infiltration and Embedding: Specimen infiltration was performed on a rotor at room temperature by incubation in gradually increasing concentrations of Durcupan (Cat. 44610 ACM FLUKA; Sigma-Aldrich, St. Louis, MO) or Epon Araldite (Cat. 13940; EMS, Hatfield, PA) epoxy resins as follows: 30% resin in acetone for 5 hrs; 70% resin in acetone overnight; 90% resin in acetone for 8 hrs. Next, specimens were transferred to 100% resin for overnight infiltration. Specimens were then transferred to fresh 100% resin. After two changes of the 100% resin over a 6 hour period the samples were polymerized at baking at 60°C for 48 hours in plastic molds. Of the two resins, Durcupan led to superior results.

Sectioning and Imaging: 45-70nm sections were obtained using a diamond knife (Diatome, Hatfield, PA) and Reichert Ultracut E microtome (Leica Microsystems). Sections were collected on coated copper grids, post-stained with 2.5% uranyl acetate for 10 minutes, and imaged at 125keV using a Hitachi 7200 electron microscope (Hitachi High Technologies America, Inc, Schaumburg, IL).

Freeze substitution procedure for correlative light/electron microscopy: Frozen larvae were quickly transferred for freeze substitution to a -90°C pre-cooled mix of 95% acetone

(EMS, RT10016), 5% distilled water, 0.1% potassium permanganate ($KMnO_4$; EMS, RT20200), and 0.001% osmium tetroxide (EMS, RT19134) (Watanabe et al., 2010), in an automated freeze substitution device (Leica Microsystems, AFS 2). Substitution was performed for 54 hours at -90°C. The samples were then gradually warmed at 5°C/hour to -50°C, washed five times for 15 minutes each in 95% acetone/5% water and once for 15 minutes in 95% acetone/5% water/0.1% uranyl acetate (addition of uranyl acetate was necessary to add contrast to the membranes.) Next, we raised the temperature of the sample at 5°C/hour to -30°C and washed six times for 15 minutes each with 95% ethanol/5% water prior to resin infiltration and embedding.

Infiltration and Embedding: Glycidyl methacrylate resin (GM; cat # 64161, Sigma Aldrich) was prepared at different concentrations in 95% ethanol/5% water. Infiltration steps were performed at -30°C in cryogenic vials as follows: 30% GM overnight; 70% GM for eight hours; 90% GM overnight. Specimens were then transferred into the caps of polypropylene BEEM capsules and the resin replaced with fresh 100% GM with two additional changes for 24 hours in total. Next, fresh 100% resin was prepared with the addition of 0.15% N,N-dimethyl-p-toluidine accelerator and added to the samples. Polymerization was performed over 48 hours at -30°C and then warmed at 5°C/hour to -20°C. Polymerized blocks were kept wrapped in aluminum foil and stored at -20°C.

Sectioning and Imaging: Blocks containing the embedded samples were thawed and imaged using a Leica MZ16 stereomicroscope equipped for epifluorescent illumination to identify the approximate location of fluorescently labeled cells within the fixed larvae. The block was trimmed to the approximate location of the cell of interest and sections of 50-100nm were collected using a Leica EM UC6 ultramicrotome onto a glass cover slip. For fluorescent imaging of CLEM sections, we found that use of TIRF (total internal reflection) microscopy

produced the best contrasting fluorescent signal. We used a Zeiss PAL-M microscope equipped with a 100x plan-apochromat objective, operating in the TIRF mode. Sections used for TIRF microscopy are directly usable for EM, but in practice we found it was more convenient to perform light and electron microscopy on pairs of adjacent sections, using tissue landmarks to correlate regions of interest. For EM, we post-stained sections with 2.5% uranyl acetate in water for 5 minutes to enhance membrane contrast, and imaged them at 125keV using a Hitachi 7200 electron microscope.

