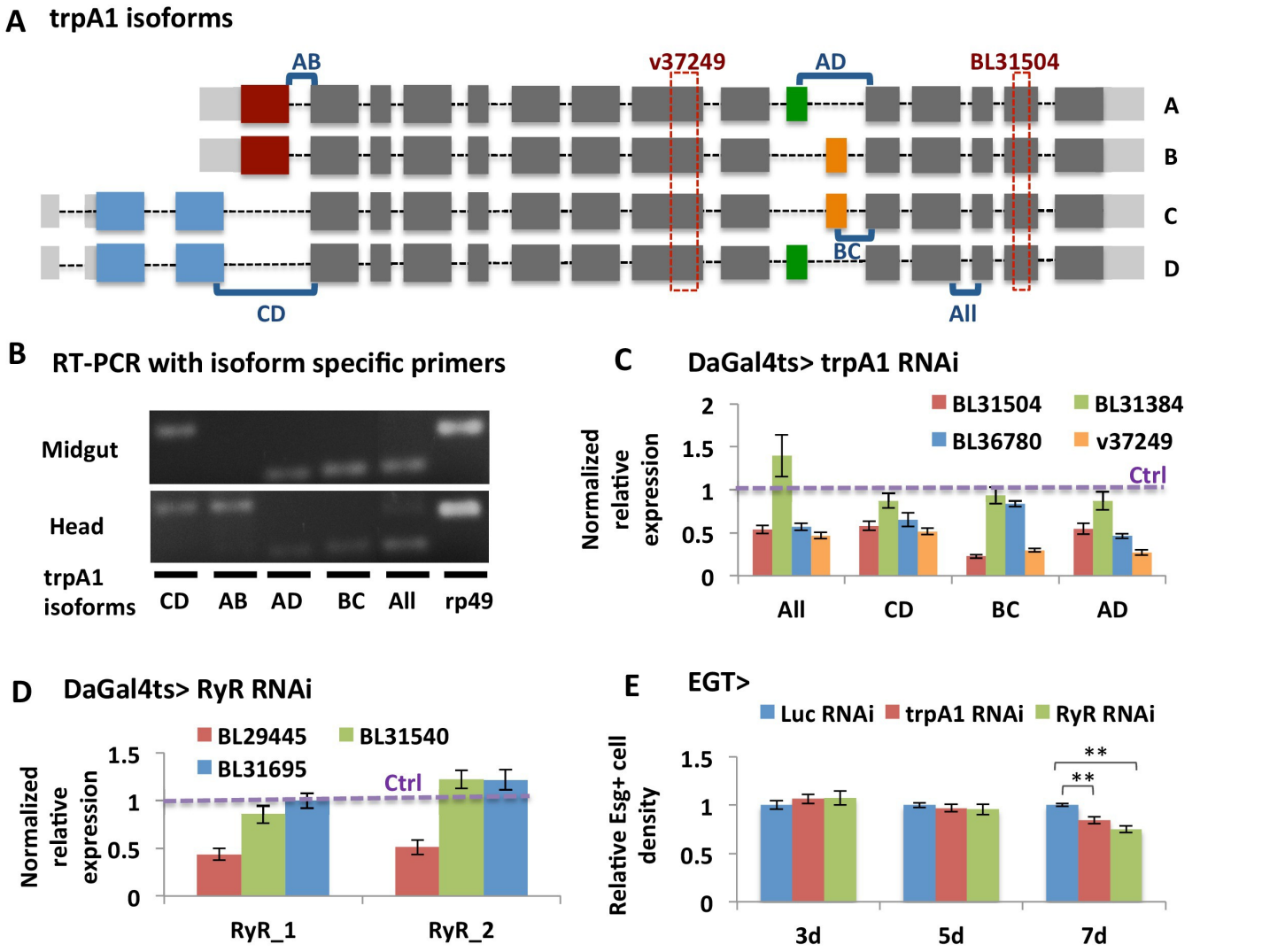

Figure 1-Figure Supplement 1

Validation of knockdown efficiency for *trpA1* RNAi and *RyR* RNAi lines.

(A) Cartoon depicting the four characterized *trpA1* isoforms. The target regions of *trpA1* RNAi lines used in this study are shown in dashed rectangles. The regions amplified with isoform-specific primers are shown in square brackets. (B) Primers spanning exons for specific splicing events are used in RT-PCR to distinguish *trpA1* isoform expression in fly midguts and heads. (C) RT-qPCR analysis of mRNAs from midguts expressing different lines of *trpA1* RNAi ubiquitously with inducible DaGal4ts for 5d, with primers designed for different types of *trpA1* isoforms. *GAPDH* and *rp49* are reference genes for normalization. To determine RNAi knockdown efficiency, the ratio of normalized *trpA1* expression to corresponding control groups expressing either *Luc* RNAi (for BL lines) or carrying an empty insertional landing site (v60100 for v37249) is calculated. The data are presented as mean ± SEM for three technical replicates. We repeated the experiments (two biological replicates) and observed consistent results. (D) RT-qPCR measurement of midguts ubiquitously expressing *Luc*RNAi or *RyR* RNAi for 5d, with two different sets of primers designed for all *RyR* isoforms. *GAPDH* and *rp49* are used for normalization. The data are presented as mean ± SEM for three technical replicates. We repeated the experiments (two biological replicates) and observed consistent results. (E) Quantification of relative esg+ cell density in the posterior midgut region. esg+ cell number is divided by the imaged area size for density calculation. N > 5 midguts per genotype per time point are analyzed. Data are represented as mean ± SEM.

