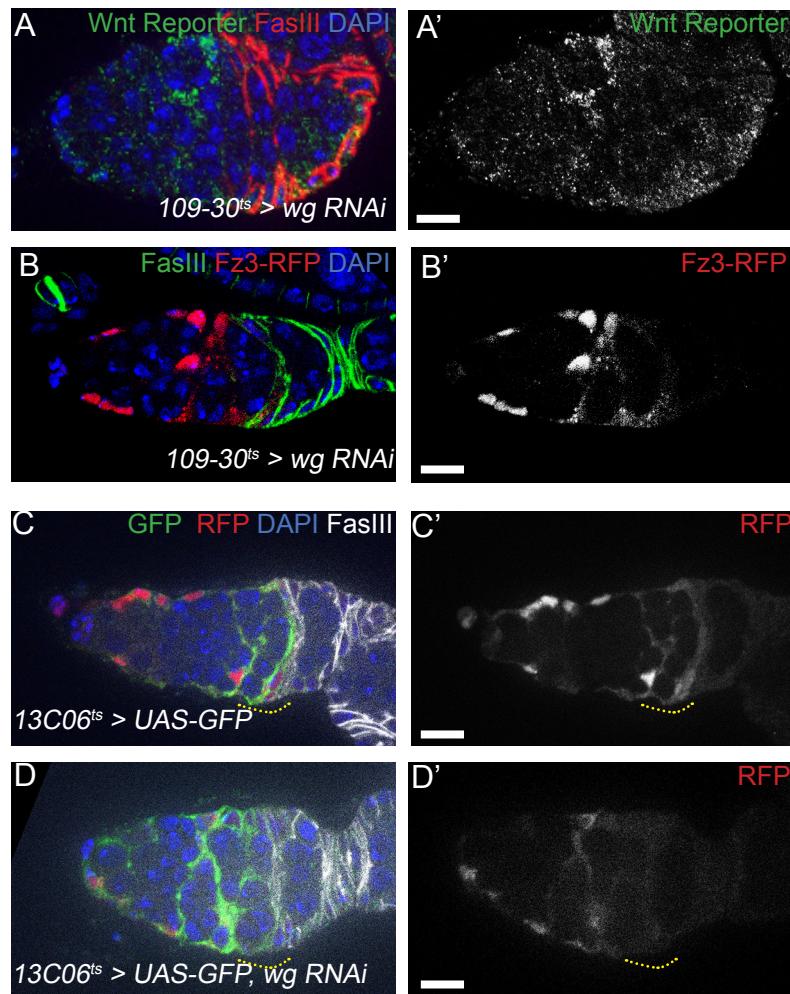
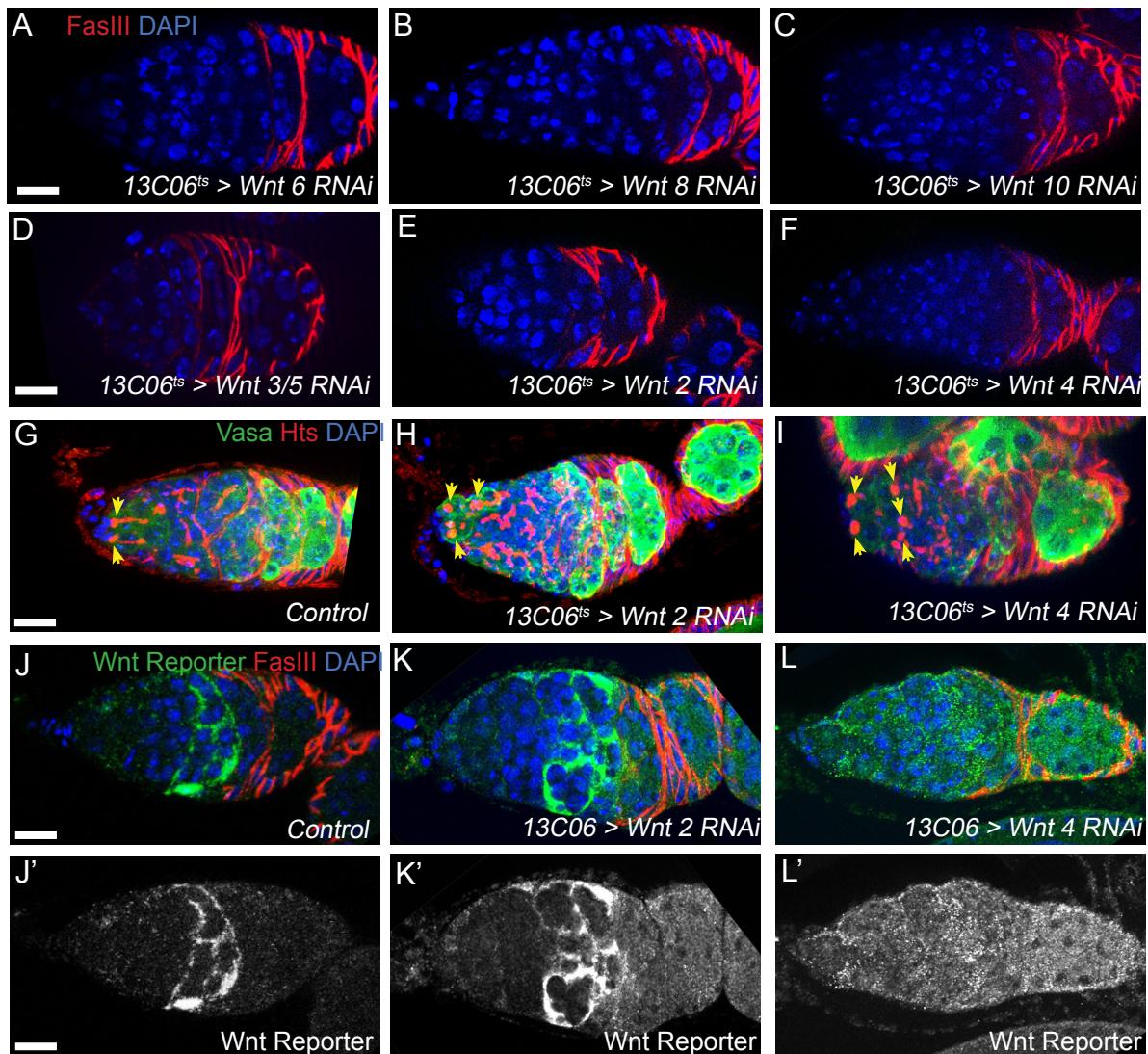


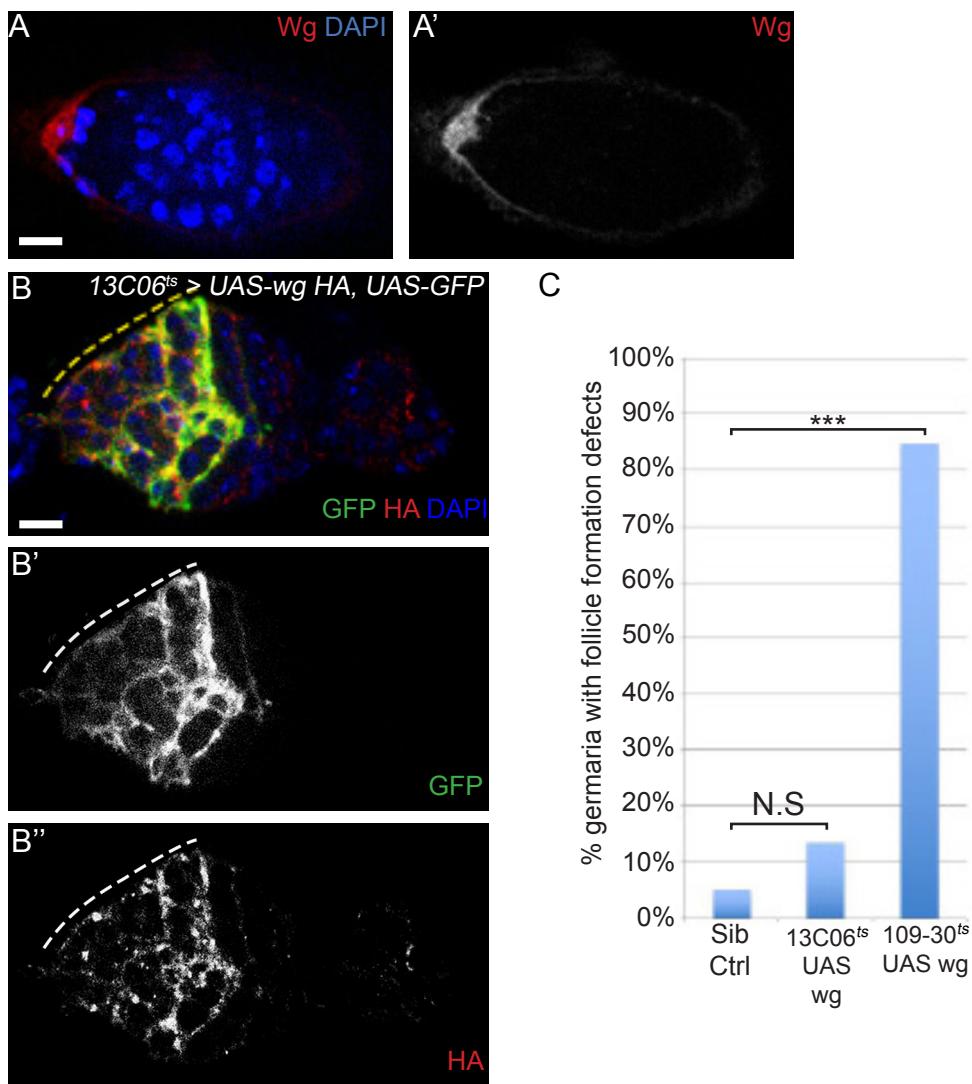
**Figure S1:** (A) A germarium with the *Fz3-RFP* Wnt reporter stained for RFP (red), FasIII (green), and DAPI (blue). The RFP and FasIII channels are shown separately for the region surrounding the anterior-most FasIII<sup>+</sup> cell (white arrow) in A' and A'' and the RFP channel for the full image is shown in A''. RFP expression is detectable in IGS cells in Regions 1 and 2a (white dashed lines) and in FSCs (white arrow in A' and A''), identified as the anterior-most