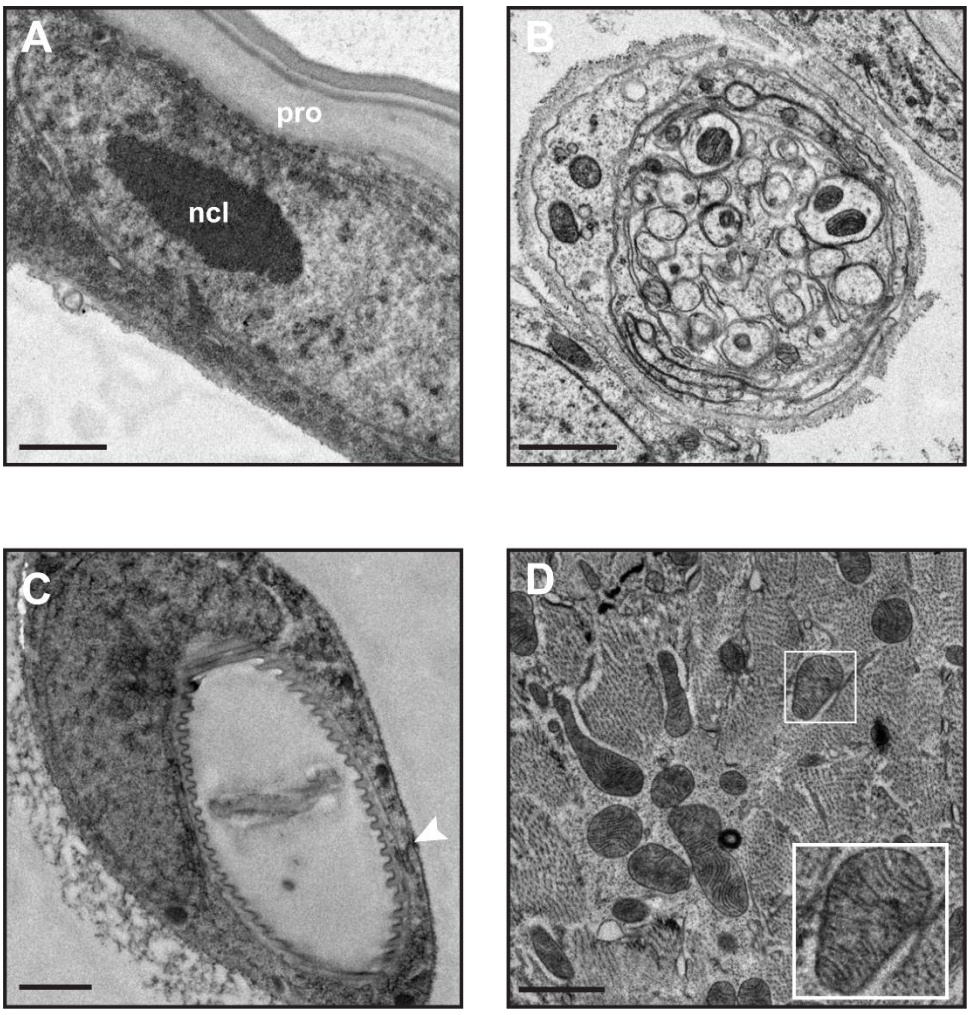
We have tested DsRed and GFP fluorescent proteins for retained activity and find DsRed expression is well maintained, but GFP fluorescence is not. We have found the DsRed fluorescence is stable in embedded samples for at least 2 years when kept at 4° in the dark.

McDonald, K. and Müller-Reichert, T. (2002). Cryomethods for thin section electron microscopy. *Meth. Enzymol.* **351**, 96–123.

