

Supplementary Information

Hh and EGFR-Ras signaling promote distinct steps of tumor progression in the *Drosophila* follicle epithelium

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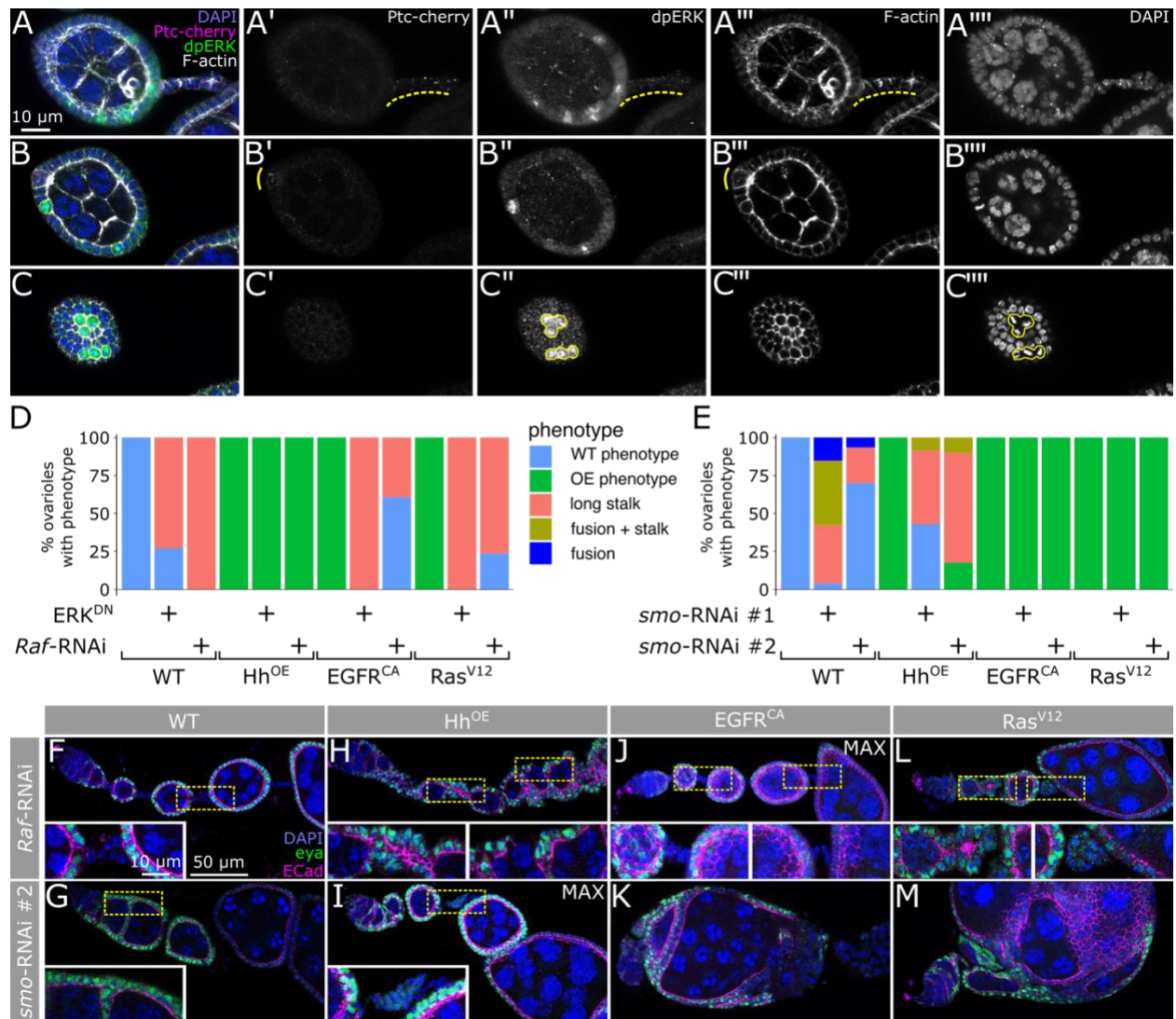
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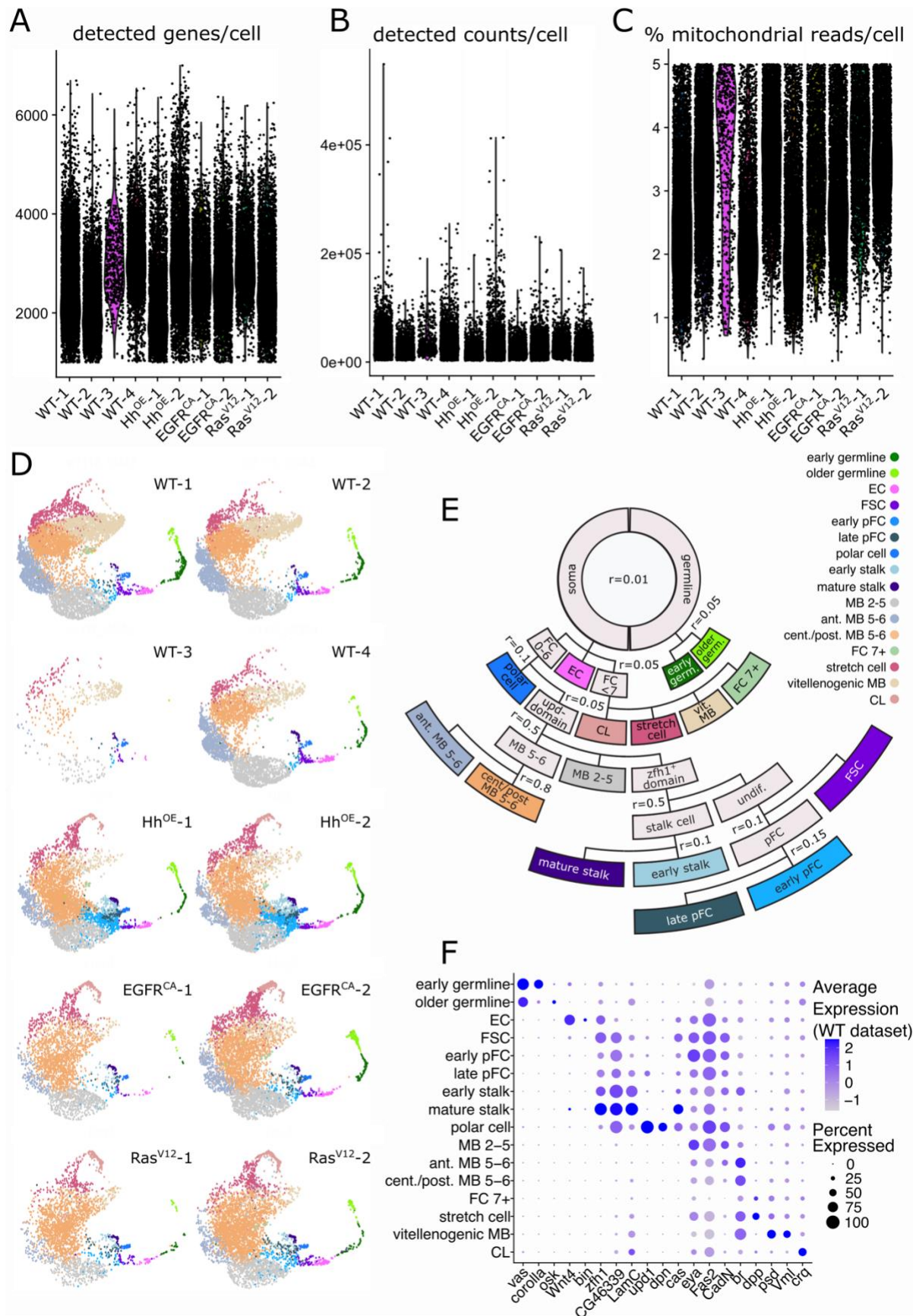
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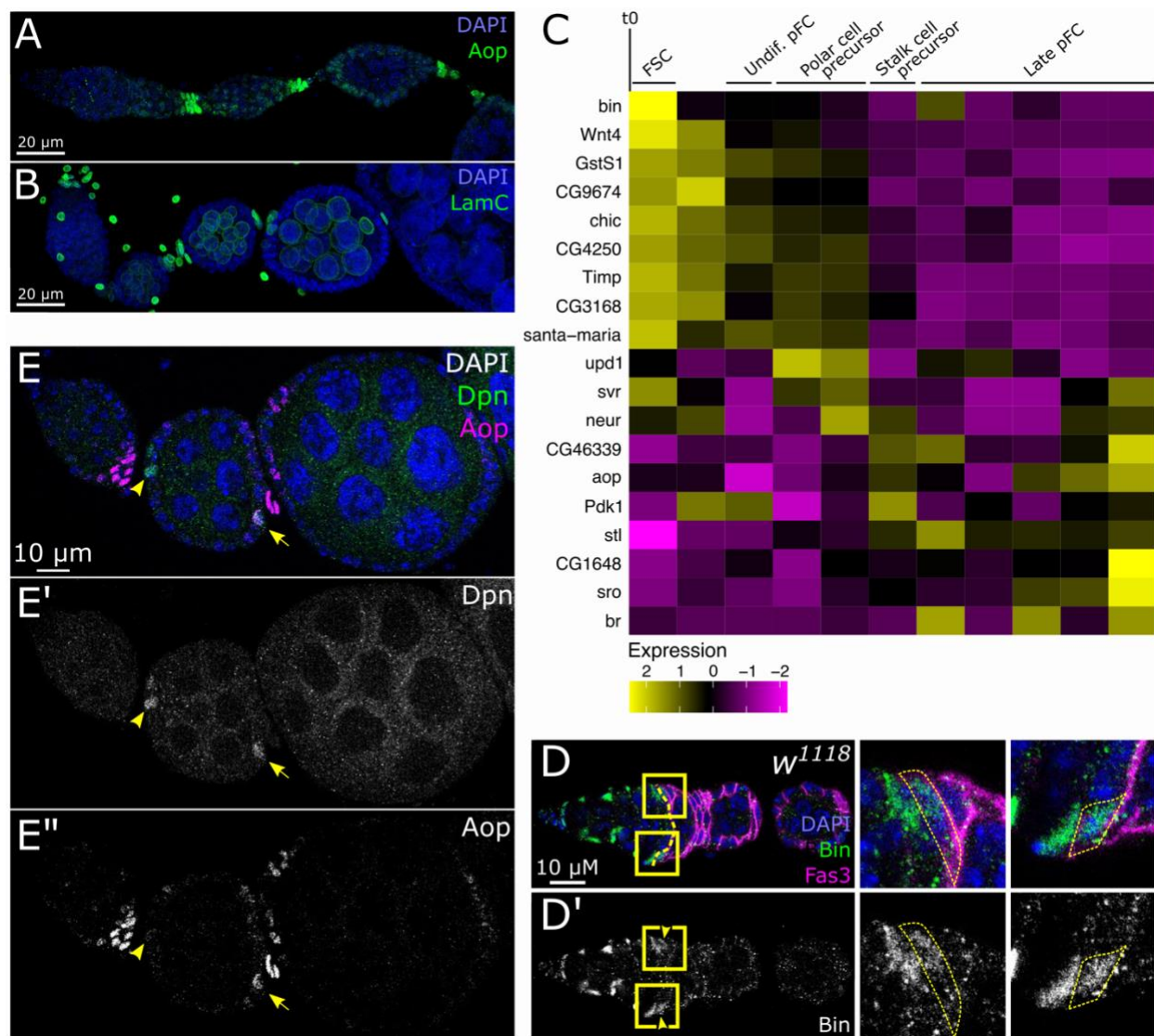
Supplementary Figure 1: Pathway-specific rescue by *Raf* and *smo* knockdown

