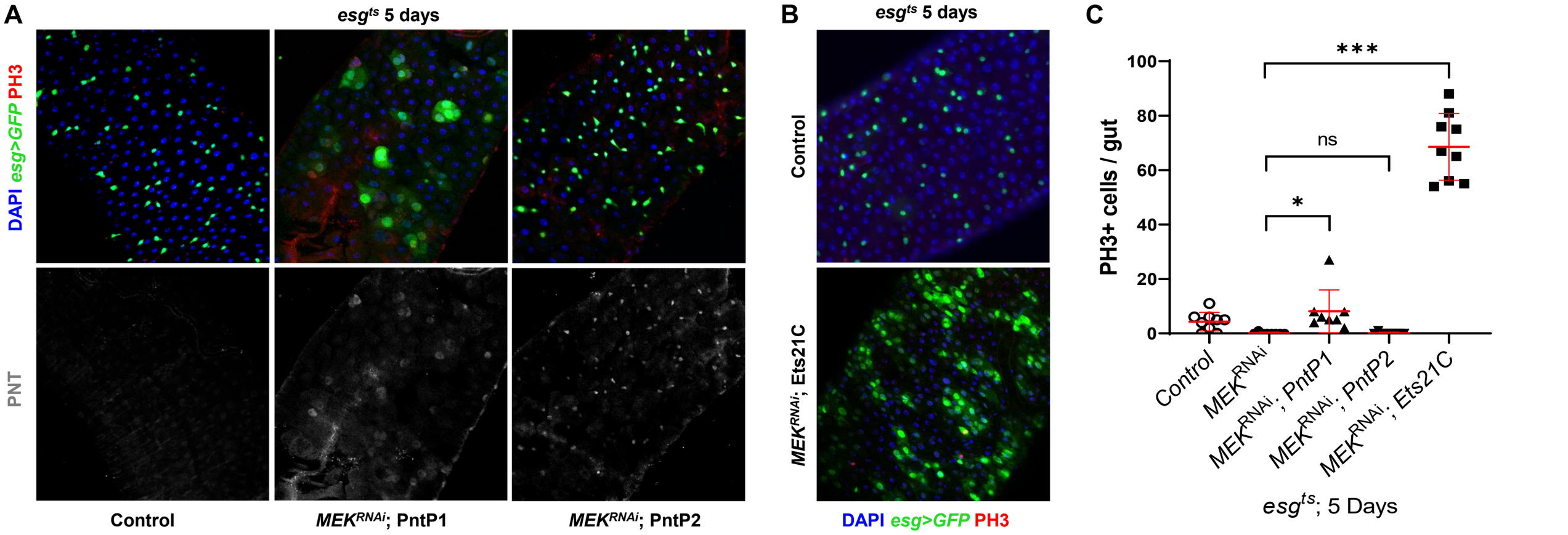
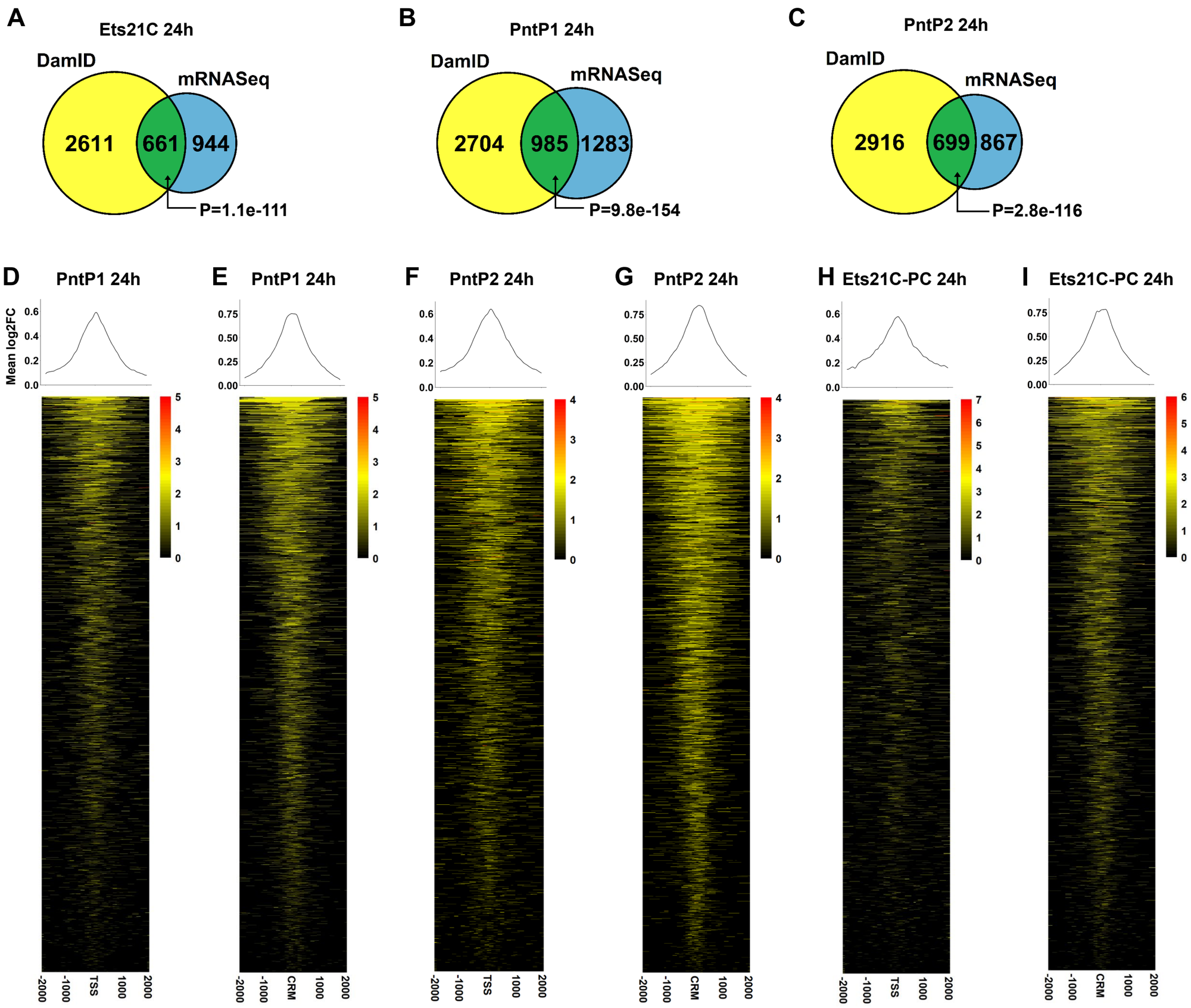
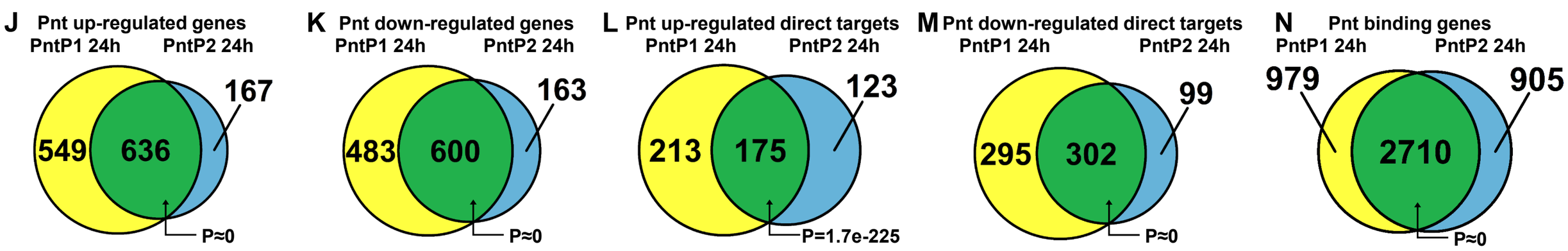


