
Figures and figure supplements

Relish plays a dynamic role in the niche to modulate *Drosophila* blood progenitor homeostasis in development and infection

Parvathy Ramesh *et al*

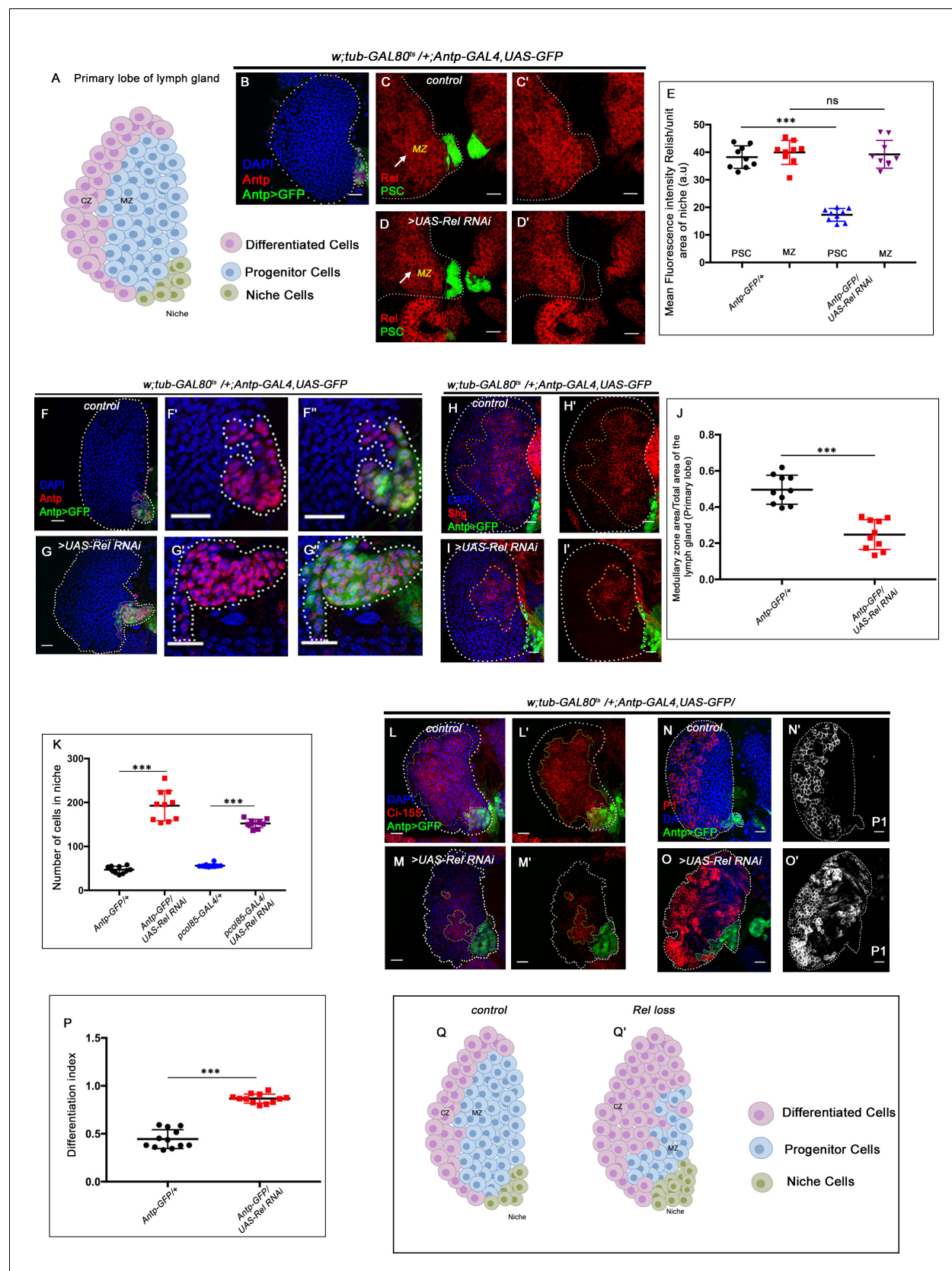


Figure 1. Relish expression and its function in hematopoietic niche of *Drosophila* larval lymph gland. Genotypes are mentioned in relevant panels. Scale bar: 20 μ m. (A) Schematic representation of *Drosophila* larval lymph gland with its different cell types. (B) Hematopoietic niche in larval lymph

Figure 1 continued on next page

Figure 1 continued

gland visualized by *Antp-Gal4,UAS-GFP* and Antennapedia (*Antp*) antibody. (C–D') Expression of Relish (antibody: red) in larval lymph gland. (C) Relish is expressed in the hematopoietic niche of lymph gland and in the progenitor population. (C') Zoomed in view of the niche showing the expression of Relish in control niche. (D–D') Relish expression is abrogated in the niche upon RNAi mediated downregulation. (E) Quantitation of Relish expression in the niche. Significant reduction in Relish expression was observed in niche ($n=10$, $p\text{-value}=7.4 \times 10^{-9}$, two-tailed unpaired Student's t-test), whereas progenitor-specific expression remained unchanged ($n=10$, $p\text{-value}=0.764$, two-tailed unpaired Student's t-test). (F–G'') Effect of Relish loss from the niche on cell proliferation (F–F''), *Antp* expression marks the niche of wild-type lymph gland. (G–G'') Loss of Relish function from niche leads to increase in niche cell number. (H–I') Hematopoietic progenitors of larval lymph gland (red, reported by DE-Cadherin [Shg] immunostaining). Compared to control (H–H'), drastic reduction in progenitor pool was observed when Relish function was attenuated from niche (I–I'). (J) Quantitation of Shg-positive progenitor population upon Relish knockdown from the niche using *Antp-GAL4* ($n=10$, $p\text{-value}=8.47 \times 10^{-6}$, two-tailed unpaired Student's t-test). (K) Quantitation of niche cell number upon Relish knockdown from the niche using *Antp-GAL4* ($n=10$, $p\text{-value}=1.3 \times 10^{-7}$, two-tailed unpaired Student's t-test) and *pcol85-GAL4* ($n=11$, $p\text{-value}=1.2 \times 10^{-12}$, two-tailed unpaired Student's t-test). (L–M') Hematopoietic progenitors of larval lymph gland (red, reported by Ci^{155} immunostaining) (L–L'). Loss of Relish from the niche resulted in reduction in Ci^{155} -positive progenitor pool (M–M'). (N–O') Compared to control (N–N'), increase in the amount of differentiated cell population (red, P1 immunostaining) was observed upon niche-specific downregulation of Relish (O–O'). (P) Quantitative analysis of (N–O') reveals significant increase in the amount of differentiated cells in comparison to control ($n=10$, $p\text{-value}=2.3 \times 10^{-9}$, two-tailed unpaired Student's t-test). (Q–Q') Scheme based on our observation. The white dotted line mark whole of the lymph gland in all cases and niche in (F–G''). Yellow dotted lines mark the progenitor zone in (H–I') and (L–M'). In all panels, age of the larvae is 96 hr AEH. The nuclei are marked with DAPI (blue). Error bar: standard deviation (SD). Individual dots represent biological replicates. Data are mean \pm SD. * $p<0.05$, ** $p<0.01$, and *** $p<0.001$.

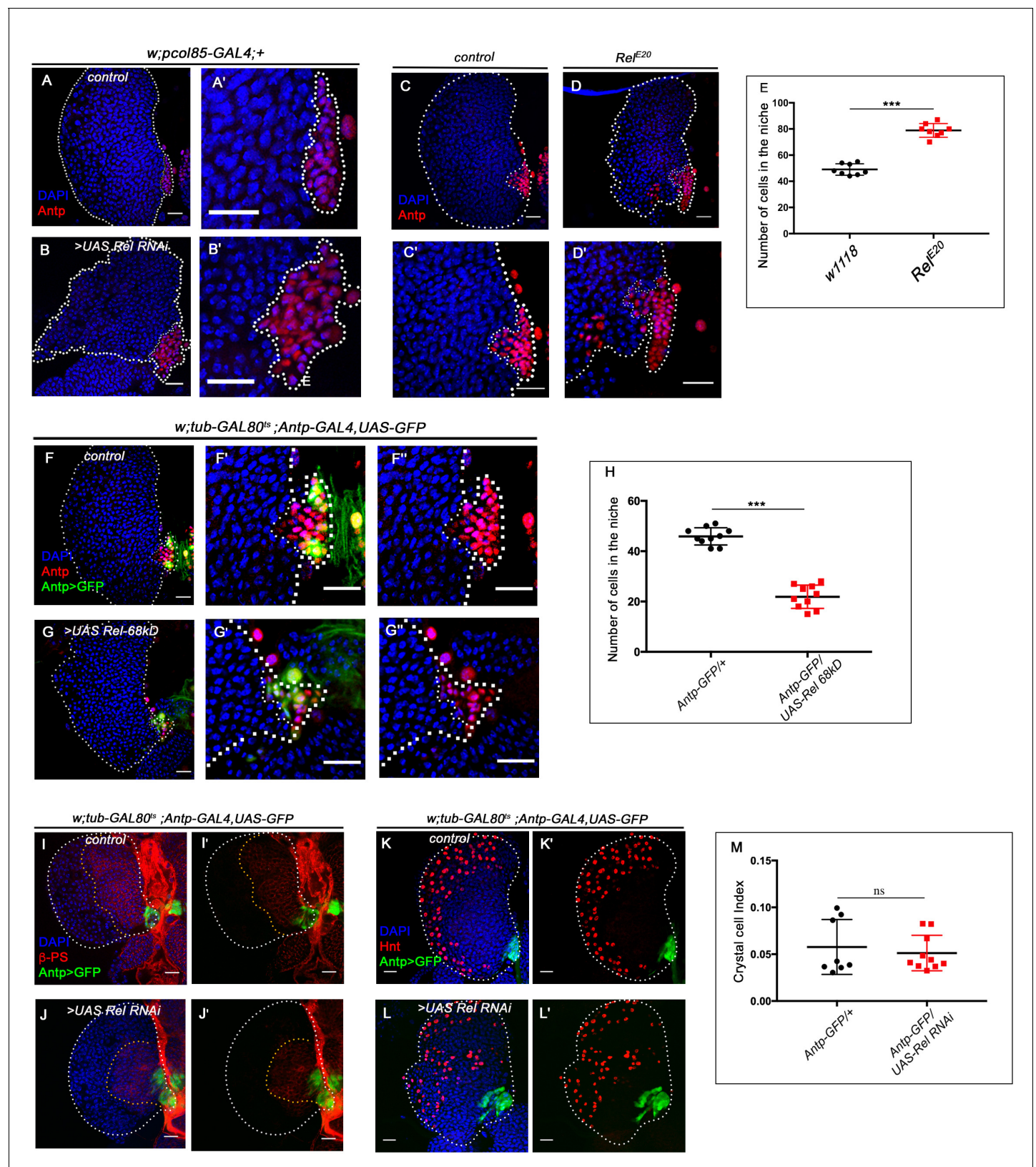


Figure 1—figure supplement 1. Relish negatively regulate niche cell proliferation. Genotypes of the larvae are mentioned in respective panels. Scale bar: 20 μ m. (A–B') Effect of Relish loss from the niche using an independent GAL4 line, *pcol85-GAL4*. Compared to control (A–A'), downregulation of Relish from the niche using *pcol85-GAL4* (B–B') also leads to increased niche cell proliferation. (C–D') A substantial increase in niche number was observed in *Rel^{E20}* larvae. (E) Quantification of the number of cells in the niche for control and *Rel^{E20}* larvae. (F) Quantification of the number of cells in the niche for control and *Antp-GFP/+* larvae. (G–I') Effect of Relish loss from the niche using an independent GAL4 line, *pcol85-GAL4*. Compared to control (G–G'), downregulation of Relish from the niche using *pcol85-GAL4* (H–H') also leads to increased niche cell proliferation. (J) Quantification of the number of cells in the niche for control and *Antp-GFP/+* larvae. (K) Quantification of the number of cells in the niche for control and *Antp-GFP/UAS-Rel 68kD* larvae. (L) Quantification of the number of cells in the niche for control and *Antp-GFP/+* larvae. (M) Quantification of the number of cells in the niche for control and *Antp-GFP/UAS-Rel RNAi* larvae. (N) Quantification of the number of cells in the niche for control and *Antp-GFP/+* larvae. (O) Quantification of the number of cells in the niche for control and *Antp-GFP/UAS-Rel RNAi* larvae.

Figure 1—figure supplement 1 continued

observed in Relish mutant (*Rel^{E20}*) (D–D') when compared to control (C–C'). (E) Quantitation of niche cell number in *Rel^{E20}* mutant in comparison to control (n=8, p-value= 9.03×10^{-9} , two-tailed unpaired Student's t-test). (F–G'') In comparison to control (F–F''), overexpression of Relish in the niche resulted in a reduction in niche cell number (G–G''). (H) Quantitation of niche cell number in Relish overexpression in comparison to control (n=10, p-value= 3.3×10^{-10} , two-tailed unpaired Student's t-test). (I–J') Lamellocytes were not observed in Relish loss scenario (red, integrin β -PS-immunostaining). Loss of β -PS-positive progenitor pool is further evident in Relish loss scenario compared to control (compare J–J' to I–I') (K–L') In comparison to the control (K–K'), no significant change in crystal cell index (number of crystal cells/total number of cells in the lobe) was observed in Relish downregulation scenario (L–L'). (M) Quantitative analysis of crystal cell index in both control and Relish loss condition (n=8, p-value=0.596, two-tailed unpaired Student's t-test). The white dotted line mark whole of the lymph gland in all cases and niche in A' and B', C' and D', F'–F'', and G'–G''. Yellow dotted lines mark the progenitor zone in (I–J') The nuclei are marked with DAPI (blue). In all panels, the age of the larvae is 96 hr AEH. Individual dots represent biological replicates. Error bar: standard deviation (SD). Data are mean \pm SD. *p<0.05, **p<0.01, and ***p<0.001.

