

1 **Tricellular junctions regulate intestinal stem cell behavior to maintain**  
2 **homeostasis**

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34 Aging results in loss of tissue homeostasis across taxa<sup>1</sup>. In the intestine of  
35 *Drosophila melanogaster*, aging is correlated with an increase in intestinal stem cell  
36 (ISC) proliferation, a block in terminal differentiation of progenitor cells, activation of  
37 inflammatory pathways, and increased intestinal permeability<sup>2</sup>. However, causal  
38 relationships between these phenotypes remain unclear. Here, we demonstrate that  
39 aging results in altered localization and expression of Septate Junction (SJ) proteins in  
40 the posterior midgut, which is quite pronounced in differentiated enterocytes (ECs) at  
41 tricellular junctions (TCJ). Acute loss of the TCJ protein Gliotactin (Gli) in ECs results in  
42 increased ISC proliferation and a block in differentiation in intestines from young flies,  
43 demonstrating that compromised TCJ function is sufficient to alter ISC behavior in a non-  
44 autonomous manner. Blocking the Jun N-terminal kinase (JNK) signaling pathway is  
45 sufficient to suppress changes in ISC behavior, but has no effect on loss of intestinal  
46 barrier function, as a consequence of Gli depletion. Our work demonstrates a pivotal  
47 link between TCJ, stem cell behavior, and intestinal homeostasis and provides new  
48 insights into causes of age-onset and gastrointestinal diseases.

49 The intestinal epithelium provides a selective barrier that permits nutrient and  
50 water transport, while preventing uptake of harmful environmental toxins and microbial  
51 contamination of interstitial tissues<sup>3</sup>. In addition to its barrier function, the intestinal  
52 epithelium serves essential roles in metabolism and immunity. Highly specialized  
53 intercellular occluding junctions, referred to as tight junctions (TJ) in chordates and  
54 septate junctions (SJ) in arthropods, maintain this critical barrier.  
55 The *Drosophila* midgut epithelium is composed of intestinal stem cells (ISCs) that self-  
56 renew to maintain the ISC pool and produce daughter cells, known as enteroblasts  
57 (EBs), which can differentiate into either secretory enteroendocrine (EE) cells or  
58 absorptive enterocytes (ECs) (Supplementary Fig. 1A)<sup>4,5</sup>. In the *Drosophila* midgut, SJ  
59 exist between adjacent ECs and between ECs and EE cells, both of which are located  
60 apically and are in contact with the intestinal lumen (Fig. 1A, Supplementary Fig. 1A).  
61 Therefore, we hypothesized that age-related changes in SJ could directly contribute to  
62 loss of intestinal barrier function. Furthermore, given the significant role that  
63 differentiated cells play in regulating ISC behavior in the intestine<sup>6,7</sup>, we speculated that  
64 changes in SJ could contribute in a non-autonomous manner to altered ISC behavior  
65 over time.

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67 Consistent with the loss of barrier function in older animals<sup>8, 9</sup>, electron  
68 microscopy revealed distinct gaps in SJ between adjacent ECs in midguts from old wild  
69 type (WT) flies, which were not observed in midguts from young flies (Fig. 1A-B). In  
70 order to determine whether the compromised SJ function in aged intestines could be due  
71 to decreased gene expression, RNAseq analysis was performed on posterior midguts  
72 from young (5do) and old (45do) flies. These data revealed that 38% (18/48) of genes  
73 annotated as being involved in SJ or SJ assembly (Gene Ontology terms GO:0005918  
74 and GO:00019991) exhibited changes in expression (Fig. 1C; Supplementary Table1),  
75 and an enriched analysis of the dataset revealed that the “cell adhesion” gene ontology  
76 (GO) category was one of the most representative GO categories that change with age  
77 (Supplementary Table1). The expression level of the majority of these genes (16 of 18)  
78 was up-regulated in old flies, indicating that decreased transcription is not a primary  
79 mechanism contributing to age-related changes in SJs in the midgut.

80 In *Drosophila*, SJ are divided into two classes based on morphological  
81 appearance: pleated SJ (pSJ) are found in ectodermally-derived tissues, such as the  
82 hindgut, while smooth SJ (sSJ) are observed in endodermally-derived tissues, including  
83 the midgut<sup>10</sup>. To evaluate possible age-related changes in SJ protein localization or  
84 expression, confocal and super-resolution immunofluorescence microscopy were used  
85 to visualize smooth and pleated SJ proteins in midguts and hindguts, respectively (Fig. 1  
86 D-M, Supplementary Fig. 1 B-O,R-S). In 45do flies, significant changes in SJ proteins  
87 Discs large (Dlg), Coracle (Cora), Scribble (Scrib), Snakeskin (Ssk) and Mesh were  
88 observed in the midgut: Dlg, Cora, and Scrib showed decreased staining intensity, due  
89 to an apparent separation of adjacent cell membranes, while Ssk and Mesh appeared to  
90 accumulate in the cytoplasm (Fig. 1D-M). These changes were quantified by measuring  
91 the ratio of staining intensities at the membrane (SJ/cytoplasm) (Fig. 1 P-T). In  
92 contrast, Dlg, Cora, and Scrib levels were not decreased in the hindgut (Supplementary  
93 Fig. 1J-O). Furthermore, levels of Armadillo (Arm), an Adherens Junctions (AJ)  
94 component, did not appear affected by age in midguts or hindguts (Fig. 1N-O,V;  
95 Supplementary Fig. 1R-S).

96 One striking and consistent age-related change in SJs was observed at tricellular  
97 junctions (TCJ) (arrowheads, Fig. 1D-I, L-M, P-T), the specialized junction at the  
98 conjunction of three cells<sup>11</sup>. Gliotactin (Gli) is one of two SJ proteins that have been  
99 described in *Drosophila* to localize to the TCJ<sup>12-14</sup>, and Gli has been demonstrated to

100 play a role in the developing nervous system<sup>15</sup> and imaginal discs<sup>12, 16</sup>. However, a role  
101 in the adult intestine has not yet been evaluated.

102 In the adult posterior midgut, Gli co-localized with Dlg (Fig. 2A-B'; Supplementary  
103 Fig. 2A-A''), as described previously in wing discs<sup>16, 17</sup>. Gli protein was clearly detected  
104 at EC-EC and EC-EE cell TCJ (Fig. 2 A,C,F); however, no Gli protein was detected in  
105 ISCs/EBs (Fig. 2D). In midguts from aged flies, Gli localization was largely absent from  
106 the TCJ, and protein levels were increased in the cytoplasm (Fig. 2F-H). In hindguts, no  
107 changes in Gli localization or protein levels were observed, similar to our observation for  
108 other SJ proteins (Supplementary Fig. 1P-Q). Interestingly, Dlg appeared cytoplasmic,  
109 rather than membrane-localized, in ISC/EB 'nests', suggesting that definitive SJ may be  
110 absent between ISC/EBs and that formation of SJ is coordinated with differentiation.  
111 Consistent with this hypothesis, SJ were not apparent between ISCs and EBs via EM  
112 (Fig. 2E).

113 Given the significant changes in TCJ (Fig. 1 D-M, P-T) and the striking loss of Gli  
114 from TCJ in older animals (Fig. 2G-H), we tested whether these changes were, indeed, a  
115 consequence of aging. Dietary restriction (DR) is the most robust and reproducible  
116 intervention known to slow aging across species<sup>18</sup>. Changes in Gli localization were  
117 delayed in long-lived, DR flies, when compared to controls, indicating that physiological  
118 age was a significant factor contributing to changes in TCJ (Fig. 2I, J)

119 To determine whether compromised TCJ function could contribute to age-related  
120 changes in the intestine, Gli was depleted from TCJs using a drug-inducible version of  
121 the GAL4-UAS system<sup>19, 20</sup>. Targeted gene expression using the 5966<sup>GS</sup> GAL4 "driver"  
122 is observed in adult ECs when flies are fed the progesterone analog RU486; addition of  
123 EtOH is used as a control, providing cohorts of isogenic individuals with or without  
124 induction of a *Gli*<sup>RNAi</sup> transgene. Efficacy of *Gli*<sup>RNAi</sup> was confirmed by immunostaining  
125 and RT-qPCR (Supplementary Fig. 2B-D).

126 Depletion of Gli from ECs resulted in an accelerated loss of barrier integrity (Fig.  
127 3A ; Supplementary Fig. 2E). Integrity of the intestinal barrier can be assayed by feeding  
128 flies a non-absorbable blue food dye. When the intestinal barrier is intact, the dye is  
129 retained within the intestine, whereas loss of barrier integrity results in spreading of blue  
130 dye throughout the hemolymph (generating "Smurf" flies)<sup>8, 9</sup>. Reduced *Gli* expression in  
131 ECs led to a significant increase in "Smurf" flies as early as 12 days after *Gli*<sup>RNAi</sup>  
132 expression, when compared to age-matched controls (Fig. 3A; Supplementary Fig. 2E).  
133 EM analysis of midguts from 23do flies revealed no noticeable changes in SJ structure



134 at bicellular junctions when Gli was depleted from ECs (Supplementary Fig.2F-G),  
135 indicating that loss of barrier function was due to disruption of TCJ, rather than disruption  
136 of the entire SJ. At 40 days, 94.7% of the *Gli<sup>RNAi</sup>* population exhibited the “Smurf”  
137 phenotype, compared to 57.1% of controls (Fig. 3A), with a concomitant decrease in  
138 median lifespan (Supplementary Fig. 2H).