Figure S1. PntP1 and Ets21C affect ISC proliferation and act in a RAS/MAPK signaling independent manner. Related to Figure 1.
(A-C) Results of the *MEK* and *PntP1, PntP2,* or *Ets21C* epistasis tests. Transgenes were induced via the *esg^{ts}* system at 29°C for 5 days. (A) *PntP1* and *MEK^{RNAi}* co-expressing midgut, showing clusters of large GFP+ ISCs and EBs (green). *PntP1* could rescue *MEK^{RNAi}*-induced inhibition of ISC proliferation. *PntP2* and *MEK^{RNAi}* co-expressing midguts did not show any difference compared to control midguts. Scale bars represent 50 μm. (B) *Ets21C* and *MEK^{RNAi}* co-expressing midgut, showing more PH3+ ISCs (red) and GFP+ ISCs/EBs (green). (C) Quantification of PH3+ cells in adult midguts of the indicated genotypes. MAPK knockdown could not inhibit ISC mitoses caused by *PntP1* or *Ets21C* overexpression. (D-E) Overexpressing Ets21C-PC isoform in ISCs induces ISC proliferation. (D) Confocal images of posterior midguts overexpressing Ets21C isoforms specifically in ISC using the *esg^{ts}; Su(H)Gal80* driver for 5 days at 29°C. Scale bar is 50μM. (E) Mitotic events per gut scored by counting PH3+ cells. (F-G) EGFR signaling and downstream transcription factors affected ISC cellular size. (F) Flow cytometry unit distribution of FSC-Area of YFP positive ISCs upon activation of EGFR signaling by EGF ligand sSpi or downstream transcription factors for 8h. Short time activation of EGFR signaling mildly promoted ISC cellular growth. (G) Flow cytometry unit distribution of FSC-Area of YFP positive ISCs upon EGFR signaling depletion by Egfr, Pnt, or Ets21C knockdown, or EGFR signaling activation by transcriptional repressor Cic knockdown. Statistical significance was determined by Student's t test corrected for multiple testing (*p<0.05, ****p<0.0001; ns, not significant). Error bars represent standard deviation.