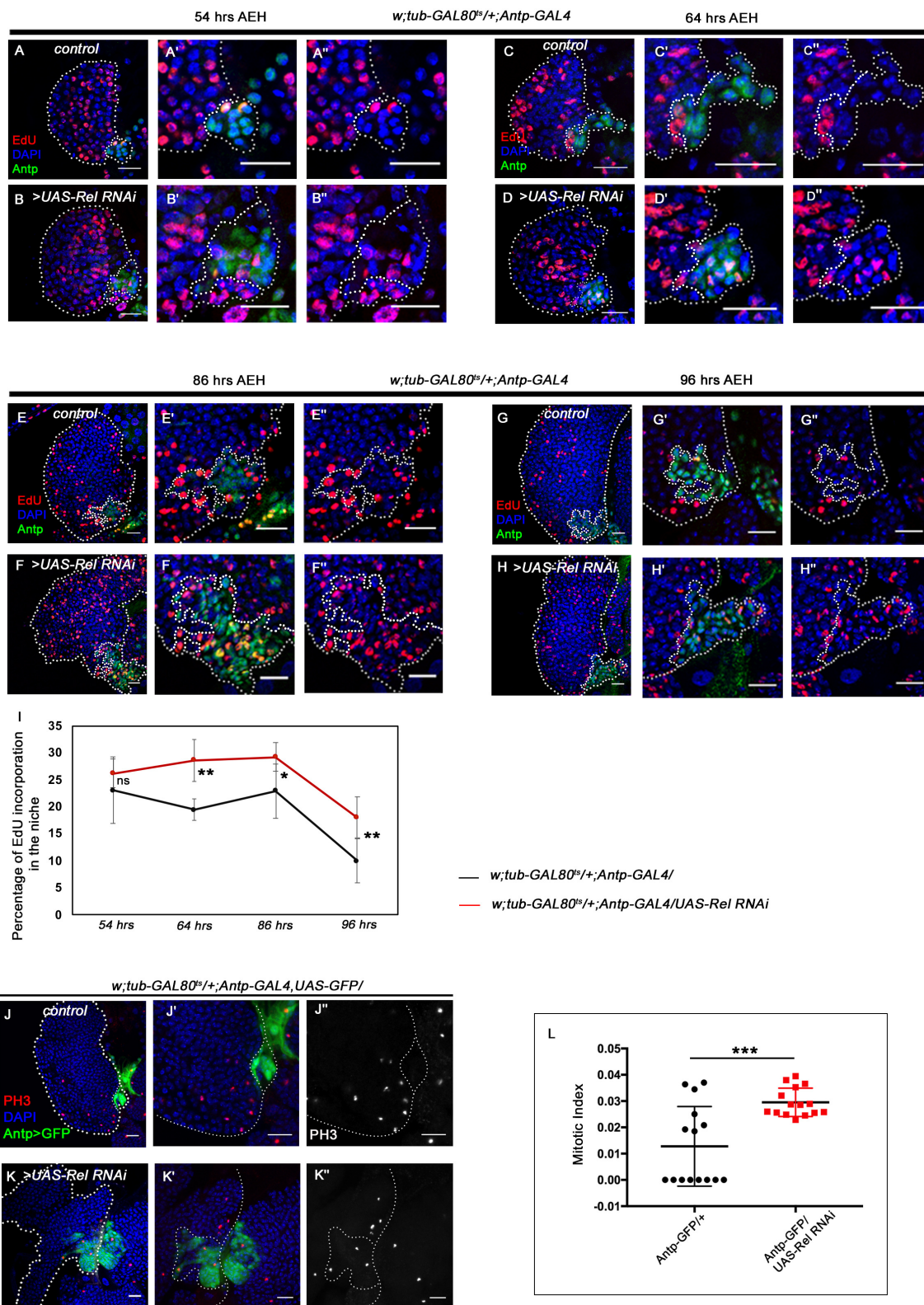


Figure 2. Loss of Relish from the niche causes niche cell hyperplasia. Genotypes are mentioned in relevant panels. Scale bar: 20 μ m. Niche is visualized by Antp antibody expression. (A–H'') EdU or 5-ethynyl-2'-deoxyuridine marks the cells in S-phase of the cell cycle. EdU profiling at 54 hr AEH (A–B''), 64 Figure 2 continued on next page

Figure 2 continued

hr AEH (C–D’), 86 hr AEH (E–F’), and 96 hr AEH (G–H’)) displayed EdU incorporation in the niche (green) in control and upon Relish downregulation. Control niches showed scanty EdU incorporation beyond 84 hr (E–E’ and G–G’), whereas loss of Relish induced niche cells to proliferate more (F–F’ and H–H’). (I) Graph representing percentage of EdU incorporation in the niche during the course of development in control (black line) and Relish loss (red line). Significant increase in the niche cell number is observed with development in Relish loss scenario. (54 hr, n=6, p-value=0.294), (64 hr, n=6, p-value=1.3 × 10^{−3}), (86 hr, n=6, p-value=2.9 × 10^{−2}), (96 hr, n=6, p-value=5.9 × 10^{−3}); two-tailed unpaired Student’s t-test. (J–K’’) Significant increase in the number of mitotic cells (phospho-histone 3 [PH3], red) was observed upon Relish loss from the niche (K–K’’) compared to the control (J–J’’). (L) Quantitation of the mitotic index of wild-type and Relish loss niche (n=15, p-value=8.1 × 10^{−4}; two-tailed unpaired Student’s t-test). The white dotted line marks whole of the lymph gland and the niches. In all panels, age of the larvae is 96 hr AEH, unless otherwise mentioned. The nuclei are marked with DAPI (blue). Individual dots represent biological replicates. Error bar: standard deviation (SD). Data are mean ± SD. *p<0.05, **p<0.01, and ***p<0.001.

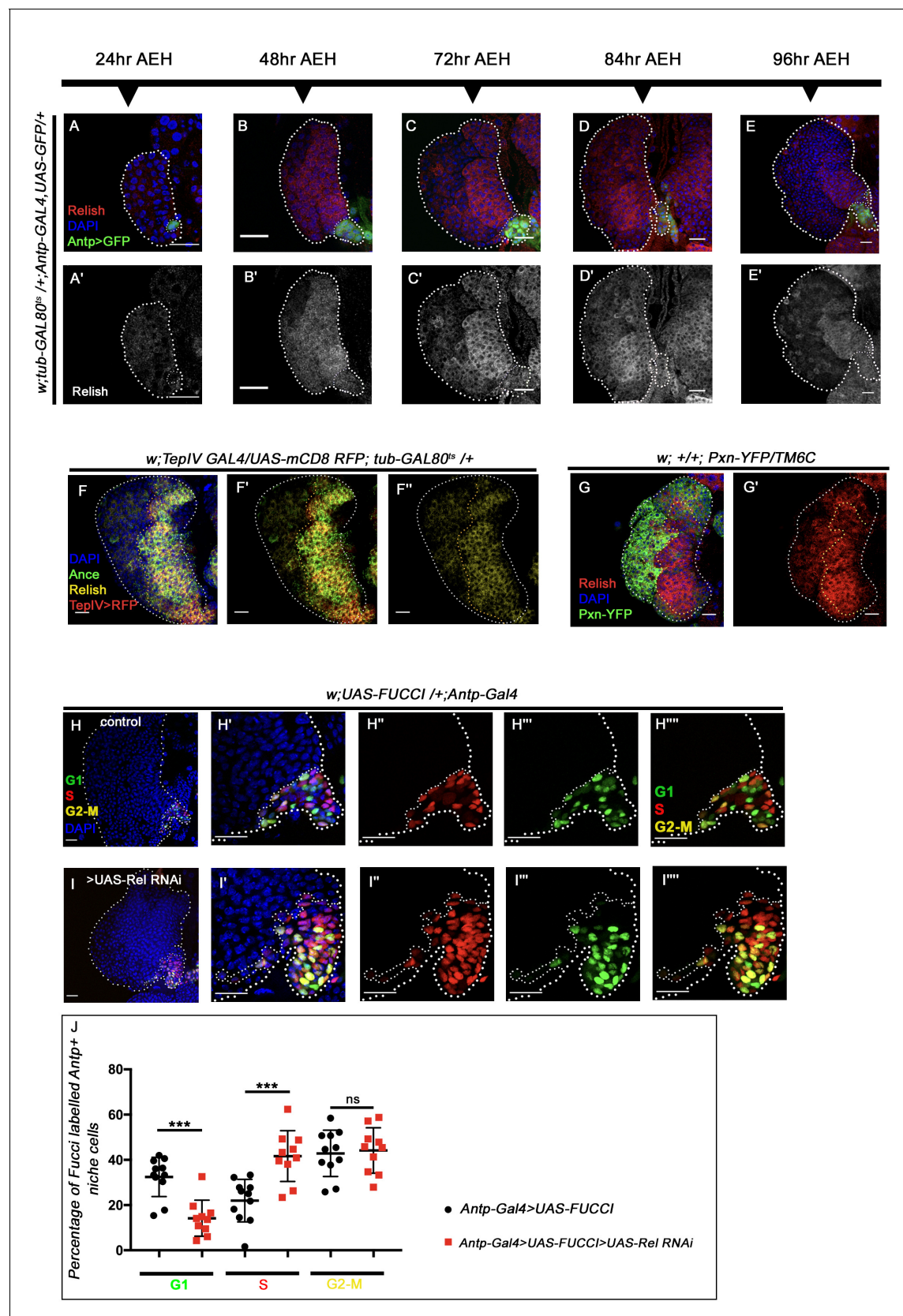


Figure 2—figure supplement 1. Relish expression starts beyond the second-instar stage in the hematopoietic niche. The genotypes are mentioned in relevant panels. Scale bar: 20 μ m. (A–E') Expression of Relish (red, by antibody) at different developmental time points in the larval lymph gland (niche). Figure 2—figure supplement 1 continued on next page

Figure 2—figure supplement 1 continued

marked with *AntpGAL4>UAS-GFP*. Observations were made at 24 hr AEH (A–A'), 48 hr AEH (B–B'), 72 hr AEH (C–C'), 84 hr AEH (D–D'), and 96 hr AEH (E–E'). Relish expression in the niche can be detected around 48 hr AEH. (F–F') Relish expression (yellow) in the progenitor cells co-localizes with prohemocyte markers *Ance* (green) and *TeplV* (red). (G–G') Relish expression (red) is restricted to progenitor cells, whereas it is downregulated in Pxn-YFP-positive differentiated cells (green). (H–I''') Cell cycle status reported by Fly-FUCCI using niche-specific GAL4: *Antp-Gal4*. In control, niche cells are mostly in G1 (green, H''') and G2-M (yellow, H''') phase, while few are in S phase (red, H''). Niche cells from where Relish function has been downregulated were mostly in S, (red, I'') and G2-M (yellow, I'''), and very less in G1 (green, I''') phase of the cell cycle. (J) Quantitative analyses of the cell cycle status of control and Relish loss niches (n=10, p-value for G1= 7.3×10^{-5} , p-value for S= 4.2×10^{-4} , p-value for G2-M = 0.657), two-tailed unpaired Student's t-test. The white dotted line marks whole of the lymph gland and the niches in (H–I'''). Yellow dotted lines mark the progenitor zone in (F–G'). In all panels. age of the larvae is 96 hr AEH, unless otherwise mentioned. The nuclei are marked with DAPI (blue). Individual dots represent biological replicates. Error bar: standard deviation (SD). Data are mean \pm SD. *p<0.05, **p<0.01, and ***p<0.001.

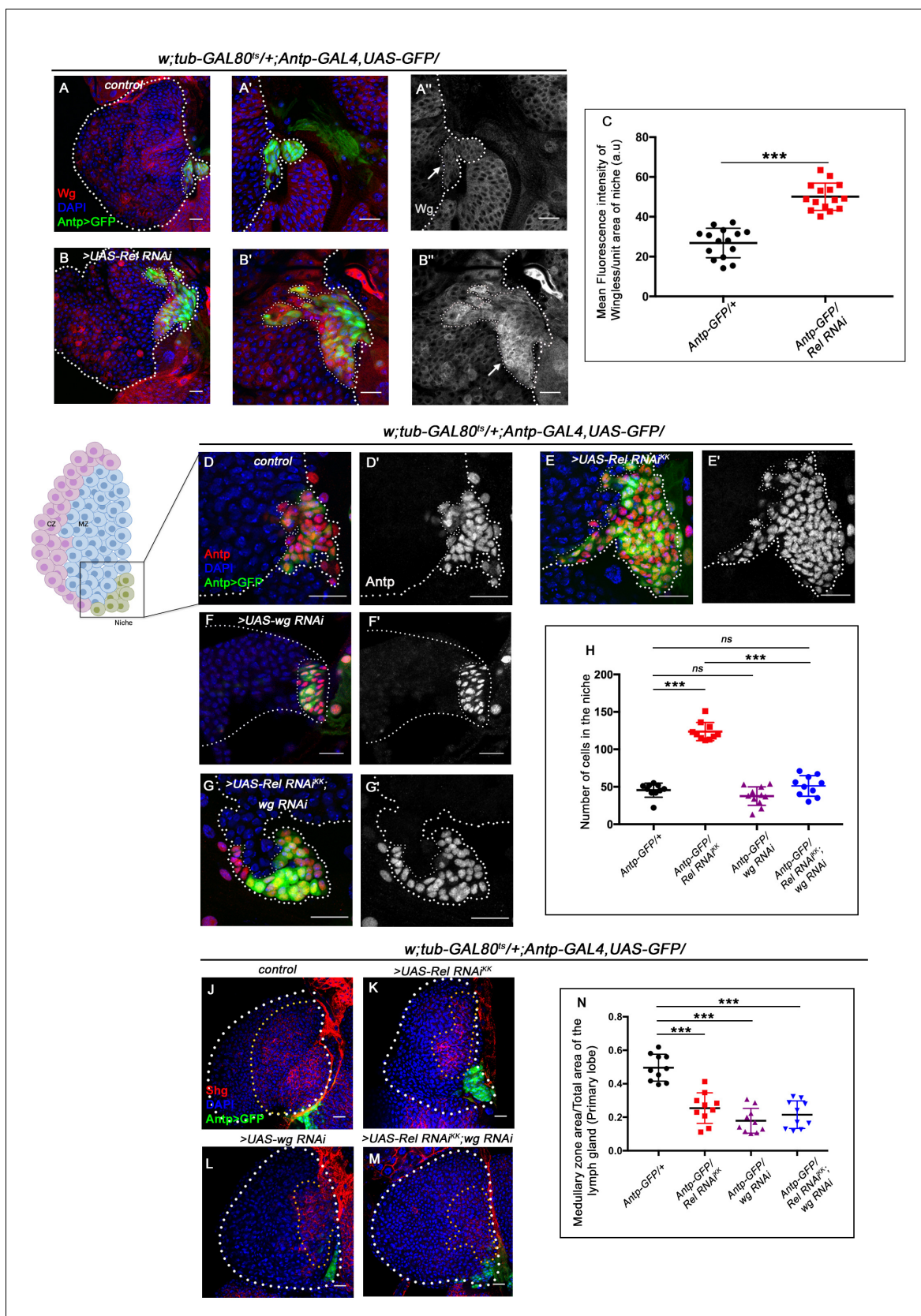


Figure 3. Upregulated Wingless signaling leads to increase in niche cell number. The genotypes are mentioned in relevant panels. Scale bar: 20 μ m. (A–B'') Expression of Wingless (antibody) in the lymph gland. The hematopoietic niche is visualized by Antp-GAL4>UAS-GFP. (A'–A'') and (B'–B'') are Figure 3 continued on next page