139 Upon Gli depletion from ECs for 23 days, the localization of SJ proteins was  
140 altered similar to what was observed in intestines from aged flies (compare Figs. 1D-M  
141 to Figs. 3 C,E,G,I). Neither protein levels nor localization was affected in controls (Fig.  
142 3B, D,F,H). In epithelia of imaginal discs, Dlg is needed to recruit Gli into TCJ, but not  
143 *vice versa*<sup>16</sup>. This relationship is maintained in the midgut, as depletion of Gli had only a  
144 modest affect on TCJ Dlg enrichment (arrowheads, Fig. 3B,C), whereas depletion of Dlg  
145 from ECs led to a complete loss of Gli from TCJ (Fig. 3J-M). Altogether, these data  
146 indicate that depletion of Gli from the TCJ, rather than mis-localization of Dlg and  
147 complete disruption of SJ, is sufficient to trigger loss of barrier integrity.

148 In the *Drosophila* midgut, aging, stress, or increased inflammation results in a  
149 dramatic increase in ISC proliferation, which is accompanied by an accumulation of mis-  
150 differentiated daughter cells that express hallmarks of both ISCs and terminally  
151 differentiated enterocytes<sup>6, 21, 22</sup>. A statistically significant increase in number of cells  
152 expressing the ISC/EB marker *Esg* was observed upon *Gli* depletion from ECs for 9  
153 days (Fig. 4C-D,E). In addition, an increase in ISC proliferation was observed, based on  
154 the mitosis marker phosphorylated histone H3 (PH3) (Fig. 4D,F). Similar results were  
155 obtained using two additional *Gli<sup>RNAi</sup>* lines (Supplementary Fig. 3A-D). After 9 days of *Gli*  
156 depletion, flies were shifted onto RU- food to re-initiate Gli expression for 11 days. Re-  
157 expression of Gli in ECs resulted in resumption of normal ISC behavior (Supplementary  
158 Fig. 3G-K), indicating that changes in ISCs are directly correlated with the presence of  
159 Gli and that loss of TCJ integrity is reversible under these conditions. Depletion of Gli  
160 specifically from EEs did not result in any changes in ISC behavior (Supplementary Fig.  
161 3L). Reduced expression of Dlg in ECs resulted in similar effects on ISC behavior  
162 (Supplementary Fig. 3E-F). However, as noted above, depletion of Dlg resulted in a  
163 complete loss of Gli from TCJ; therefore, the effects of Dlg depletion cannot be  
164 uncoupled from an effect on Gli. These data indicate that depletion of Gli from  
165 differentiated ECs is sufficient to induce age-related phenotypes, including loss of barrier  
166 function and changes in ISC behavior, in intestines of young flies.

167 Finally, to address whether increased ISC proliferation, in response to Gli  
168 depletion, was due to death of ECs via apoptosis, anti-apoptotic factors dIAP and P35  
169 were individually co-expressed with *Gli<sup>RNAi</sup>* in ECs. Co-expression of dIAP or P35 with  
170 *Gli<sup>RNAi</sup>* was not sufficient to suppress the increase of *esg*+ cells or ISC proliferation; no  
171 effect on ISC behavior was observed in controls (Supplementary Fig. 4A-F). Therefore,  
172 our data indicate that apoptosis does not play a critical role downstream of Gli to trigger  
173 non-autonomous changes in ISC behavior.

174 Increased microbial loads and a disruption in the composition of commensal  
175 bacteria in aged flies, collectively referred to as dysbiosis, has been shown to contribute  
176 to cellular changes in the aging intestine, including an increase in ISC proliferation  
177 <sup>23-26</sup>. To test whether the presence of bacteria could impact ISC behavior, upon Gli  
178 depletion, we generated *5966<sup>GS</sup>GAL4/UAS-Gli<sup>RNAi</sup>* flies under axenic conditions and  
179 examined ISC behavior in young flies (Supplementary Fig. 4I). Axenic flies in which Gli  
180 was depleted exclusively from ECs showed a similar increase in the number of cells  
181 expressing the ISC/EB marker *Esg*, as well as an increase in ISC mitoses, when  
182 compared to flies reared under conventional, non-sterile conditions (Fig. 4G-J).  
183 Altogether, our data suggest that changes in bacterial populations are not a major  
184 contributing factor to accelerated ISC proliferation and intestinal dysplasia observed  
185 upon depletion of Gli.

186 To determine possible mechanisms by which Gli depletion could elicit alterations  
187 in stem cell behavior, changes in gene expression were assessed in posterior midguts  
188 from control flies or flies depleted of Gli for 2 or 9 days. At the 9-day time point, *Gli*  
189 expression was decreased and *esg* expression increased, presumably due to the  
190 increase in the *esg*+ cells, compared to controls (Supplementary Table 2).  
191 Comprehensive analysis of the RNAseq dataset revealed an increase in activity of  
192 pathways known to stimulate ISC proliferation in response to damage, infection, or  
193 stress, including the Jun-N-terminal Kinase (JNK), Hippo, Epidermal Growth Factor  
194 (EGF), Wingless (Wg) and Janus kinase-Signal Transducer and Activator of  
195 Transcription (JAK/STAT) pathways <sup>2</sup> (Supplementary Table 2). Upregulation of genes  
196 encoding SJ proteins, such as *dlg*, *mesh*, *cora* and *Ssk*, was observed, similar to the  
197 age-related changes in expression detected in midguts from aged flies (Fig. 1C;  
198 Supplementary Tables 1, 2). However, an increase in expression of AMPs was not  
199 detected, suggesting that the increase in ISC proliferation is not due to an inflammatory

200 response as a result of bacterial dysbiosis<sup>23,26</sup> (Supplementary Fig. 3M-N and  
201 Supplementary Table 2).

202 The JNK pathway is a highly conserved stress-sensing pathway that regulates  
203 gene expression, regeneration, apoptosis and metabolic adaptation in response to both  
204 extrinsic and intrinsic stressors<sup>27</sup>. In the intestine, activation of the JNK pathway in ECs  
205 leads to increased expression of cytokine-like molecules that, in turn, activate the JAK-  
206 STAT pathway in ISCs to stimulate ISC proliferation<sup>2, 6, 7, 28</sup>. Therefore, we wanted to  
207 determine whether JNK pathway activation could be responsible for driving the ISC  
208 response downstream of *Gli* depletion.

209 Activation of the JNK pathway was observed in ECs as early as 2 days after *Gli*  
210 depletion, in comparison to controls, as determined by expression of a downstream  
211 target of the pathway, *puckered* (*puc*) (Fig. 5A-F'). Similar results were observed when  
212 the experiment was conducted under axenic conditions (Supplementary Fig. 4G-H'). An  
213 increase in *puc* expression upon *Gli* depletion was confirmed by RT-qPCR and RNAseq  
214 analysis (Supplementary Fig. 4J and Table 2). Suppression of JNK signaling in ECs,  
215 achieved by ectopic expression of a dominant negative version of the single *Drosophila*  
216 JNK, *basket* (*bsk<sup>DN</sup>*), had no effect on ISC behavior in controls (Fig. 5 G-H,K-L,O-P).  
217 However, expression of *bsk<sup>DN</sup>* was sufficient to suppress the increase in *esg*-expressing  
218 cells and ISC proliferation in response to EC-specific *Gli* depletion (Fig. 5I-J,M-P). In  
219 contrast, co-expression of *bsk<sup>DN</sup>* was not sufficient to suppress the loss of barrier  
220 integrity, nor the decrease in survival, observed upon *Gli<sup>RNAi</sup>* (Fig. 5Q; Supplementary  
221 Fig. 4K), suggesting that activation of JNK at early time points does not contribute to loss  
222 of barrier function. Altogether, these data indicate that JNK signaling is activated  
223 downstream of TCJ to drive changes in ISC behavior in a non-autonomous manner.

224 Our data indicate that aging results in changes to occluding junctions that likely  
225 contribute to the loss of barrier integrity described previously<sup>8, 9</sup>. However, acute  
226 depletion of the TCJ protein *Gli* in young flies quickly led to hallmarks of aging, including  
227 an increase in ISC proliferation and a block in terminal differentiation. Surprisingly,  
228 changes in ISC behavior were observed prior to loss of the permeability barrier,  
229 activation of AMPs, and increases in bacterial populations (Figs. 3,4). While not all  
230 phenotypes associated with aging are observed upon downregulation of *Gli* at early time  
231 points, our data suggest that altered TCJ function during the course of normal aging  
232 could contribute directly to the changes in ISC behavior observed in older animals.