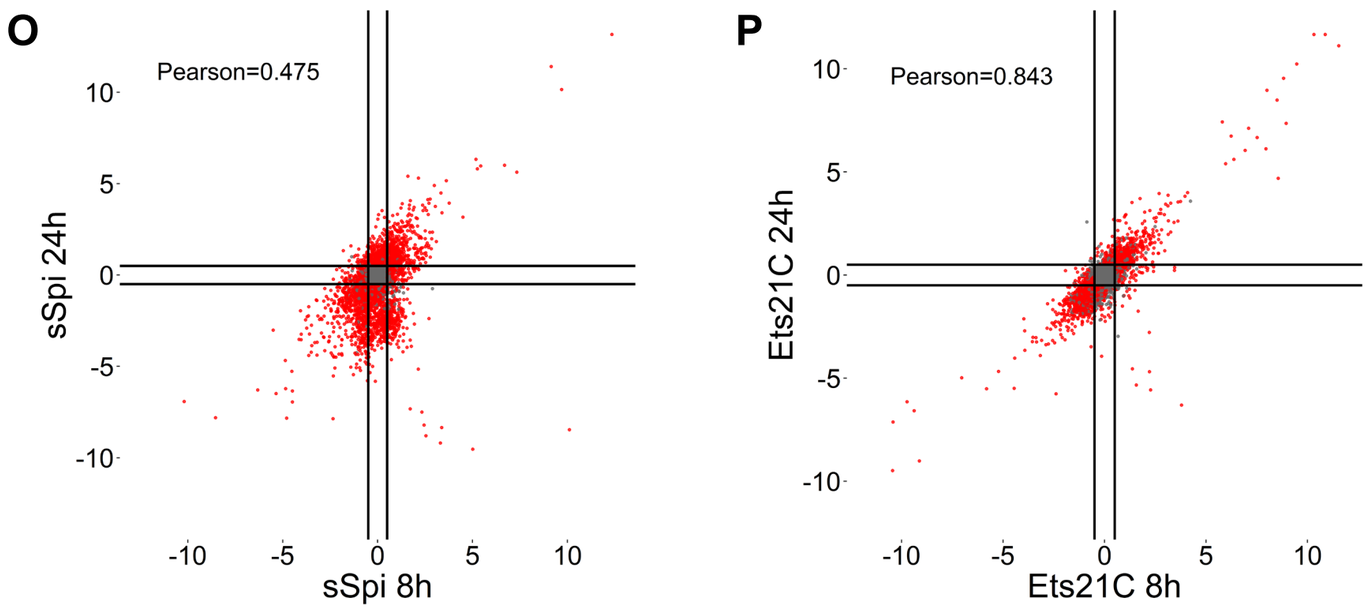


Figure S2. ISC specific DamID Seq and mRNA Seq of PntP1, PntP2, and Ets21C. Related to Figure 2.
(A-C) Venn diagram showing the significant overlap between DamID Seq and mRNA Seq of Ets21C, PntP1, and PntP2 OE for 24h in ISC. (D-I) DamID binding peaks align nicely at TSS and CRM. (J-N) PntP1 and PntP2 showed great similarity in their transcriptional function as shown by the significant overlap between their (J) up-regulated genes, (K) down-regulated genes, (I) up-regulated direct targets, (M) down-regulated direct targets, and (N) DamID bound genes. (O-P) Comparison of the Log2FC expression of genes differentially expressed by 8h induction versus 24h induction of (O) Spitz or (P) Ets21C. Black bars indicate the Log2FC cutoff (0.5 and -0.5). The gene expression profile of (O) sSpi changes dramatically between the 8h and 24h time-points, while the gene profile induced by (P) Ets21C OE strongly correlates between the 8h and 24h time-points.

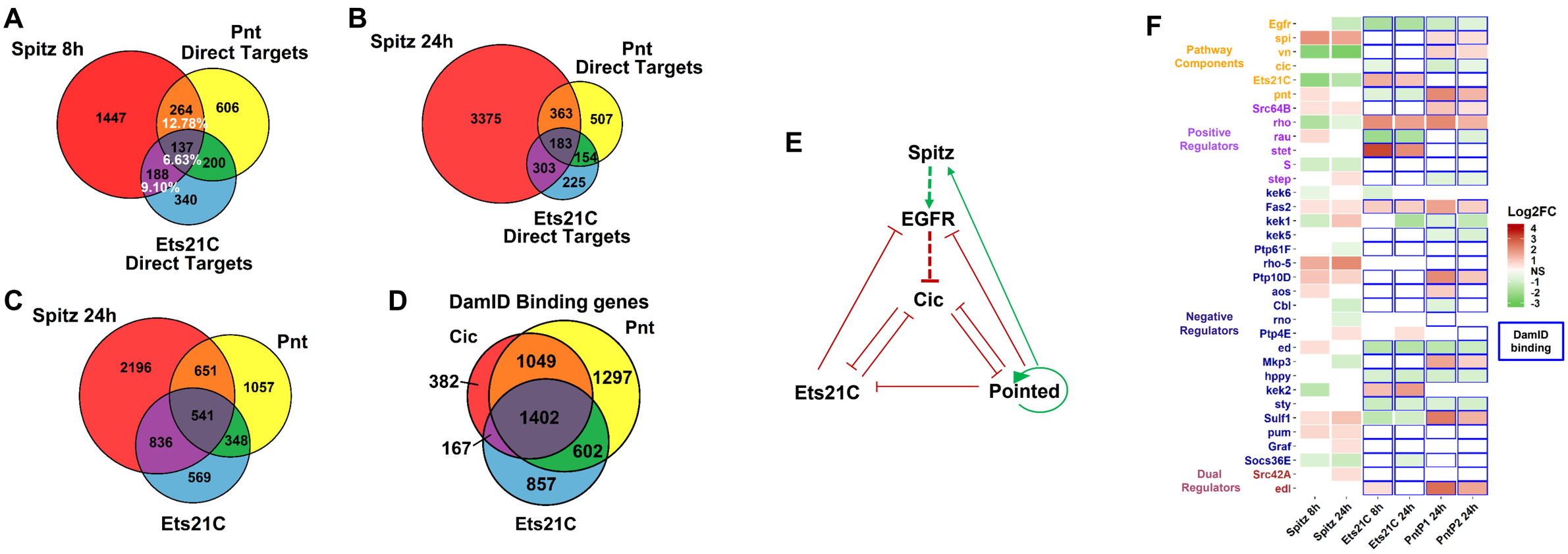


Figure S3. Pnt and Ets21C are essential downstream transcriptional mediators of EGFR signaling. Related to Figure 3.