Figure 3 continued

higher magnifications of (A) and (B), respectively. In comparison to the wild-type niche (A–A''), Wingless protein levels were substantially high in Relish loss of function (B–B''). (C) Statistical analysis reveals elevated wingless expression upon Relish knockdown in niche ($n=15$; p -value= 5.8×10^{-9} , two-tailed unpaired Student's t-test.) (D–G') The increased niche number observed upon Relish loss (E–E') is rescued upon reducing Wingless level by the *wg RNAi* (F–F') in Relish loss genetic background (G–G'). The rescued niche cell number is comparable to control (D–D'). (H) Statistical analysis of the data in (D–G') ($n=10$, p -value= 1.1×10^{-11} for control versus *Rel RNAi^{KK}*, p -value= 3.15×10^{-10} for *Rel RNAi^{KK}* versus *Rel RNAi^{KK}; wg RNAi*, $n=10$, p -value=0.10 for control versus *wg RNAi*, $n=10$, p -value=0.29 for control versus *Rel RNAi^{KK}; wg RNAi*; two-tailed unpaired Student's t-test). (J–M) Hematopoietic progenitors of larval lymph gland (red, reported by DE-Cadherin [Shg] immunostaining). Knocking down wingless function from the niche resulted in loss of Shg-positive progenitors (L). Downregulating wingless using *wg RNAi* in Relish loss genetic background was unable to restore the reduction in prohemocyte pool (M) observed in Relish loss (K) scenario in comparison to control (J). (N) Statistical analysis of the data in (J–M) ($n=10$, p -value= 6.74×10^{-6} for control versus *Rel RNAi^{KK}*, p -value= 4.03×10^{-7} for control versus *wg RNAi*; *Rel RNAi^{KK}*, p -value= 3.42×10^{-8} for control versus *wg RNAi*; two-tailed unpaired Student's t-test). The white dotted line marks whole of the lymph gland and the niches in (A–G') Yellow dotted lines mark the progenitor zone in (J–M). In all panels, age of the larvae is 96 hr AEH. The nuclei are marked with DAPI (blue). Individual dots represent biological replicates. Error bar: standard deviation (SD). Data are mean \pm SD. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

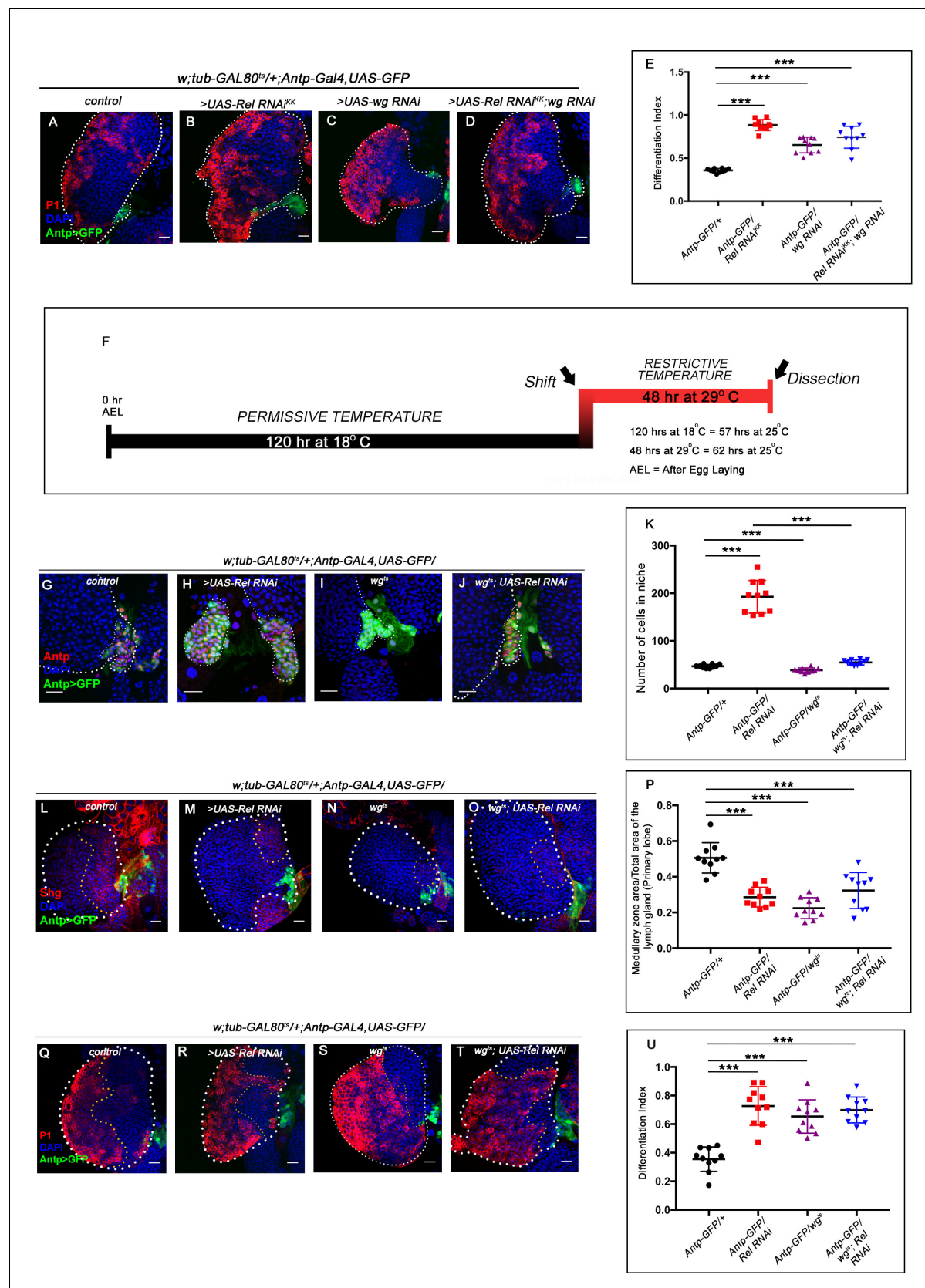


Figure 3—figure supplement 1. Downregulating wingless in Relish loss condition rescues niche cell proliferation, but not differentiation. The genotypes are mentioned in relevant panels. Scale bar: 20 μ m. (A–D) Increase in plasmatocyte population (marked by P1, red) was observed upon

Figure 3—figure supplement 1 continued on next page

Figure 3—figure supplement 1 continued

Relish (B) and wingless downregulation (C) from the niche compared to the control (A). Simultaneous downregulation of wingless function in Relish loss genetic background did not rescue the increased differentiation (D). (E) Statistical analysis of the data in (A–D) ($n=10$, $p\text{-value}=2.97 \times 10^{-9}$ for control versus *Rel RNAi^{KK}*, $p\text{-value} = 4.18 \times 10^{-5}$ for control versus *wg RNAi*; *Rel RNAi^{KK}*, $p\text{-value}=2.8 \times 10^{-4}$ for control versus *wg RNAi*; two-tailed unpaired Student's t-test). (F) Scheme depicting the temperature regime followed for the rescue experiments (G–U) for wingless mutant (*wg^{ts}*). (G–J) The increased niche number observed upon Relish loss (H) is rescued upon reducing Wingless level by the temperature-sensitive allele *wg^{ts}* (I) in Relish loss genetic background (J). The rescued niche cell number is comparable to control (G). (K) Statistical analysis of the data in (G–J) ($n=10$; $p\text{-value}=2.4 \times 10^{-7}$ for control versus Relish RNAi, $p\text{-value}=4.3 \times 10^{-4}$ for control versus *wg^{ts}* and $p\text{-value} = 3.4 \times 10^{-7}$ for *wg^{ts}*; Relish RNAi versus Relish RNAi; two-tailed unpaired Student's t-test). (L–O) Hematopoietic progenitors of larval lymph gland (red, reported by DE-Cadherin [Shg] immunostaining). Knocking down wingless function using *wg^{ts}* resulted in loss of Shg-positive progenitors (N). Downregulating *wg* function in Relish loss genetic background was unable to restore the reduction in prohemocyte pool (O) observed in Relish loss (M) scenario in comparison to control (L). (P) Statistical analysis of the data in (L–O) ($n=10$; $p\text{-value}=4.80 \times 10^{-6}$ for control versus *Rel RNAi*, $p\text{-value}=3.8 \times 10^{-4}$ for *wg^{ts}*; *Rel RNAi* versus control, $p\text{-value}=2.18 \times 10^{-7}$ for control versus *wg^{ts}*; two-tailed unpaired Student's t-test). (Q–T) Increase in plasmacyte population (marked by P1, red) was observed upon wingless (S) and Relish down regulation from the niche (R) compared with the control (Q). Simultaneous downregulation of wingless function using *wg^{ts}* in Relish loss genetic background did not rescue the increased differentiation (T). (U) Statistical analysis of the data in (Q–T) ($n=10$, $p\text{-value}=2.1 \times 10^{-6}$ for control versus *Rel RNAi*; $p\text{-value}=5.9 \times 10^{-6}$ for control versus *wg^{ts}*, $p\text{-value}=6.8 \times 10^{-8}$ for control versus *wg^{ts}*; *Rel RNAi*; two-tailed unpaired Student's t-test). The white dotted line marks whole of the lymph gland and the niches in (A–D) and (G–J). Yellow dotted lines mark the progenitor zone in (L–O) and (Q–T). In all panels, age of the larvae is 96 hr AEH. The nuclei are marked with DAPI (blue). Individual dots represent biological replicates. Error bar: standard deviation (SD). Data are mean \pm SD. * $p<0.05$, ** $p<0.01$, and *** $p<0.001$.

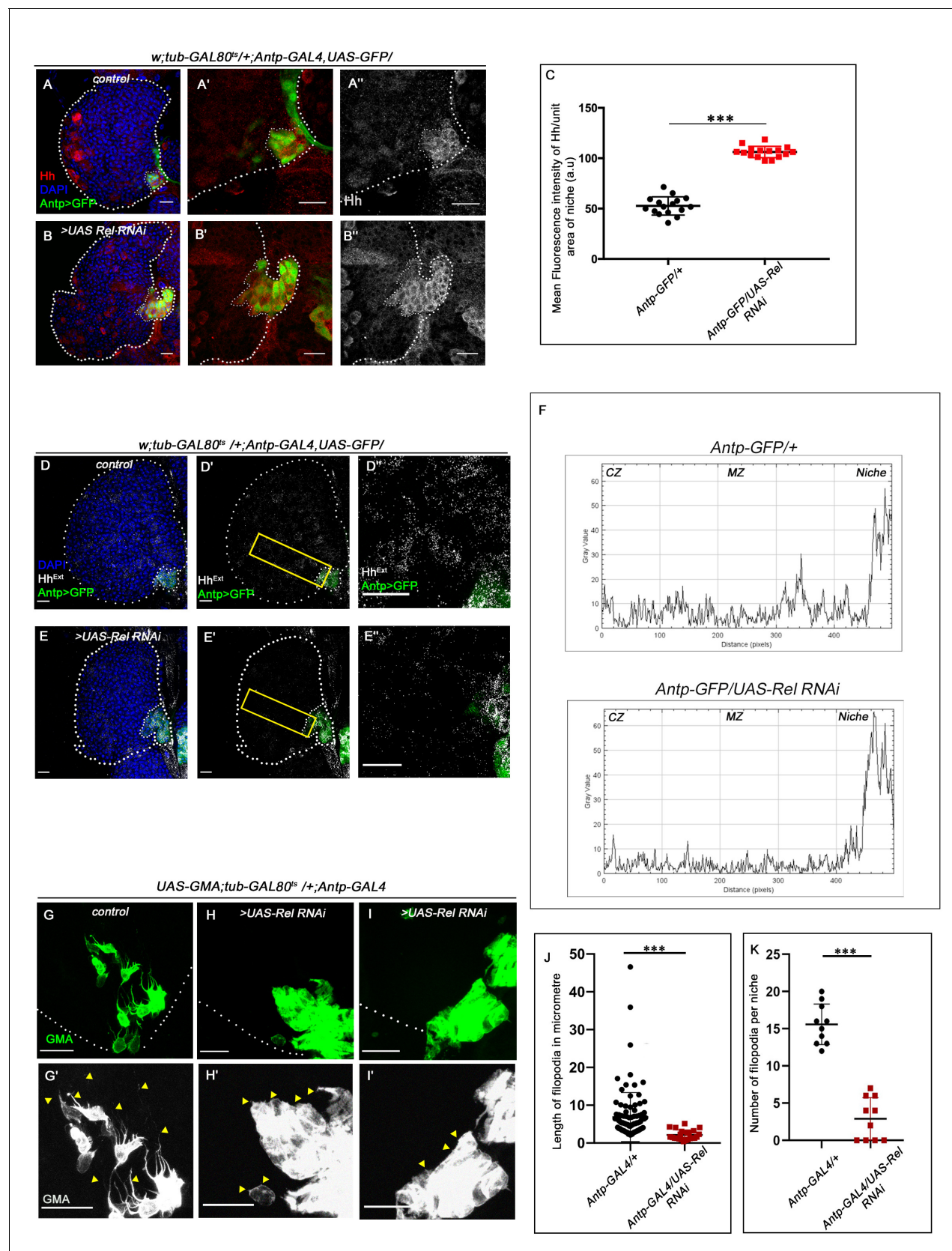


Figure 4. Hedgehog release from the niche is affected in Relish loss of function. The genotypes are mentioned in relevant panels. Scale bar: 20 μ m. (A–B'') Hedgehog (Hh) antibody staining in the lymph gland shows Hh enrichment in the niche. The hematopoietic niche in Relish loss of function (B–B'')

Figure 4 continued on next page

Figure 4 continued

exhibits higher level of Hh in comparison to the control (**A–A''**). (**C**) Statistical analysis of fluorescence intensity revealed more than 2.5-fold increase in Hh levels compared to control ($n=15$, $p\text{-value}=2.5 \times 10^{-17}$, two-tailed Students t-test). (**D–E''**) Progenitors in Relish loss of function exhibits lower level of Extracellular Hh (Hh^{Extra}) (**E–E''**) in comparison to those of control (**D–D''**). (**E''** and **D''**) are zoomed in view of niche and the neighboring progenitor cells of (**E'** and **D'**), respectively. The yellow box denotes the area quantified in (**F**). (**F**) The intensity profile of Hh^{Extra} in progenitors (along the rectangle drawn from PSC to cortical zone housing differentiated cells in **D'** and **E'**) reflects a stark decline in the level of Hh^{Extra} in Relish loss scenario compared to control. (**G–I'**) Cellular filopodia emanating from the niche cells were stabilized by using untagged phalloidin. The filopodia in Relish loss of function niches were found to be smaller in length and fewer in number (**H–H'**, **I–I'**) as compared to control (**G–G'**). (**J–K**) Significant reduction in filopodial length (**J**, $n=10$, $p\text{-value}=6.64 \times 10^{-9}$, two-tailed Student's t-test) and number (**K**, $n=6$, $p\text{-value}=9.19 \times 10^{-10}$, two-tailed Student's t-test) were observed in Relish loss scenario compared to control. The white dotted line marks whole of the lymph gland and niches in **A–B''**, **D–E'**. In all panels, age of the larvae is 96 hr AEH. The nuclei are marked with DAPI (blue). Individual dots represent biological replicates. Error bar: standard deviation (SD). Data are mean \pm SD. * $p<0.05$, ** $p<0.01$, and *** $p<0.001$.