FasIII<sup>+</sup> cells (A''), but expression rapidly decreases in pFCs downstream from the niche. (B) A germarium with *Fz3-RFP* stained for RFP (red), Lamin C (green) to mark the cap and terminal filament cells, and DAPI (C). *Fz3-RFP* is not detectable in Lamin C<sup>+</sup> cells (solid yellow line). (C) A germarium with the *3xGRH-4TH-GFP* reporter and a LacZ negatively marked clonal marking system to identify FSCs stained for LacZ (clone marker, red), GFP (green), and DAPI (blue). FSCs are marked as the anterior-most cell in a LacZ negative clone (C', C'' arrow). *3xGRH-4TH-GFP* is detectable in FSCs (C'', C''' arrow) but not pFCs downstream. (D) A negative control for all FISH experiments in Figure 1. The probe matches the *spitz* sense strand sequence. Scale bar represents 5  $\mu$ m.



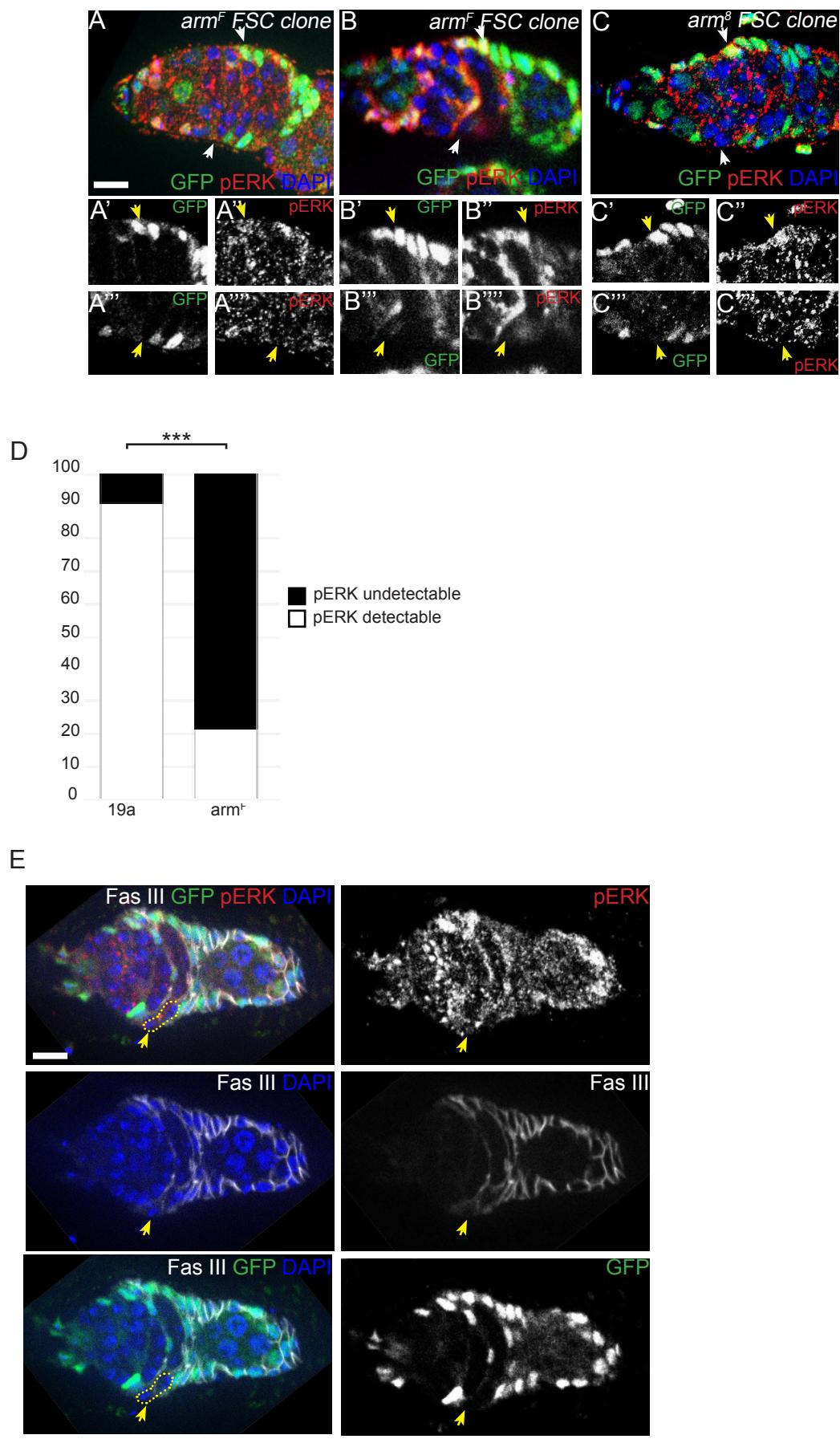
**Figure S2:** (A-B) The *3xGRH-4TH-GFP* reporter (A) or the *Fz3-RFP* reporter (B) combined with *UAS-wg RNAi*, *109-30-Gal4*, and *tub-Gal80<sup>ts</sup>* to restrict expression to adulthood stained for GFP (green in A) or RFP (red in B), FasIII (red in A, green in B), and DAPI (blue). Wg knockdown with this driver reduces but does not eliminate *3xGRH-4TH-GFP* expression, and does not affect *Fz3-RFP* expression. (C-D) Germaria with either *13C06-Gal4* driving *CD8-GFP* as a control (C) or *13C06-Gal4* driving *CD8-GFP* and *wg RNAi* (D) stained for FasIII (white), GFP (green), RFP (red), and DAPI (blue). In germaria with *wg RNAi* expressed using *13C06-Gal4*, RFP expression is reduced overall and undetectable in the posterior region of *13C06-Gal4* expression domain (dotted yellow line), which includes FSCs, identified as the anterior most FasIII<sup>+</sup> cells, and posterior IGS cells. Scale bar represents 5  $\mu$ m.