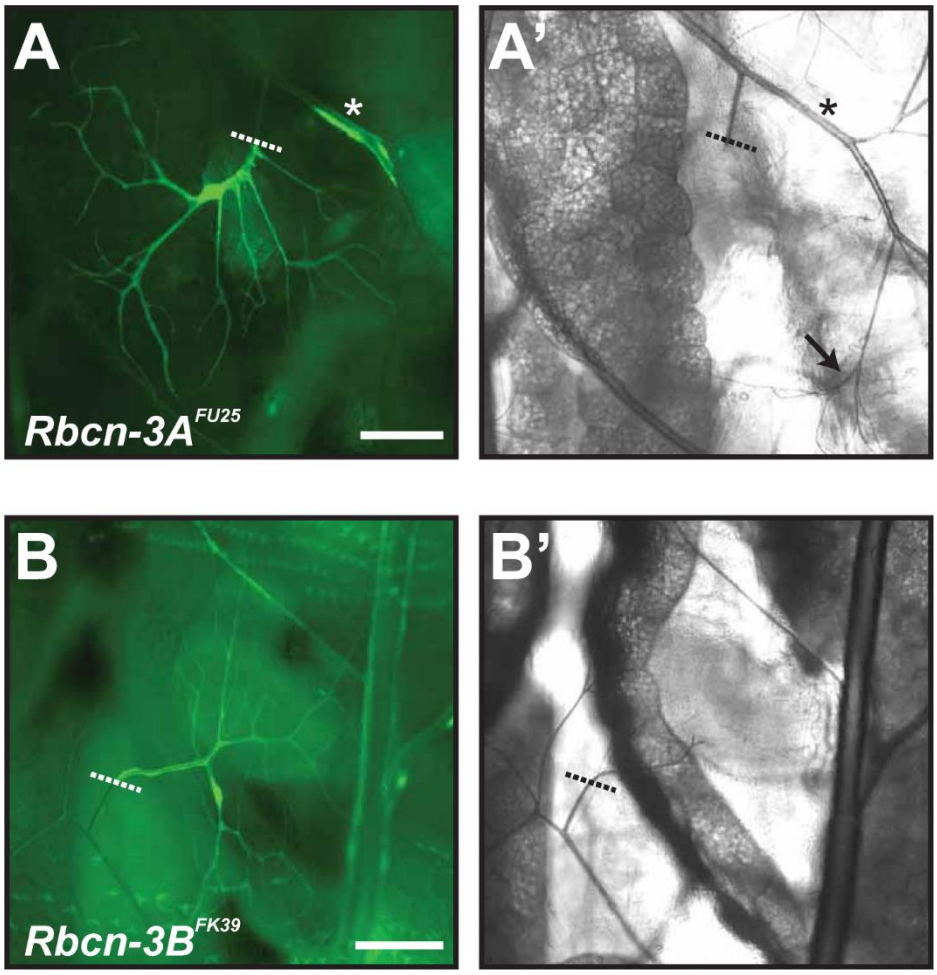
Walther, P. and Ziegler, A. (2002). Freeze substitution of high-pressure frozen samples: the visibility of biological membranes is improved when the substitution medium contains water. *Journal of Microscopy* **208**, 3–10.

Watanabe, S., Punge, A., Hollopeter, G., Willig, K. I., Hobson, R. J., Davis, M. W., Hell, S. W. and Jorgensen, E. M. (2010). Protein localization in electron micrographs using fluorescence nanoscopy. *Nature methods* **8**, 80–84.