233           Although age-dependent remodeling of epithelial junctions has been described in  
234 mammalian models<sup>29-31</sup>, no prior link between changes in occluding junctions and age-  
235 related changes in stem cell behavior has been established in other systems. If this  
236 connection is conserved in mammalian systems, epithelial remodeling could be a driver  
237 of a host of intestinal diseases, including colon cancer. Furthermore, if age-related  
238 changes to occluding junctions also occur within tissues in which low or no turnover  
239 takes place, loss of barrier function could be a substantial contributing factor to age-  
240 onset or degenerative diseases in tissues such as the nervous system, ear, or the  
241 kidney.

242

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### 255 **Author Contributions**

256 M.R.D. designed, performed, and analyzed experiments and wrote the manuscript.  
257 C.L.K., R.I.C, J.M.S, D.M.W., V.S., S.L., and C.D. designed, performed and analyzed  
258 experiments. D.W.W. designed and analyzed experiments. D.L.J. designed and  
259 analyzed experiments and wrote the manuscript.

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348 **Figure Legends:**

349 **FIG. 1: Age-related changes in septate junctions (SJ) in posterior midguts from**  
350 **aged flies.** (A-B) EM images showing a gap at the SJ (arrowhead) between ECs in an  
351 intestine from a 45 days old (do) fly (B) compared to SJ between adjacent ECs in a gut  
352 from a 5do fly (young) (A); n=6 midguts per condition (n=10 EC/EC SJs were observed  
353 per midgut). Scale bars 0.1 $\mu$ m. (C) Expression heatmap of representative changes in  
354 gene expression (old/young) of genes encoding SJ or SJ assembly components that  
355 change with age. (D-O) STED images comparing SJ protein localization in ECs in young  
356 (10do) or aged (45 do) midguts. SJ protein mis-localization is observed in old ECs,  
357 represented by thicker bicellular junctions, disappearance of enriched SJ protein  
358 localization at TCJ (arrowheads, D, F, H, L compared to E, G, I, M respectively) and an  
359 increase in cytoplasmic localization (K, M). (N-O) STED images showed the AJ protein  
360 Arm is not affected by aging. n=>14 midguts per condition; n=10 ECs were observed per  
361 midgut. Samples were dissected and stained in parallel under same conditions, pictures  
362 taken at same laser intensity. (P-V) TCJ/Cytoplasm (red) and bicellular SJ/Cytoplasm  
363 (grey) fluorescence ratios for different SJ and AJ components. Data analyzed with ONE-  
364 way ANOVA/Tukey's multiple comparisons test and the error bars are the SEM range of  
365 those averages. \*\*\*\* = P<0.0001 , \*\*\* = P<0.001, \*\* = P< 0.01, \* = P<0.05 represent a  
366 statistically significant difference. (P) Discs large (5do n=20; 45do n=19). (Q) Coracle  
367 (5do n=27; 45do n=22). (R) Scribble (5do n=20; 45do n=21). (S) Snakeskin (5do n=21;  
368 45do n=20). (T) Mesh (5do n=21; 45do n=21). (V) Armadillo (5do n=20; 45do n=21).  
369 Each data point (n= midguts) represents an average fluorescence intensity ratio from 2-7  
370 independent measurements per midgut and the error bars are the S.E.M of those  
371 averages. Scale bars 1 $\mu$ m.

372

373 **FIG. 2: Gliotactin is located at the Tricellular junction (TCJ) in differentiated cells**  
374 **in the intestine.** (A) Confocal image of posterior midgut showing the localization of Gli-  
375 GFP (green) at the TCJ. Scale bar 10 $\mu$ m; n=11. (B-B') Protein localization along the  
376 apical-basal axis; note co-localization of Gli-GFP (green) with Dlg (red). Scale bar, 5 $\mu$ m.  
377 (C) Gli (green) is localized at TCJ of EC (arrows) and in EE (arrowhead, marked by  
378 Prospero in red). Arm (red) localizes to the adherens junction (AJ) in all cells. Scale bar  
379 5 $\mu$ m; n=10 (D) Gli does not co-localize with Dlg, which appears cytoplasmic, in ISC/EB  
380 nests (dashed line). Scale bar, 5 $\mu$ m; n=10. (E) EM of an ISC/EB nest. SJs are apparent  
381 between ECs (black box), but not between ISC/EBs (red box). Blue box= ISC/EB-EC

382 contacts. n=11 midguts (n=5 ISC/EB nests were observed per midgut), Scale bar, 1µm.  
383 (F-G') Gli is mis-localized from TCJ in posterior midguts from 50do flies; Scale bar, 5µm.  
384 Samples dissected and stained in parallel under same conditions, pictures taken at  
385 same laser intensity; (F) n=27 midguts, (G) n=22. (H) Fluorescence intensity ratio of Gli  
386 at TCJ/Cytoplasm in ECs from young and old flies. Asterisks represent statistically  
387 significant difference using an unpaired Student's t-test, two-tailed (\*\*\*\* = P < 0.0001).  
388 Error bars are SEM range of those averages. (I) Lifespan curves for female flies raised  
389 on conventional food (red)(n=294) or DR food (yellow)(n=223). T50 and total lifespan  
390 were significantly lower in controls compared with DR. Data analyzed with non  
391 parametric Log-Rank (Mantel-Cox) test; \*\*\*\* = P<0.0001, represent statistically  
392 significant difference. (J) TCJ/Cytoplasm fluorescence ratio for Gli shows a slower  
393 decrease in DR flies (red) than in control (yellow). Data analyzed with unpaired Student's  
394 t test, two tailed, and error bars are the SEM range of those averages. \*\*\*\*= P<0,0001.  
395 Each data point (n=midguts) represents an average fluorescence intensity ratio from 2-7  
396 independent measurements per midgut and bars are SEM range of those averages.  
397 10do Ctrl n=20 midguts; 10do DR n=18; 20do Ctrl n=35; 20do DR n=29; 30do Ctrl n=22;  
398 30do DR n=22; 40do Ctrl n=15; 40do DR n=23.

399

400 **FIG. 3: Loss of Gliotactin in ECs leads to loss of intestinal barrier integrity.**

401 (A) Flies with reduced *Gli* expression ( $5966^{GS} GAL4/UAS-Gli^{RNAi}$ , RU+, n=265) show  
402 acceleration of loss of barrier integrity, when compared to controls ( $5966^{GS} GAL4/UAS$   
403  $Gli^{RNAi}$  EtOH fed, RU-, n=240 flies). Asterisks represent a statistically significant  
404 difference in pairwise post-test comparisons, indicated by the corresponding bars (\*\*\*\* P  
405 < 0.0001; \*\* P < 0.001 and \* P < 0.05; Fisher's exact test; two tailed). (B-I) Confocal  
406 images of posterior midguts from 23do flies. Loss of Gli in ECs did not affect the levels of  
407 SJ proteins Dlg (C; n=18), Cora (E; n=18), Mesh (G; n=18) or Ssk (I; n=18), although  
408 disruption of the TCJ and mis-localization of SJ proteins was observed, compared to  
409 respective controls (TCJ marked by arrowheads in B, n=16; D, n=16; F, n=19; H, n=16).  
410 Samples were dissected and stained in parallel under the same conditions, pictures  
411 taken at same laser intensity. (J-M) Depletion of *dlg* induced in ECs with  $Myo1A-GAL4$   
412  $GAL80^{ts} UAS-dlg^{RNAi}$  for 7 days. At 29°C Dlg is reduced from ECs but maintained in EEs  
413 (L), while Gli is completely lost from TCJ (M) compared to controls maintained at 18°C  
414 (J-K). Scale bars 10 µm. J-K n=19; L-M n=20.