A-C) A wildtype follicle stained for DAPI (blue), dpERK (green), F-actin stained by Phalloidin (white) and expressing Ptc-cherry (magenta). A'-C) Ptc-cherry; A''-C'') dpERK; A'''-C''') F-actin; A'''' - C''''') DAPI in white. A) shows a maximum intensity projection of all stalk cells, B-C) are different focal planes of the same follicle. Dotted line in (A) marks the stalk, line in (B) denotes polar cells identified by apical F-actin accumulation and lines in C) mark dpERK positive cells with chromosomes aligned in metaphase planes. D-E) Barplots showing quantification of phenotypes of ovarioids shown in Fig. D) n = 20, 30, 29, 28 ovarioids for WT, Hh^{OE}, EGFR^{CA}, Ras^{V12}. With ERK^{DN}: n = 26, 53, 39, 21 ovarioids for WT, Hh^{OE}, EGFR^{CA}, Ras^{V12}. With *Raf*-RNAi: n = 34, 26, 33, 32 ovarioids for WT, Hh^{OE}, EGFR^{CA}, Ras^{V12}. E) n = 20, 30, 29, 28 ovarioids for WT, Hh^{OE}, EGFR^{CA}, Ras^{V12}. With *smo*-RNAi #1: n = 26, 35, 24, 20 ovarioids for WT, Hh^{OE}, EGFR^{CA}, Ras^{V12}. With *smo*-RNAi #2: n = 30, 40, 39, 48 ovarioids for WT, Hh^{OE}, EGFR^{CA}, Ras^{V12}. F-M) Ovarioids where the respective genotype was combined with *Raf*-RNAi (F,H,J,L) or a second *smo*-RNAi (G,I,K,M).



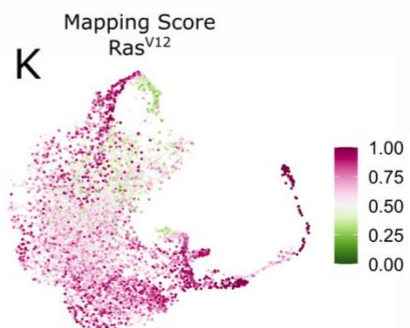
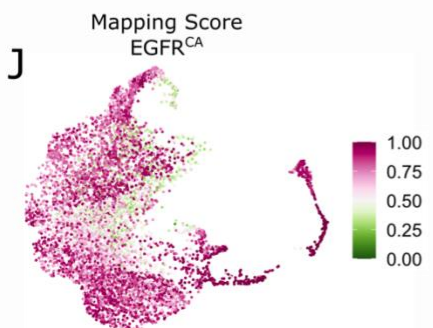
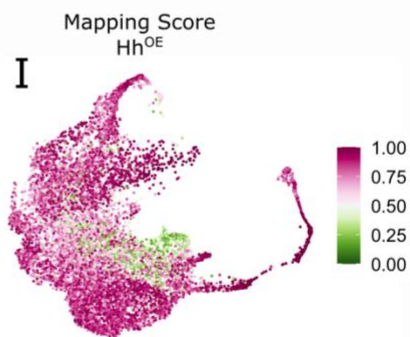
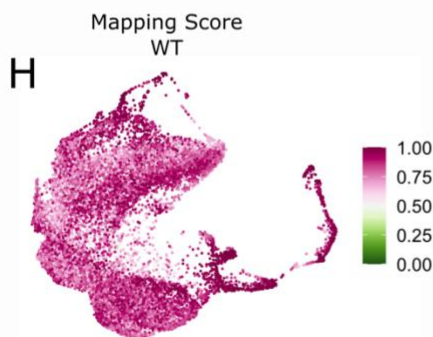
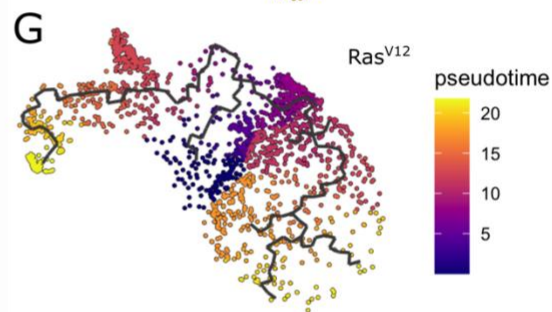
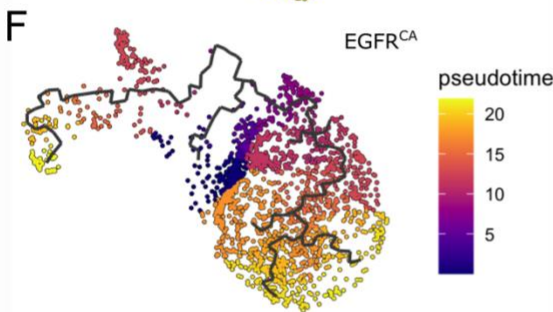
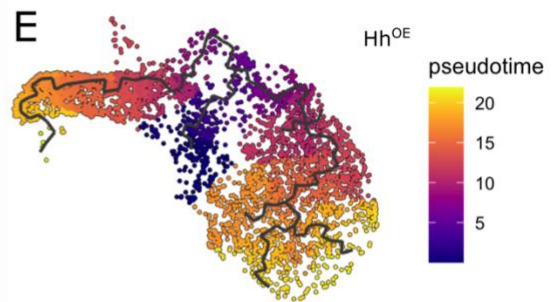
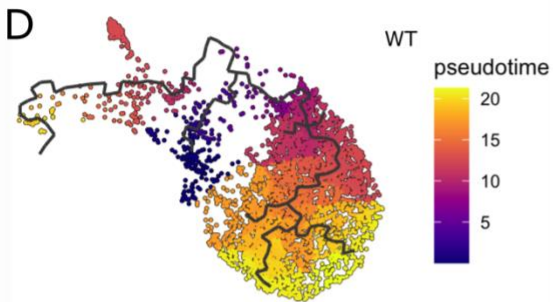
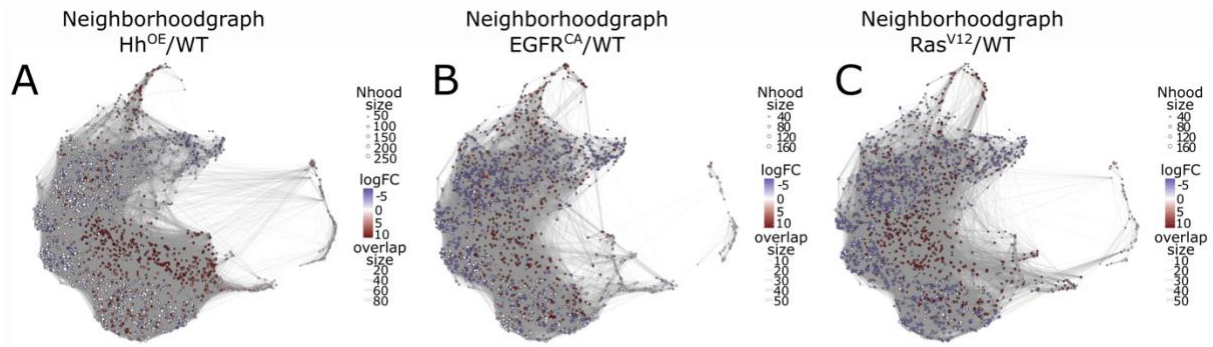
Supplementary Figure 2: Single-cell RNA-sequencing dataset quality