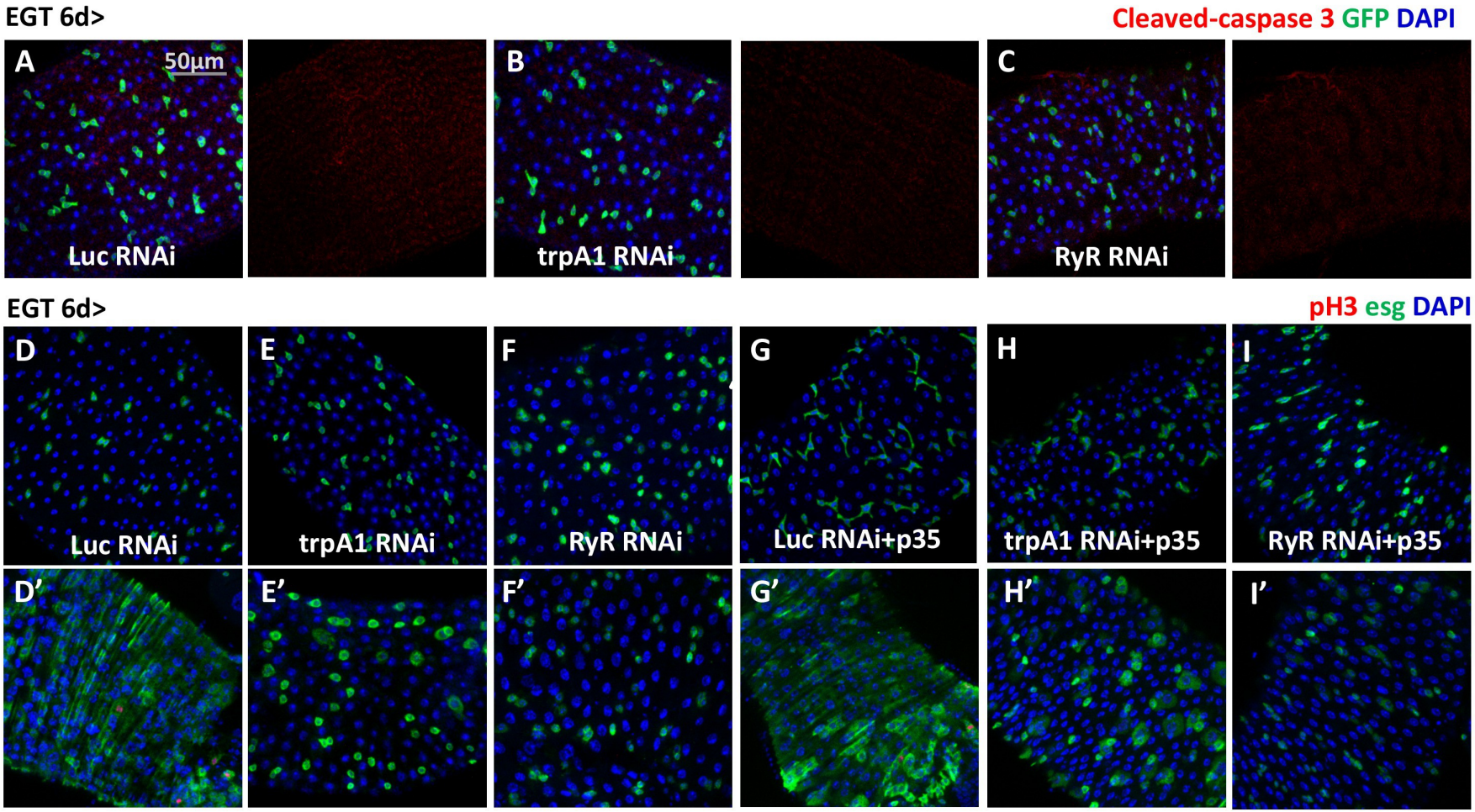

Figure 1-Figure Supplement 2

***trpA1* RNAi and *RyR* RNAi do not cause ISC apoptosis.**

(A–C) Immunostaining of midguts expressing *Luc* RNAi, *trpA1* RNAi or *RyR* RNAi in ISCs for apoptosis marker cleaved-caspase 3. The channels of cleaved-caspase 3 signal are shown to the right of the merged images. No signal can be detected except some background staining in the trachea and muscle. (D–F) Midguts expressing *Luc* RNAi, *trpA1* RNAi or *RyR* RNAi in ISCs with the last 2d feeding 2 mM paraquat (D', E') are stained for

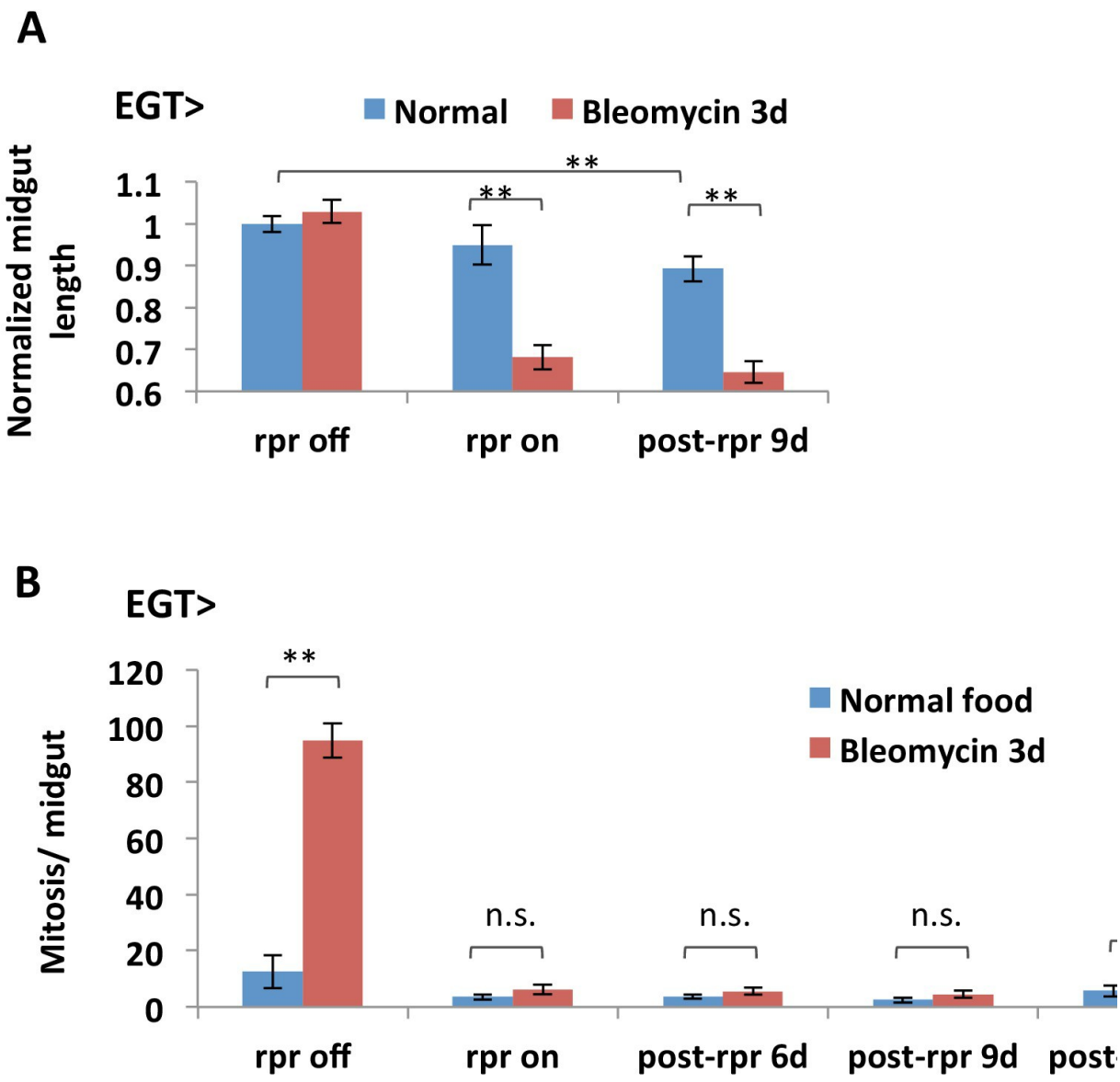

Figure 1-Figure Supplement 3

ISC depletion results in midgut shortening.

(A) Length quantification of midguts with inducible expression of rpr in ISCs. In the 'rpr off' group, t
kept at 18°C to prevent Gal4 activity and rpr expression; in the 'rpr on' group, rpr expression is ind
for 4d and turned off at 18°C for 3d feeding normal food or bleomycin; in the 'post-rpr' group, after
expression of rpr, the flies are placed back at 18°C for specified days before bleomycin treatment.
midguts are analyzed per genotype per treatment. Data are represented as mean ± SEM. (B) Mitc
quantification of midguts with inducible expression of rpr in ISCs. Mitosis activity in the midgut can
after depletion of the esg+ cell population by 4d rpr expression, N > 7 midguts are analyzed per ge
treatment. Data are represented as mean ± SEM.