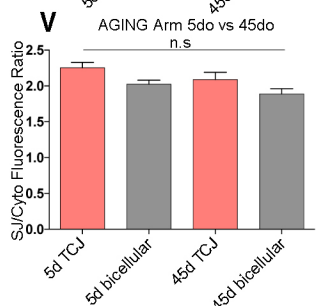
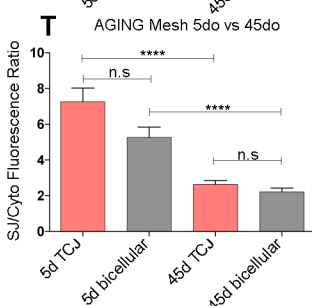
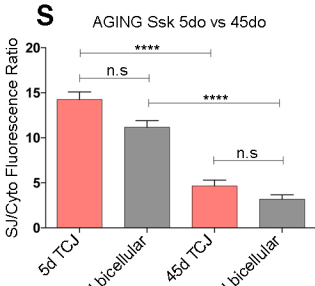
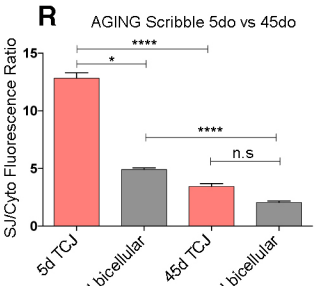
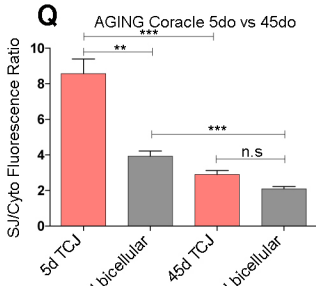
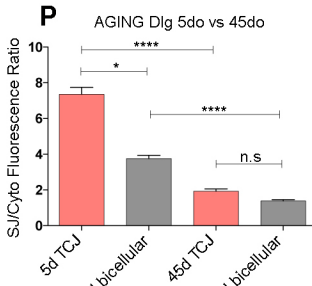
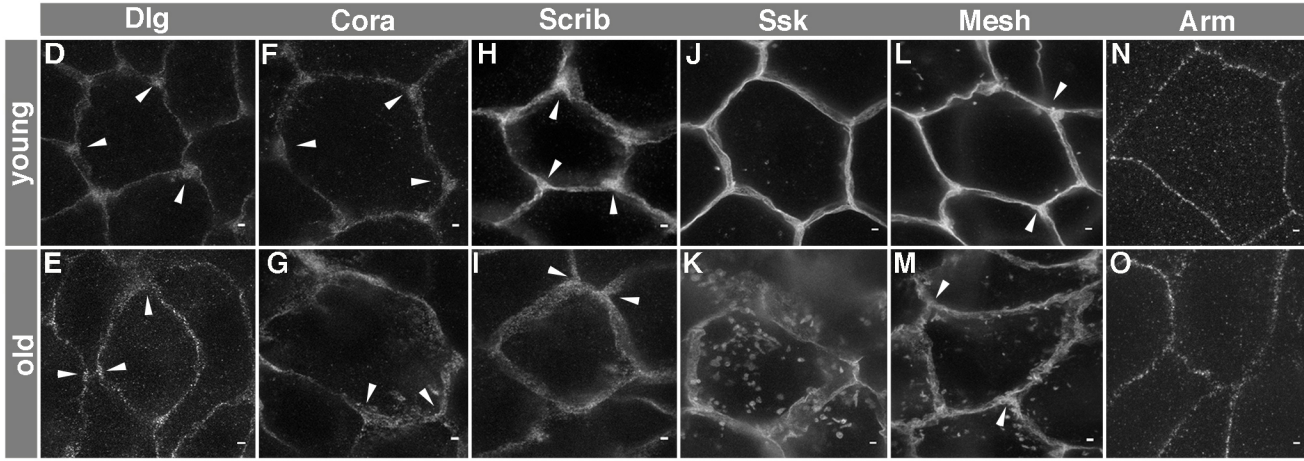
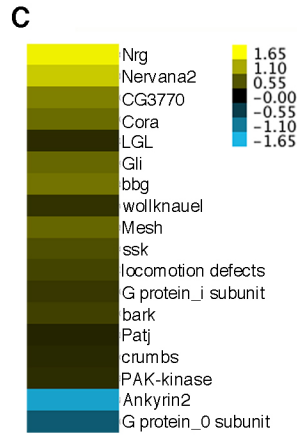
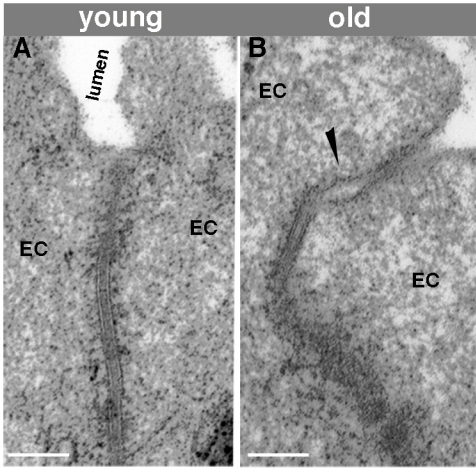


415 **FIG. 4: Loss of Gliotactin in ECs induces ISC proliferation and accumulation of**  
 416 **esg+ cells.** (A-F) Posterior midguts from *Su(H)LacZ*; *5966<sup>GS</sup>GAL4*, *esg:GFP/ UAS-*  
 417 *Gli<sup>RNAi</sup>* flies after 2d (A-B) or 9d (C-D). *Gli* knockdown causes an increase in *esg+*  
 418 ISC/EBs (marked by *esg:GFP*, green) and ISC proliferation (marked by arrowheads  
 419 PH3, red) after 9days (D), comparing to RU- controls (C). (A) n=64 images (4 images  
 420 taken per midgut n=16 midguts); (B) n=76 (4 images taken per midgut n=19); (C) n=72  
 421 (4 images were taken per midgut n=18); (D) n=60 (4 images taken per midgut n=15). (E-  
 422 F) Graphical summary showing changes in ISC/EB number (E) and mitosis counts (F)  
 423 after 5 days. (E) Each data point is an average proportion calculated from 4 independent  
 424 images per midgut and bars are the mean +/- S.E.M of those averages (ONE-way  
 425 ANOVA/Tukey's multiple comparisons test) \*\* = P< 0.01, represents a statistically  
 426 significant difference. (F) Each data point is an average proportion calculated from 4  
 427 independent images per midgut and bars are the median with interquartile range of  
 428 those averages (Kruskal-Wallis/Dunn multiple comparisons test). \*\* = P< 0.01,  
 429 represents a statistically significant difference. (G-J). Posterior midguts from *Su(H)LacZ*;  
 430 *5966<sup>GS</sup>GAL4*, *esg:GFP/ UAS-Gli<sup>RNAi</sup>* flies raised under axenic conditions. After 7 days of  
 431 *Gli* depletion, effects on *esg+* cell number and ISC mitoses were still observed. (G-H) *Gli*  
 432 knockdown caused an increase in the ISC/EBs cell number (marked by *esg:GFP*, green)  
 433 and proliferation (marked by arrowheads PH3, red) after 7days (H), comparing to RU-  
 434 controls (G). (G) n=80 (4 images taken per midgut n=20); (H) n=80 (4 images taken per  
 435 midgut n=20) (I-J) Graphical summary showing the statistical significance in ISC/EB  
 436 number (I) and mitosis counts (J) after 7 days. (I) Each data point is an average  
 437 proportion calculated from 4 independent images per midgut and the bars are the mean  
 438 +/- S.E.M of those averages (unpaired Student's t-test, two tailed) \*\*\*\* = P< 0.0001,  
 439 represents a statistically significant difference. (J) Each data point is an average  
 440 proportion calculated from 4 independent images per midgut and bars are the median  
 441 with interquartile range of those averages (Mann-Whitney non-parametric test). \*\* = P<  
 442 0.01, represents a statistically significant difference. Scale bars 10µm.