A-C) Quality features by dataset and per cell. A) Number of detected genes. B) Number of reads. C) Percent of mitochondrial reads. D) UMAP plot and cluster annotation of each dataset. E) Plot showing results from iterative unsupervised clustering. Resolution factor (r) is indicated for each round of clustering. F) Average and percent expression of cell type specific markers in WT cells.



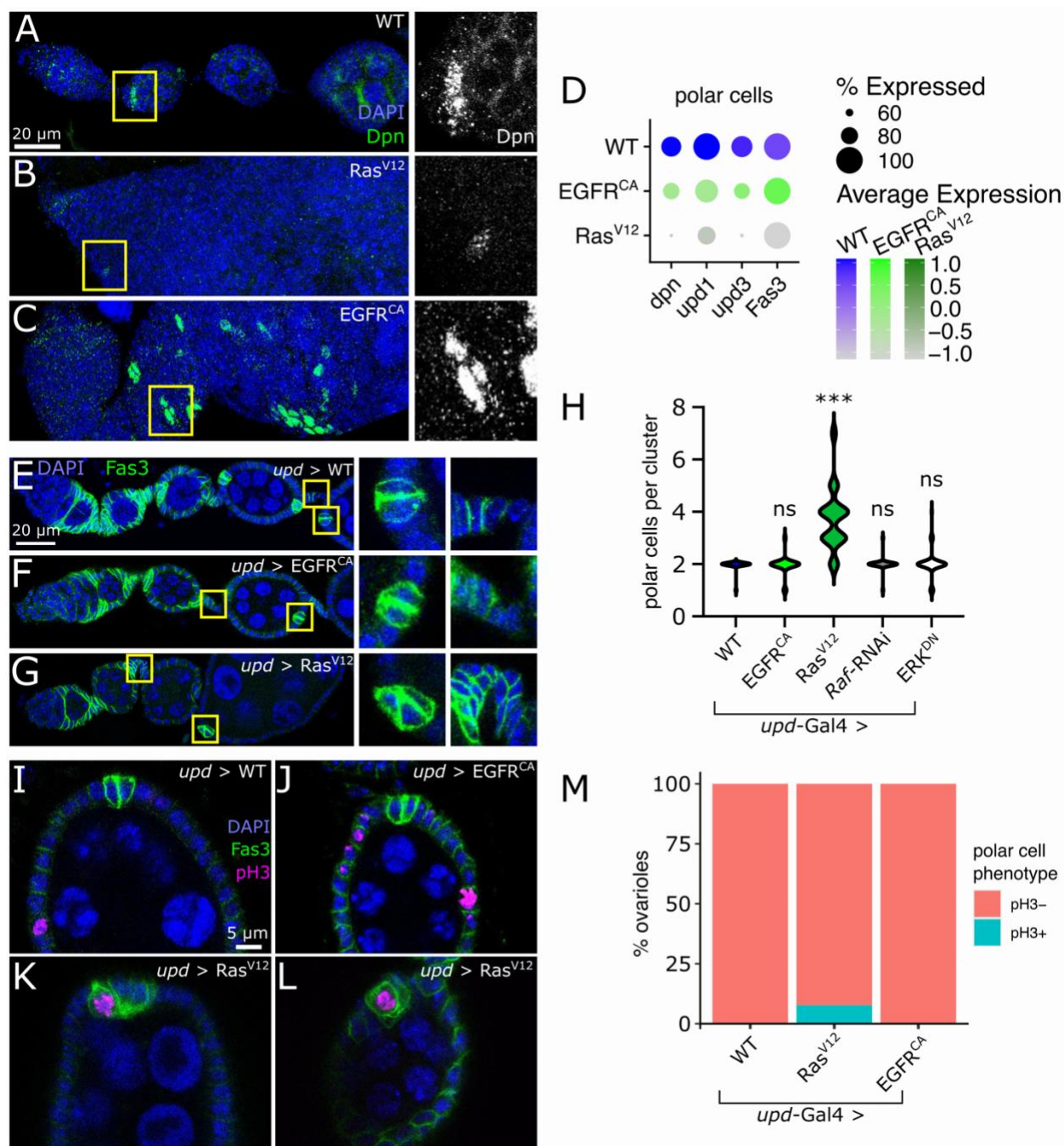
Supplementary Figure 3: Identification of Dpn and Bin as novel cell-type specific markers

A-B) Expression of the stalk cell markers in wildtype ovaries with the genotype $109-30^{ts} > w^{1118}$ stained for DAPI (blue) and A) Aop or B) LamC (green). C) Pseudotime analysis of wildtype FSCs and pFCs as reported in ¹². *bin* marks FSCs at the beginning of pseudotime. D) Wildtype ovary of the genotype $109-30^{ts} > w^{1118}$ stained for DAPI (blue), Bin (green) and Fas3 (magenta). Note that a small number of Fas3 positive cells express Bin (arrows). E) Expression of the polar cell marker Dpn (green, white in E') and the stalk cell marker Aop (magenta, white in E'') in wildtype ovaries ($109-30^{ts} > w^{1118}$). DAPI (blue) marks nuclei. Young stages contain cells co-expressing Dpn and Aop (arrowheads).



Supplementary Figure 4: Monocle analysis identifies developmental delay in MB differentiation