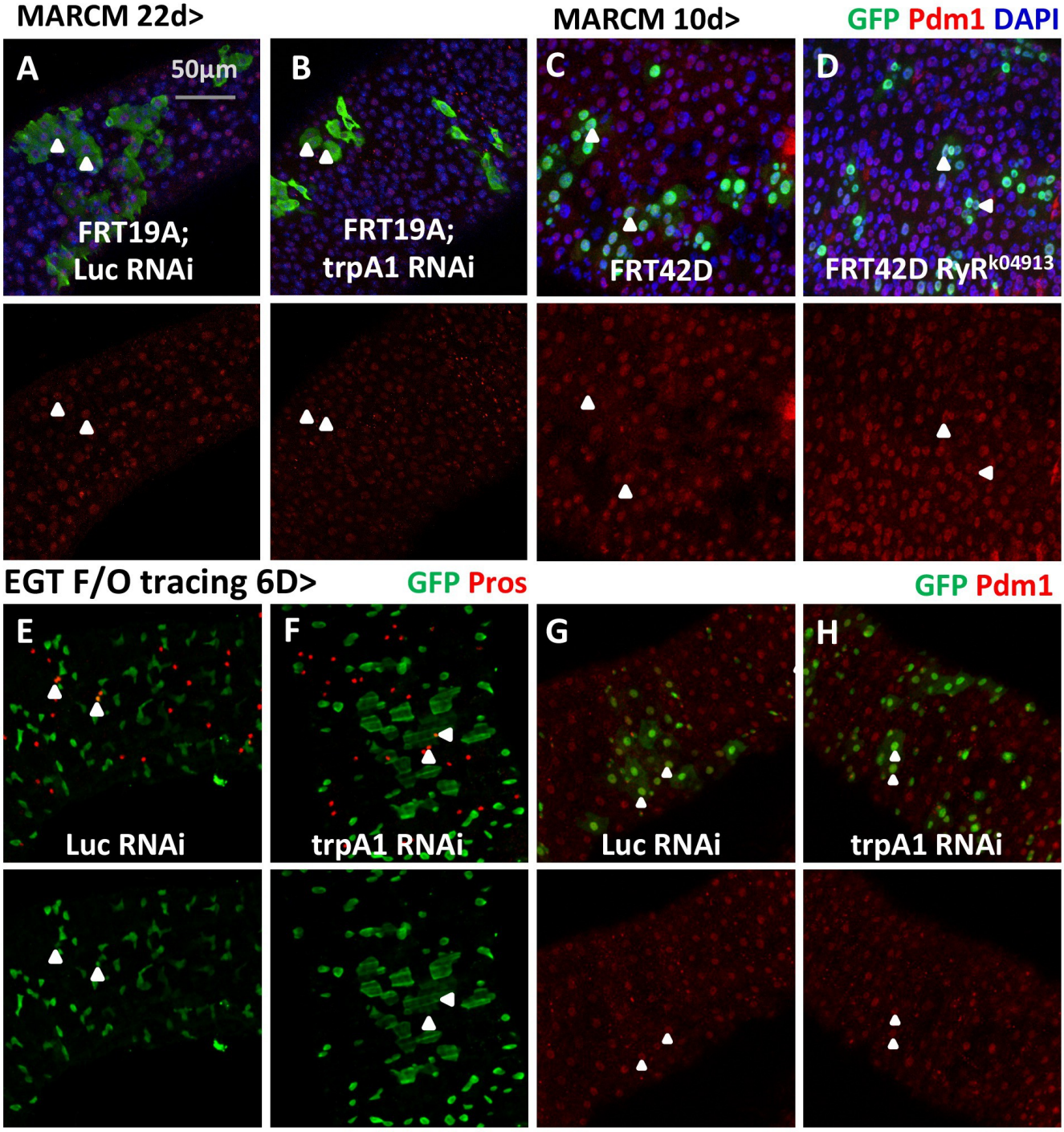

Figure 2-Figure Supplement 1

Lineage-tracing experiments provide evidence that TRPA1 or RyR-deficient ISCs can differentiate.

(A–B) MARCM clones expressing *Luc* RNAi or *trpA1* RNAi for 22d are examined for their survival and stained with anti-Pdm1 antibody to examine EC differentiation. Arrowheads highlight examples of ECs generated from ISCs expressing *Luc*RNAi or *trpA1* RNAi. (C–D) MARCM clones that are homozygous RyR mutant are stained with anti-Pdm1 antibody. Arrowheads highlight examples of ECs in the MARCM clones that are Pdm1+. (E–F) Immunostaining of midguts expressing *Luc* RNAi or *trpA1* RNAi in the ISC lineage (labeled by GFP) for EE marker, Pros. Arrowheads highlight examples of EEs generated by ISCs during the 6d period of tracing. (G–H) Immunostaining of midguts expressing *Luc* RNAi or *trpA1* RNAi in the ISC lineage (labeled by GFP) for EC marker, Pdm1. Arrowheads highlight examples of ECs generated by ISCs during the 6d period of tracing.

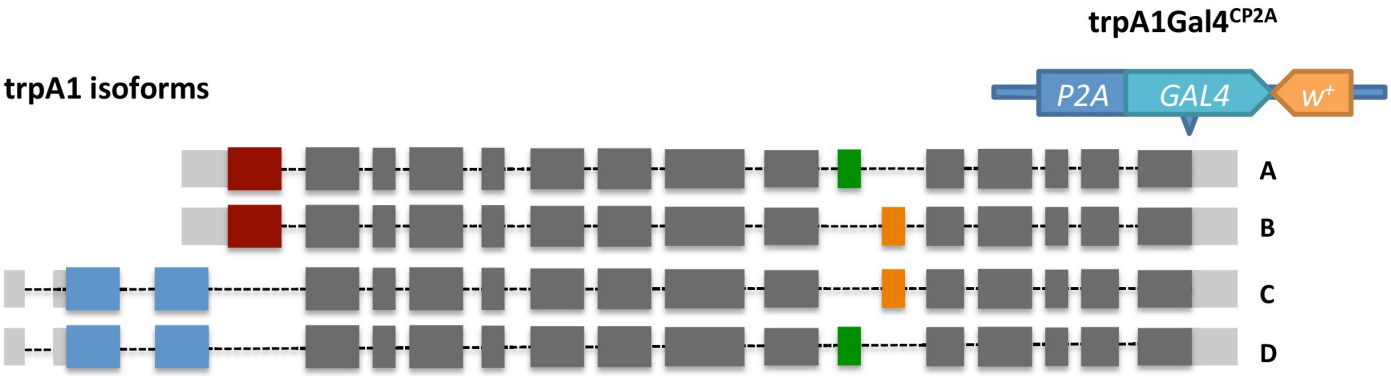

Figure 3-Figure Supplement 1

The knock-in design of *trpA1*Gal4^{CP2A}.

By CRISPR/Cas9-induced homologous recombination, Gal4 is inserted into the shared C terminal of *trpA1* isoforms right before the stop codon. The knock-in could result in bi-cistronic transcripts expressing both TRPA1 and Gal4.