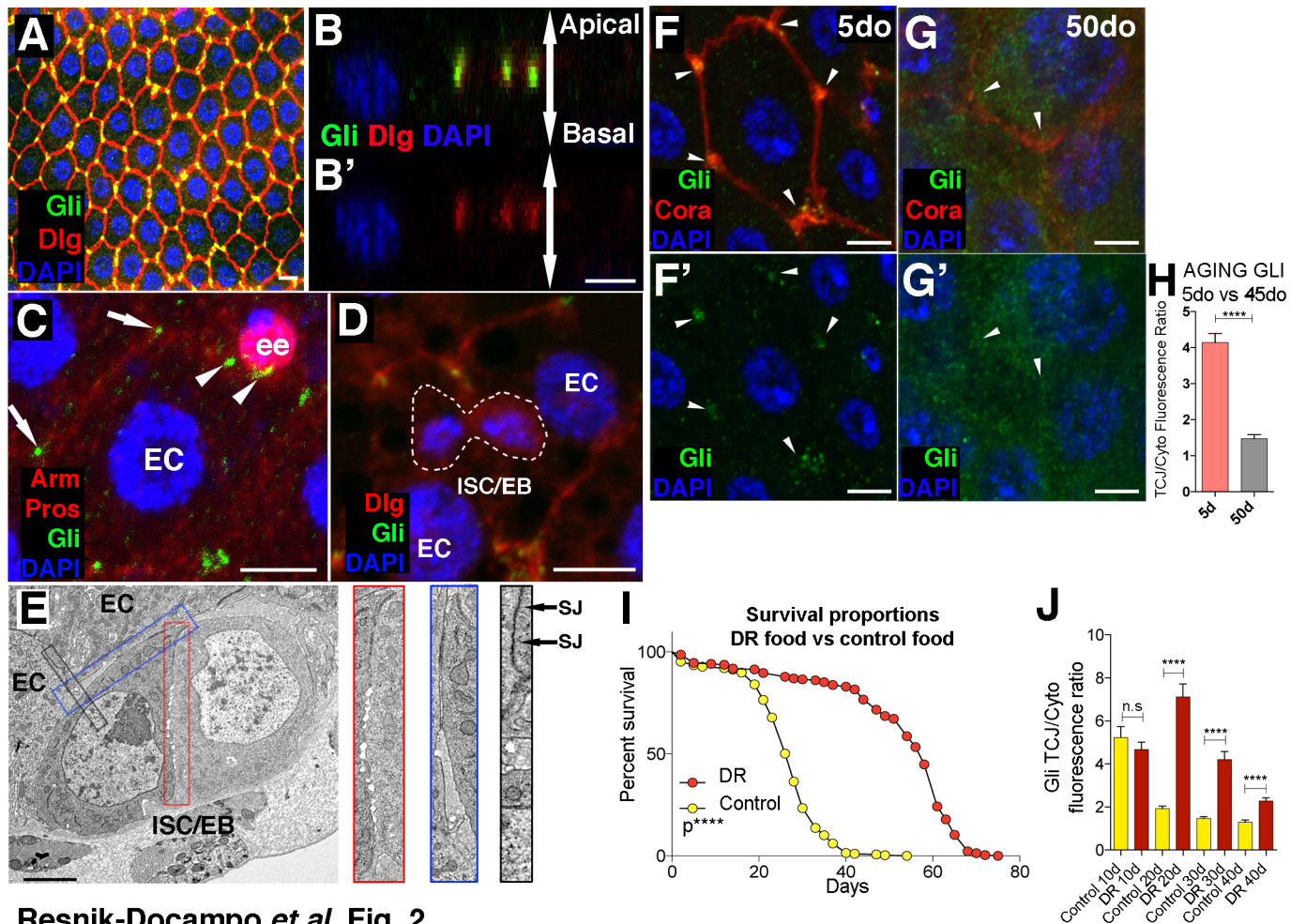
443

444 **FIG. 5: JNK signaling is activated downstream of Gli to drive changes in ISC**  
 445 **behavior in a non-autonomous manner.** (A-F') Reduction of *Gli* expression in ECs  
 446 using *5966 GAL4<sup>GS</sup>* triggers JNK pathway activation, reported by *puc-lacZ* expression  
 447 (red or grey) in ECs 2 (B-B'; n=11 midguts), 5 (D-D'; n=8) and 9 (F-F'; n=8) days after  
 448 reducing *Gli* expression, compared to RU- controls (A-A', n=7; C-C', n=8; E-E', n=7).

449 ISC/EB marked by *esg*-GFP (green), cell nuclei by DAPI (blue). (G-Q) Epistasis analysis  
450 between *Gli* (*Gli<sup>RNAi</sup>*) and *Bsk* (*Bsk<sup>DN</sup>*). Midguts were stained with DAPI (nuclei, blue),  
451 GFP (*esg*+ cells, green) and PH3 (mitotic cells, red) following 9 days of incubation in  
452 RU+ or RU. We observe that blocking JNK signaling (*Bsk<sup>DN</sup>*) (*5966<sup>GS</sup> GAL4/UAS-Gli<sup>RNAi</sup>*  
453 *UAS-Bsk<sup>DN</sup>*, RU+) (N;O-P) rescues the non-autonomous effect on ISC proliferation  
454 produced by *Gli<sup>RNAi</sup>* (*5966<sup>GS</sup> GAL4/UAS-Gli<sup>RNAi</sup>*, RU+)(J, O-P). (O) ISC/EB counts in  
455 midguts. Each data point is an average proportion calculated from 4 independent images  
456 per midgut and bars are the mean +/- S.E.M of those averages (ONE-way  
457 ANOVA/Tukey's multiple comparisons test). (P) Quantification of mitotic ISCs. Each data  
458 point is an average proportion calculated from 4 independent images per midgut and  
459 bars are the median with interquartile range of those averages (Kruskal-Wallis/Dunn  
460 multiple comparisons test) \*\*\*\* =  $P < 0.0001$ , represent statistically significant difference.  
461 (Q) Quantification of loss of barrier function. Flies with reduced *Gli* expression (*5966<sup>GS</sup>*  
462 *GAL4/UAS-Gli<sup>RNAi</sup>*, RU+, n=259) present the same increase on Smurf proportion than  
463 the combination *Gli<sup>RNAi</sup> Bsk<sup>DN</sup>* (*5966<sup>GS</sup> GAL4/UAS-Gli<sup>RNAi</sup> UAS-Bsk<sup>DN</sup>*, RU+, n=264),  
464 compared to *Bsk<sup>DN</sup>* (*5966<sup>GS</sup> GAL4/UAS-Bsk<sup>DN</sup>*, RU+, n= 277) and controls flies (*5966<sup>GS</sup>*  
465 *GAL4/UAS-Gli<sup>RNAi</sup>* EtOH, RU-, n=290). Fisher's exact test; two tailed. \*\*\*\* =  $P < 0.0001$ ,  
466 \*\*\* =  $P < 0.001$ , \*\* =  $P < 0.01$ , \* =  $P < 0.05$  represent statistically significant difference.  
467 Scale bars, 10  $\mu$ m. (M) n=80 (4 images taken per midgut n=20); (H) n=80 (4 images  
468 taken per midgut n=20); (I) n=76 (4 images taken per midgut n=19); (J) n=80 (4 images  
469 taken per midgut n=20); (K) n=72 (4 images taken per midgut n=18); (L) n=64 (4 images  
470 taken per midgut n=16); (M) n=88 (4 images taken per midgut n=22); (N) n=96 (4  
471 images taken per midgut n=24).



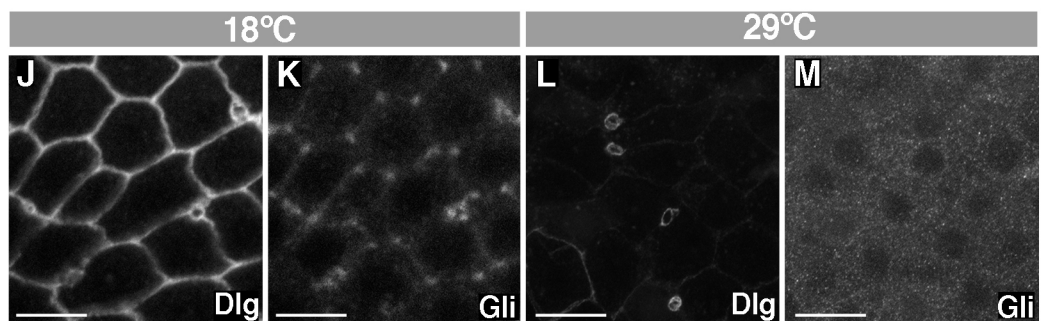
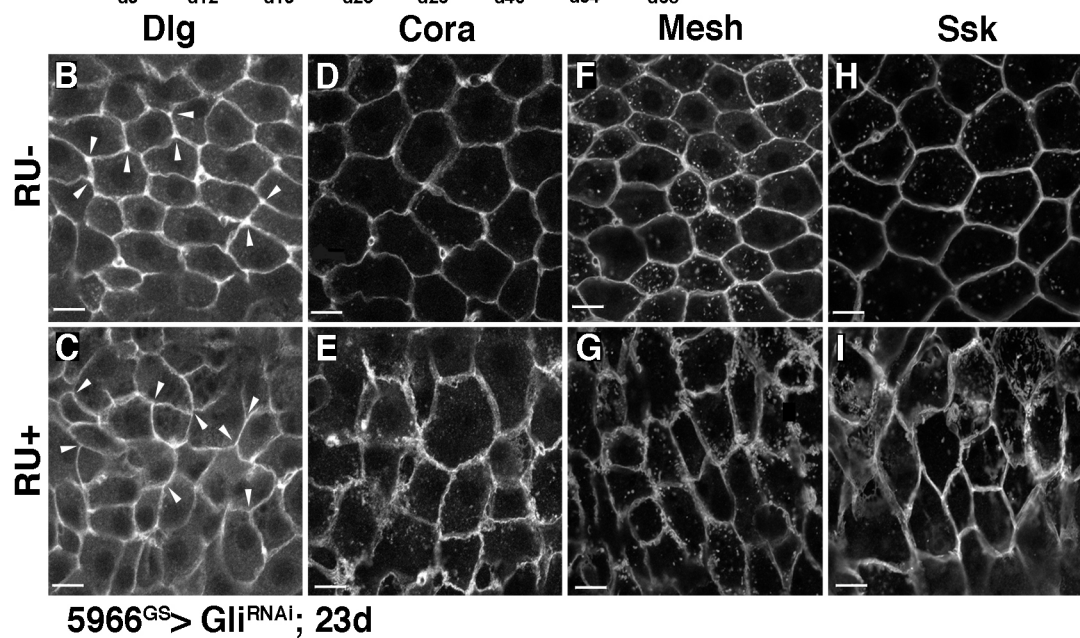
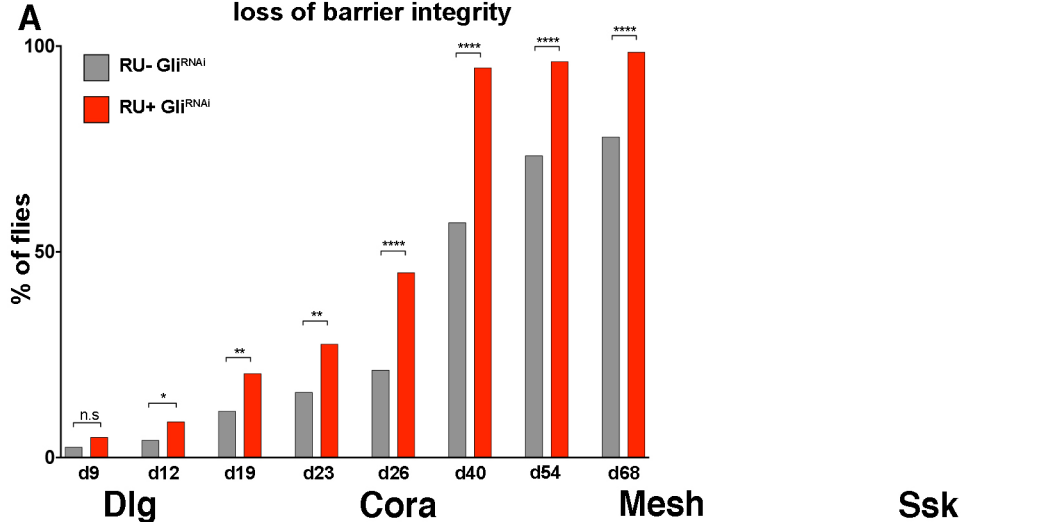
Resnik-Docampo *et al.* Fig. 1