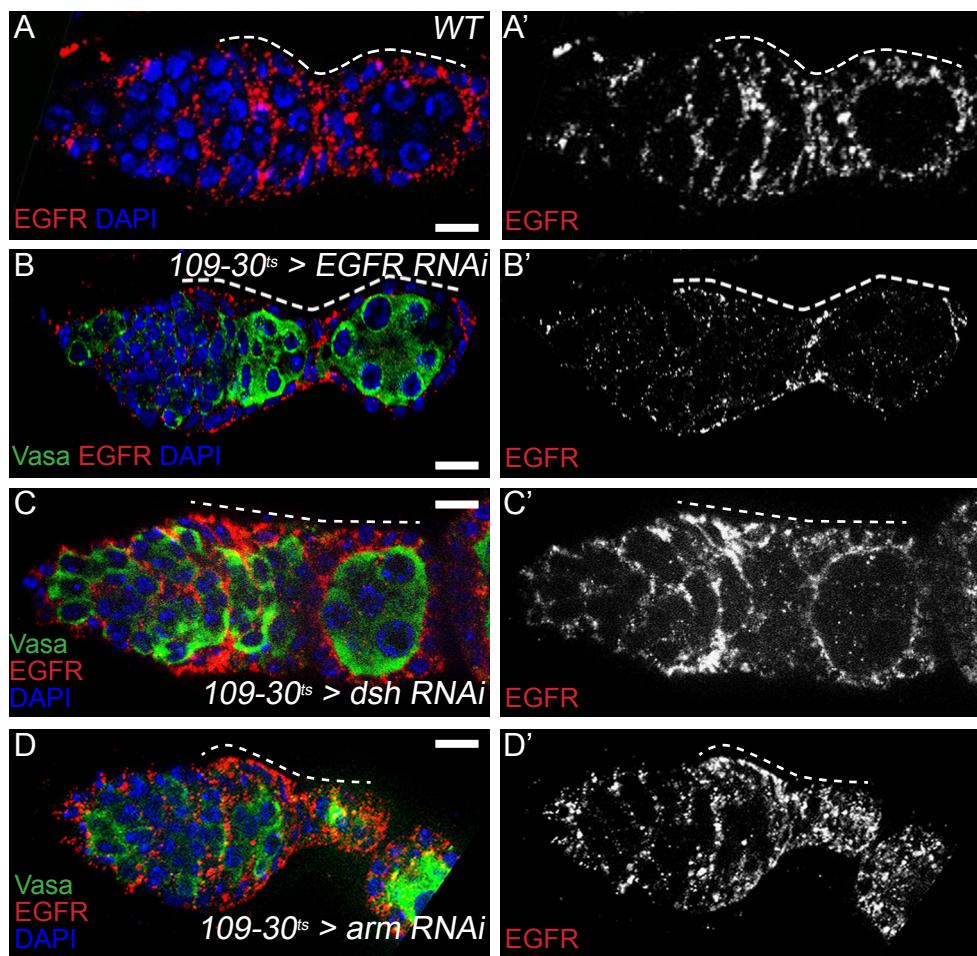
**Figure S3:** (A-F) Germaria with RNAi against *Wnt6* (A), *Wnt8* (B), *Wnt10* (C), *Wnt3/5* (D), *Wnt2* (E) or *Wnt4* (F) driven with *13C06-Gal4* and combined with *tub-Gal80<sup>ts</sup>* to restrict expression to adulthood stained for FasIII (red) and DAPI (blue). No significant morphological phenotypes, as visualized with FasIII staining, were observed. (G-H) Maximum intensity projections of 5-10 optical sections of wildtype control (G), *Wnt2* RNAi (H), or *Wnt4* RNAi (I) stained for Hts (red) to identify spectrosomes (yellow arrows), vasa (green) and DAPI (blue). *Wnt2* and *Wnt4* knockdown exhibit more spectrosomes compared to control. (J-L) Germaria with *3xGRH-4TH-GFP*, *13C06-Gal4*, and either no RNAi as a control (J), *Wnt2* RNAi (K), or *Wnt4* RNAi (L) stained for FasIII (red), GFP (green), and DAPI (blue). Expression of *Wnt2* RNAi had no effect on *3xGRH-4TH-GFP* reporter levels whereas expression of *Wnt4* RNAi caused in a reduction in the *3xGRH-4TH-GFP* reporter levels. The GFP channels are shown in J'-L'. Scale bar represents 5 μm.