(A) Venn diagrams showing the significant overlap (P=7.87e-134) between 8h sSpi induced differentially expressed genes, Pnt direct target genes, and Ets21C direct target genes. (B) Venn diagrams showing the significant overlap (P= 3.16e-147) between 24h sSpi induced differentially expressed genes, Pnt direct target genes, and Ets21C direct target genes. (C) Venn diagrams showing the significant overlap (P= 1.35e-301) between 24h sSpi induced differentially expressed genes, Pnt differentially expressed genes, and Ets21C differentially expressed genes. (D) Venn diagrams showing the significant overlap (P≈0) between Cic, Pnt, and Ets21C DamID binding target genes. . (E) Network of core transcription factors downstream of EGFR signaling. Red solid line blunt arrow indicates transcriptional down-regulation, while green indicates up-regulation. Previous study showed upon activation, PntP2 up-regulates itself, but PntP1 isoform[S1]. Red dash line blunt arrow indicates post-translational repression, while green indicates activation. Pnt and Ets21C directly bound and down-regulated both Cic and EGFR, suggesting positive and negative feedback, respectively. Similarly, long-term induction of sSpi also down-regulated EGFR. Pnt and Ets21C had binding sites at each other's loci, but, while Pnt down-regulated Ets21C gene expression, Ets21C did not affect Pnt expression. Interestingly, DamID indicated that Pnt and Ets21C both bound to the Spi gene, but only Pnt up-regulated Spi. (F) Heatmap of pathway components and feedback regulators of EGFR signaling. Due to its potent mitogenic action, tight control of EGFR activity is essential for intestinal homeostasis. EGFR signaling pathway has many accessory regulators that modulate its spatial and temporal responses, using both positive and negative feedback mechanisms[S2]. When looking at the known feedback regulators of EGFR signaling, a majority of them were responsive to sSpi. Positive feedback regulators included *Src64B*, *rho*, *rau*, *stet*, *S*, *step*, and two EGFR ligands, *vn*[S3–S5] and *spi*[S3], while negative feedback regulators included *Fasciclin2*[S6], *kek1*[S7], *kek5*, *kek6*, *pum*[S8], *aos*[S3, S9–S11], *rho-5*, *Chmp1*, *ed*, *Sulf1*[S12], *Socs36E*, *geminin*[S13], *Ptp10D*, *sty*[S14], *Mkp3* (MAPK Phosphatase 3) [S15, S16], and *d-Cbl*[S17]. Dual regulators *edl*[S18, S19] and *Src42A* were also present. Interestingly, this set of feedback regulators was significantly enriched in our DamID data for PntP1, PntP2, and Ets21C with p-value 1.28e-9, 7.75e-10, and 4.43e-10, respectively, meaning they are potential targets of Pnt and Ets21C. In short, our data suggest that the ETS-TFs directly influence a complex network of feedback in the EGFR signaling pathway.