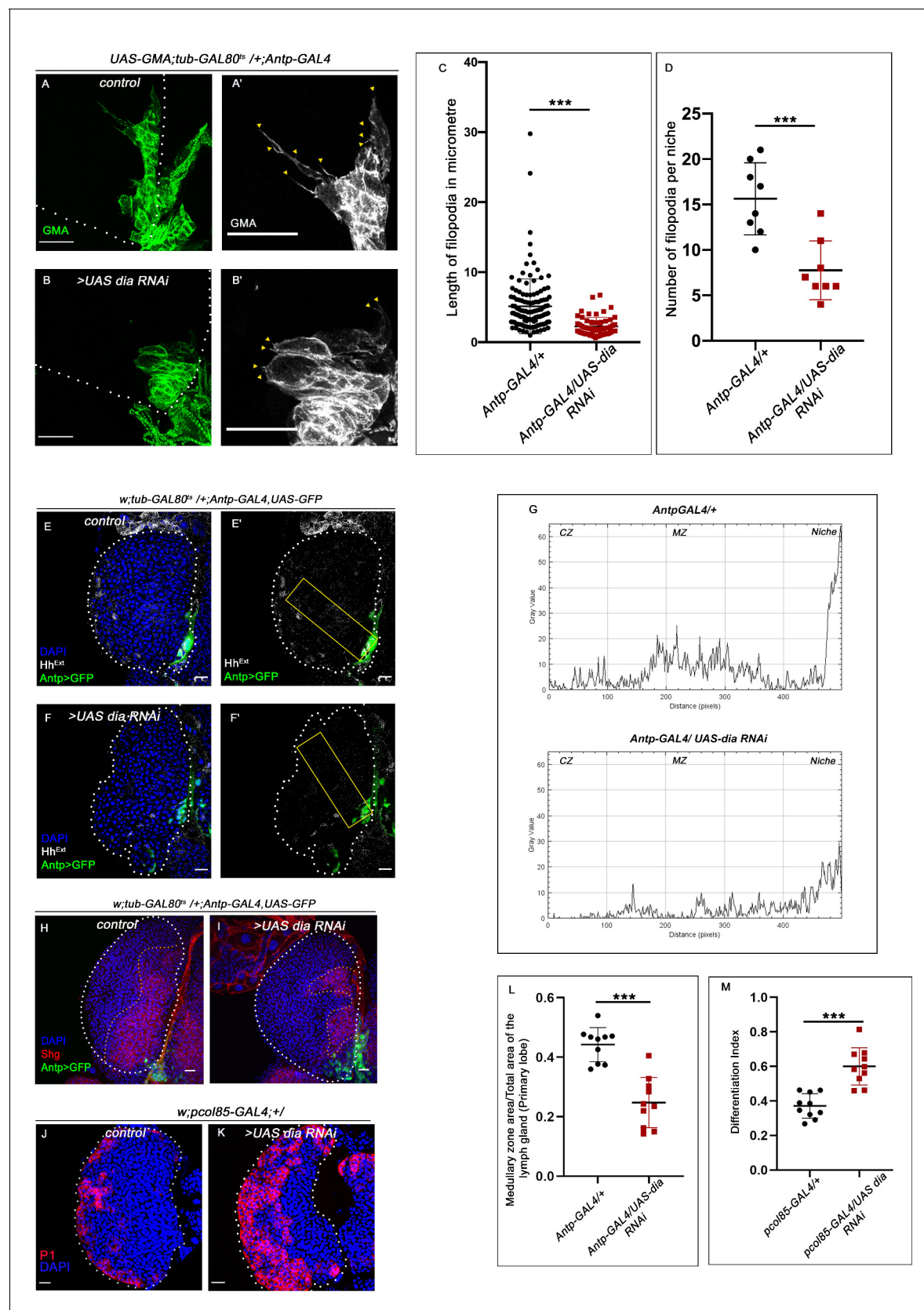


Figure 4—figure supplement 1. Loss of Diaphanous from the niche resulted in defect in filopodial formation and enhanced differentiation. The genotypes are mentioned in relevant panels. Scale bar: 20 μ m. (A–B') The filopodia in *dia* loss of function niches were found to be smaller in length and Figure 4—figure supplement 1 continued on next page

Figure 4—figure supplement 1 continued

fewer in number (**B–B'**) as compared to control (**A–A'**). (**C–D**) Significant reduction in filopodial lengths (**C**, $n=8$, $p\text{-value}=3.73 \times 10^{-12}$, two-tailed Student's t-test) and number (**D**, $n=8$, $p\text{-value}=7.2 \times 10^{-4}$, two-tailed Student's t-test) was observed in *dia* loss scenario compared to control. (**E–F'**) Progenitors in *dia* loss of function from niche exhibits lower level of extracellular Hh (Hh^{Extra}) (**F–F'**) in comparison to those of control (**E–E'**). The yellow box denotes the area quantified in (**G**). (**G**) The intensity profile of Hh^{Extra} in progenitors (along the rectangle drawn from niche to Cortical zone housing differentiated cells in **Figure 4E' and F'**) reflects a stark decline in the level of Hh^{Extra} in *dia* loss scenario compared to control. (**H–I**) Knocking down *dia* function resulted in loss of Shg-positive progenitors (**I**) compared to control (**H**). (**L**) Statistical analysis of the data in (**H–I**) ($n = 10$, $p\text{-value}=1.8 \times 10^{-5}$; two-tailed Student's t-test). (**J–K**) Loss of *dia*, from the niche caused ectopic differentiation of progenitors (**K**) compared to control (**J**). (**M**) Differentiation index for *dia* loss niches compared to control ($n=10$, $p\text{-value}=4.28 \times 10^{-5}$; two-tailed Student's t-test). The white dotted line mark whole of the lymph gland in all cases. Yellow dotted lines mark the progenitor zone in (**H**)–(**I**). In all panels, age of the larvae is 96 hr AEH. The nuclei are marked with DAPI (blue). Data are mean \pm SD. * $p<0.05$, ** $p<0.01$, and *** $p<0.001$.

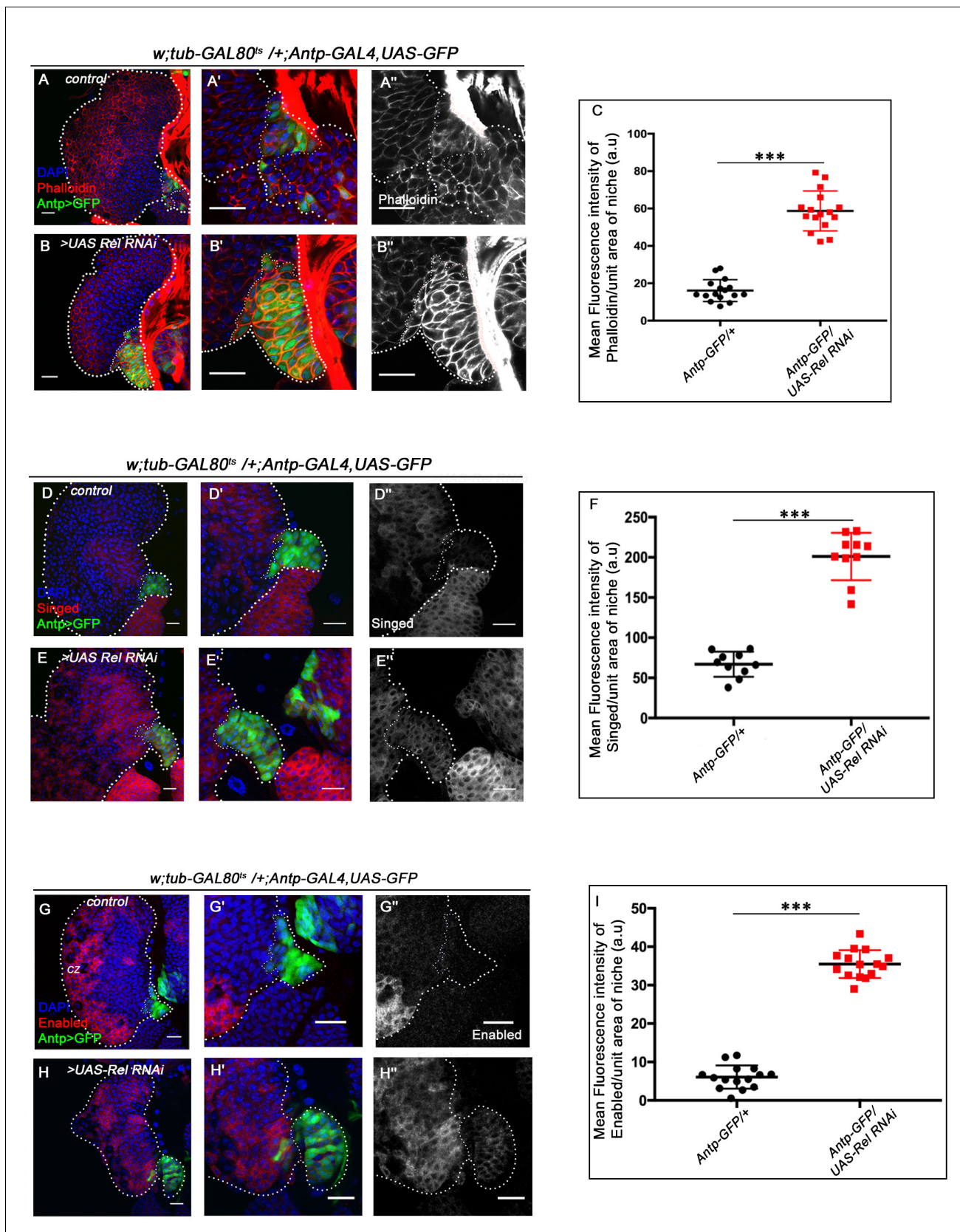


Figure 4—figure supplement 2. Loss of Relish from the niche resulted in upregulation of actin remodellers. The genotypes are mentioned in relevant panels. Scale bar: 20 μ m. (A–B'') F-actin (visualized by Phalloidin, red) highly enriched in the plasma membrane of niche cells where Relish function is

Figure 4—figure supplement 2 continued on next page

Figure 4—figure supplement 2 continued

downregulated (**B–B''**) in comparison to that of control (**A–A''**). (**C**) Statistical analysis of fluorescence intensity showed significant increase in F-actin in Relish loss niches compared to control ($n=10$, $p\text{-value}=5.6 \times 10^{-9}$, two-tailed Student's t-test). (**D–E''**) Expression of Singed, an actin-bundling protein, is significantly upregulated in Relish loss niches (**E–E''**) compared to control (**D–D''**). (**F**) Statistical analysis of fluorescence intensity showed significant increase in Singed expression in Relish loss niches compared to control ($n=15$, $p\text{-value}=7.0 \times 10^{-13}$, two-tailed Student's t-test). (**G–H''**) Enabled an actin polymerase, which is normally absent from the niche cells of control (**G–G''**) is upregulated upon Relish downregulation (**H–H''**). (**I**) Statistical analysis of fluorescence intensity showed significant increase in Ena expression in Relish loss niches compared to control ($n=15$, $p\text{-value}=8.1 \times 10^{-20}$, two-tailed Student's t-test). The white dotted line mark whole of the lymph gland and the niches in all cases. In all panels age of the larvae is 96 hr AEH. The nuclei are marked with DAPI (blue). Individual dots represent biological replicates. Error bar: standard deviation (SD). Data are mean \pm SD. * $p<0.05$, ** $p<0.01$, and *** $p<0.001$.