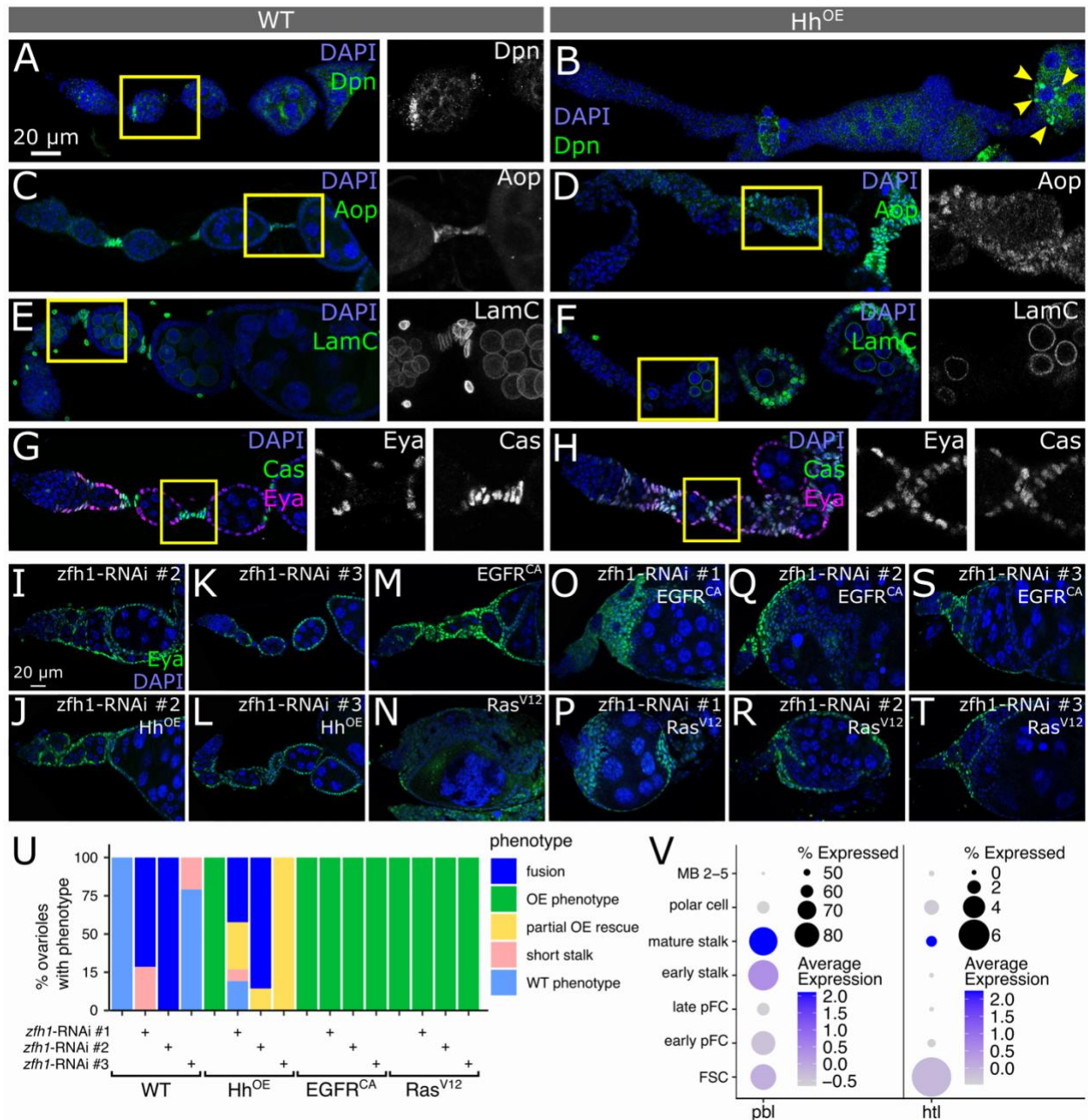
A-C) Neighborhoodgraphs from differential abundance analysis comparing Hh^{OE} (A), EGFR^{CA} (B) or Ras^{V12} (C) to the WT dataset. D-G) UMAP plots showing pseudotime of the respective dataset in the FSC, pFC and stalk clusters, polar cells and MB 2-5 clusters. H-K) UMAP plots with mapping scores for each cell of the respective genotypes.



Supplementary Figure 5: Ras^{V12} induces ectopic mitosis in polar cells

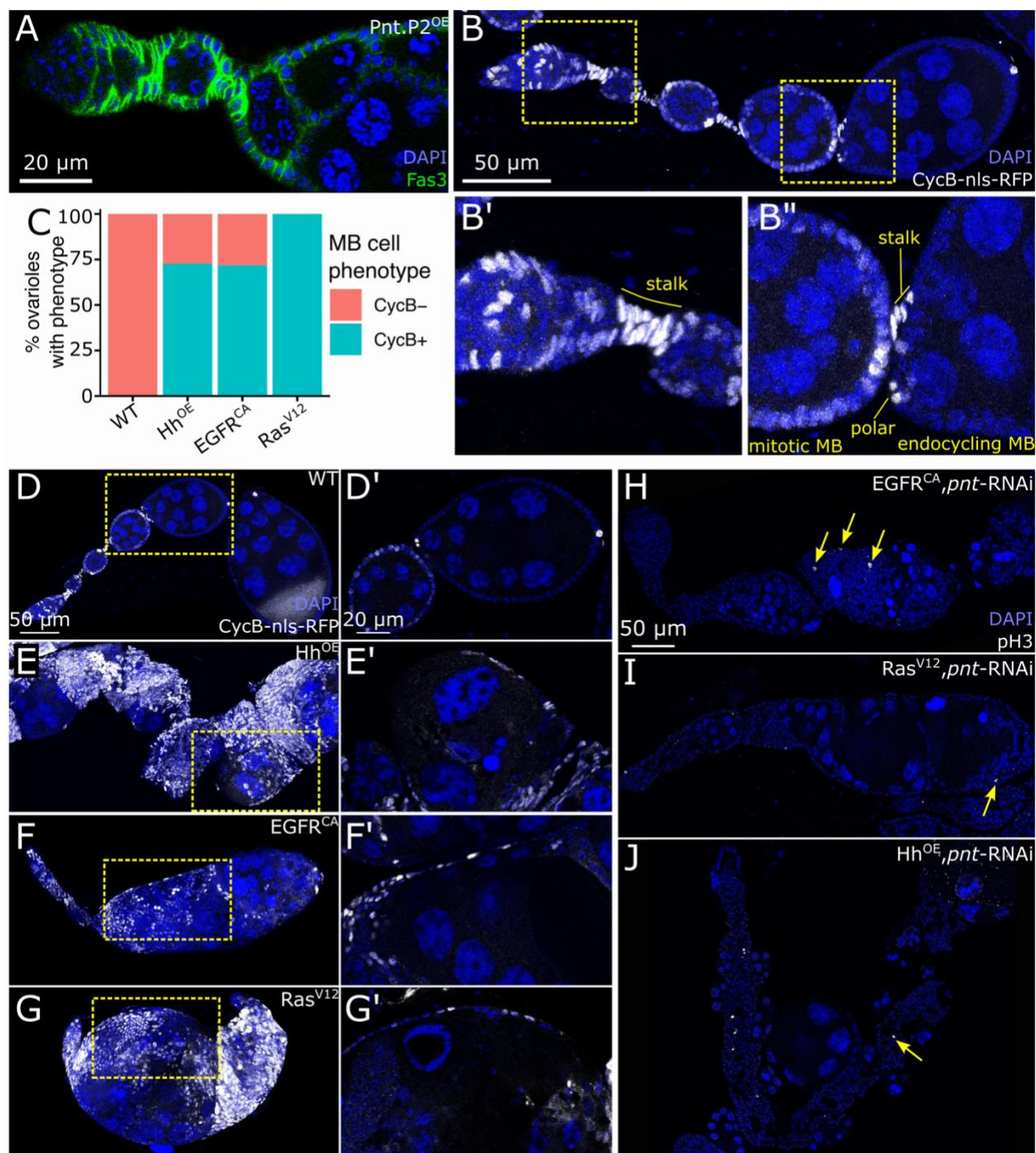
A-C) Ovarioles of *109-30^{ts}* driving *w¹¹¹⁸* as a WT control (A), Ras^{V12} (B) or EGFR^{CA} (C) stained for DAPI (blue) and Dpn (green, white in insets). D) Average and percent expression of polar cell markers in polar cells of the indicated datasets. E-G) Ovarioles driving Gal4 in polar cells under the *upd* promoter and combined with (E) WT, (F) EGFR^{CA} or (G) Ras^{V12} and stained for DAPI (blue) and Fas3 (green). Insets show a mature polar cell cluster (left) and a mature stalk (right). Note that polar cell specific expression of Ras^{V12} but not EGFR^{CA} or the control results in more than 2 polar cells per cluster and double row stalks. H) Violinplot showing the quantification of

polar cells per cluster (St. 5-7) of *upd^{ts}* inducing the respective genotype. n = 25, 18, 23, 61, 59 ovarioles for WT, Ras^{V12}, EGFR^{CA}, *Raf*-RNAi and ERK^{DN} respectively. p-values from Kruskal-Wallis test for EGFR^{CA} = 0.9485 (ns), Ras^{V12} = 1.791e-08 (***), *Raf*-RNAi = 0.6461 (ns), ERK^{DN} = 0.4965 (ns). I-L) Immunostaining of follicles of *upd* inducing the respective genotype stained for DAPI (blue). Polar cells were identified by Fas3 expression (green) and mitotic cells marked by pH3 (magenta). M) Quantification associated with I-L). We quantified the number of ovarioles containing mitotic polar cells. n = 44, 39, 42 ovarioles for WT, Ras^{V12} and EGFR^{CA} respectively.