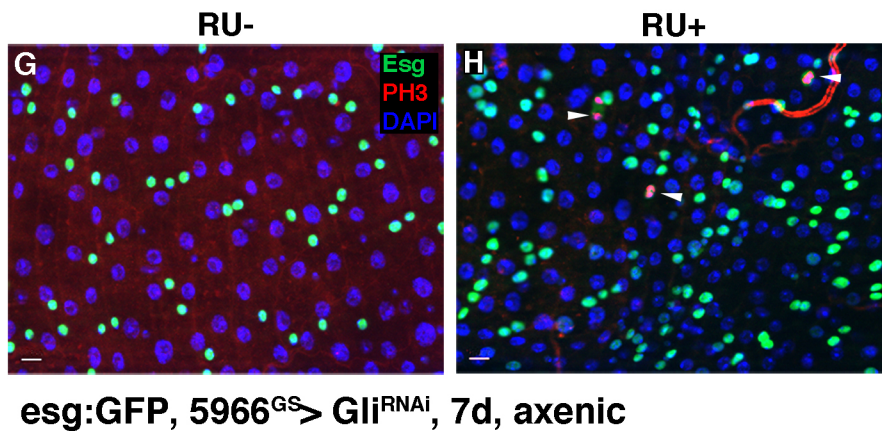
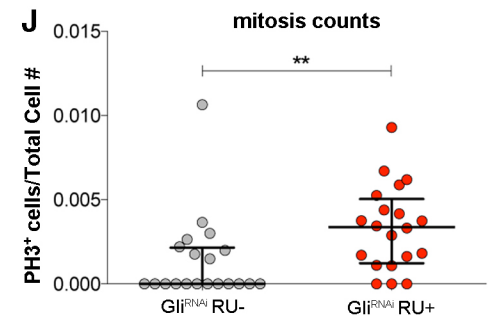
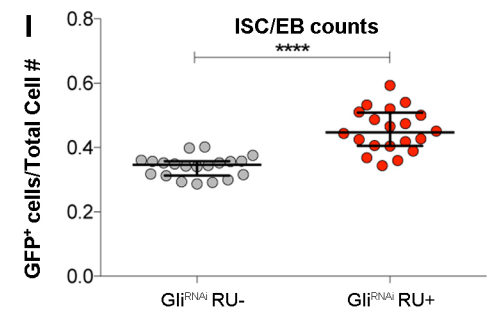
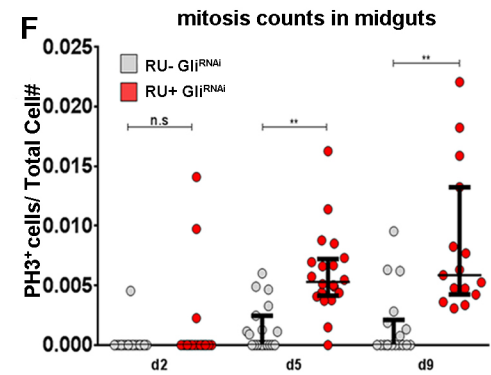
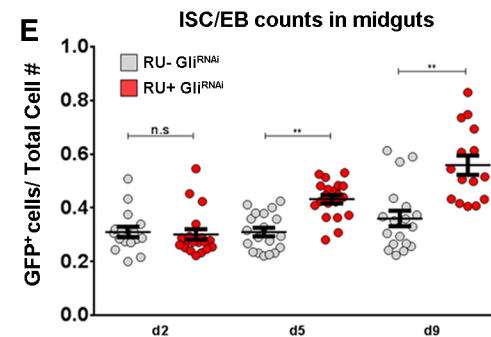
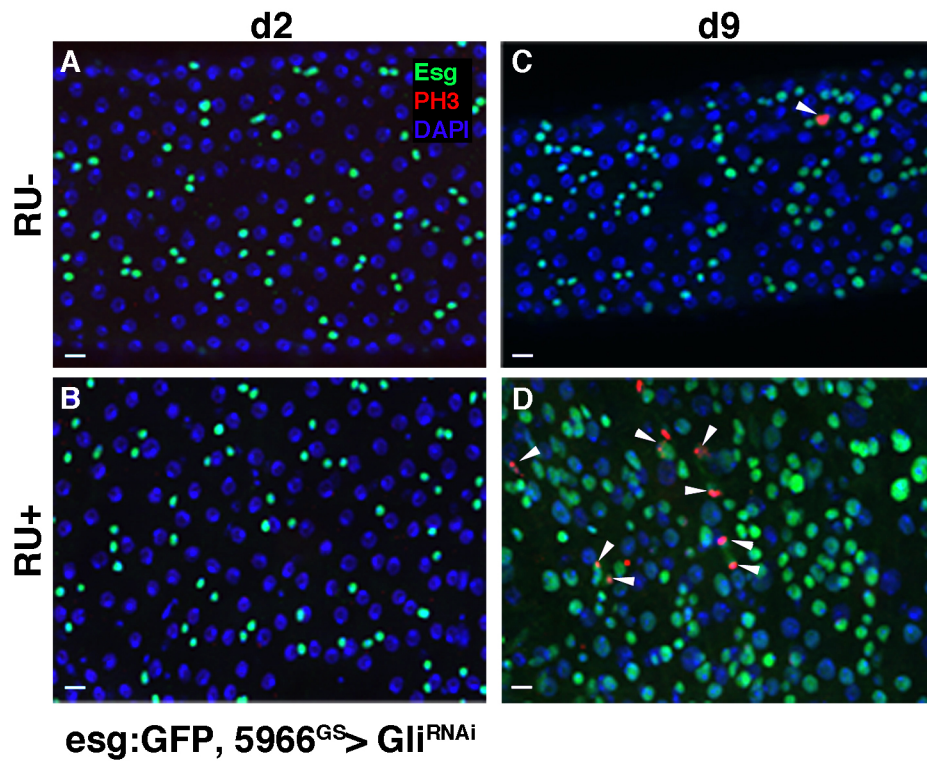
Resnik-Docampo *et al.* Fig. 2