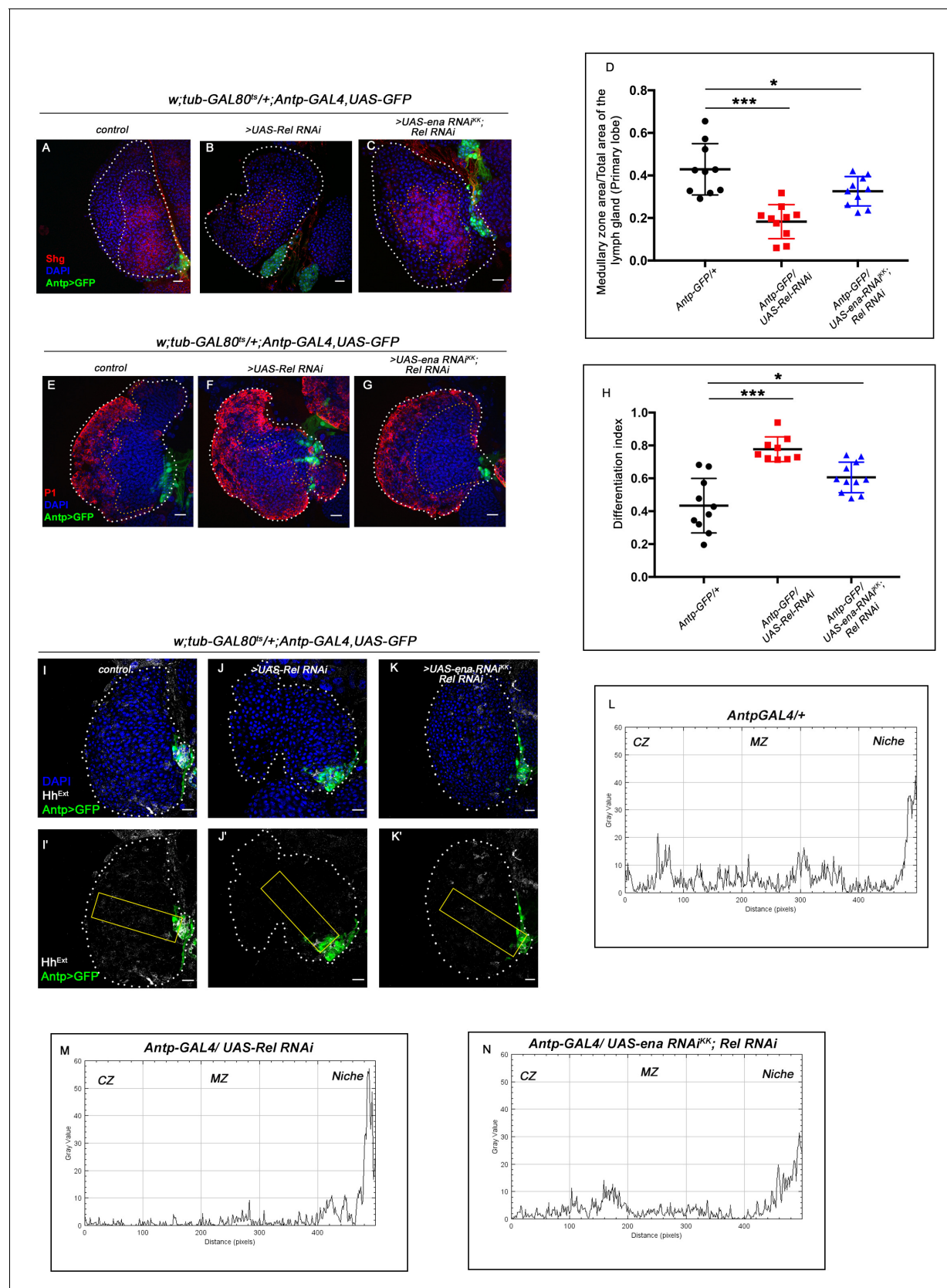


Figure 4—figure supplement 3. Downregulation of Ena in Rel loss genetic condition partially rescues the differentiation and Hh^{Extra} dispersal defects. The genotypes are mentioned in relevant panels. Scale bar: 20 μ m. (A–C) Upon simultaneous knockdown of both Rel and Ena from the niche, the

Figure 4—figure supplement 3 continued on next page

Figure 4—figure supplement 3 continued

decrease in Shg-positive progenitors observed in Relish loss (**B**) was partially rescued (**C**) compared to control (**A**). (**D**) Statistical analysis of the data in (**A–C**) ($n=10$, $p\text{-value}=6.8 \times 10^{-5}$ for control versus *Rel RNAi*, $p\text{-value}=3.4 \times 10^{-2}$ for *ena RNAi^{KK}*; *Rel RNAi* versus control; two-tailed unpaired Student's t-test). (**E–G**) Differentiation defects observed in Rel loss (**F**) was partially rescued when both Rel and Ena was simultaneously downregulated from the niche (**G**) compared to the control (**E**). (**H**) Statistical analysis of the data in (**E–G**) ($n=10$, $p\text{-value}=5.5 \times 10^{-5}$ for control versus *Rel RNAi*, $p\text{-value}=1.1 \times 10^{-2}$ for *ena RNAi^{KK}*; *Rel RNAi* versus control; two-tailed unpaired Student's t-test). (**I–K'**) Reduced extracellular Hh observed in the progenitors (Hh^{Ext}) of Rel loss of function condition (**J–J'**), in comparison to those of control (**I–I'**), is partially rescued in simultaneous loss of both Rel and Ena from the niche (**K–K'**). The yellow box in **I'**, **J'**, and **K'** denotes the area quantified in **L**, **M**, and **N**, respectively. (**L–N**) The intensity profile of Hh^{Extra} in progenitors (along the rectangle drawn from niche to Cortical zone housing differentiated cells in **I'–K'**) reflects a stark decline in the level of Hh^{Extra} in Rel loss scenario (**M**) compared to control (**L**) and a partial rescue when both Rel and Ena was downregulated simultaneously (**N**). The white dotted line mark whole of the lymph gland in all cases. Yellow dotted lines mark the progenitor zone in (**A–C** and **E–G**). In all panels, age of the larvae is 96 hr AEH. The nuclei are marked with DAPI (blue). Individual dots represent biological replicates. Error bar: standard deviation (SD). Data are mean \pm SD. * $p<0.05$, ** $p<0.01$, and *** $p<0.001$.

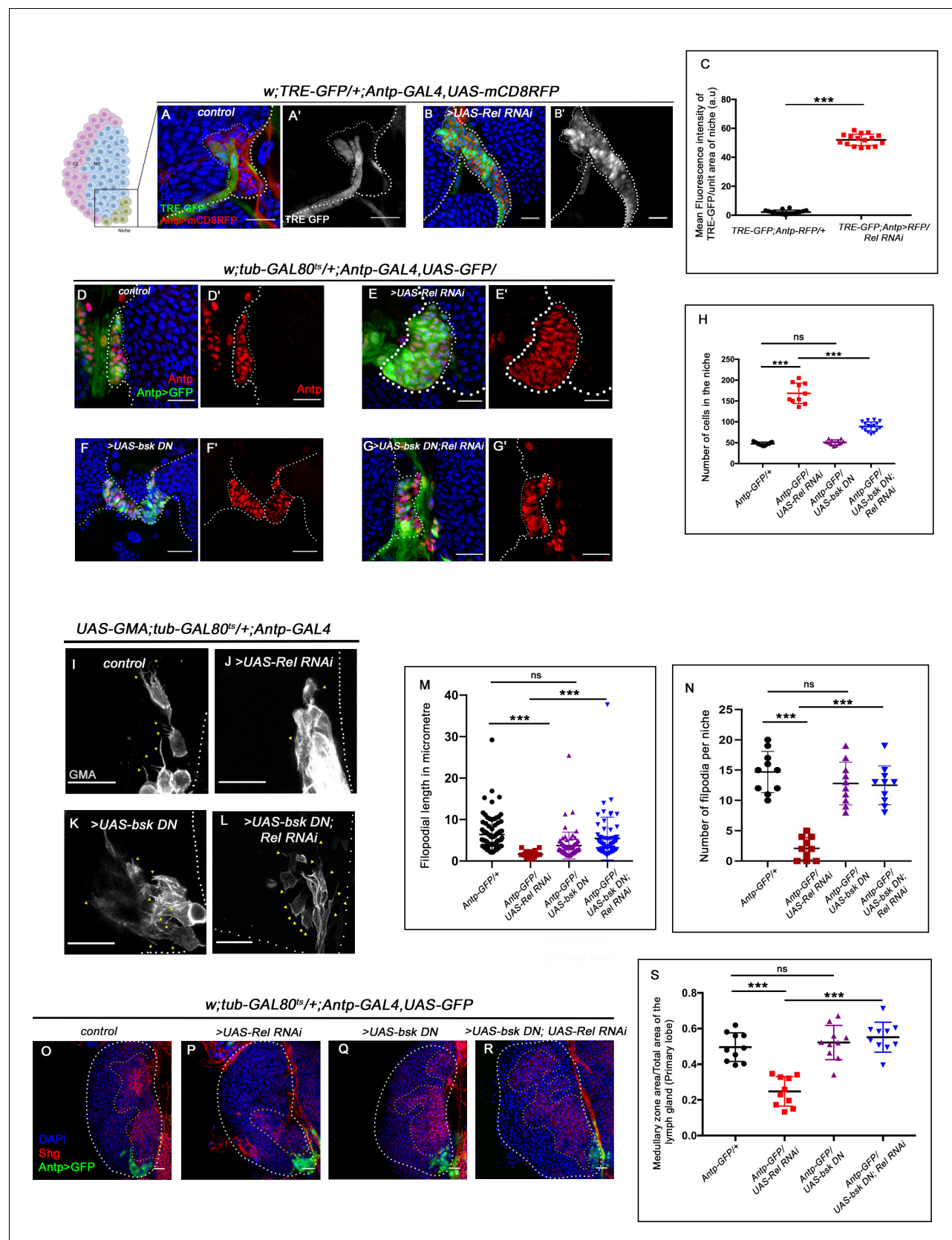
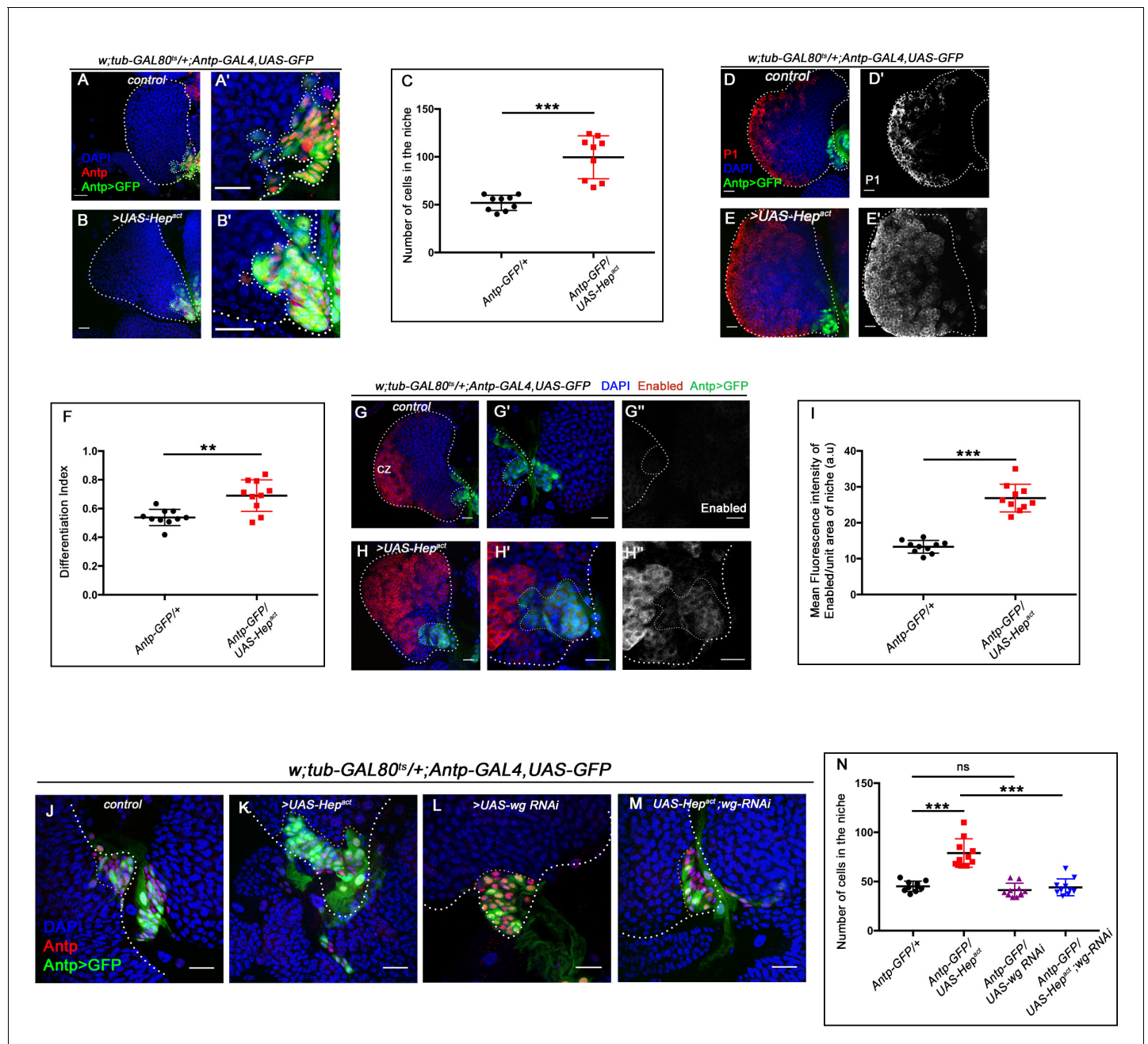


Figure 5. Loss of Relish from the niche activated JNK causing niche hyperplasia. The genotypes are mentioned in relevant panels. Scale bar: 20 μ m. (A–B') Upregulation of JNK signaling visualized by its reporter TRE-GFP (green) in Relish knockdown (B–B') compared with WT niche (A–A'). (C) Statistical analysis of TRE-GFP intensity. (D–G') Antp-GFP expression in control, Rel knockdown, and bsk knockdown niches. (H) Statistical analysis of cell numbers. (I–L) GMA staining in control, Rel knockdown, and bsk knockdown niches. (M–N) Statistical analysis of filopodial length and number. (O–R) DAPI, Shg, and Antp-GFP staining in control, Rel knockdown, and bsk knockdown niches. (S) Statistical analysis of medullary zone area.