Supplementary Figure 6: *zfh1* is a Hh pathway target gene

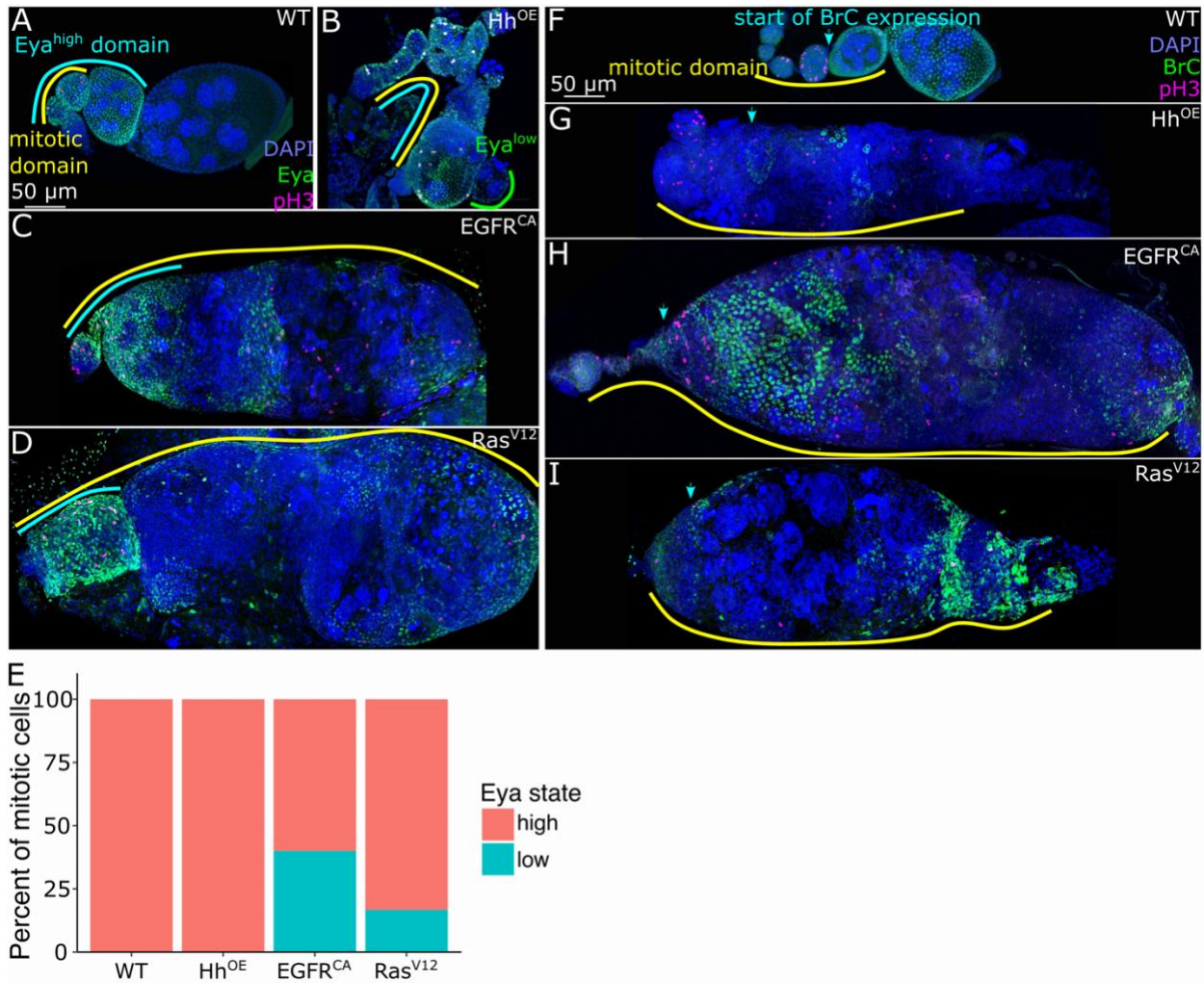
A-H) Expression of cell type specific markers in WT ovarioles (A,C,E,G) and ovarioles with Hh^{OE} (B,D,F,H). We stained for DAPI (blue) and (A-B) Dpn (green), (C-D) Aop (green), (E-F) LamC (green) and (G-H) Cas (green) and Eya (magenta). Insets in (G-H) show stalk regions with Eya or Cas in white. I-T) Immunostaining for DAPI (blue) and Eya (green) in ovarioles with the respective genotypes. U) Quantification of phenotypes in WT, Hh^{OE}, EGFR^{CA} and Ras^{V12} with or without *zfh1*-RNAi #1. n = 20, 30, 29, 28 ovarioles for WT, Hh^{OE}, EGFR^{CA}, Ras^{V12}. With *zfh1*-RNAi #1: n = 21, 26, 24, 19 ovarioles for WT, Hh^{OE}, EGFR^{CA}, Ras^{V12}. With *zfh1*-RNAi #2: n = 22, 35, 29, 19 ovarioles for WT, Hh^{OE}, EGFR^{CA}, Ras^{V12}. With *zfh1*-RNAi #3: n = 24, 18, 48, 35 ovarioles for WT, Hh^{OE}, EGFR^{CA}, Ras^{V12}. V) Average and percent expression of *pbl* and *htl* in WT clusters.



Supplementary Figure 7: EGFR-Ras and Hh signaling induce ectopic CycB expression in MB cells

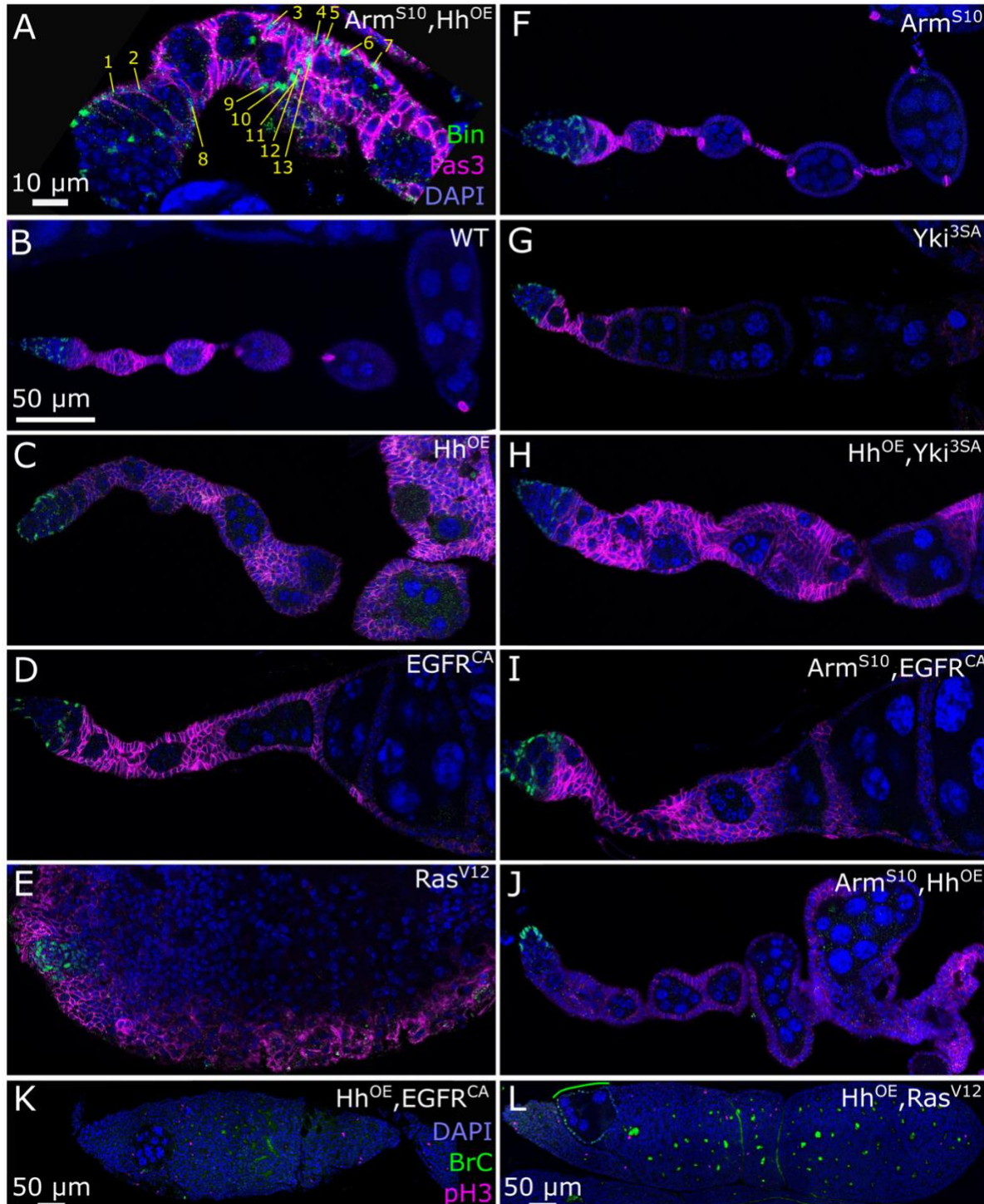
A) Ovariole expressing Pnt.P2 under the *109-30* promoter and stained for DAPI (blue) and Fas3 (green). B) WT ovariole expressing CycB-nls-RFP (white) and stained for DAPI (blue). B') Some follicle cells in Region 2b express CycB-nls-RFP while stalk cells express high levels. B'') Follicle cells up to Stage 5 express CycB-nls-RFP in a "salt and pepper" pattern but at low levels while no CycB-nls-RFP can be detected in MB cells of later stages. Polar and stalk cells express high CycB-nls-RFP levels. C-G) Quantification and immunostaining of CycB-nls-RFP expression. C)

We quantified the presence of ovarioles past Stage 6 with CycB-nls-RFP positive MB cells. n = 31, 32, 32, 4 ovarioles for WT, Hh^{OE}, EGFR^{CA}, Ras^{V12}. Note that we examined well over 100 ovarioles for Ras^{V12} but only rarely identified ovarioles with cysts later than Stage 6. D-G) Ovarioles stained for DAPI (blue) and CycB-nls-RFP (white) in the respective genotypes. D'-G') show insets in follicles past Stage 6. H-J) Ovarioles of indicated genotypes stained for DAPI (blue) and pH3 (white). Note the presence of pH3 positive cells past Stage 6 (arrows).