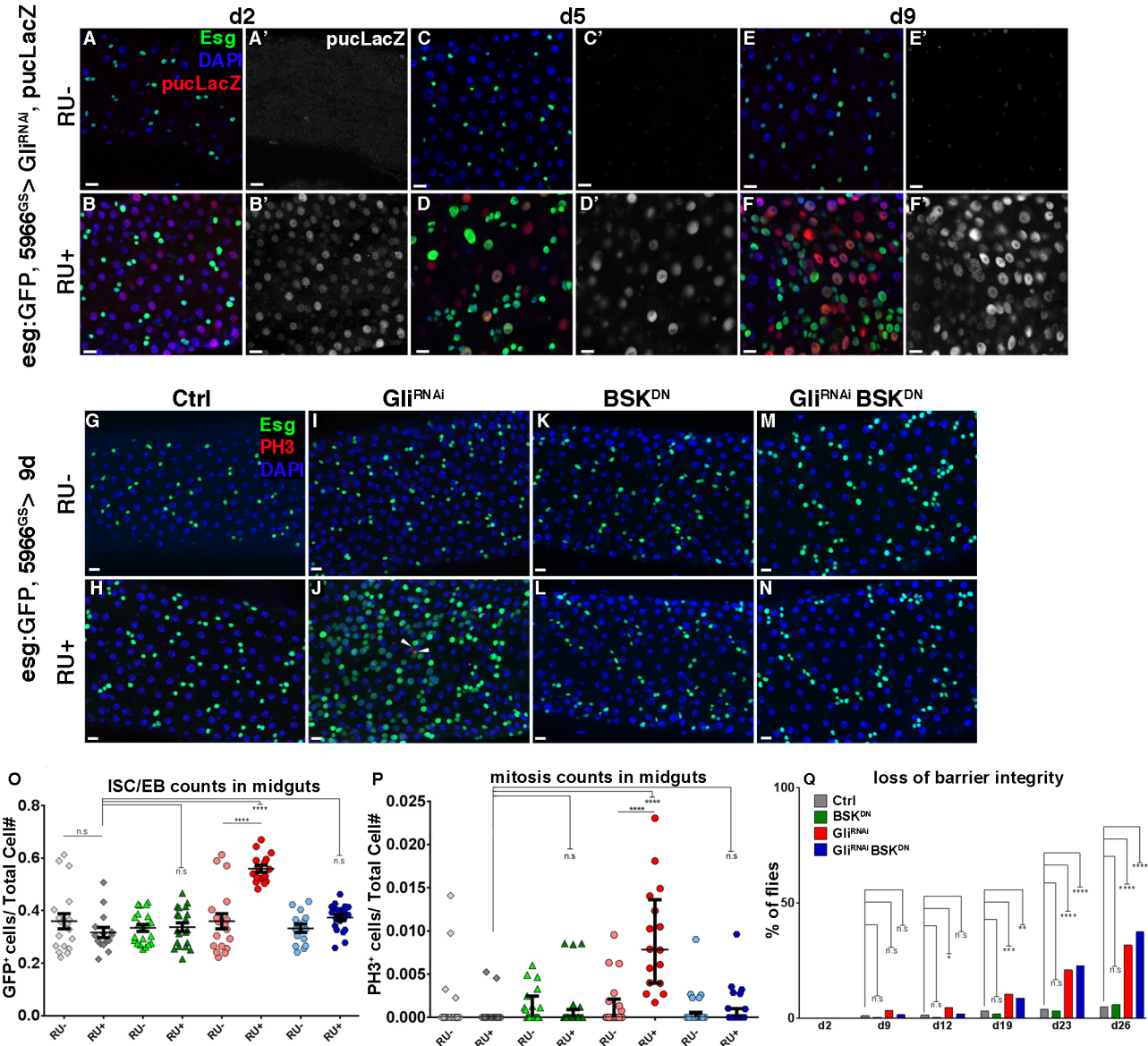


loss of barrier integrity



Myo1A > tub Gal80<sup>ts</sup>, Dlg<sup>RNAi</sup>, Gli:GFP 7d





**Resnik-Docampo et al. Fig. 5**

## **Materials & Methods**

### **Fly food and husbandry**

Flies were cultured in vials containing standard cornmeal medium (1% agar, 3% brewer's yeast, 1.9% sucrose, 3.8% dextrose, and 9.1% cornmeal; all concentrations given in wt/vol). Dietary restriction diet<sup>9</sup>: 1% agar, 0.55% brewer's yeast, 5% sucrose, and 8.6 % cornmeal; all concentrations given in wt/vol. Control food for DR diet: 1% agar, 5.5% brewer's yeast, 5% sucrose, and 8.6 % cornmeal; all concentrations given in wt/vol. Two inducible GAL4/UAS systems were used in this study: the GeneSwitch system<sup>19, 20</sup>, and the Target system<sup>32</sup>. All crosses with the GeneSwitch driver were carried out at 25°C. Crosses with the TARGET system were set up and maintained at 18°C until eclosion. In both cases, adults were kept for an additional 2-3 days, and induced at 3-4 days after eclosion by placement on food containing the steroid hormone mifepristone (RU486; Sigma M8046) in a 25µg/mL concentration, and flipped every 2 days thereafter. All analyses for these studies were performed on female flies, as age-related gut pathology has been well established in females<sup>6, 9</sup>.

### **Fly Stocks used**

Unless otherwise stated, crosses were done at 25°C. Lines not described in the text can be found in Flybase. *UAS-Bsk<sup>DN</sup>*, *UAS-dIAP*, *UAS-P35* and *puc-lacZ*<sup>33</sup>. Gal4 lines: *Su(H)lacZ*; *esg:GFP, 5966GAL4<sup>GS</sup>* (gift from B. Ohlstein); *Myo1AGAL4*, *UAS-GAL80<sup>TS</sup>*; *Sal<sup>EP</sup>GAL4*<sup>(34)</sup>, gift from JF. de Celis); *Rab3GAL4*, *tubGAL80<sup>TS</sup>*. Lines used for RNAi-mediated knock-down: *UAS-Gli<sup>RNAi</sup>* (37115GD, VDRC); *UAS-Gli<sup>RNAi</sup>* (37116GD, VDRC); *UAS-Gli<sup>RNAi</sup>* (107258KK, DSRC); *UAS-Ssk<sup>RNAi</sup>* (11906GD, VDRC); *UAS-mesh<sup>RNAi</sup>* (11906GD, VDRC); *UAS-bbg<sup>RNAi</sup>* (15975GD, VDRC); *UAS-cora<sup>RNAi</sup>* (9787GD, VDRC); *UAS-scrib<sup>RNAi</sup>* (29552, BDSC); *UAS-dlg<sup>RNAi</sup>* (25780, BDSC). GFP protein trap lines: Gli:GFP (115-332, DGRC); Scribble:GFP, Neuroglian:GFP, NeurexinIV:GFP, Nervana:GFP (gifts from G. Tanentzapf).

### **Transmission Electron Microscopy**

Tissues were fixed in 2.5% glutaraldehyde, in 0.1M phosphate buffer, 0.9% sodium chloride (PBS) and washed. The tissues were treated with 1% OsO<sub>4</sub> with 0.3 % potassium ferrocyanide in PB for 1 hour, followed by 2% UA for 1 hour. The tissues then were dehydrated in a graded series of ethanol, treated with propylene oxide and embedded in Eponate 12 (Ted Pella). Approximately 50-60 nm thick sections were cut



on a TMC ultramicrotome and picked up on formvar coated copper grids. The sections were stained with uranyl acetate and Reynolds lead citrate and examined on a JEOL 100CX electron microscope at 60kV.

#### Fluorescence Microscopy and Antibody staining

Our observations were carried out in the P3-P4 regions of the *Drosophila* intestine located by centering the pyloric ring in a 40× field of view (fov) and moving 1–2 fov toward the anterior. Posterior midguts were dissected into ice-cold PBS/4% formaldehyde and incubated for 1hr in fixative at room temperature. Samples were then washed three times, for 10 min each, in PBT (PBS containing 0.5% Triton X-100), 10 min in Na-deoxycholate (0.3%) in PBT (PBS with 0.3% Triton X-100), and incubated in block (PBT-0.5% bovine serum albumin) for 30 min. Samples were immunostained with primary antibodies overnight at 4°C, washed 4 × 5 min at RT in PBT, incubated with secondary antibodies at RT for 2 h, washed three times with PBT and mounted in Vecta-Shield/DAPI (Vector Laboratories, H-1200).

The following antibodies were obtained from the Developmental Studies Hybridoma Bank, developed under the auspices of the NICHD and maintained by The University of Iowa, Department of Biology, Iowa City, IA 52242. Discs large (mouse, 1:20, 4F3) and Coracle (mouse, 1:20, C615.16). GFP (rabbit, 1:3,000, Molecular Probes A-11122); GFP (mouse, 1:200, Molecular Probes A-11120); GFP (chicken, 1:500, Aves Labs GFP-1010); β-GAL (rabbit, 1:2000, Cappel/ MPbio 559761); Phospho-histone3 (rabbit, 1:200, Millipore 06-570). Gliotactin (mouse, 1:50, gift from V. Auld); Snakeskin (rabbit, 1:1000) and Mesh (rabbit, 1:1000) (gifts from M. Furuse).

Images were acquired on a Zeiss LSM710 inverted confocal microscope, and on a Zeiss Axio Observer Z1 and processed with Fiji/ImageJ and Zen from Zeiss. Super resolution images were obtained from a custom build Stimulated Emission Depletion (STED) super resolution microscope currently reaching a resolution of about 30-40nm<sup>35</sup>. The STED system was built in the Dept of Anesthesiology, UCLA. Supported an NIH grant from the National Heart, Lung, and Blood Institute BRG R01 HL088640. Final figures were assembled using Adobe Photoshop.

#### TCJ and Bicellular septate junction fluorescence quantification

To measure and compare the TCJ or Bicellular SJ fluorescence intensity of Gli-GFP, Dlg, Cora, Scribble-GFP, Mesh and Ssk in posterior midguts ECs, 100x + 3X of digital magnification confocal z-stack maximum projections at the level of the TCJ and SJ were generated using Zen 2 pro Blue software edition (Zeiss). TCJ were manually localized using Gli-GFP and a SJ marker (Ssk, Cora, Dlg or Mesh). TCJ and SJ fluorescence intensity were measured using a mask of 25.5 pixels diameter. Then cytoplasm fluorescence intensity were calculated using the same mask. Average fluorescence intensity at TCJ or SJ was divided by the cytoplasmic average intensity. Between 3 to 7 measurements were taken per picture and a minimum of 20 posterior midguts were analyzed per experimental condition.