Figure 5 continued

analysis of fluorescence intensity (**A–B'**) revealed a significant increase in TRE-GFP levels compared to control ($n=15$, $p\text{-value}=4.2 \times 10^{-19}$, two-tailed Student's t-test). (**D–G'**) Upon niche-specific simultaneous knockdown of Rel and JNK, the niche hyperplasia observed upon loss of Relish (**E–E'**) is rescued (**G–G'**) and is comparable to control (**D–D'**) whereas loss of *bsk* from the niche does not alter niche cell number (**F–F'**). (**H**) Statistical analysis of the data in (**D–G'**) ($n=10$, $p\text{-value}=5.6 \times 10^{-8}$ for control versus *Rel RNAi*, $p\text{-value}=8.0 \times 10^{-7}$ for *bsk DN*; *Rel RNAi* versus *Rel RNAi*, $p\text{-value}=0.10$ control versus for *bsk DN*; two-tailed unpaired Student's t-test). (**I–N**) Cellular filopodia from the niche cells in Rel loss of function is found to be smaller in length and fewer in numbers (**J and M–N**). Simultaneous loss of both JNK using *bsk DN* and Relish (**L and M–N**) rescued the stunted, scanty filopodia to control state (**I and M–N**), whereas loss of JNK did not affect filopodia formation (**K and M–N**). (**M–N**) Statistical analysis of the data in (**I–L**) (Filopodia number: $n=10$, $p=6.96 \times 10^{-8}$ for control versus *Rel RNAi*, $p\text{-value}=8.11 \times 10^{-7}$ for *bsk DN*; *Rel RNAi* versus *Rel RNAi*, $p\text{-value}=0.153$ for *bsk DN* versus control. Filopodia length: $n=6$, $p\text{-value}=2.78 \times 10^{-16}$ for control versus *Rel RNAi*, $p\text{-value}=1.84 \times 10^{-6}$ for *bsk DN*; *Rel RNAi* versus *Rel RNAi*, $p\text{-value}=0.22$ for *bsk DN* vs control; two-tailed unpaired Student's t-test). (**O–R**) Knocking down JNK function from the niche did not have any effect on progenitors (visualized by Shg) (**Q**). Downregulating *bsk* function in Rel loss genetic background was able to restore the reduction in prohemocyte pool (**R**) observed in Relish loss (**P**) scenario in comparison to control (**O**). (**S**) Statistical analysis of the data in (**O–R**) ($n=10$, $p\text{-value}=2.26 \times 10^{-6}$ for control versus *Rel RNAi*, $p\text{-value}=1.94 \times 10^{-7}$ for *bsk DN*; *Rel RNAi* versus *Rel RNAi*, $p\text{-value}=0.521$ for control versus *bsk DN*; two-tailed unpaired Student's t-test) The white dotted line marks whole of the lymph gland in all cases and niches in (**A–G'**). Yellow dotted lines mark the progenitor zone in (**O–R**). In all panels, age of the larvae is 96 hr AEH. The nuclei are marked with DAPI (blue). Individual dots represent biological replicates. Error bar: standard deviation (SD). Data are mean \pm SD. * $p<0.05$, ** $p<0.01$, and *** $p<0.001$.



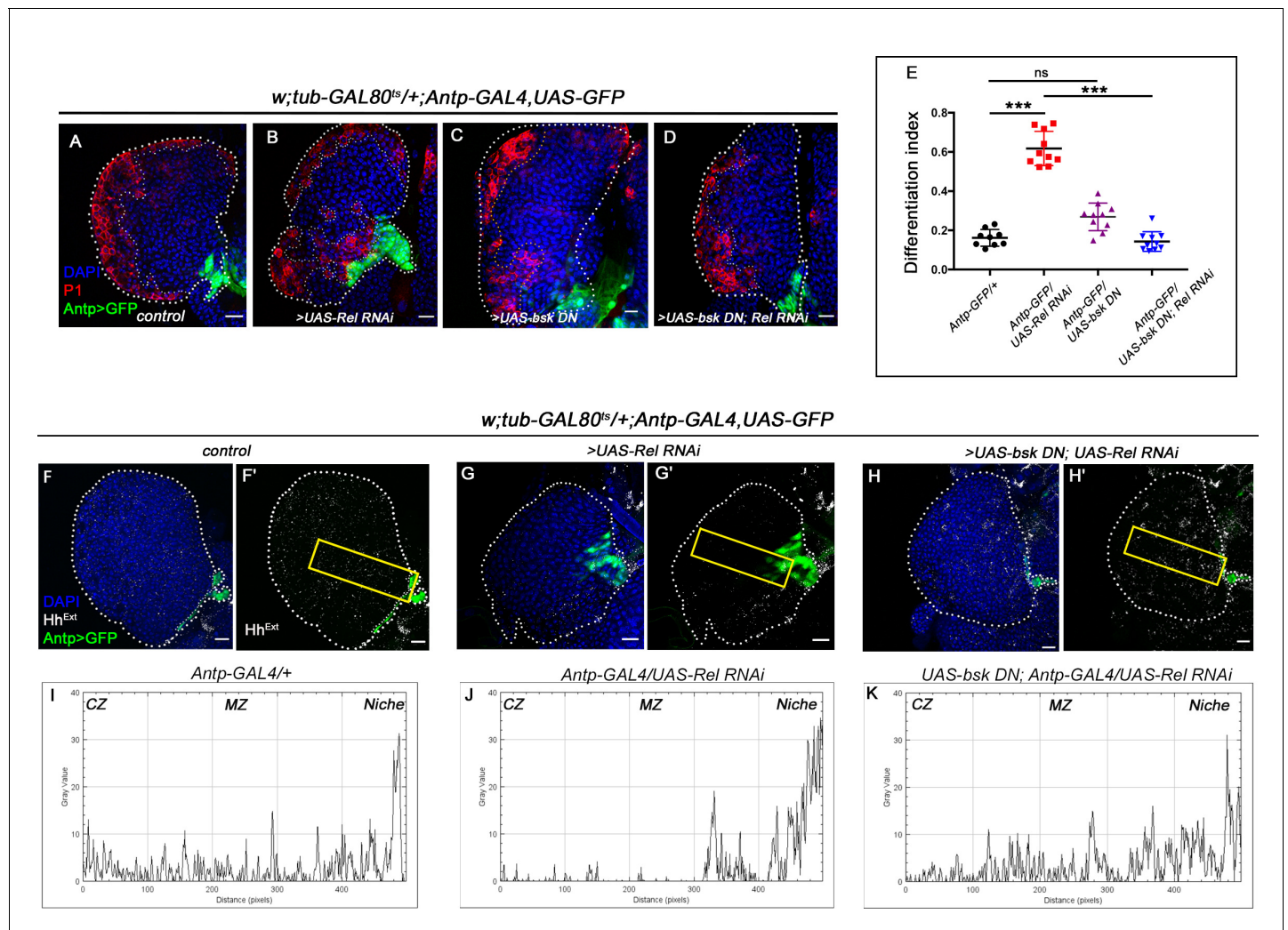


Figure 5—figure supplement 2. Downregulating JNK in Relish loss genetic background rescues progenitor loss and precocious differentiation. The genotypes are mentioned in relevant panels. Scale bar: 20 μ m. (A–D) Differentiation defect observed in Relish loss (B) was reverted to control (A) in a simultaneous knockdown of both Relish and JNK (D) from the niche. Loss of JNK alone from the niche had no significant effect on differentiation (C). (E) Statistical analysis of the data in (A–D) ($n = 10$, p -value = 1.5×10^{-9} for control versus *Rel RNAi*, p -value = 1.79×10^{-8} for *bsk DN; Rel RNAi* versus *Rel RNAi*, p -value = 0.392 for *bsk DN* versus control; two-tailed unpaired Student's *t*-test). (F–H') Reduced Extracellular Hh observed in the progenitors (Hh^{Ext}) of Relish loss of function condition (G–G') in comparison to those of control (F–F'), is significantly rescued in simultaneous loss of both Rel and JNK from the niche (H–H'). The yellow box in (F', G', and H') denotes the area quantified in (I, J, and K) respectively. (I–K) The intensity profile of Hh^{Extra} in progenitors (along the rectangle drawn from niche to Cortical zone housing differentiated cells in F', G', and H') reflects a stark decline in the level of Hh^{Extra} in Rel loss scenario (J) compared to control (I) which is rescued upon simultaneous loss of both Rel and JNK from the niche (K). The white dotted line mark whole of the lymph gland in all cases. Yellow dotted line indicates the boundary between CZ and MZ in (A)–(D). In all panels, age of the larvae is 96 hr AEH. The nuclei are marked with DAPI (blue). Individual dots represent biological replicates. Error bar: SD. Data are mean \pm (SD). * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

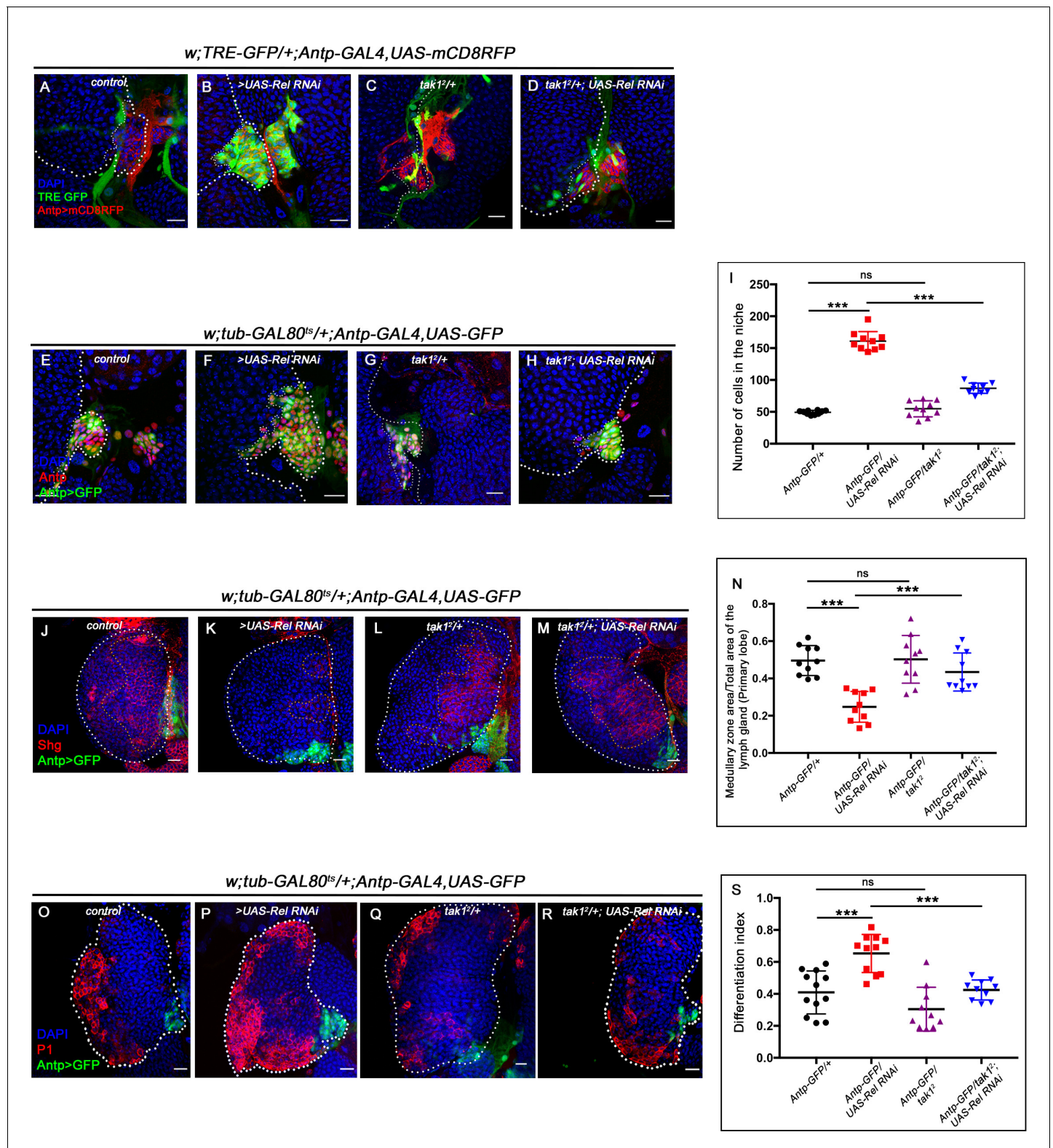


Figure 5—figure supplement 3. Relish inhibits JNK signaling by restricting *tak1* activity in the niche during development. The genotypes are mentioned in relevant panels. Scale bar: 20 μ m. (A–D) Up regulation of JNK signaling visualized by its reporter TRE-GFP (green) in Rel knockdown (B) compared with WT niche (A) is rescued in simultaneous loss of both the function of *tak1* and Rel (D) whereas JNK activation was not observed in *tak1* loss (C). (E–H) Increase in niche cell numbers observed upon loss of Rel from the niche (F) is rescued to control levels (E) in a simultaneous loss of both Rel and *tak1* function from the niche (H) whereas no significant change in niche cell number was observed in *tak1* loss (G). (I) Statistical analysis of the Figure 5—figure supplement 3 continued on next page

Figure 5—figure supplement 3 continued

data in (E–H) ($n=10$, p -value= 6.9×10^{-10} for control versus *Rel* RNAi, p -value= 1.9×10^{-9} for *tak1*²; *Rel* RNAi versus *Rel* RNAi, p -value=0.201 for control versus *tak1*²; two-tailed unpaired Student's *t*-test). (J–M) Loss of *tak1* function from the niche did not have any effect on progenitors (Shg) (L). Downregulating *tak1* function in *Rel* loss genetic background could restore the reduction in prohemocyte pool (M) observed in *Rel* loss (K) scenario in comparison to control (J). (N) Statistical analysis of the data in (J–M) ($n = 10$, p -value= 2.26×10^{-6} for control versus *Rel* RNAi, p -value = 3.1×10^{-4} for *tak1*²; *Rel* RNAi versus *Rel* RNAi, p -value=0.891 for control versus *tak1*²; two-tailed unpaired Student's *t*-test). (O–R) Differentiation defects observed in *Rel* loss (P) was comparable to control (O) in simultaneous loss of both *Rel* and *tak1* function (R) from the niche. No significant change in differentiation was observed in *tak1* loss from the niche (Q). (S) Statistical analysis of the data in (O–R) ($n=10$; p -value= 1.5×10^{-4} for control versus *Rel* RNAi, p -value = 4.7×10^{-5} for; *Rel* RNAi versus *tak1*²; *Rel* RNAi, p -value=0.115 for control versus *tak1*²; two-tailed unpaired Student's *t*-test). The white dotted line mark whole of the lymph gland in all cases and niches in (A–D and E–H). Yellow dotted lines marks the progenitor zone in (J–M). In all panels, age of the larvae is 96 hr AEH. The nuclei are marked with DAPI (blue). Individual dots represent biological replicates. Error bar: standard deviation (SD). Data are mean \pm SD. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