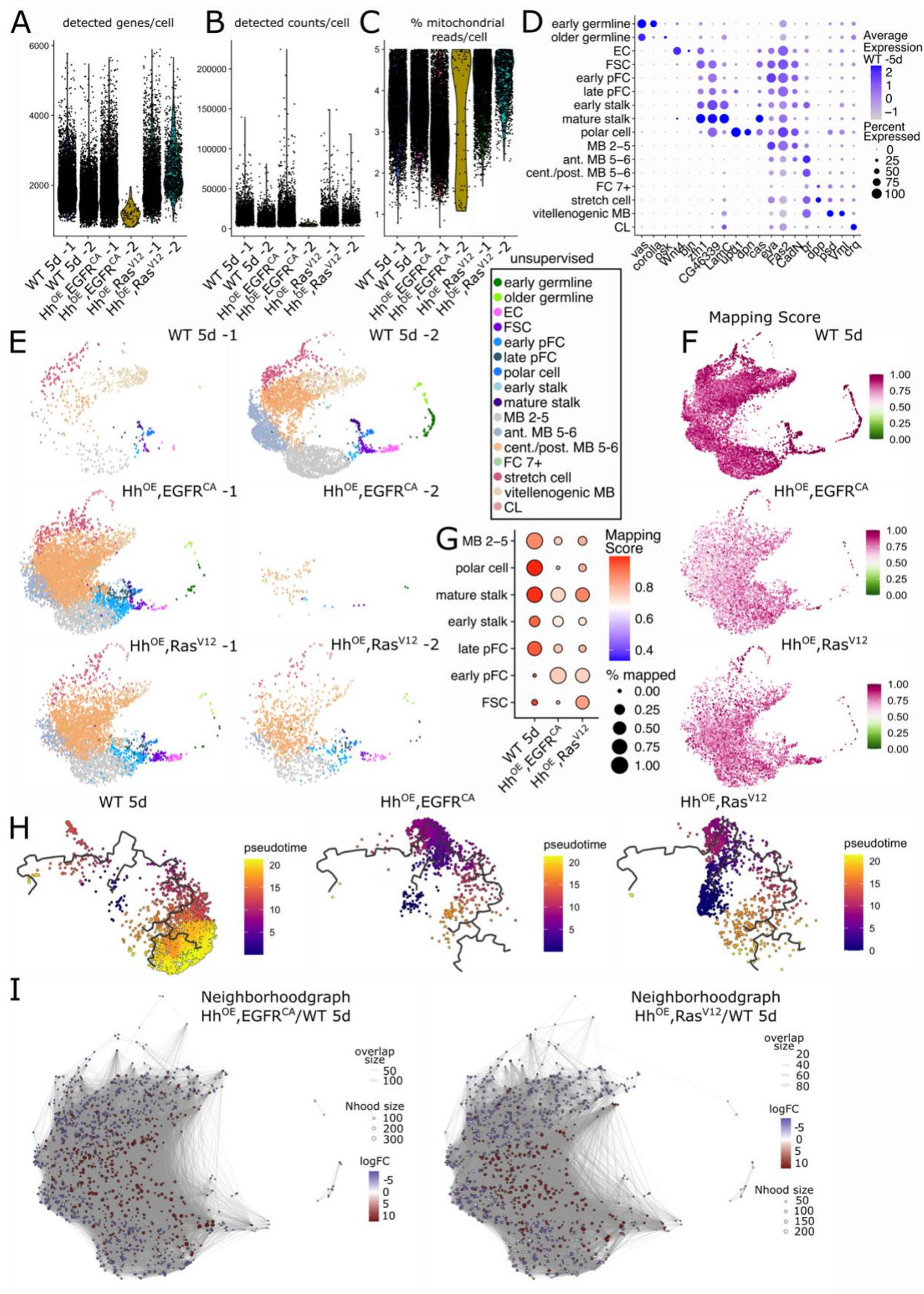
Supplementary Figure 8: MB cells with active Hh or EGFR-Ras signaling are competent to differentiate

A-D) Maximum intensity projections of ovarioles immunostained for DAPI (blue), pH3 (magenta) and Eya (green) of *109-30^{ts}* driving the respective genotype. Follicle stages containing mitotic cells are marked by a yellow line. Stages containing high expression of Eya are marked by a cyan line. Green line in (B) marks a cyst with low Eya expression. E) Quantification of mitotic cells with and without high levels of Eya expression. $n = 35, 20, 25, 18$ cells for WT, Hh^{OE}, EGFR^{CA} and Ras^{V12} respectively. F-I) Maximum intensity projections of immunostained ovarioles of *109-30^{ts}* driving the respective genotype. We stained for DAPI (blue), pH3 (magenta) and BrC (green). Areas with mitotic cells are marked with yellow lines, the first cyst expressing BrC is marked by a cyan arrow.