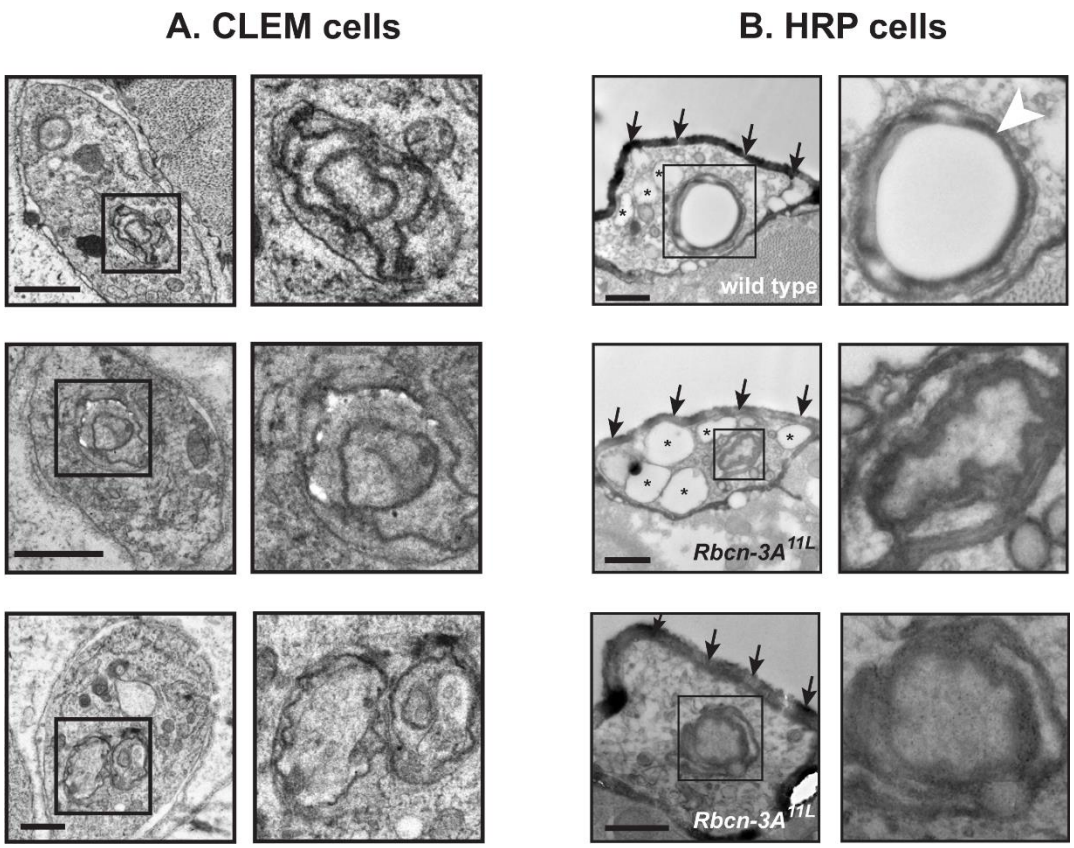
Supplemental Figures



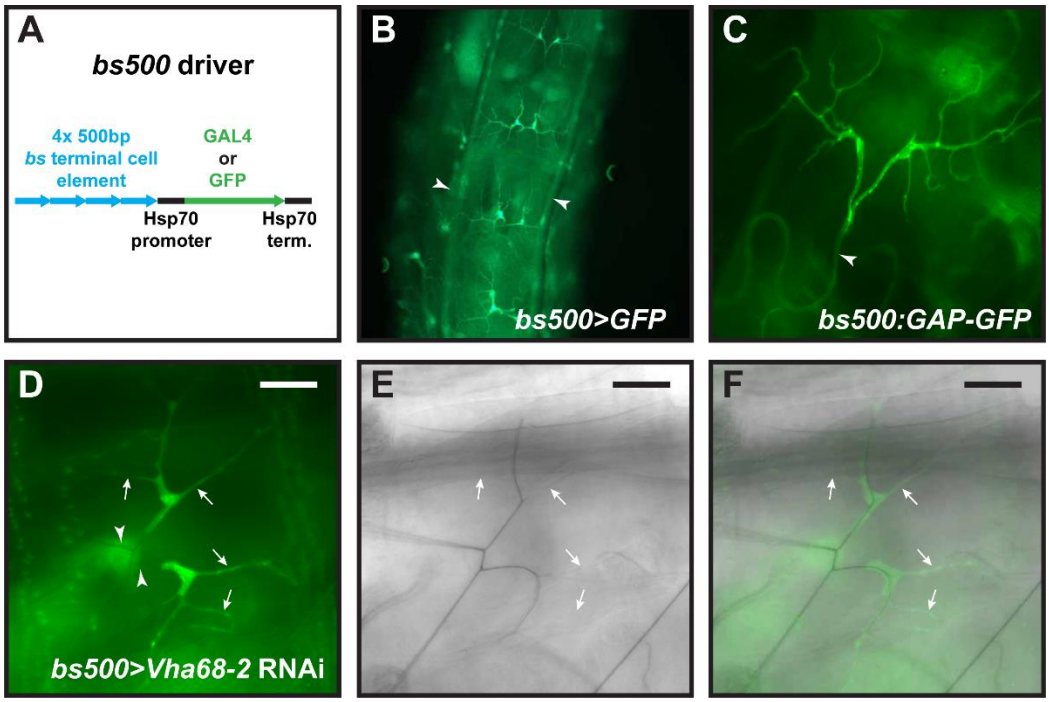
Supplemental Figure 1. Examples of ultrastructural preservation in *Drosophila* larvae fixed for correlative light/electron microscopy. Late L1/early L2 larvae were fixed using HPF/potassium permanganate/osmium tetroxide and embedded in GM resin. Ultrastructure was examined in 90nm sections by TEM. (A) Epidermal cell nucleus. The nuclear envelope has a smooth appearance, indicative of minimal tissue shrinkage during fixation. Chitin layers can be observed in the external procuticle (pro). Note that the epidermal microvilli are not preserved with this fixation technique (compare to Fig. 2B). ncl, nucleolus. (B) Ensheathed nerve showing neurite cross sections. (C) Unicellular tracheal branch. Chitinous taenidial folds line the lumen of the cell. Arrowhead indicates the autocellular junction forming the unicellular tube. (D) Muscles containing mitochondria. The myofibers and mitochondrial cristae (inset) are well preserved. Scale bars: 1µm.