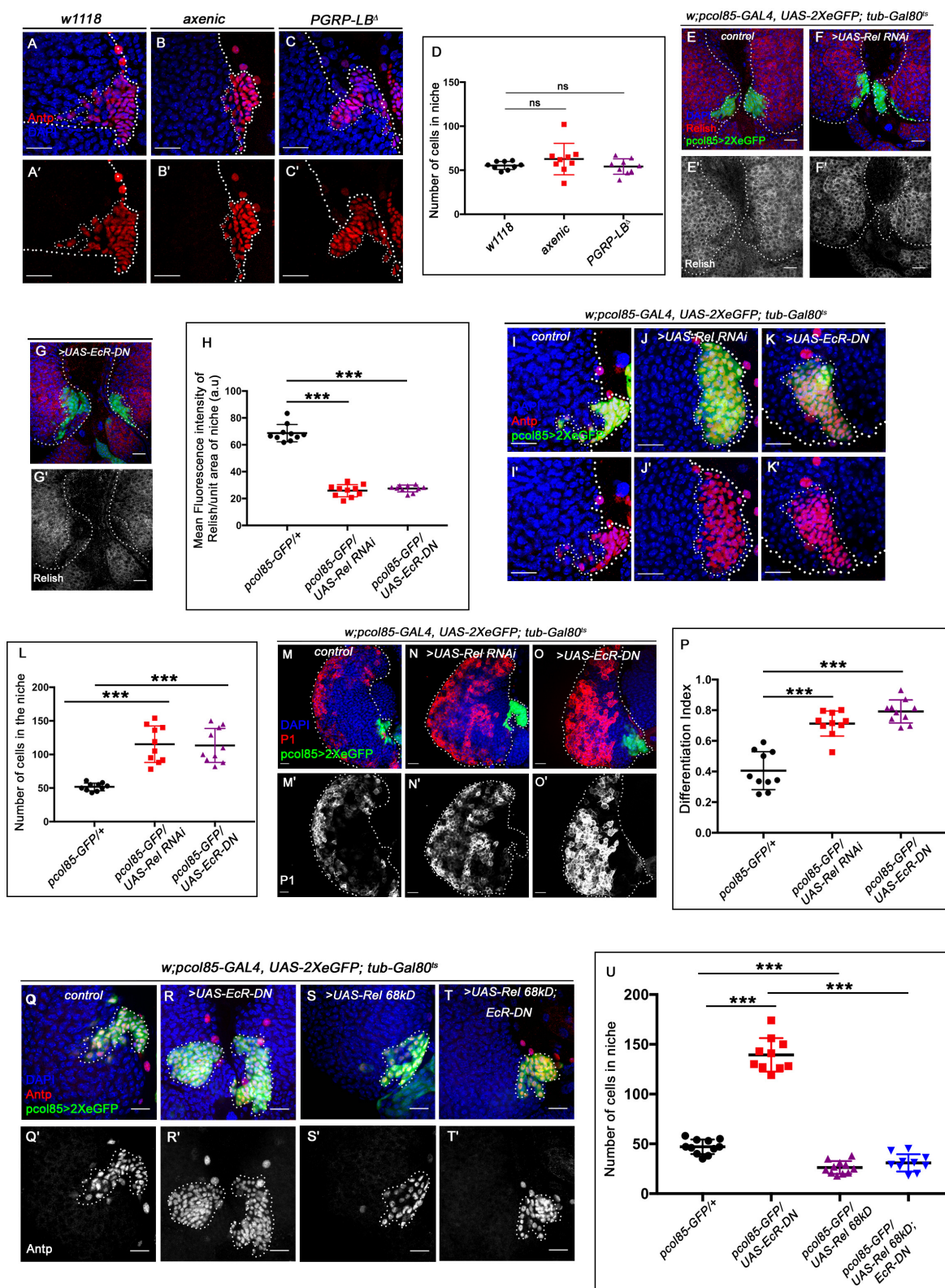


Figure 6. Ecdysone regulates Relish expression and functionality in the niche. The genotypes are mentioned in relevant panels. Scale bar: 20 μ m. (A–C') Niche number remains comparable to control (A–A') both in axenic larval lymph gland (B–B') and in PGRP-LB mutant where there is upregulation in Figure 6 continued on next page

Figure 6 continued

systemic peptidoglycan levels (C–C'). (D) Statistical analysis of the data in (A–C') (n=9; p-value = 0.262 for control versus germ free and 0.392 for control versus PGRP-LB mutant; two-tailed unpaired Student's t-test). (E–G') Compared to that of control (E–E') Rel expression is significantly downregulated both in EcR loss (G–G') as well as in Rel loss from the niche (F–F'). (H) Statistical analysis of the data in (E–G') (n=10, p-value=7.81 × 10⁻¹² for control versus *Rel RNAi* loss and p-value = 3.76 × 10⁻¹⁰ for control versus *EcR-DN*; two-tailed unpaired Student's t-test). (I–K') Similar to Rel loss from the niche (J–J'), EcR loss also results in increase in niche cell numbers (K–K') compared to that of control (I–I'). (L) Statistical analysis of the data in I–K' (n=10, p-value=6.6 × 10⁻⁵ for control versus *EcR-DN* and p-value = 3.1 × 10⁻⁵ for control versus *Rel RNAi*; two-tailed unpaired Student's t-test). (M–O') Compared to control (M–M'), both loss of Rel (N–N') and EcR (O–O') from the niche results in increase in differentiation. (P) Statistical analysis of the data in (M–O') (n=10, p-value=4.3 × 10⁻⁵ for control versus *Rel RNAi* and p-value=2.2 × 10⁻⁶ for control versus *EcR-DN*; two-tailed unpaired Student's t-test). (Q–T') Increase in niche cell numbers observed upon EcR loss from the niche (R–R') is rescued to control levels (Q–Q') when Relish was overexpressed in an EcR loss genetic background (T–T'). Overexpression of Relish in the niche reduced the cell number compared to control (compare S–S' and Q–Q'). (U) Statistical analysis of the data in (Q–T') (n=10; p-value=1.7 × 10⁻⁹ for control versus *EcR-DN*, p-value=7.8 × 10⁻¹¹ for *EcR-DN* versus *UAS-Rel 68kD*; *EcR-DN*, p-value=3.63 × 10⁻⁶ for control versus *UAS-Rel 68kD*; two-tailed unpaired Student's t-test). The white dotted line marks whole of the lymph gland and niches in all the cases. In all panels, age of the larvae is 96 hr AEH. The nuclei are marked with DAPI (blue). Individual dots represent biological replicates. Error bar: standard deviation (SD). Data are mean ± SD. *p<0.05, **p<0.01, and ***p<0.001.

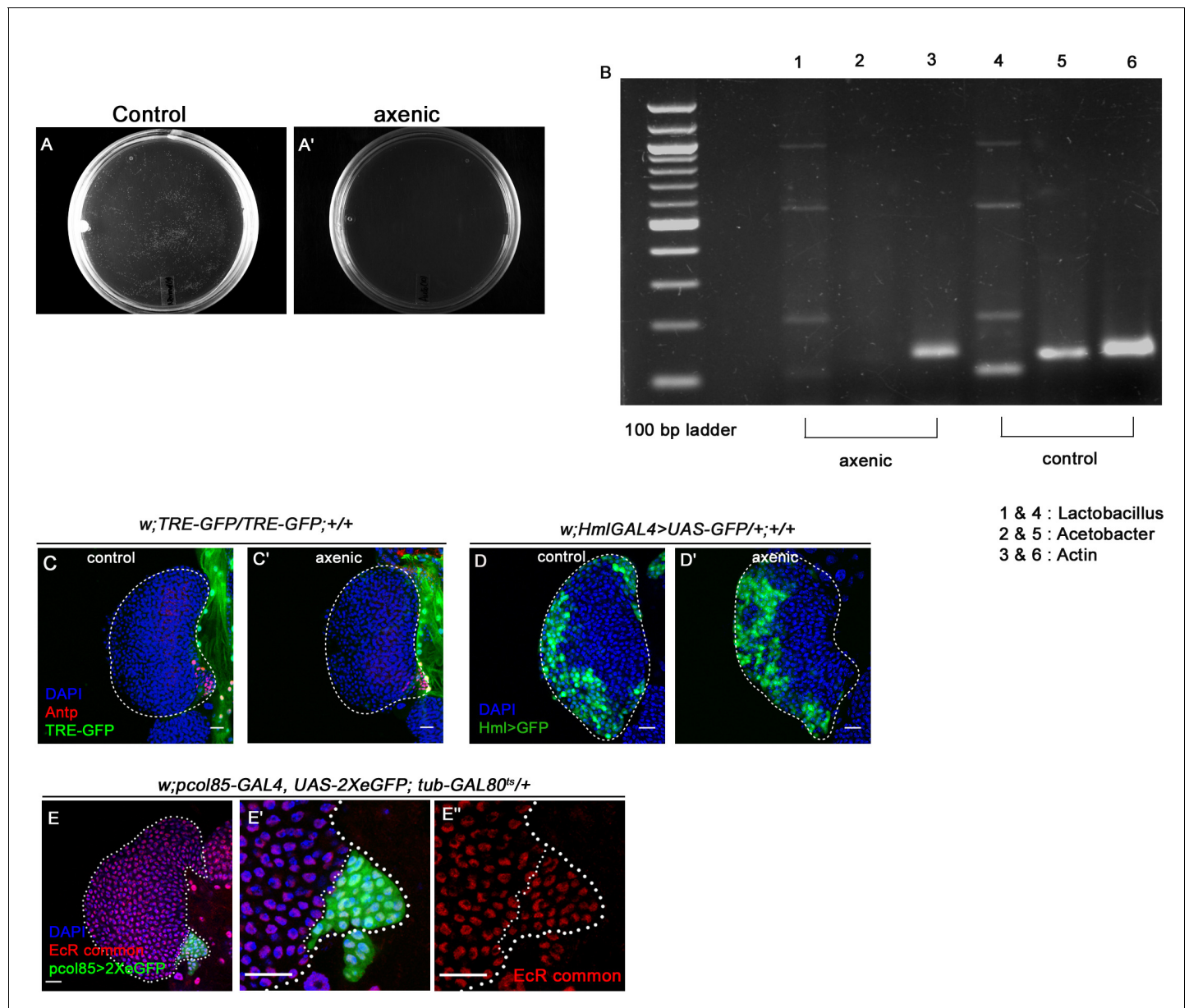


Figure 6—figure supplement 1. Ecdysone signaling is active in the hematopoietic niche. Genotypes of the larvae are mentioned in respective panels. Scale bar: 20 μ m (A–A'). Larval homogenates were spread on LB Agar plates to check the presence of commensal gut microbiota. In control scenario (A) bacterial colonies were visible post incubation whereas in axenic condition no growth was observed on the plates (A'). (B) The efficacy of removal of gut microflora was further checked by performing PCR analysis on DNA isolated from larval guts using 16S rDNA primers. *Drosophila* actin was used as control. Significant reduction in the amount of both *Lactobacillus* (compare lane 1 (axenic) with 4 (control)) and *Acetobacter* (compare lane 2 [axenic] with 5 [control]) species was observed in axenic condition compared to control scenario (compare lane 3 [axenic] and 6 [control]). (C–C') TRE-GFP expression in the hematopoietic niche (visualized by Antp, red) in axenic condition (C') is comparable to that of control (C). (D–D') Differentiation status (visualized by Hml>GFP, pan plasmatocyte marker) in axenic condition (D') is comparable to control (D). (E–E'') Nuclear expression of Ecdysone receptor (red, EcR common) in the hematopoietic niche (green). The white dotted line marks whole of the lymph gland and the niches in (E–E''). In all panels, age of the larvae is 96 hr AEH. The nuclei are marked with DAPI (blue).

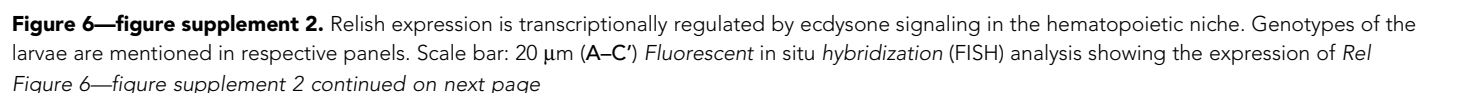


Figure 6—figure supplement 2 continued

transcript in the lymph gland of the control larvae (**A–A'**). Loss of EcR from the niche resulted in loss of Re-positive progenitors (**B–B'**). *Rel* transcripts were also detected in salivary gland of the control larvae (**C–C'**). (**D–E**) Sense probe (negative control) showing nonspecific background expression in the control lymph gland (**D**) and salivary gland (**E**). (**F–G''**) Whole-mount immunofluorescence (IF) and FISH on third-instar lymph gland. Compared to control (**F–F''**), drastic reduction of the *Rel* transcript was observed in the niche from where EcR levels were downregulated (**G–G''**). Please note the smaller size of the LG in **G–G'** reflects the peeling off of the cortical zone due to excessive differentiation around 96 hr AEH in EcR loss from the niche. The increased differentiation renders fragility to the LG, which is unable to withstand harsh in situ process. (**H**) Statistical analysis of the data in (**F'–G''**) ($n=10$, $p\text{-value}=1.56 \times 10^{-10}$ for control versus *EcR-DN*; two-tailed unpaired Student's t-test). (**I–L**) Differentiation defects observed in EcR loss (**J**) was reverted to control (**I**) when Relish was overexpressed in EcR loss genetic background (**L**). Slight decrease in differentiation of progenitors were observed upon Relish overexpression in the niche (compare **I** and **K**). (**M**) Statistical analysis of the data in (**I–L**) ($n=10$; $p=3.8 \times 10^{-7}$ for control versus *EcR-DN*, $p=3.3 \times 10^{-6}$ for *EcR-DN* versus *UAS-Rel 68kD*; *EcR-DN*, $p=7.2 \times 10^{-2}$ for control versus *UAS-Rel 68kD*; two-tailed unpaired Student's t-test). (**N**) Model depicting the developmental role of Relish in hematopoietic niche maintenance. Downregulation of Relish affects the proliferation and primary function of the niche by upregulated JNK signaling. Upregulated JNK disturbs niche homeostasis through wingless and cytoskeletal remodeling, thereby affecting progenitor maintenance. The white dotted line mark whole of the lymph gland in all cases. Yellow dotted line marks the niche in (**F–G''**) and the boundary between CZ and MZ in (**I–L**). In all panels, age of the larvae is 96 hr AEH. The nuclei are marked with DAPI (blue). Individual dots represent biological replicates. Error bar: standard deviation (SD). Data are mean \pm SD. * $p<0.05$, ** $p<0.01$, and *** $p<0.001$.