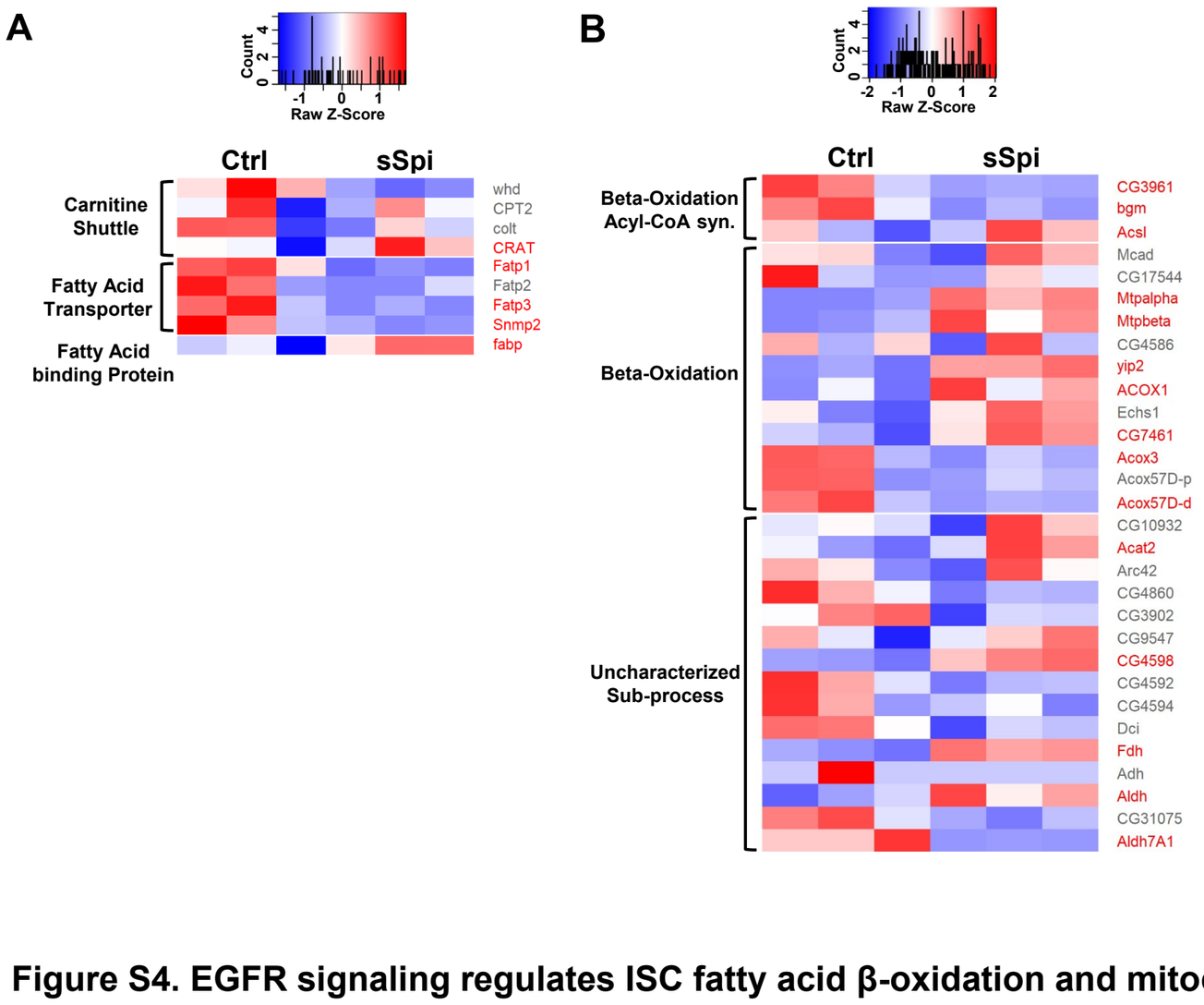


Figure S4. EGFR signaling regulates ISC fatty acid β-oxidation and mitochondrial biogenesis. Related to Figure 4.
Heatmap of (A) fatty acid transport related genes, (B) fatty acid degradation genes (KEGG dme00071), and (C) mitochondrial localized genes and mitochondria master regulators for control and sSpi 24h OE. Genes whose name is in red color are significantly differentially expressed according to our cutoff.