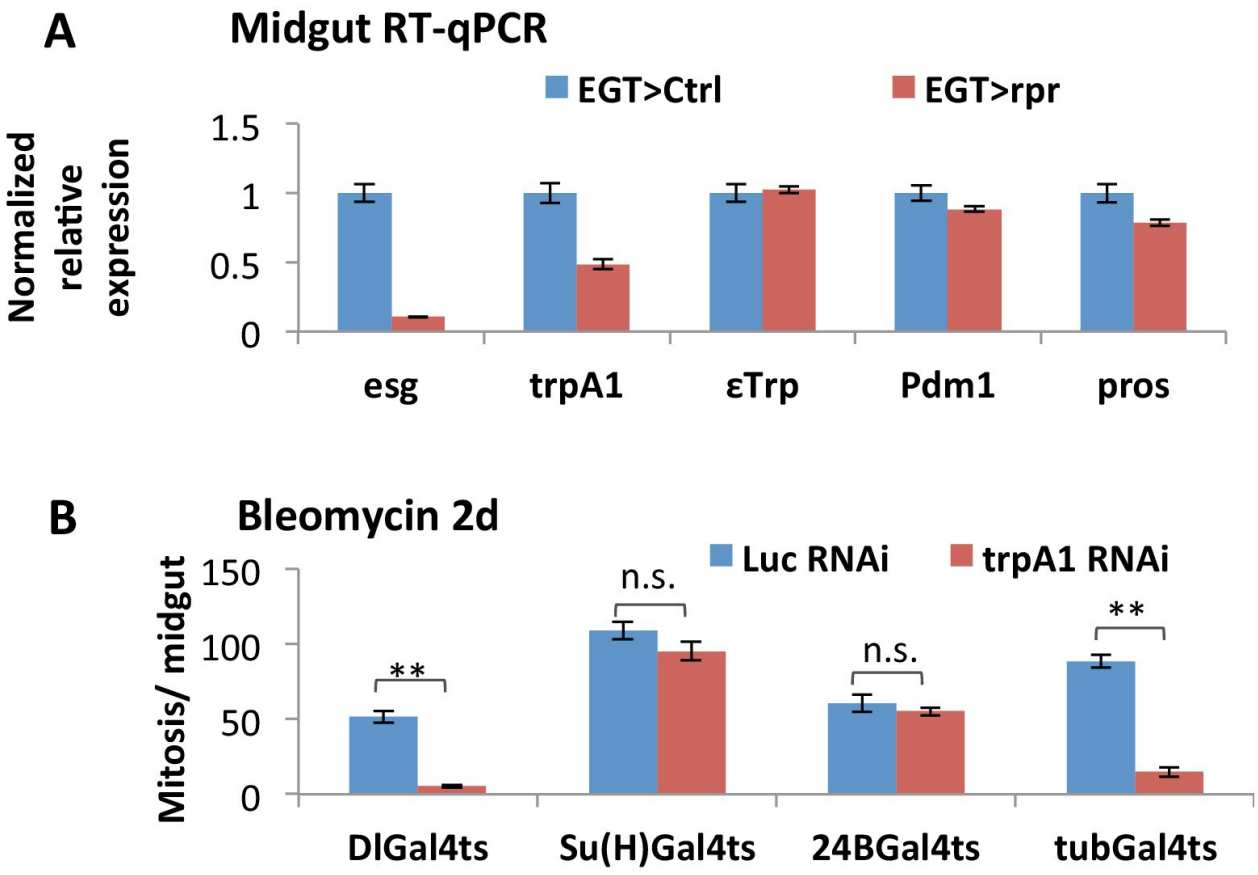

Figure 3-Figure Supplement 2

Additional evidence for *trpA1* expression and function in ISCs.

(A) RT-qPCR measurement of midguts expressing the cell death gene *rpr* in ISCs for 4d for the expression of *esg*, *trpA1*(using primers for *CD* isoforms), EC marker *ε-Trpsin* (*εTrp*), *Pdm1*, or *pros. GAPDH* and *rp49* are used for normalization. The data are presented as mean ± SEM for three technical replicates. We repeated the experiments (two biological replicates) and observed consistent results. (B) Mitosis quantification of midguts expressing *Luc* RNAi or *trpA1* RNAi in self-renewing ISCs (DlGal4ts), differentiating ISCs or enteroblasts (Su(H)Gal4ts), muscles (24BGal4ts), or ubiquitously (tubGal4ts) for 9d, with last 2d feeding bleomycin. DlGal4ts and Su(H)Gal4ts are used to drive gene expression in ISCs and enteroblasts, respectively (Zeng et al., 2010). Data are represented as mean ± SEM.

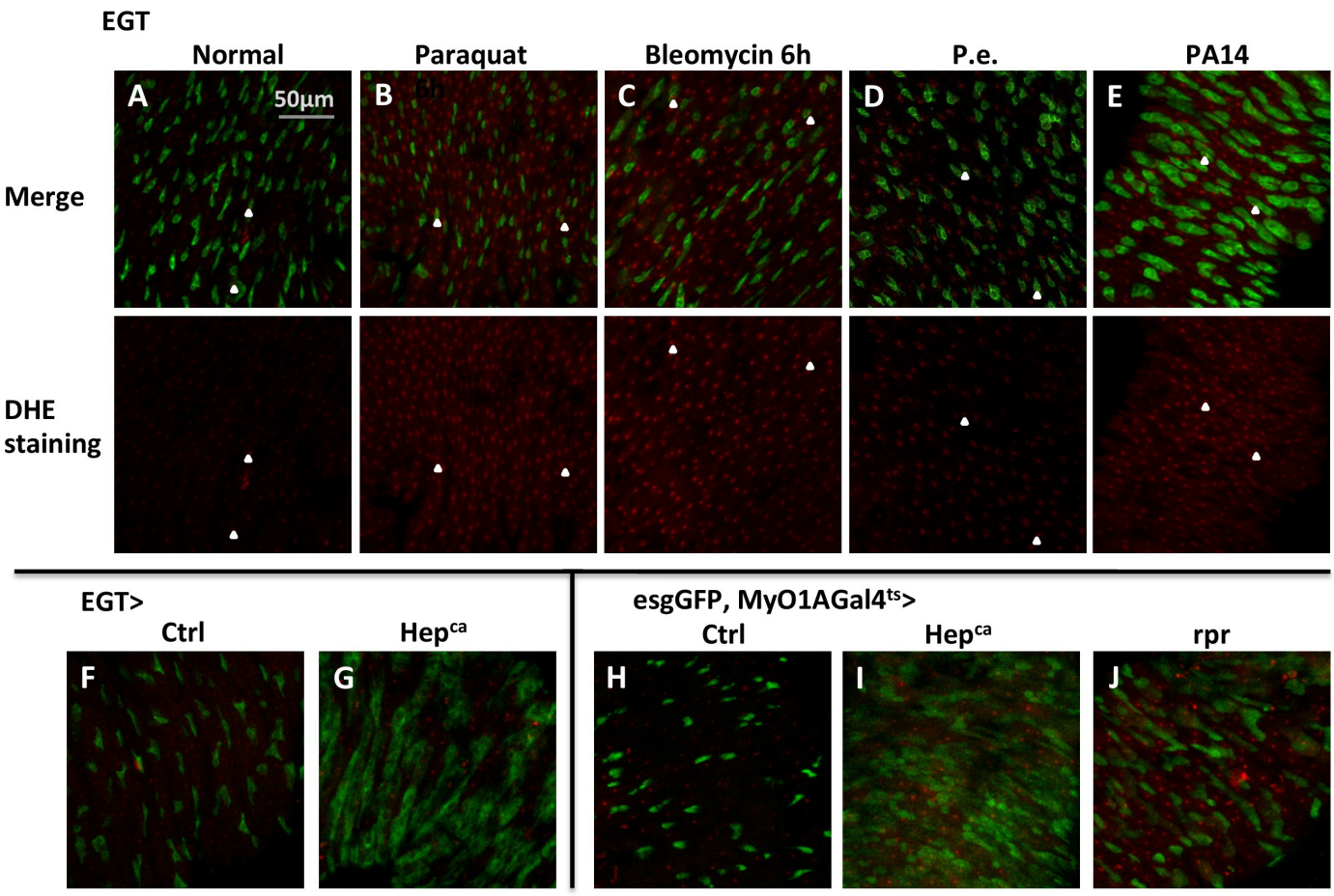

Figure 4-Figure Supplement 1

Elevated ROS is a common stress signal in various midgut damage conditions.

(A–E) Dihydro-ethidium (DHE) stainings of live midguts expressing GFP in ISCs for 3d from flies fed with normal food, food containing 2 mM paraquat, 25 µg/ml bleomycin for 6 hr, pathogens *Pseudomonas entomophila* (P.e.), or *Pseudomonas aeruginosa* (PA14) for 18 hr. DHE signal channel is shown below the merged image. The control group for *Pseudomonas*infection, using Bacteria-free LB media, is not shown in the figure because the midgut DHE staining looks the same as flies fed with normal food. Arrowheads highlight examples of stem cells that have high ROS concentration. (F–G) DHE staining of midguts overexpressing active JNKK (*Drosophila* Hep^{ca}) for 1d in ISCs with EGT. (H–J) DHE staining of midguts overexpressing Hep^{ca} or cell death gene *reaper* (*rpr*) for 2d in ECs with MyO1AGal4ts. esgGFP labels the ISCs.