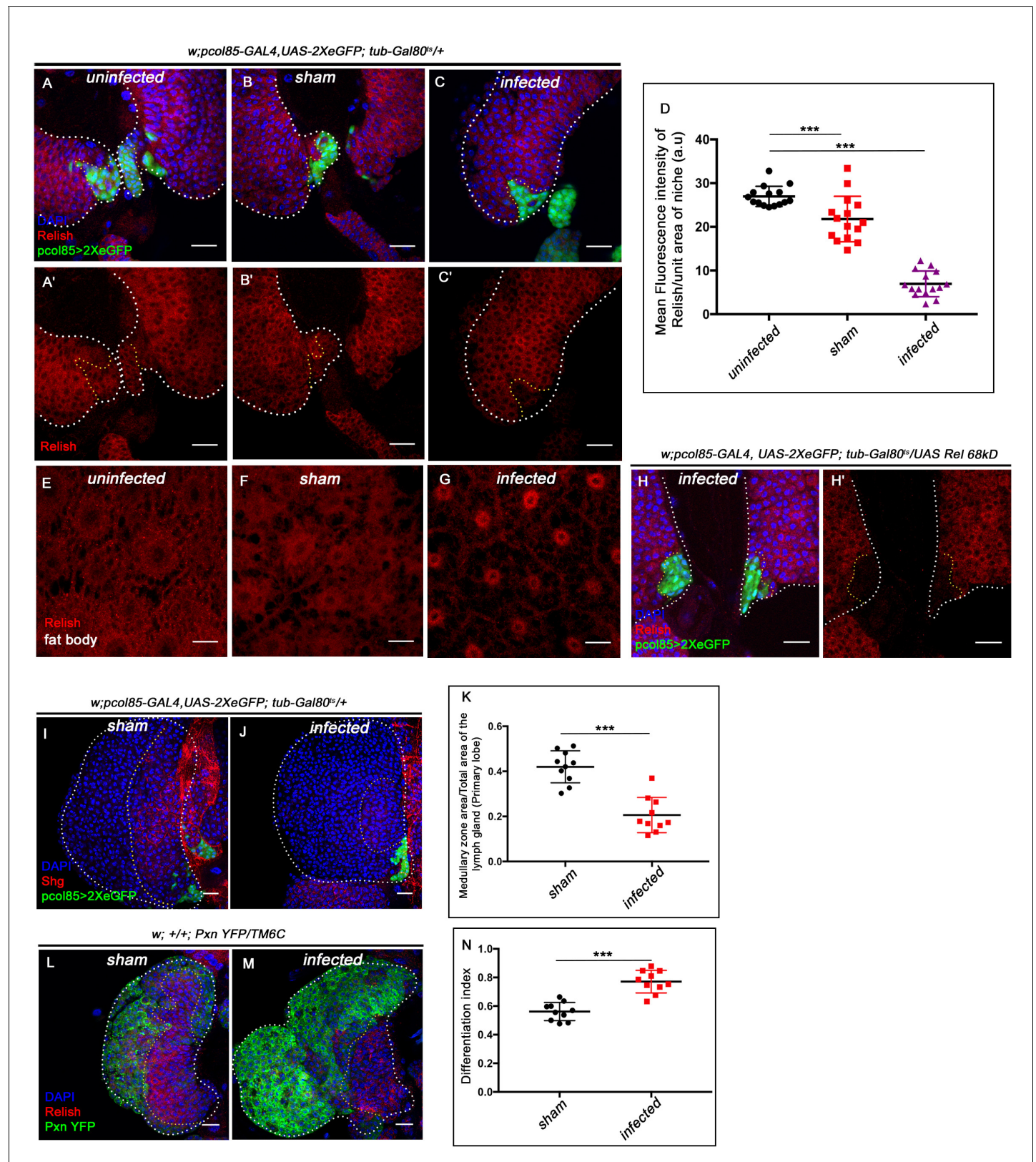


Figure 7. Niche-specific expression and function of Relish is susceptible to pathophysiological state of the organism. The genotypes are mentioned in relevant panels. Scale bar: 20 μ m. (A–C') Compare to uninfected conditions (A–A') and sham (B–B'), significant reduction in Relish expression was observed in the hematopoietic niche 4 hr post-infection (C–C'). (D) Statistical analysis of the data in (A–C') ($n=15$; $p=6.62 \times 10^{-18}$ for unpricked versus infected). (E–G) Relish expression in fat bodies. (H–H') Relish expression in the lymph gland. (I–J) Relish expression in the medullary zone of the lymph gland. (K) Statistical analysis of the data in (I–J) ($n=15$; $p=0.0001$ for sham versus infected). (L–M) Relish expression in the lymph gland. (N) Statistical analysis of the data in (L–M) ($n=15$; $p=0.0001$ for sham versus infected). Figure 7 continued on next page

Figure 7 continued

infected, $p=2.5 \times 10^{-7}$ for sham versus infected, two-tailed unpaired Student's t-test). (E–G) Nuclear expression of Relish was observed in infected (G) fat body cells 4 hr post in contrast to uninfected (E) and sham (F) larval fat body. (H–H') Overexpressing Relish N-terminus (*UAS-Rel-68kD*) could not rescue loss of Relish expression post-infection. (I–J) Compared to sham (I), significant reduction in Shg-positive progenitors (red) were observed in infected lymph glands (J). (K) Statistical analysis of the data in (I–J) ($n=10$; $p\text{-value}=5.2 \times 10^{-6}$ for sham versus infected, two-tailed unpaired Student's t-test). (L–M) Drastic increase in differentiation (visualized by *Pxn-YFP*, green) was observed in infected lymph glands (M) compared to sham (L). (N) Statistical analysis of the data in (L–M) ($n=10$; $p\text{-value} = 4.65 \times 10^{-6}$ for sham versus infected, two-tailed unpaired Student's t-test). The white dotted line mark whole of the lymph gland in all cases. Yellow dotted line marks the niche in (A–C' and H–H') and the boundary between CZ and MZ in (L–M). In all panels, age of the larvae is 96 hr AEH. The nuclei are marked with DAPI (Blue). Individual dots represent biological replicates. Error bar: standard deviation (SD). Data are mean \pm SD. * $p<0.05$, ** $p<0.01$, and *** $p<0.001$.

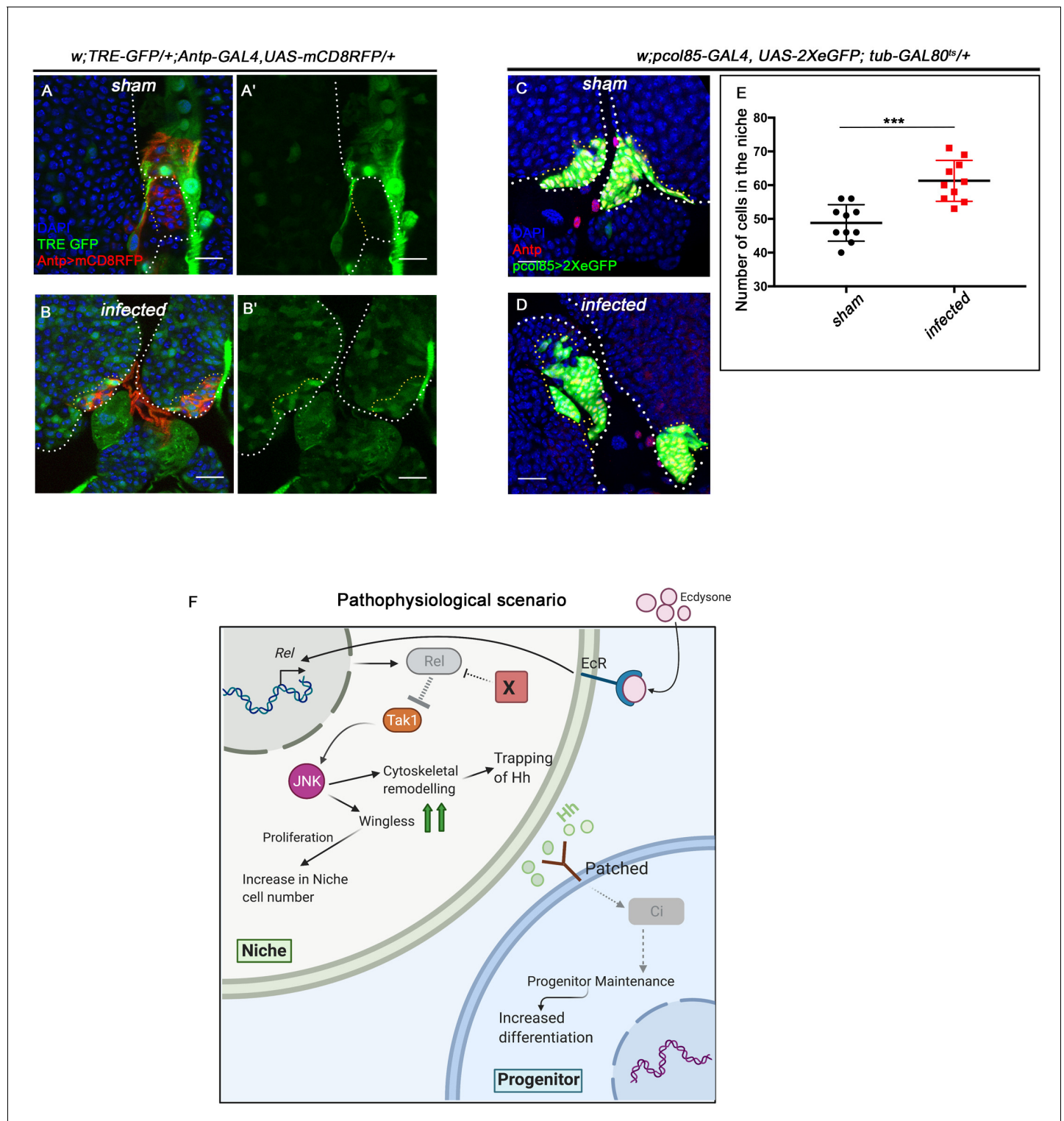


Figure 7—figure supplement 1. Upregulation in JNK signaling and increase in cell proliferation was observed in the niche during infection. The genotypes are mentioned in relevant panels. Scale bar: 20 μ m. (A–B') An overall up regulation in JNK signaling (visualized by its reporter *TRE-GFP* [green]) was observed in infected lymph glands (B–B') compared to sham (A–A'). (C–D) Significant increase in niche proliferation was observed in infected lymph gland niches (D) compared to sham infected (C). (E) Statistical analysis of the data in (C–D) ($n=10$; p -value= 1.1×10^{-4} for sham versus infected, two-tailed unpaired Student's t -test). (F) Model based on current results depicting how upon bacterial challenge Relish expression is differentially modulated in the niche to bolster the cellular immune response by eliciting precocious differentiation of the lymph gland hemocytes. The white dotted line mark whole of the lymph gland and yellow dotted lines marks the niches in all cases. In all panels, age of the larvae is 96 hr AEH. The Figure 7—figure supplement 1 continued on next page

Figure 7—figure supplement 1 continued

nuclei are marked with DAPI (blue). Individual dots represent biological replicates. Error bar: standard deviation (S.D). Data are mean \pm SD. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

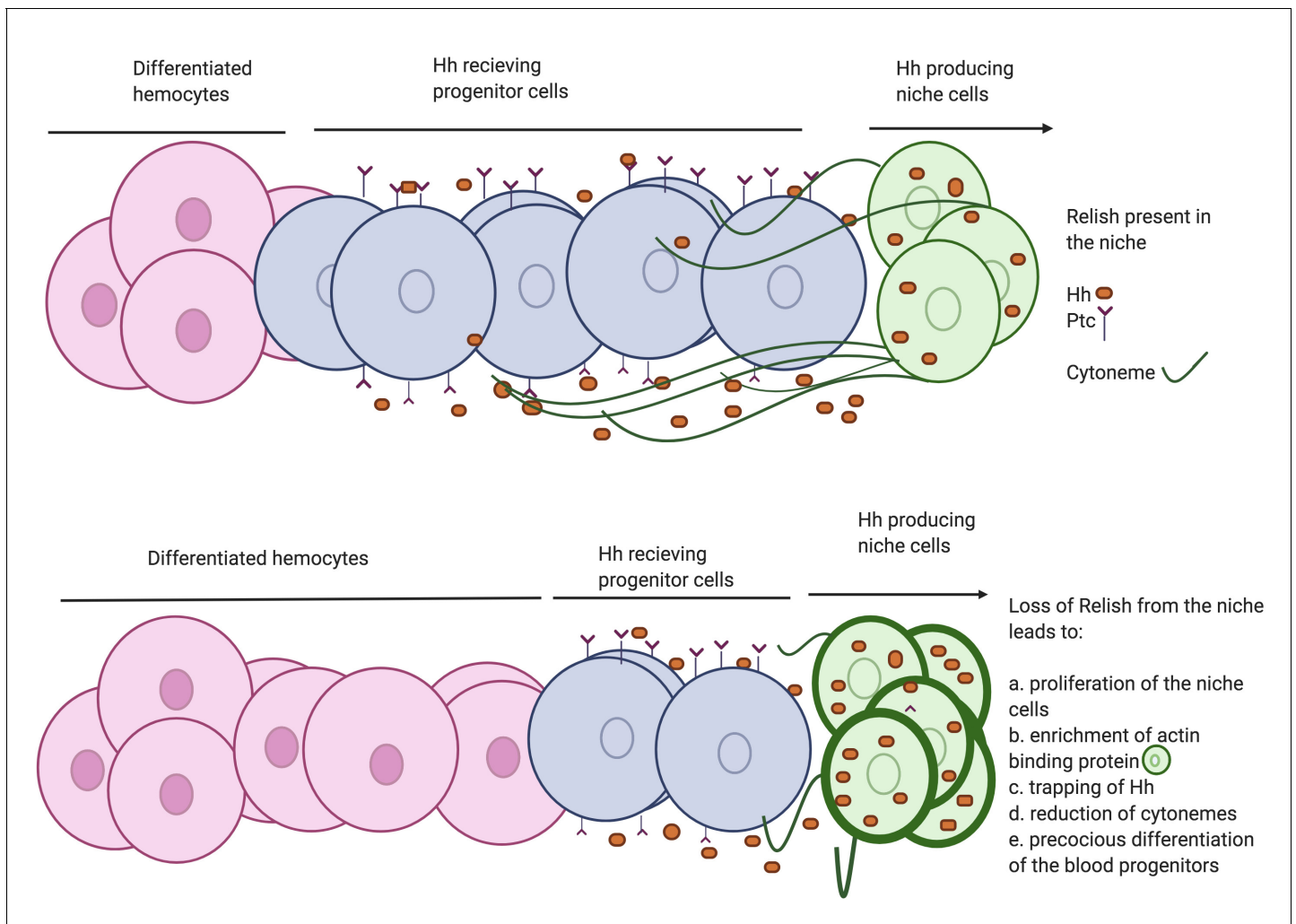


Figure 8. Developmental requirement of Relish in the niche for progenitor maintenance. Scheme describing how loss of Relish from the niche alters cytoskeletal elements of the cells. The change in cytoskeletal architecture affects cytoneme-like filopodial formation thereby trapping Hedgehog within the niche. The failure of Hh delivery in turn interferes with progenitor maintenance and pushes them toward differentiation.