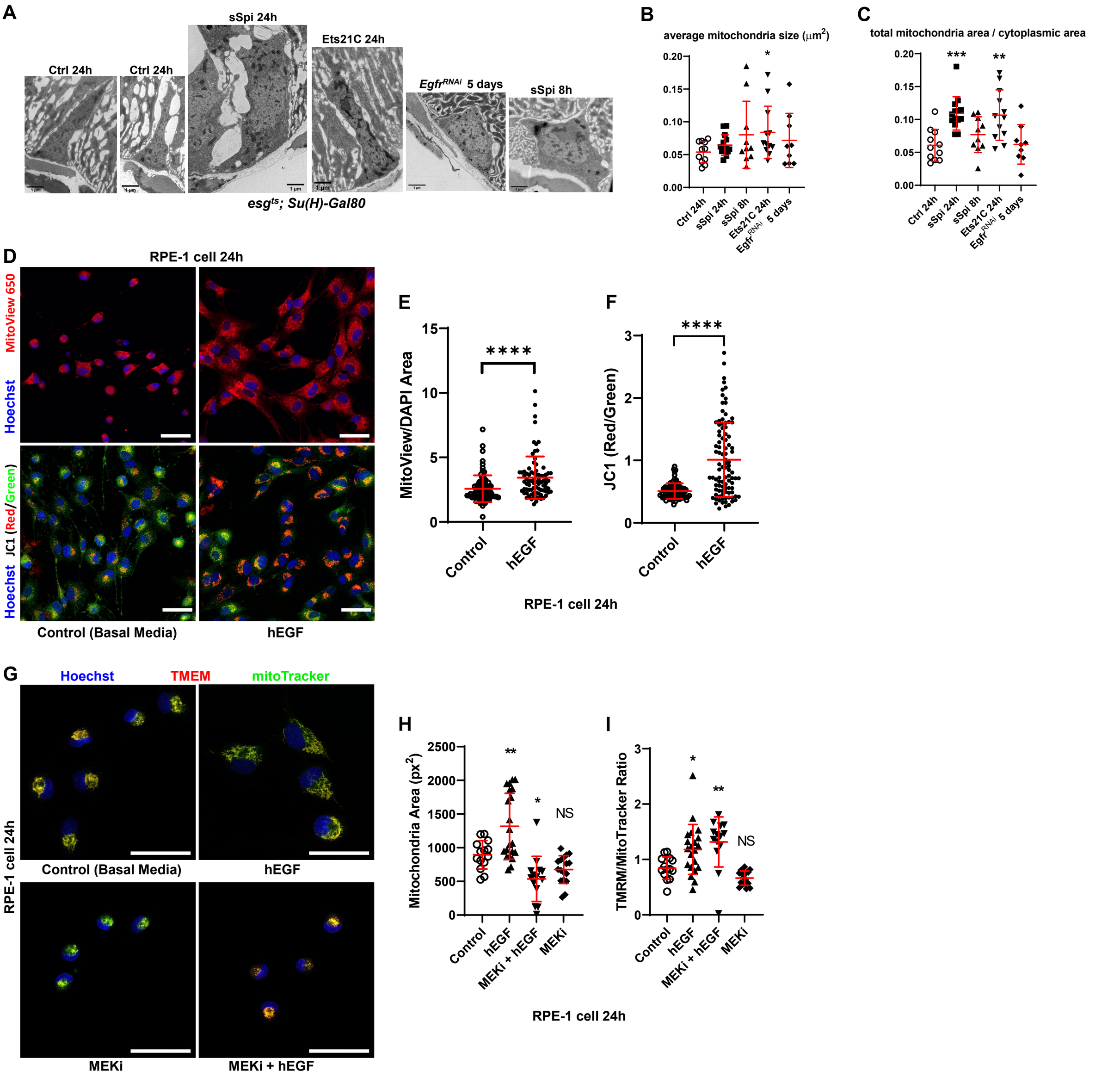


Figure S5. EGFR signaling promotes mitochondrial biogenesis and activity of human RPE-1 cells. Related to Figure 5.
(G-I) Transmission Electron Microscopy (TEM) images reveal EGFR signaling activation promotes *Drosophila* ISC mitochondrial biogenesis. (A) TEM images of ISCs in posterior midguts. Scale bar is 1μM. (B-C) Quantification of the (B) average area of mitochondria in an ISC section, and (C) the proportion of total mitochondria area in cytoplasm (cytoplasm area is cell area minus nuclear area). (C) Live-imaging of RPE-1 cells upon 24h hEGF treatment. For top panels, blue is Hoechst dye, and red is mitoview650 dye. For bottom panels, blue is Hoechst dye, red is JC1 aggregates, and green is JC1 monomers. The ratio of JC1 red/green indicates mitochondrial membrane potential. Scale bar is 50μM. (D) Mitochondria mass increased upon EGF activation. (E) Mitochondria activity increased upon EGF activation. (G) Live-imaging of RPE-1 cells upon 24h hEGF, MEKi, and hEGF+MEKi treatment. Cells were stained with Hoechst dye (blue), TMRM (red), and MitoTracker dye (green). Scale bar is 50μM. (H) Quantification of the average mitochondria pixel area across the Z-stacks of each RPE-1 cell. hEGF treatment significantly increased the mitochondria mass of RPE-1 cells in a MEK-dependent manner. (I) Quantification of average ratio between TMRM intensity and MitoTracker intensity across the Z-stacks of a RPE-1 cell, as an indication of mitochondrial activity. hEGF treatment significantly increased mitochondrial activity of RPE-1 cells in a MEK-independent manner. (*p<0.05, **p<0.01, ****p<0.0001; ns, not significant)

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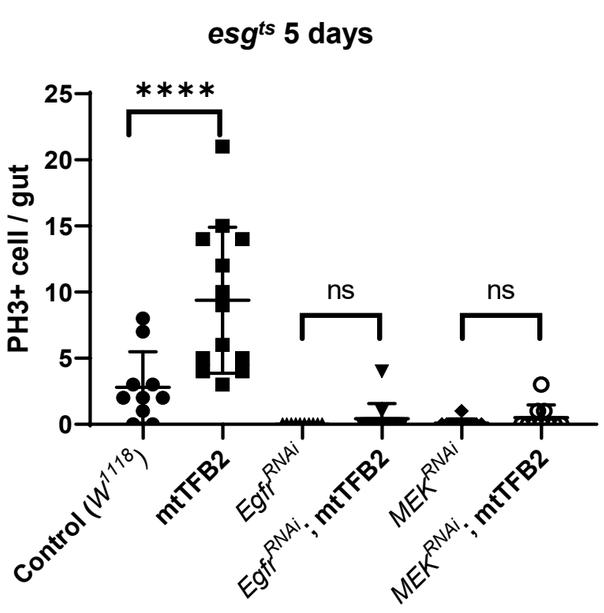

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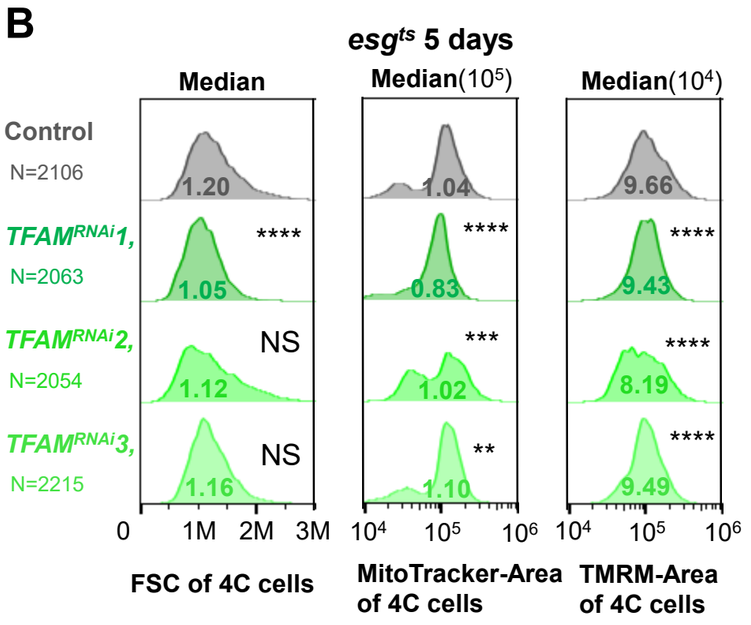