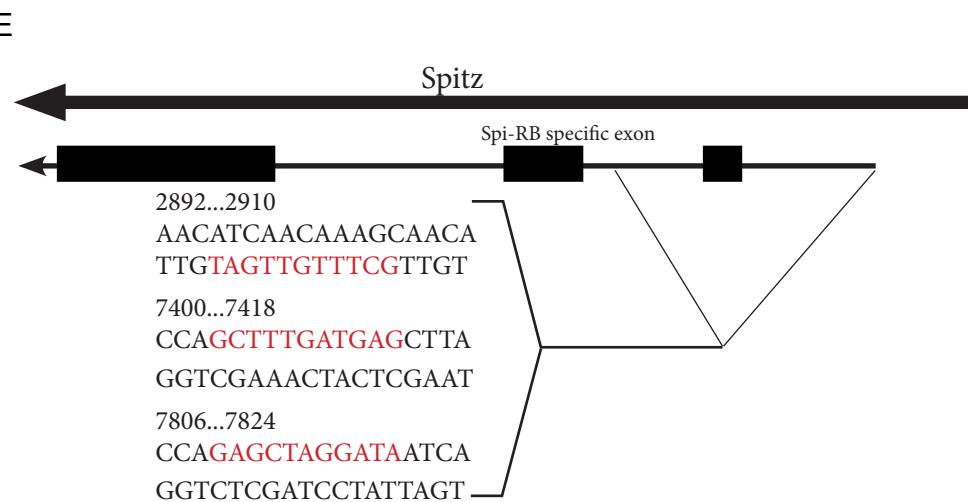
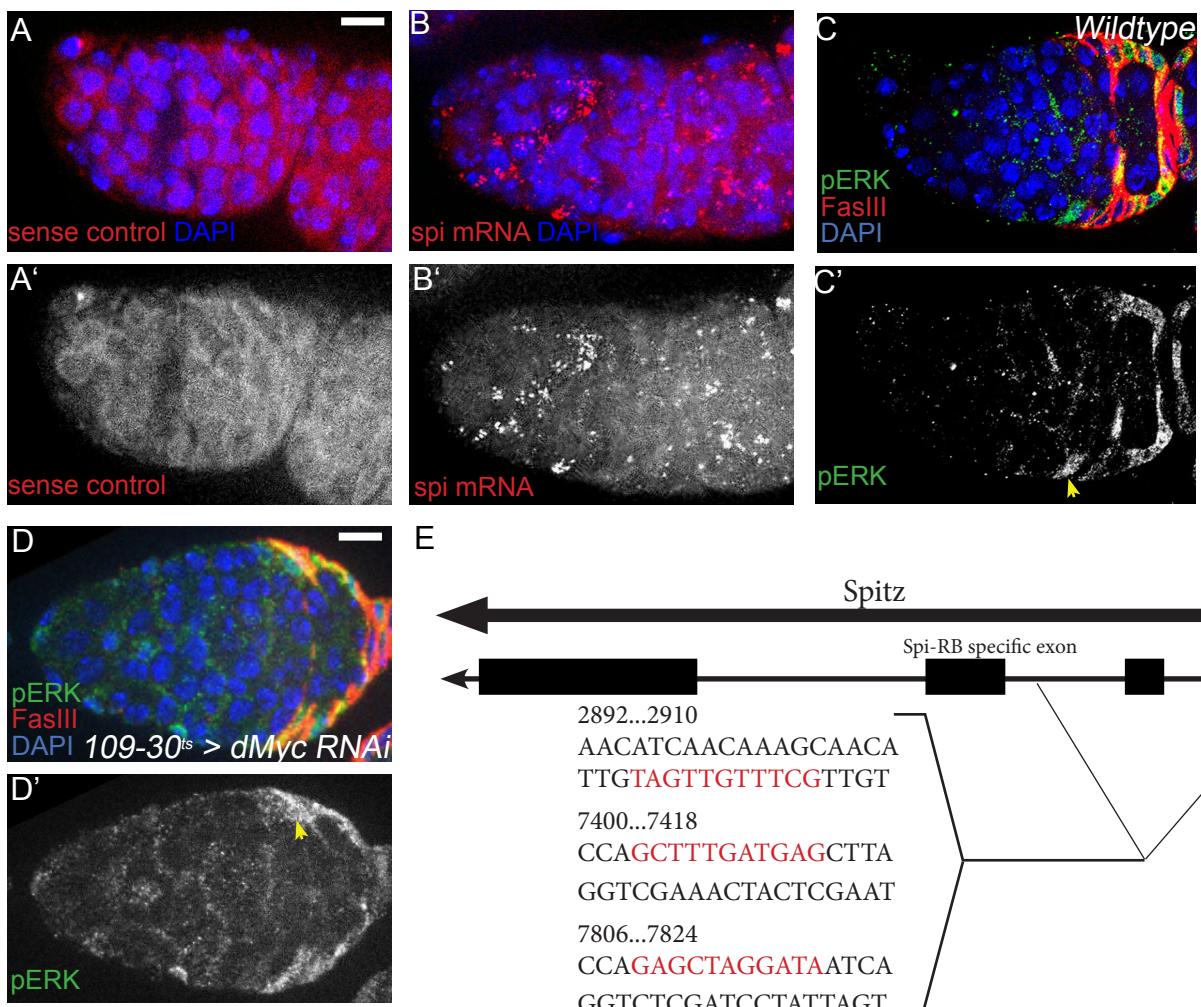
**Figure S4:** (A) A wildtype germarium stained for Wg (red) and DAPI (blue). The Wg signal is strongest at the anterior tip of the germarium, and tapers off toward the posterior in Regions 1 and 2a. The Wg channel is shown separately in A'. (B) Panels showing the merged image (B), GFP only channel (B'), and HA::Wg channel (B'') from the image in Figure 3D. The HA::Wg signal overlaps precisely with the GFP signal (dotted lines) (C) Quantification of follicle formation defects in flies with *tub-Gal80<sup>ts</sup>, UAS-HA::Wg*, and either *13C06-Gal4*, *109-30-Gal4*, or CyO siblings that lack a Gal4 driver shifted to 29°C for 7 days during adulthood to inactivate Gal80 and allow for HA::Wg expression. \*\*\*p<0.001 and N.S. indicates not significant with a chi-squared test. n>50 germaria. Scale bar represents 5 μm.



**Figure S5:** (A-C) Germaria with *arm<sup>F</sup>* (A-B) or *arm<sup>8</sup>* (C) clones stained for pERK (red), GFP (clonal marker, green) and DAPI (blue). Images are oriented with a GFP<sup>+</sup> (wildtype) FSC on top and a GFP<sup>-</sup> (homozygous mutant) FSC on the bottom. FSCs (arrows) are identified as the anterior-most cell in an FSC clone and by their position at the Region 2a/2b border. Wildtype control FSCs are pERK<sup>+</sup> in each case whereas *arm<sup>F</sup>* and *arm<sup>8</sup>* FSCs are pERK<sup>-</sup>. (D) Quantification