Supplemental Figure 2. Other alleles of *Rbcn-3A* and *Rbcn-3B* are required for terminal cell lumen formation. Fluorescent (A, B) and brightfield (A', B') images of homozygous mutant GFP-labeled *Rbcn-3A*^{FU25} (A) and *Rbcn-3B*^{FK39} (B) terminal cells in mosaic L3 larva, generated using the MARCM system. Dashed lines indicates the proximal end of mutant cells where they join the rest of the tracheal system. The proximal portion of this *Rbcn-3B*^{FK39} mutant cell shows gas filling. The arrow in A' indicates a nearby non-GFP labeled (thus wild-type) terminal cell in which a gas-filled lumen can be clearly observed; the asterisk indicates two adjacent GFP-labeled homozygous *Rbcn-3A*^{FU25} unicellular branches. These show apparently normal gas-filled lumens. Scale bars: 100µm



Supplemental Figure 3. Further examples of *Rbcn-3A^{11L}* mutant cell ultrastructure. (A) Profiles of tracheoles in three different *Rbcn-3A^{11L}* homozygous mutant cells fixed using HPF/FS. Abnormal luminal structures are shown magnified on the right and appear as multiple membrane layers. (B) EM analysis of wild-type and *Rbcn-3A^{11L}* terminal cells labeled with HRP. L3 mosaic larvae were generated using the MARCM, chemically fixed with glutaraldehyde, and positively labeled with GFP and HRP::CD2. Homozygous wild-type or mutant *Rbcn-3A^{11L}* terminal cells were identified by GFP fluorescence (not shown) and HRP activity was used to generate an electron dense stain on the plasma membrane of the cell (arrows). Note the lumen membranes are also darker than usual due to the HRP staining. The magnified view of the lumen in the wild-type cell shows a single membrane surrounding the chitinous lining (arrowhead), while in *Rbcn-3A^{11L}* terminal cells, the presumptive lumen has multiple membranous layers, but no obvious cuticle or gas-filled space is present. Asterisks indicate artifacts, often observed in glutaraldehyde fixed samples. Scale bars: 1μm (A); 500nm (B).



Supplemental Figure 4. *bs500-GAL4* constructs and *Vha68* RNAi in tracheal terminal cells. (A) Diagram of 4 x multimerized 500bp *bs* (*bs500*) enhancer transgene used to express GAL4 or GFP. (B) Dorsal view of L2 larva with *bs500:GAL4* driving expression of *UAS:GFP* in terminal cells. Note lack of GFP expression in the multicellular tracheal dorsal trunks (arrowheads). (C) Pair of dorsal terminal cells with *bs500* directly driving expression of membrane localized GAP:GFP. (D-F) *bs500:GAL4* driving expression of *UAS:GFP* and *UAS:Vha68 RNAi* in dorsal terminal cells. (D) GFP shows growth and branching of cells. (E) Brightfield image showing lack of a gas-filled lumen in branches, indicated by arrows. (F) Merged image. In C & D, autofluorescence but no GFP expression is present in the unicellular stalk or fusion cells (arrowheads). Scale bars: 50µm.