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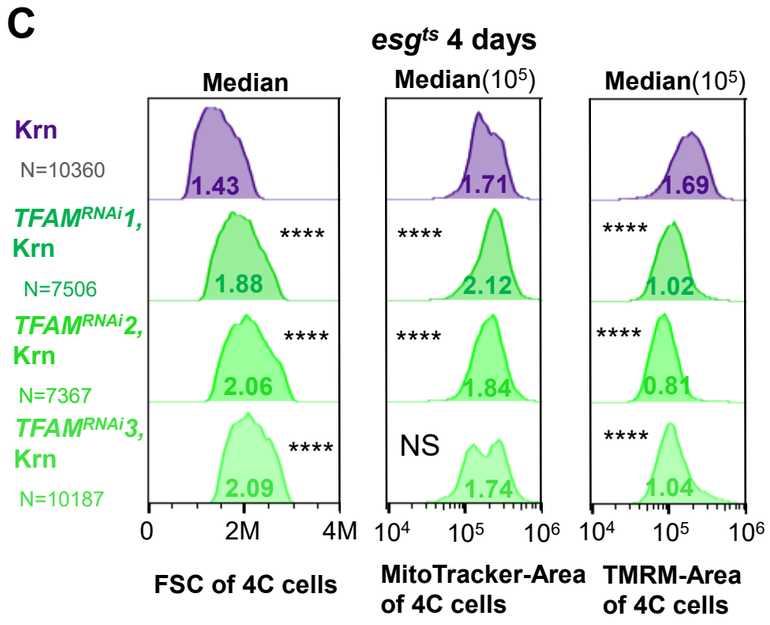

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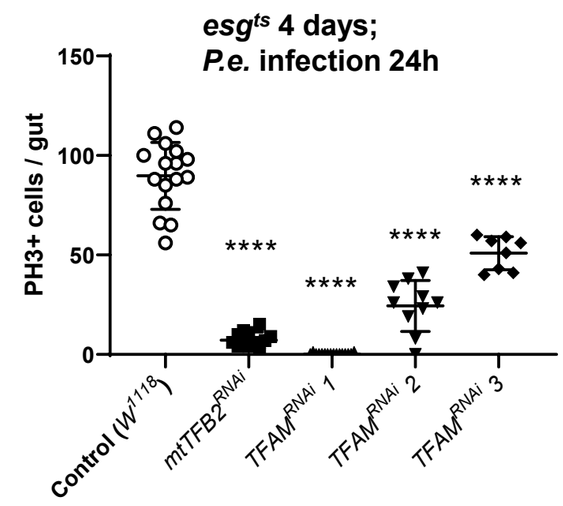

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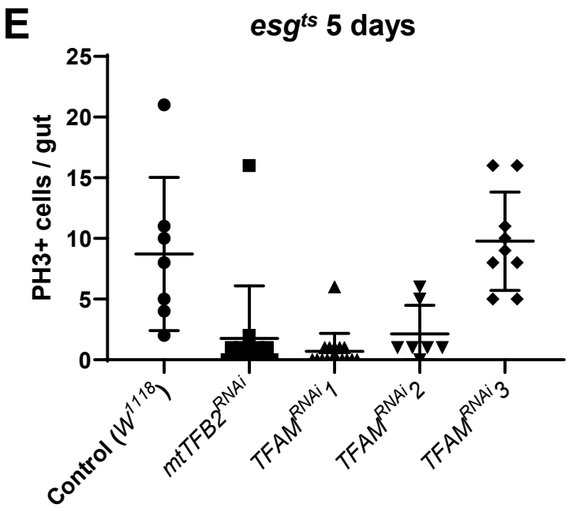

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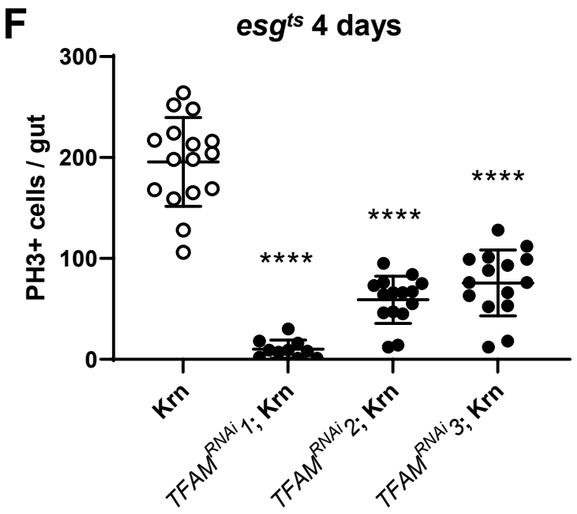
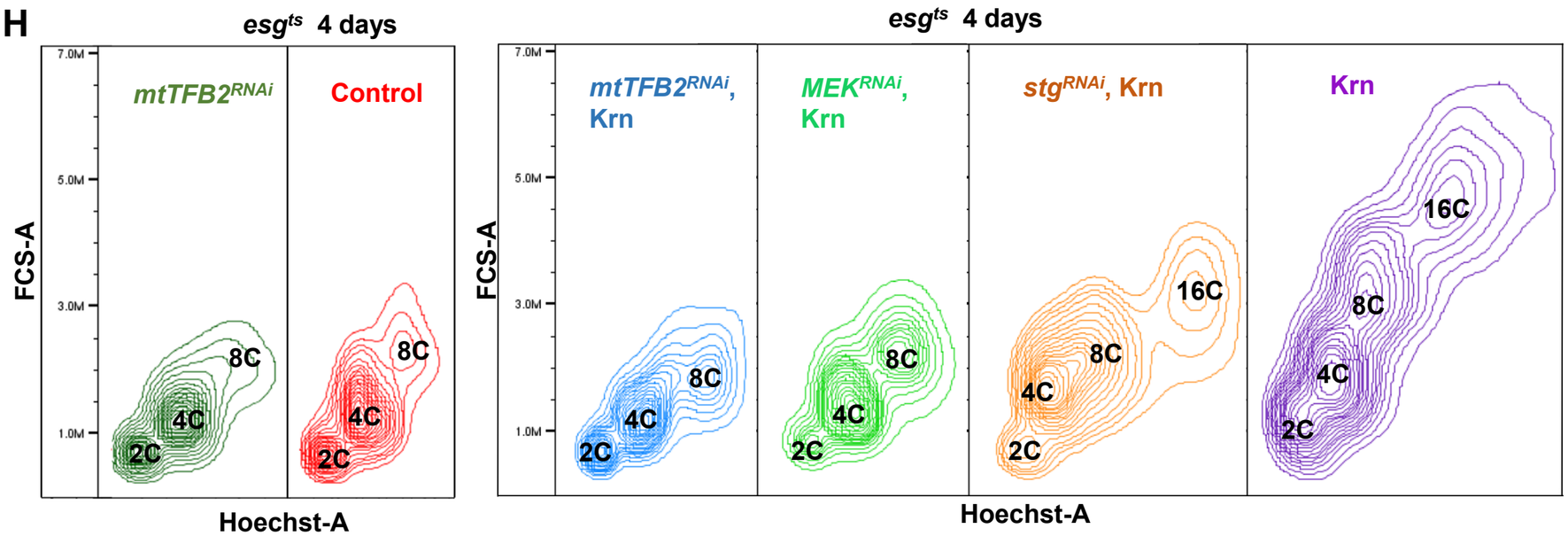