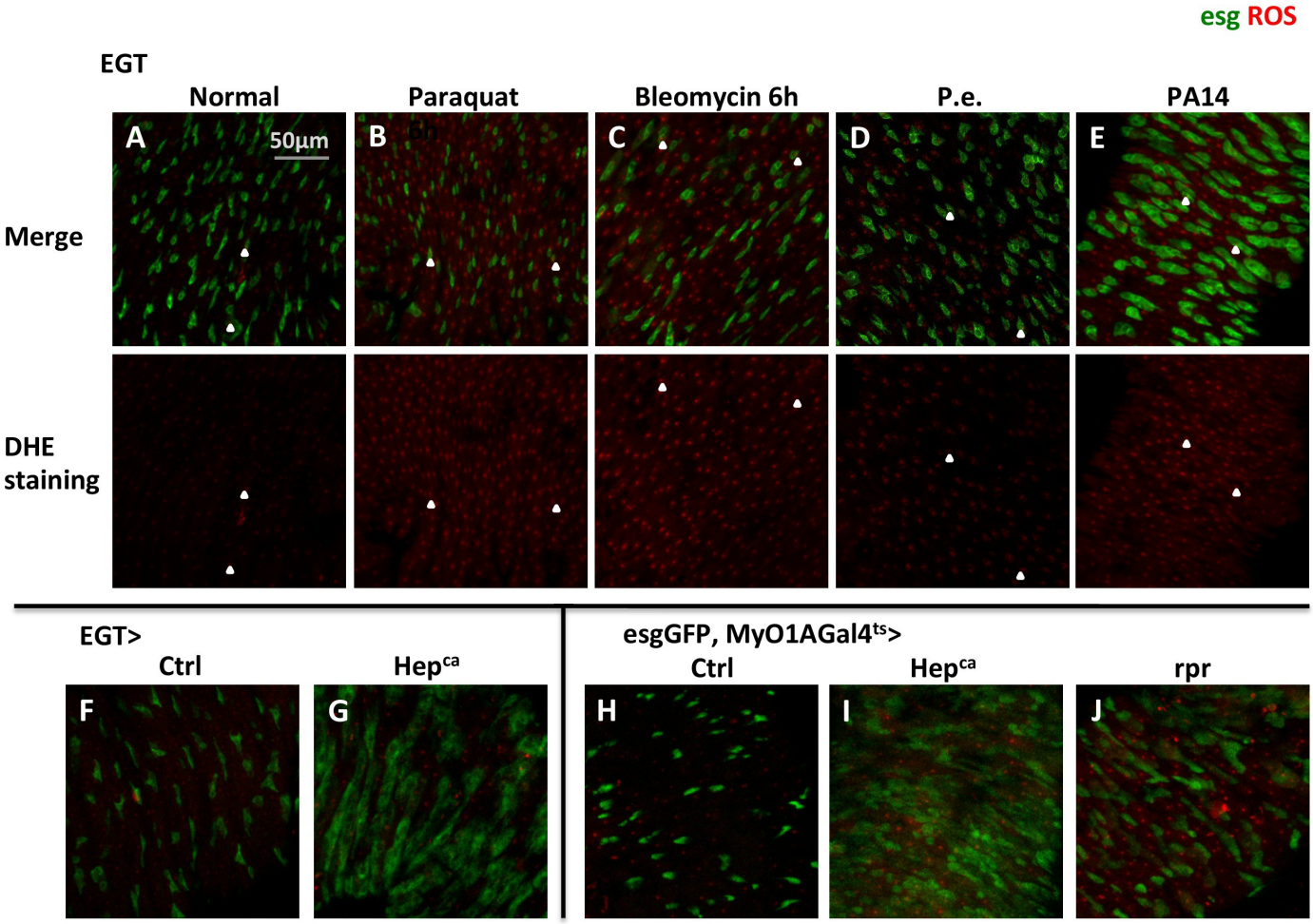

Figure 5-Figure Supplement 1

The total number of ISCs expressing esgGal4>GCamP6s reporter is not significantly reduced by *trpA1* RNAi or *RyR* RNAi.

(A–C) Anti-GFP staining of midguts expressing GCamP6s alone, or together with *trpA1* RNAi or *RyR* RNAi in ISCs detects GCamP6s expression. The images are acquired on a confocal microscope. The relative density of GCamP6s+ (driven by esgGal4) cells, imaged on a Keyence microscope covering most of the posterior midgut region, is quantified in (D) while the total number of GCamP6s+ cells in the posterior midgut region is quantified in (E). N > 8 midguts are analyzed for each genotype. Data are represented as mean ± SEM.

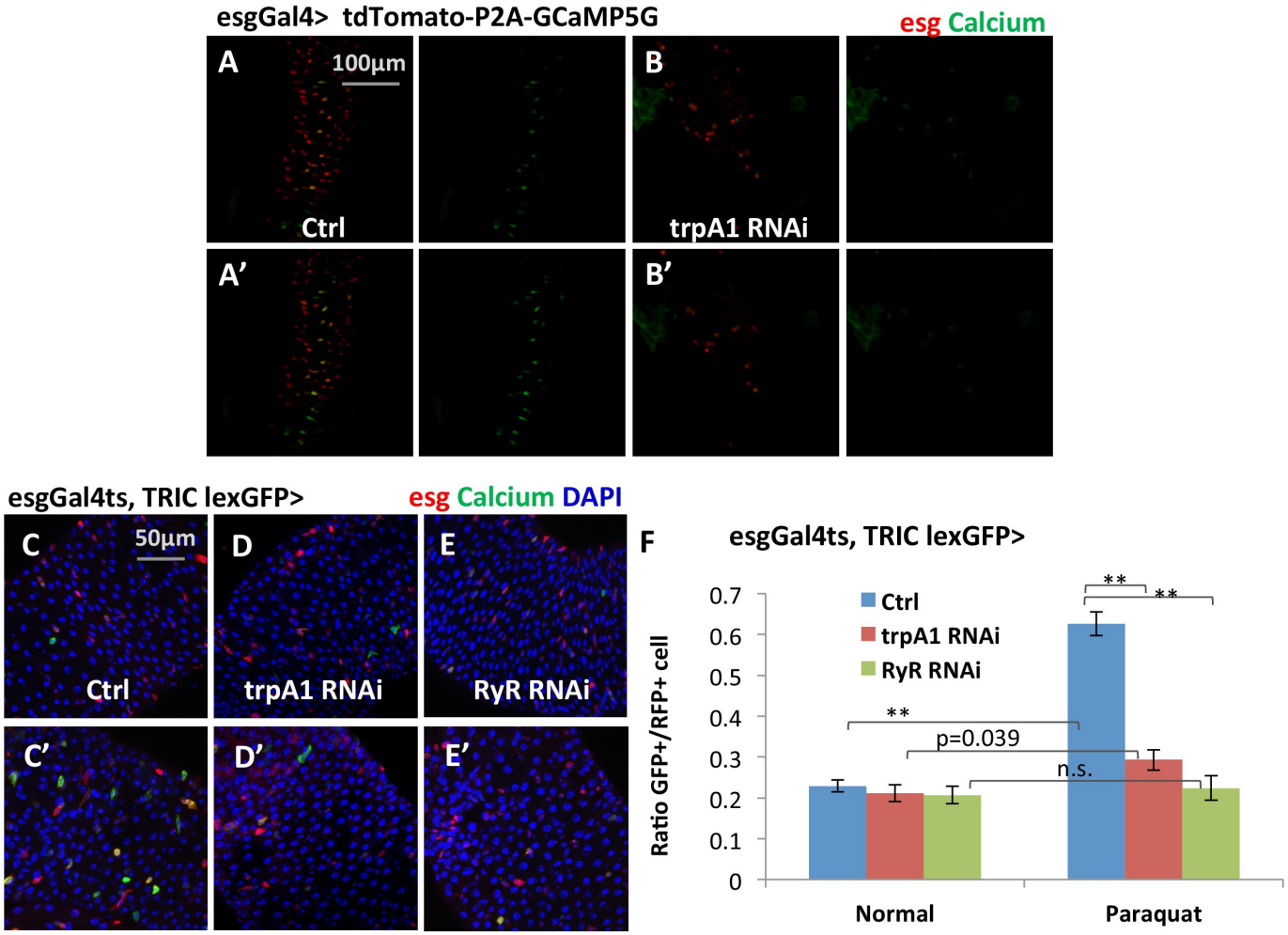

Figure 5-Figure Supplement 2

Additional reporters showing that TRPA1 and RyR are required for ROS-mediated Ca^{2+} increases in ISCs.