of germaria with pERK<sup>+</sup> GFP<sup>-</sup> FSCs in germaria in which the wildtype (GFP<sup>+</sup>) FSC is pERK<sup>+</sup> for the indicated genotypes. \*\*\*P<0.001 using a two-sided Fisher Exact test. (E) A demonstration of the criteria used to identify FSCs using the image of the germarium with a *dsh<sup>3</sup>* mutant clone shown in Figure 5B. FasIII is white, GFP is green, pERK is red, DAPI is blue, and the FasIII, GFP, and pERK channels are shown separately. FSCs are identified by scrolling through the optical sections to find the anterior-most marked cell (GFP<sup>-</sup>) in a clone. Because this cell also the anterior-most FasIII<sup>+</sup> cell, we used the border of FasIII staining to identify the FSCs in other cases when a clone is not present. The FSC is indicated with a yellow arrow and the GFP<sup>-</sup> clone is outlined with a yellow dotted line. Scale bar represents 5 μm.



**Figure S6:** (A-D) A wildtype germarium (A) or germarium with *UAS-EGFR RNAi* (B), *UAS-dsh RNAi* (C), or *UAS-arm RNAi* (D) combined with *109-30-Gal4* and *tub-Gal80<sup>ts</sup>* to restrict expression to adulthood (B) stained for EGFR (red), vasa (panels B-D, green) and DAPI (blue). EGFR expression is detectable in FSCs and pFCs (dotted lines) of wildtype germaria and is unaffected by expression of *dsh* RNAi or *arm* RNAi, but is substantially reduced by expression of *EGFR* RNAi. Scale bar represents 5  $\mu$ m.