G

Cell ploidy state of *esg^ts^>GFP* cells

Ploidy	*mtTFB2^RNAi^*		Control		*mtTFB2^RNAi^*, *Krn*		*MEK^RNAi^*, *Krn*		*stg^RNAi^*, *Krn*		*Krn*	
	N	Percentage	N	Percentage	N	Percentage	N	Percentage	N	Percentage	N	Percentage
2C	832	15.15%	788	15.04%	1239	22.70%	400	7.77%	278	5.60%	912	11.96%
4C	3485	63.44%	3249	62.03%	2884	52.84%	2718	52.80%	2882	58.02%	3273	42.91%
8C	1176	21.41%	1201	22.93%	1335	24.46%	2030	39.43%	1415	28.49%	1876	24.59%
16C	0	0.00%	0	0.00%	0	0.00%	0	0.00%	392	7.89%	1567	20.54%

H



I

Median of Cell Size (FSC) (E^6)

Ploidy	*mtTFB2^RNAi^*	Control	*mtTFB2^RNAi^*, Krn	*MEK^RNAi^*, Krn	*stg^RNAi^*, Krn	Krn
2C	0.64	0.74	0.82	1.07	0.969	1.16
4C	1.05	1.21	1.49	1.68	1.93	1.91
8C	1.69	1.96	2.26	2.39	3.30	3.25
16C	-	-	-	-	4.26	4.89

J

Median of Mitochondria Size (MitoTracker)

Ploidy	*mtTFB2^RNAi^*	Control	*mtTFB2^RNAi^*, Krn	*MEK^RNAi^*, Krn	*stg^RNAi^*, Krn	Krn
2C	59828	65532	74791	81202	89467	118608
4C	99445	101809	143558	163362	180518	204218
8C	229677	183189	291366	287963	345479	379524
16C	-	-	-	-	4463854	5352973

K

Median of Mitochondria Activity (TMRM)

Ploidy	*mtTFB2^RNAi^*	Control	*mtTFB2^RNAi^*, Krn	*MEK^RNAi^*, Krn	*stg^RNAi^*, Krn	Krn
2C	13028	13575	74791	47161	15732	38510
4C	99445	101809	143558	163362	180518	204218
8C	37067	50904	291366	115514	123951	174779
16C	-	-	-	-	189204	328652

Figure S6. mtTFB2 and TFAM are required for ISC proliferation and cellular growth. Related to Figure 6.
(A) mtTFB2 mediated ISC proliferation is EGFR signaling dependent. (B,C) FASC showed the effects of TFAM knockdown on cell size, mitochondria size and activity. In quiescent esg cells, TFAM knockdown did not show a strong effect (B). Upon EGFR activation, *TFAM*^{RNAi} only decreased mitochondrial activity, but increased cell size and mitochondria mass (2 out of 3 *TFAM*^{RNAi} lines) (C). (D-F) Similar with *mTFB2*^{RNAi}, *TFAM*^{RNAi} lines blocked EGFR mediated ISC proliferation. (G-H) mtTFB2 and MEK are required for EB endoreplication and differentiation. Cell ploidy state of *esg*^{ts}*>GFP* cells with different genotypes (H). *esg*^{ts}*>GFP* driver drives the expression of UAS transgenes in both ISC and EB. When the guts are undergoing fast regeneration, for instance during EGFR signaling activation, differentiating EBs or newly differentiated ECs still retain some GFP protein and would be sorted as GFP positive cells by FACS. For this reason, we found that it is important to distinguish the cell ploidy when using the *esg*^{ts}*>GFP* driver. Flow cytometry gating scheme (H), *esg-GFP* cells were gated to 2C, 4C, 8C, and 16C sub-populations based their cell size (FCS-Area) and DNA content (Hoechst-Area). (I-K) Median of FSC-Area (I), MitoTracker-Area (J), and TMRM-Area (K) of *esg*^{ts}*>GFP* cells in different ploidy states and genotypes.

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