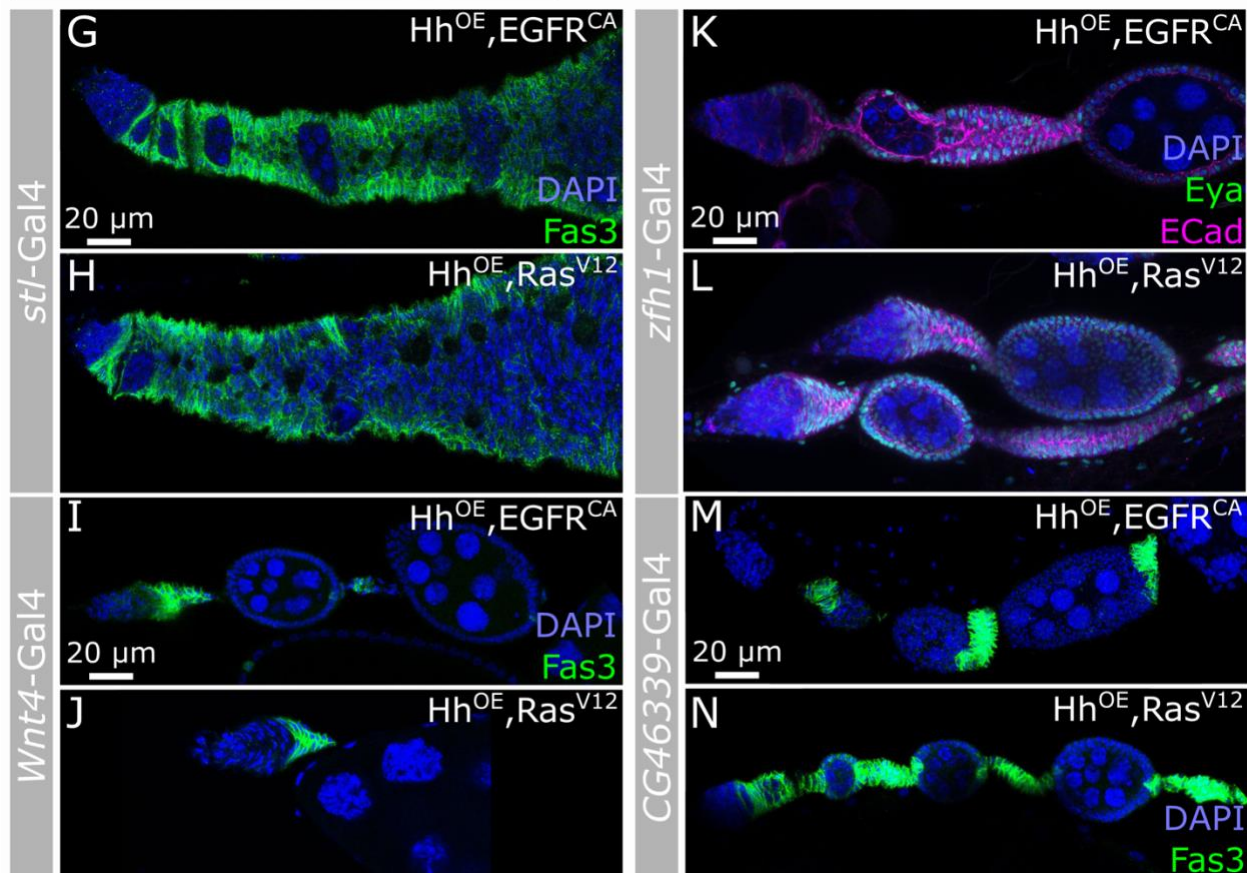
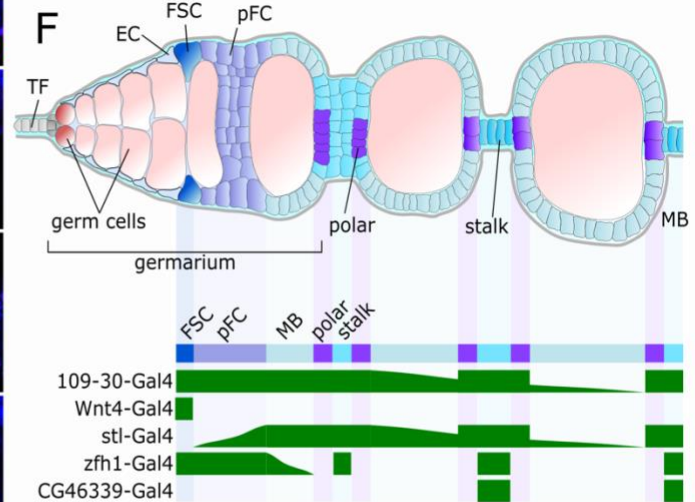
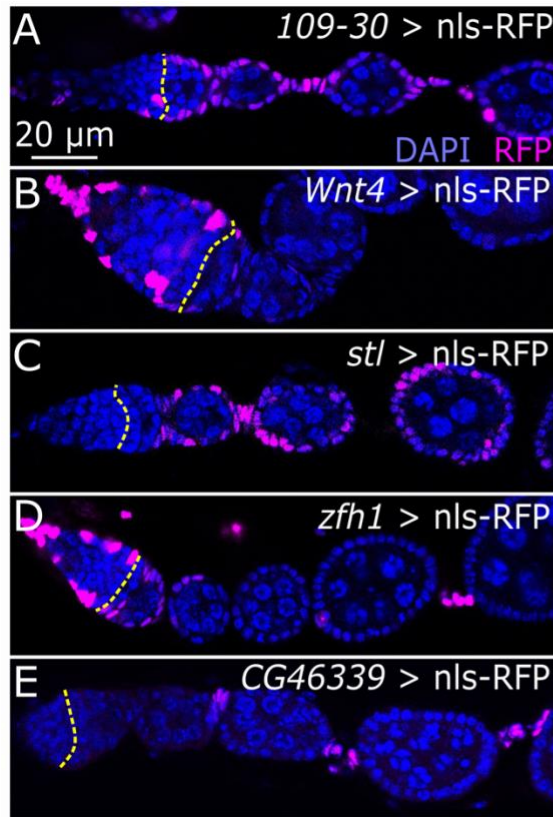
Supplementary Figure 9: Pathway overactivation promotes morphological defects in the follicle epithelium

A-L) Ovarioles of respective genotypes stained for DAPI (blue), Fas3 (magenta) and Bin (green) (A-J) or DAPI (blue), pH3 (magenta) and BrC (green) (K-L). Fas3/Bin positive cells are numbered in (A). Rarely observed BrC positive cells in the co-overactivation background are marked by a green line in (L).



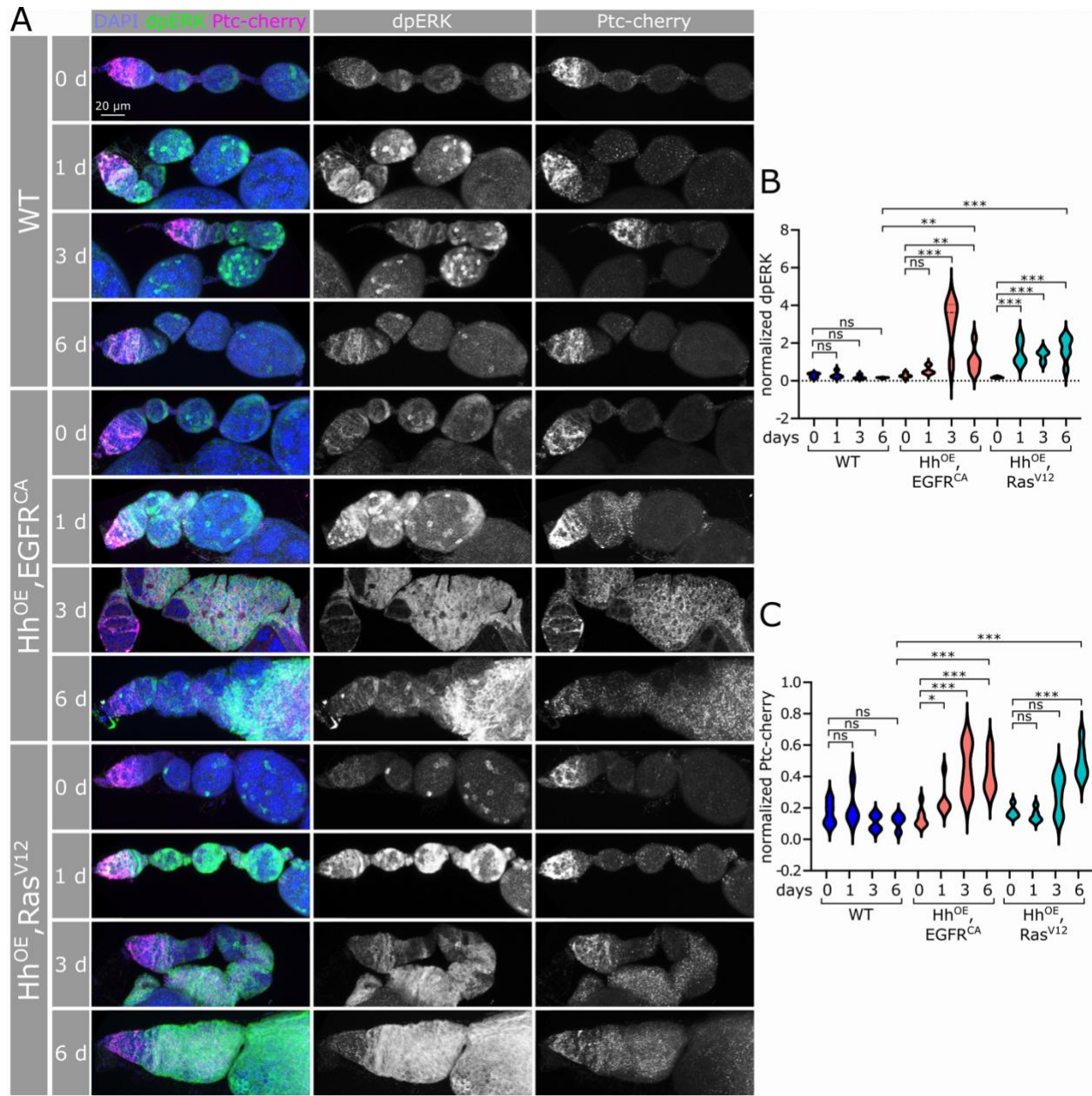
Supplementary Figure 10: scRNA-sequencing of Hh-EGFR/Ras co-overactivated follicle cells

A-C) Violin plots showing the number of genes (A), counts (B) and percentage of mitochondrial reads (C) per cell in the indicated genotypes. D) Dotplot of average and percent expression of cell type specific markers in the WT 5d dataset. E) UMAP plots of each 5d dataset labeled by cluster. F) Mapping scores for datasets as indicated. G) DotPlot showing the average mapping score and percentage of cells per cluster mapped to the expected fate. H) UMAP plot showing the pseudotime of FSCs, pFC, stalk, polar and MB 2-5 clusters. I) Neighborhoodgraphs comparing co-overexpressions to WT associated with differential expression analysis.