(A–B) Confocal calcium imaging of midguts that express bi-cistronic UAS-tdTomato-P2A-GCaMP5G reporter alone or together with *trpA1* RNAi in ISCs with exposure to 2 mM paraquat for 5 min (A'–B'). The bi-cistronic reporter consists of tdTomato that labels all cells expressing the reporter, and GCaMP5G whose GFP signal intensity reflects intracellular calcium concentration (Daniels et al., 2014). (C–E) Midguts expressing the TRIC lexGFP calcium reporter alone or together with *trpA1* RNAi or *RyR* RNAi in ISCs for 6d, with the last 1d feeding 2 mM paraquat (C'–E') are stained to examine stem cell Ca^{2+} concentration in vivo. The conventional *UAS*-RFP reporter is used to label all stem cells. (F) Quantification of high Ca^{2+} stem cells (GFP+) ratio to all stem cells (RFP+) for midguts in experiments (C–E). N = 16, 10, six images are analyzed for the genotypes of control, *trpA1* RNAi, and *RyR* RNAi (half feeding normally, half feeding paraquat for 1d). Data are represented as mean ± SEM.

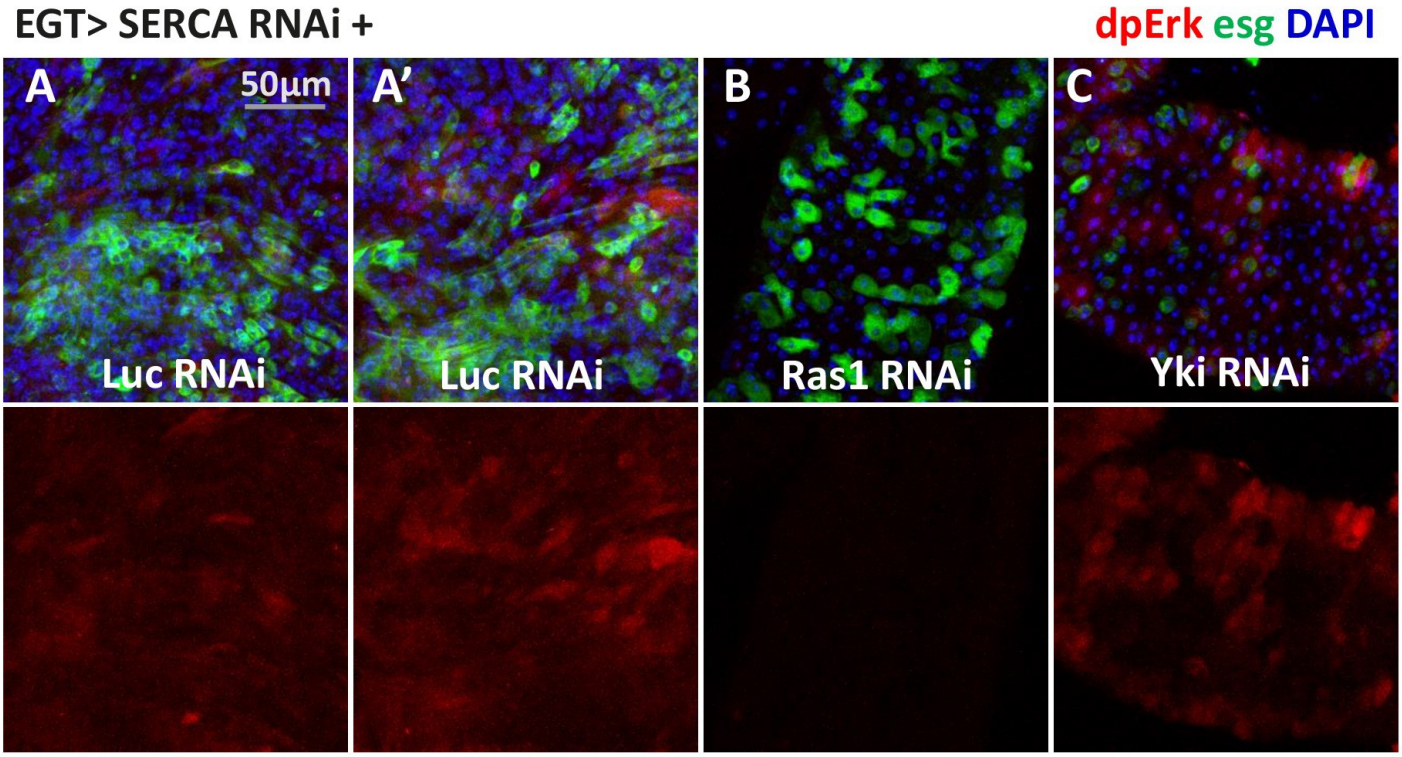

Figure 6-Figure Supplement 1

Prolonged induction of high cytosolic Ca^{2+} in ISCs results in a nonspecific and variable pattern of Ras/MAPK activation.

(A–C) Midguts expressing *SERCA* RNAi together with *Luc* RNAi, *Ras1* RNAi, or *Yki* RNAi in ISCs for 5d are stained for dpErk. The channel of dpErk signal is shown below the merged image. A and A' are biological replicates of the same genotype showing examples of variation. Although pErk induction is still detectable in some ISCs, the signal intensity varies among different ISCs and different midguts.

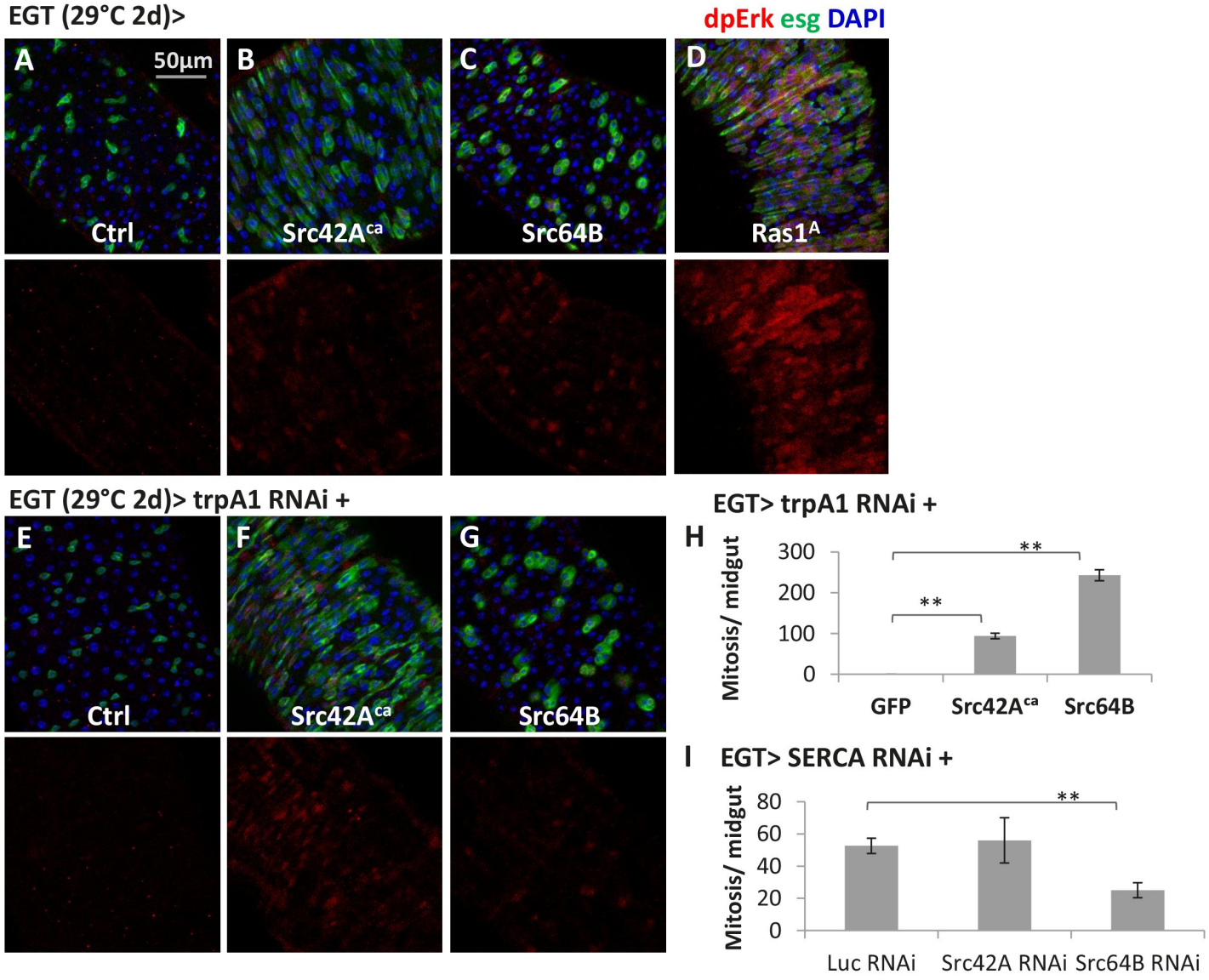

Figure 6-Figure Supplement 2

Src might be a mechanism by which cytosolic Ca^{2+} can activate Ras/MAPK in ISCs.