D. mel. chrII coords. 19574403	19574445	D. mel. chrII coords. 19569499	19569527
D. melanogaster	gttctgttgc-ttttgtgat	gttacaatttgtgcacttgttgc	gttcataaagctggctgtatgcgt
D. simulans	gttctgttgc-ttttgtgat	gttacaatttgtgcacttgttgc	gttcataaagctggctgtatgcgt
D. sechellia	gttctgttgc-ttttgtgat	gttacaatttgtgcacttgttgc	gttcataaagctggctgtatgcgt
D. yakuba	gttctgttgc-ttttgtgat	gttacaatttgtgcacttgttgc	gttcataaagctggctgtatgcgt
D. erecta	gttccgttgc-ttttgtgat	gttacaatttgtgcacttgttgc	gttcataaagctggctgtatgcgt
D. biarmipes	gtctgttcccttttgtgat	gttacaatttgtgcacttgttgc	gttcataaagctggctgtatgcgt
D. suzukii	gttctgttcccttttgtgat	gttacaatttgtgcacttgttgc	gttcataaagctggctgtatgcgt
D. ananassae	----gttg- tt-----	caatttgtgcacttgttgc	gttcataaagctggctgtatgcgt
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D. eugracilis	gttctgttgc-ttttgtgat	gttacaatttgtgcacttgttgc	gttcataaagctggctgtatgcgt
D. elegans	gttctgttgc-ttttgtgat	gttacaatttgtgcacttgttgc	gttcataaagctggctgtatgcgt
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D. bipectinata	ctaagctatcaaaggctggctgtata	gttacaatttgtgcacttgttgc	gttcataaagctggctgtatgcgt
D. eugracilis	ctaagctatcaaaggctggctgtata	gttacaatttgtgcacttgttgc	gttcataaagctggctgtatgcgt
D. elegans	ctaagctatcaaaggctggctgtata	gttacaatttgtgcacttgttgc	gttcataaagctggctgtatgcgt
D. kikkawai	ctaagctatcaaaggctggctgtata	gttacaatttgtgcacttgttgc	gttcataaagctggctgtatgcgt
D. takahashii	ctaagctatcaaaggctggctgtata	gttacaatttgtgcacttgttgc	gttcataaagctggctgtatgcgt
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M. domestica	ctaagctatcaaaggctggctgtata	gttacaatttgtgcacttgttgc	gttcataaagctggctgtatgcgt
A. mellifera	ctaagctatcaaaggctggctgtata	gttacaatttgtgcacttgttgc	gttcataaagctggctgtatgcgt
T. castaneum	ctaagctatcaaaggctggctgtata	gttacaatttgtgcacttgttgc	gttcataaagctggctgtatgcgt

**Figure S7:** (A-B) Wildtype germaria incubated with a FISH probe (red) that matches either the *spitz* sense strand as a negative control (A) or the *spitz* anti-sense strand to detect *spitz* transcript (B) and stained with DAPI (blue). (C-D) A wildtype (Canton-S) germarium or germarium with *dMyc* RNAi driven with *109-30-Gal4* and with *tub-Gal80<sup>ts</sup>* to restrict expression to adulthood stained for FasIII (red), pERK (green), and DAPI (blue). *dMyc* knockdown does not eliminate the pERK signal in FSCs (yellow arrow), identified as the anterior most FasIII<sup>+</sup> cells. Scale bar represents 5 μm. (E) Putative TCF binding sites in the TSS of two *spitz* isoforms: Spi-RB and Spi-RE. Predicted High Mobility Group (HMG) recognition motifs are highlighted in red. (F) Alignments of HMG binding sites shown in (E) throughout multiple dipteran insect 75 species performed using the UCSC genome browser. Conserved sequences are highlighted in yellow.

**Supplementary Data Files:** The raw data for all of the quantifications reported in the paper (Figures 5, 6, 7, S4, and S5) are included as an Rdata file. The statistical analyses of these data are provided as an Rmd file and an HTML file. The Rdata file and Rmd file can be opened in RStudio ([www.rstudio.com](http://www.rstudio.com)) and the HTML file can be opened in standard web browsers.

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