Supplementary Figure 11: MB transformation drives Hh-EGFR/Ras follicle cell tumors

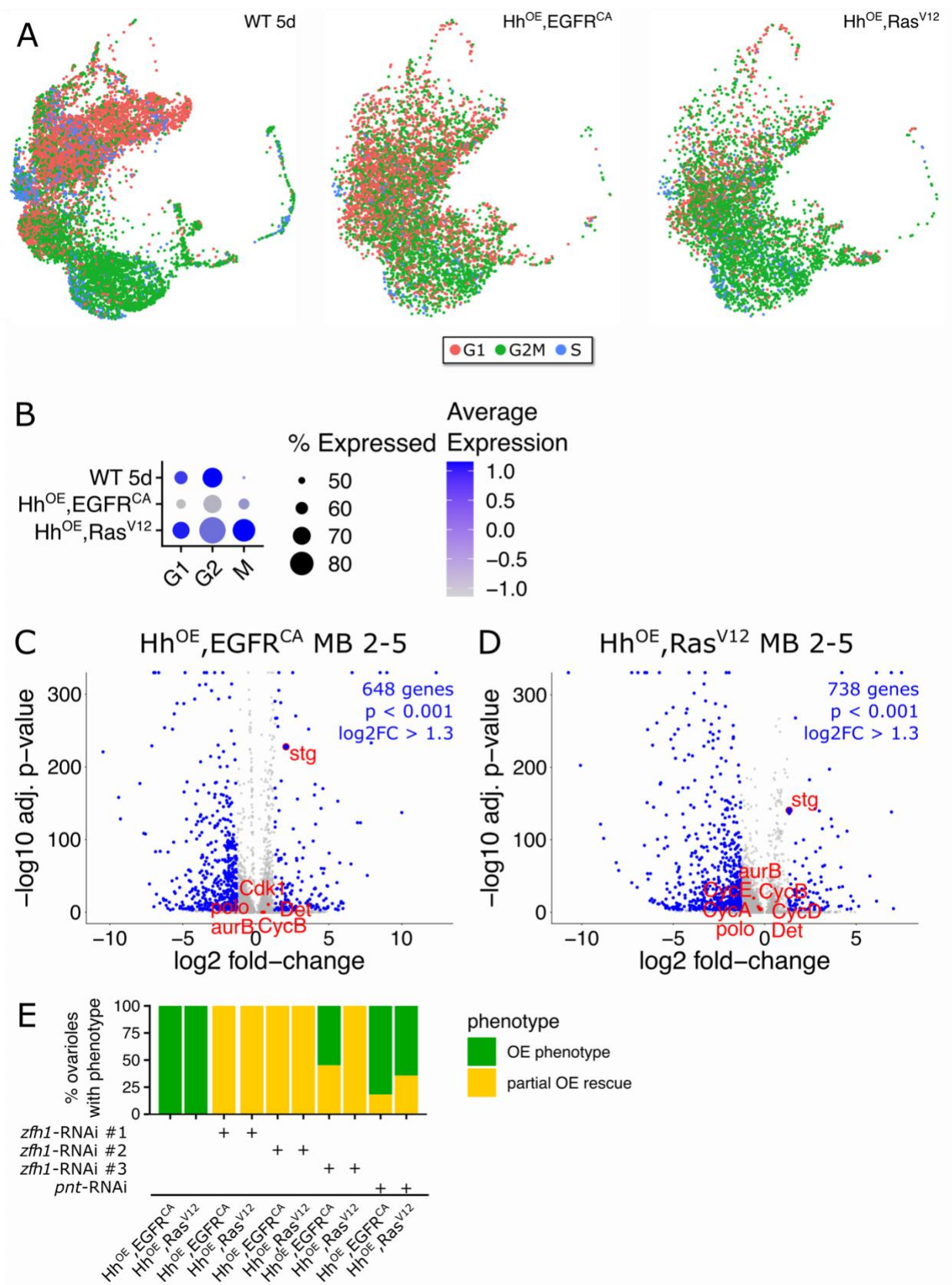
A-E) Expression of nls-RFP under different Gal4 drivers. Yellow dotted line marks the 2a/b border. A) *109-30*-Gal4 induces expression in follicle cells. MB cells reduce Gal4 activity as they differentiate. B) *Wnt4*-Gal4 is active in ECs and in a very narrow range of cells after the 2a/b boundary. C) *stl*-Gal4 is inactive at the 2a/b boundary but drives expression in differentiated follicle cells. D) *zfh1*-Gal4 promotes expression in follicle cells in the germarium. It remains active in stalk cells and rapidly turns off in MB and polar cells. E) *CG46339* expression is specific for stalk cells. F) Graph of the early ovariole stages summarizing Gal4 activity patterns. G-N) Ovarioles expressing Hh^{OE} with EGFR^{CA} (G,I,K,M) or Ras^{V12} (H,J,L,N) under (G-H) *stl*-Gal4, (I-J) *Wnt4*-Gal4, (K-L) *zfh1*-Gal4, (M-N) *CG46339*-Gal4. Note that only expression with *stl*-Gal4 induces a morphological phenotype similar to induction by *109-30*-Gal4.



Supplementary Figure 12: Tumor progression involves rapid tissue expansion

A) Ovarioles of the indicated genotype induced by *109-30^{ts}*, combined with Ptc-cherry and stained for DAPI (blue), cherry (magenta) and dpERK (green). dpERK and Ptc-cherry channels are also shown in white. Ovarioles were dissected at 0, 1, 3 or 6 days post temperature shift. All images were taken with similar settings. B-C) Quantification of dpERK levels (B) and Ptc-cherry levels (C). We measured activity in stalk-regions for the WT and similar stages for the co-overactivation genotypes and normalized each measurement to the levels in Region 1. WT: n = 4,4,6,4 for 0,1,3,6 days respectively. Hh^{OE},EGFR^{CA}: n = 7,6,5,6 for 0,1,3,6 days respectively. Hh^{OE},Ras^{V12}: n = 5,5,6,6 for 0,1,3,6 days respectively. p-values from student's t-test: B) WT in comparison to 0 days: 1 days: 0.93 (ns), 3 day : 0.81 (ns), 6 days: 0.73 (ns). Hh^{OE},EGFR^{CA} in comparison to 0 days: 1 days: 0.3 (ns), 3 day : 1.93E-12 (***) , 6 days: 0.009 (**). Hh^{OE},Ras^{V12} in comparison to 0

days: 1 days: 0.0002 (***), 3 day: 0.0005 (***), 6 days: 1.29E-05 (***). 6 days comparison to WT: Hh^{OE},EGFR^{CA}: 0.01 (**), Hh^{OE},Ras^{V12}: 2.62E-05 (***). C) WT in comparison to 0 days: 1 days: 0.56 (ns), 3 day : 0.36 (ns), 6 days: 0.43 (ns). Hh^{OE},EGFR^{CA} in comparison to 0 days: 1 days: 0.044 (*), 3 day: 1.57E-06 (***), 6 days: 2.41E-06 (***). Hh^{OE},Ras^{V12} in comparison to 0 days: 1 days: 0.71 (ns), 3 day : 0.05 (ns), 6 days: 1.65E-06 (***). 6 days comparison to WT: Hh^{OE},EGFR^{CA}: 7.83E-06 (***), Hh^{OE},Ras^{V12}: 1.55E-07 (***).



Supplementary Figure 13: Hh-EGFR/Ras follicle tumors upregulate the cell cycle regulator *string*

A) UMAP plots showing results from cell cycle scoring. B) DotPlot showing the expression of cell cycle specific genes per all cells of the indicated datasets. G1-phase: *Cdk1*, *CycD* and *CycE*, G2-phase: *stg*, *CycA* and *CycB*, M-phase: *polo*, *aurB* and *Det* as reported in ⁵⁵. C-D) Volcano plot showing all differentially expressed genes of the MB 2-5 of Hh^{OE},EGFR^{CA} (C) and Hh^{OE},Ras^{V12} (D) compared to WT 5d MB 2-5. Genes with a p-value under 0.001 and a log2FC > ± 1.3 are labeled in blue. Cell cycle markers are marked in red. Note that *stg* is among the significantly regulated genes in both datasets. E) Quantification of phenotypes of ovarioles with co-overexpression of Hh^{OE} with EGFR^{CA} or Ras^{V12} combined with different *zfh1*- or *pnt*-RNAi lines. Compare to phenotypes shown in Fig. 7T-W. n = 33, 41 ovarioles for Hh^{OE}, EGFR^{CA} and Hh^{OE}, Ras^{V12} respectively. With *zfh1*-RNAi #1: n = 36, 32 ovarioles for Hh^{OE}, EGFR^{CA} and Hh^{OE}, Ras^{V12} respectively. With *zfh1*-RNAi #2: n = 39, 40 ovarioles for Hh^{OE}, EGFR^{CA} and Hh^{OE}, Ras^{V12} respectively. With *zfh1*-RNAi #3: n = 31, 38 ovarioles for Hh^{OE}, EGFR^{CA} and Hh^{OE}, Ras^{V12} respectively. With *pnt*-RNAi: n = 28, 33 ovarioles for Hh^{OE}, EGFR^{CA} and Hh^{OE}, Ras^{V12} respectively.