(A–D) Midguts expressing the constitutive active form of Src42A (Src42A^{ca}), Src64B, or Ras1^{A} in ISCs for 2d are stained for the Ras/MAPK activity marker dpErk. The channel of dpErk signal is shown below the merged image. *w-* genetic background is used as the control for transgenic expression. (E–G) Midguts expressing *trpA1* RNAi alone or together with Src42A^{ca}/ Src64B in ISCs for 2d are stained for dpErk. The channel of dpErk signal is shown below the merged image. (H) Mitosis quantification of midguts expressing *trpA1* RNAi with GFP, Src42A^{ca}, or Src64B in ISCs for 3d. N > 5 midguts are analyzed for each genotype. Data are represented as mean ± SEM. (I) Mitosis quantification of midguts expressing *SERCA*RNAi with *Luc* RNAi, *Src42A* RNAi, or *Src64B* RNAi in ISCs for 4d. N > 4 midguts are analyzed for each genotype. Data are represented as mean ± SEM.

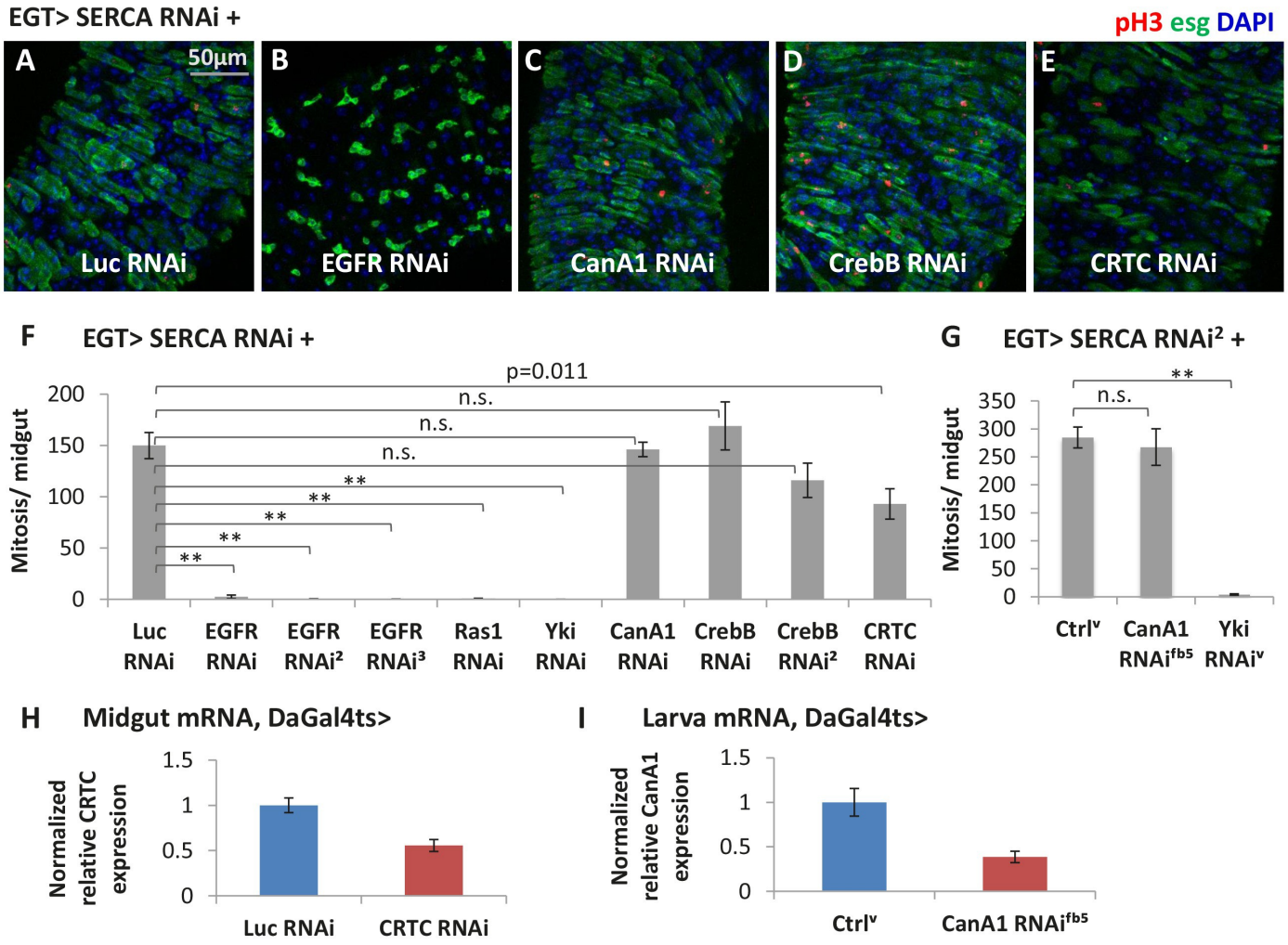

Figure 7-Figure Supplement 1

Ras/MAPK activity, but not CanA1/CrebB, is required for ISC proliferation induced by calcium influx.

(A–E) Midguts expressing *SERCA* RNAi together with *Luc* RNAi, *EGFR* RNAi, *CanA1* RNAi, *CrebB* RNAi, or *CRTC* RNAi in ISCs for 3d are stained for mitosis marker pH3. (F) Mitosis quantification of midguts expressing *SERCA* RNAi together with *Luc* RNAi, *EGFR* RNAi (three different lines), *Ras1* RNAi, *Yki* RNAi, *CanA1* RNAi, *CrebB* RNAi (two different lines), or *CRTC*RNAi in ISCs for 5d. N > 7 midguts are analyzed for each genotype. Data are represented as mean ± SEM. (G) Mitosis quantification of midguts expressing *SERCA* RNAi^{2} alone, or together with *CanA1* RNAi^{fb5}, or *Yki* RNAi^{v} in ISCs for 5d. N > 5 midguts are analyzed for each genotype. Data are represented as mean ± SEM. For *CanA1* RNAi line 'fb5', flies with a similar w- genetic background carrying an empty insertional landing site (v60100) are used as the control (Ctrl^{v}). (H) RT-qPCR measurement of *CRTC* expression in midguts ubiquitously expressing *Luc* RNAi or *CRTC* RNAi for 5d. *GAPDH* and *rp49* are used for normalization. The data are presented as mean ± SEM for three technical replicates. (I) RT-qPCR measurement of *CanA1* RNAi ^{fb5} knockdown efficiency. L3 larvae expressing RNAi for 2 days are used for better quantification because midgut *CanA1* expression is barely detectable. *GAPDH* and *rp49* are used for normalization. The data are presented as mean ± SEM for three technical replicates.

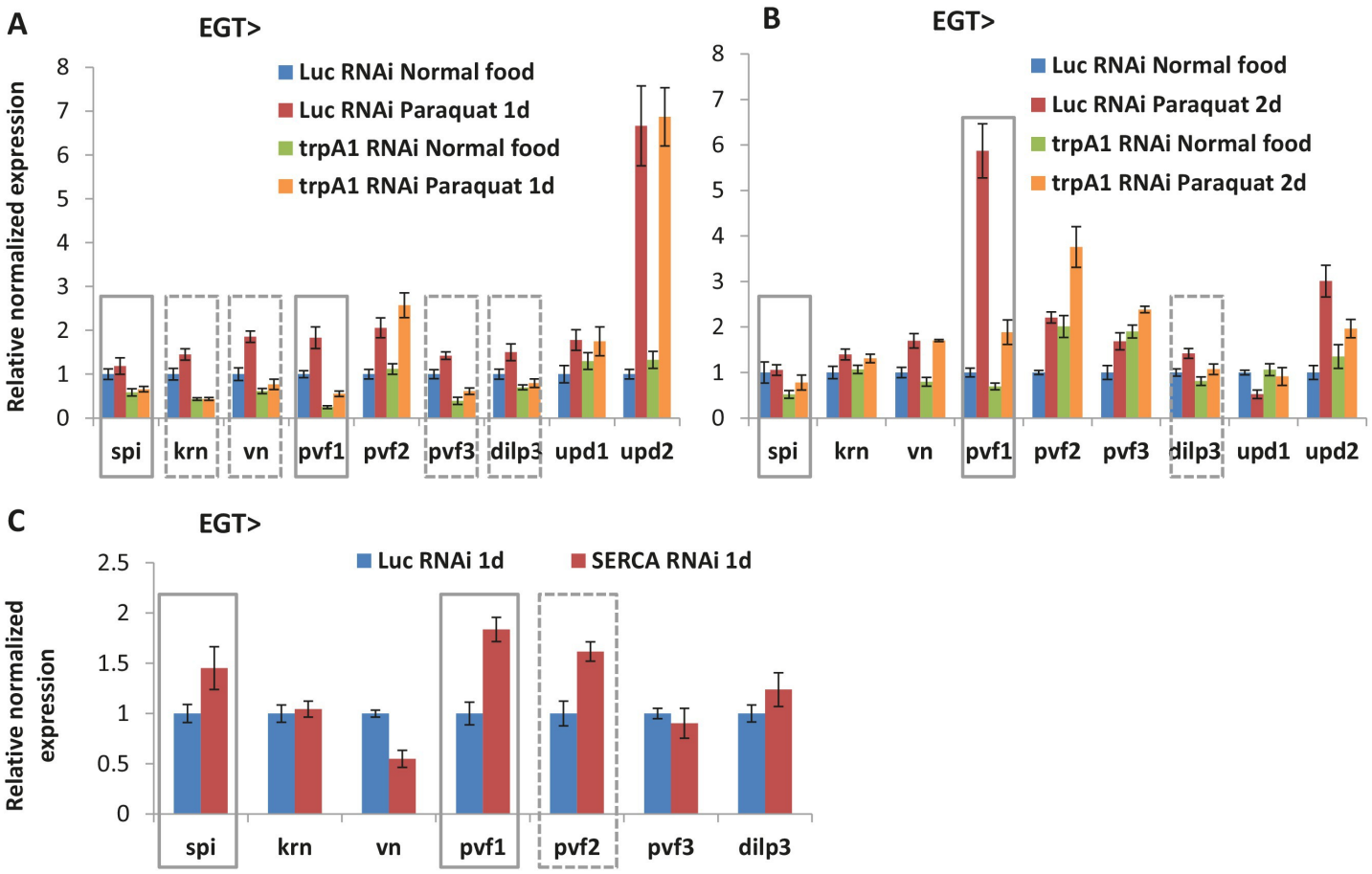

Figure 7-Figure Supplement 2

Ligands for receptor tyrosine kinases (RTKs) are affected by cytosolic Ca^{2+} signaling.

(A) RT-qPCR measurement of midguts expressing *Luc* RNAi or *trpA1* RNAi in ISCs for 6d, with the last day feeding on normal food or paraquat. *rp49* is used for normalization. The data are presented as mean ± SEM for three technical replicates. While expression of JAK/ Stat pathway ligands *upd1* and *upd2* remains unaffected, multiple RTK ligands (*spi*, *krn*, *vn*, *pvf1*, *pvf3*, *dilp3*, highlighted by gray square boxes) are down-regulated by *trpA1* RNAi. (B) RT-qPCR measurement of midguts expressing *Luc* RNAi or *trpA1* RNAi in ISCs for 9d, with the last 2d feeding on normal food or paraquat. *rp49* is used for normalization. The data are presented as mean ± SEM for three technical replicates. Note that only *spi*, *pvf1*, and *dilp3* are consistently down-regulated in *trpA1* RNAi groups in both (A) and (B). Different trends of *krn*, *vn*, and *pvf3*expression observed in (B) compared to (A) might suggest a delay, rather than reduction of these ligands by *trpA1* RNAi. (C) RT-qPCR measurement of midguts expressing *Luc* RNAi or *SERCA* RNAi in ISCs for 1d. Such early time point is chosen empirically to allow for efficient knockdown and avoid confounding effects of signaling pathway cross-activation at later stages of ISC proliferation. *rp49* is used for normalization. The data are presented as mean ± SEM for three technical replicates.

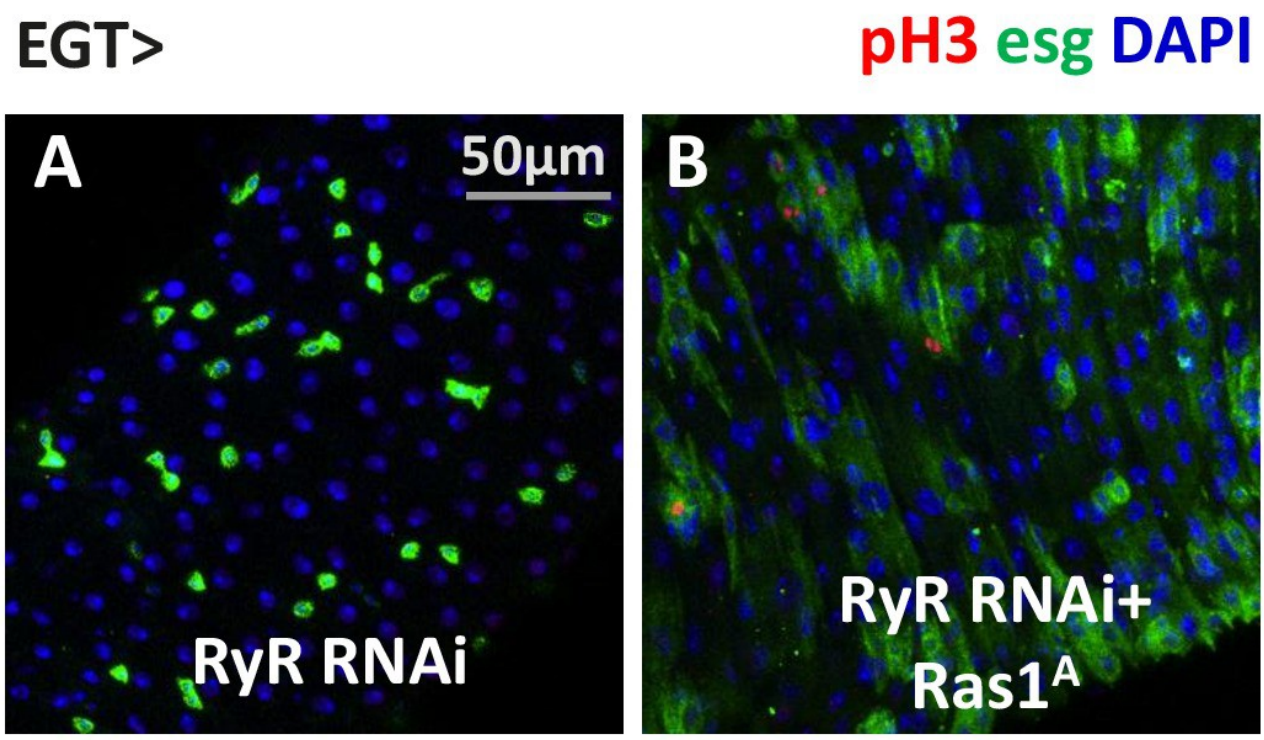

Figure 7-Figure Supplement 3

Ras/MAPK activity is sufficient for ISC proliferation in the absence of RyR.

(A–B) Midguts, expressing *RyR* RNAi alone, or together with *SERCA* RNAi in ISCs for 5d, are stained for mitosis marker pH3.