#### Quantification using CellProfiler

For statistical significance four images were taken as z-stacks with a typical slice thickness of 750 nm. per posterior midgut; two on each side (top and bottom); from contiguous field of view, starting at 1 fov from the pylorus (using a minimum of 20 guts). The images were then processed using CellProfiler<sup>36</sup> pipeline developed in the Jones lab. ISC number and mitotic events were obtained from *esg:GFP/total cell* and *PH3/total cell* ratios respectively. Average ratios from the four images corresponding to a single gut were used in subsequent statistical analyses.

#### Statistics and reproducibility

Statistical analysis and graphical display of the data were performed using Prism6 (GraphPad). Significance, expressed as p values, was determined with a two-tailed test, the following parametric or non-parametric tests were used as indicated in figure legends: ANOVA/Tukey's multiple comparisons test or Student's t-test were used when data met criteria for parametric analysis (normal distribution, equal variances), Kruskal-Wallis/Dunn multiple comparisons test was used when data were non-parametric, Fisher's exact test, Log-rank (Mantel-Cox) test. Experiments were repeated at least two times.

#### Barrier integrity assays ('Smurf' assay)

Flies were maintained on standard medium prepared with FD&C Blue Dye n°1 from Spectrum added at a concentration of 2.5% (wt/vol). Loss of intestinal barrier function

was determined when dye was observed outside of the digestive tract as described in <sup>8, 9</sup>.

### Generation of axenic flies

Embryos were treated as described previously <sup>26, 37</sup>. Eight to fourteen hour old embryos were collected on grape agar plates using commercially available cages (flystuff.com). Embryos were dechorionated in 3% sodium hypochlorite (50% v/v regular bleach) for 20 min, rinsed in 70% ethanol for 5 min, and then washed three times with in PBS + 0.01% Triton X-100. Axenic embryos were transferred to autoclaved medium (50 embryos/vial) in a laminar flow cabinet. Axenic conditions were confirmed via plating fly homogenate, as well as by plating of swabs from spent vials, on de Man, Rogosa, Sharpe (MRS) bacterial agar, prior to each time point. Microbe-associated controls were generated by adding 60 uL whole fly homogenate (1 fly equivalent, from a glycerol stock of conventionally reared flies) per vial to embryos post sterilization, as described previously<sup>26</sup>.

### RNA extraction

Seventy-five female posterior midguts or 5 whole flies per condition after dissection were frozen at -80°C in fresh trizol buffer (TRizol Reagent, Life Technologies, 15596026; 5µg Linear Poly-Acrylamide Sigma 56575, 100ng of tRNA). Total RNA was extracted pooling midgut samples, followed by 5 rounds of freezing (liquid nitrogen)/thawing at 37°C in a water bath. Samples then underwent 5 rounds of vortexing at RT for 30", letting stand at RT for 5 min to disrupt all RNA-protein complexes. Finally RNA was isolated by phenol/chloroform extraction. Purified RNA was treated with DNase Q1 (Promega, M610A).

### Quantitative RT-PCR and PCR

RNA (2µg) from posterior midguts dissected from RU486 (RU+) or EtOH (RU-) fed flies (genotypes: *Su(H)lacZ*; *esgGFP*, *5966GAL4<sup>GS</sup>* crossed to *UAS-Gli<sup>RNAi</sup>*) was reverse-transcribed using the iScriptkit (Bio-Rad, 170-8841). Standard qPCRs were carried out on a Bio-Rad CFX96/C1000 Touch system (Bio-Rad), using Sso Advanced SYBR Green (Bio-Rad, 1725-264). The following primer sequences were used: RpL32 Fwd: ATCGTGAAGAAGCGCACCAA; RpL32Rev: TGTCGATACCCTTGGGCTTG; Gli Fwd: GCCGAATCGTCCAATTACAG; Gli Rev: ACTTTAAAGAAAAATTCCAGGAGAAA; Puc

Fwd: CGACTTTATCGAAGATGCACGG; PUC Rev: CAGGGAGAGCGACTTGTACC.  
Expression levels of targets analyzed were calculated relative to Rpl32 expression, using the  $\Delta\Delta C_t$  method.

#### Transcriptome analysis (RNA sequencing)

RNA-Seq libraries were prepared from three biological replicates for each experimental condition. The NEBNext poly(A)-mRNA magnetic isolation kit (NEB E7490S) was used to isolate poly(A)-mRNA from 4  $\mu$ g of whole mRNA. cDNA libraries were generated using the NEBNext RNA Library Prep Kit for Illumina (NEB E6110S), and NEBNext Multiplex Oligos for Illumina (NEB E7335S) were used for multiplexing. All steps were performed according to manufacturer's directions. RNA sequencing was performed on a Hi-Seq2000 (Illumina) with 50 bp single-end read length.

#### Bioinformatics analysis

Genomatix software from was used for mapping spliced reads, making transcript assemblies, and for differential expression analysis. Reads were first trimmed by removing adapter and Illumina-specific sequences. Next, trimmed reads were aligned against the *Drosophila* genome (NCBI el dorado version 08.2011) with default settings. Finally, a differential expression analysis was performed using the DESeq package method with a P-value threshold of  $p < 0.05$ . Differentially expressed genes were interrogated for overrepresented biological themes using database for annotation visualization and integrated discovery (DAVID) and categorized based on GO terms. The DAVID functional annotation clustering tool highlights the most relevant GO terms associated with a differentially expressed gene list. Details of the DAVID algorithm can be found at <http://david.abcc.ncifcrf.gov/>.

#### Data availability

The RNAseq data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus and are accessible through GEO Series accession number GSE74168, GSE74171, GSE74172.

<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE74168>

<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE74171>

<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE74172>

Source data for Fig. 1C have been provided as a Supplementary table 1. All other data supporting the findings of this study are available from the corresponding author on request.

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1 **Extended Data:**

2

3 **Supplementary Fig. 1:** Localization of septate junction (SJ) proteins in the intestinal  
4 tract in young and aged flies.. Related to Fig1 and Fig2F-G

5

6 **Supplementary Fig.2:** Verification of Gli reagents. Data related to Figs. 3-5.

7

8 **Supplementary Fig. 3:** Effects of Gli depletion in the posterior midgut. Extended data  
9 related to Fig.4

10

11 **Supplementary Fig. 4:** Neither changes in bacteria nor death of ECs induces changes  
12 in ISC behavior downstream of Gli. Extended data related to Fig. 5

13

14 **Supplementary Table 1:** RNAseq data of gene expression changes in posterior  
15 midguts from young and old flies

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17 **Supplementary Table 2:** RNAseq data of gene expression changes in posterior  
18 midguts from flies in which *Gliotactin* is reduced specifically in enterocytes for 2 or 9  
19 days

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36 **Figure Legends**

37

38 **FIG. 1:** Localization of septate junction (SJ) proteins in the intestinal tract in young and  
39 aged flies. (A) Schematic representation of a *Drosophila melanogaster* midgut (B-I)  
40 Localization of SJ proteins in posterior midgut and hindgut of young flies. (B; n=4  
41 midguts) Nervana:GFP, (C; n=5) Neurexin IV:GFP, (D; n=11) Neuroglian:GFP, (E; n=7)  
42 Snakeskin, (F; n=6) Scribble:GFP, (G; n=10) Discs large, (H; n=6) Coracle, (I; n=6)  
43 Mesh. Scale bars, 0.1mm. (J-S) No changes in SJ protein levels were observed in  
44 hindguts from aged flies. STED (J-O) and confocal (P-Q) images comparing SJ proteins  
45 localization of 10do hindguts to 45do, scale bars 1 $\mu$ m and 5 $\mu$ m. (R-S) STED images  
46 showed the AJ protein Arm localization is not affected by aging, scale bars 1 $\mu$ m. n=>14  
47 hindguts per condition (n=10 ECs were observed per hindgut). Samples were dissected  
48 and stained in parallel under same conditions, pictures taken at same laser intensity.

49

50 **FIG. 2:** Verification of Gli reagents. (A-C) GLI:GFP and anti-GLI co-localization (wing  
51 discs). GliRNAi efficiency demonstrated by immunofluorescence (IF) microscopy (wing  
52 discs) and qPCR (posterior midguts). (A-A'') Wing disc peripodial membrane showing  
53 the co-localization of Gli-GFP (green) with Gli (red), DAPI (blue); Scale bars, 10 $\mu$ m;  
54 n=16. (B-C) Gli protein is localized at the TCJ between wing cells (red signal in B; n=14)  
55 in wild type discs. Gli is strongly reduced in discs expressing GliRNAi (*SalPE-GAL4*  
56 *UAS-GFP UAS-Gli<sup>RNAi</sup>*, C; n=12). Scale bars, 50 $\mu$ m. (D) RT-qPCR of posterior midguts  
57 *5966<sup>GS</sup> GAL4/UAS-Gli<sup>RNAi</sup>* flies RU+ and RU- showing a decrease of *Gli* expression after  
58 5 and 9 days post Gli<sup>RNAi</sup> expression. n=75 females posterior midguts per condition. Bars  
59 are the mean +/- S.E.M (two tailed, unpaired Student's t-test). \*\*\* = P<0.001, \*\* = P<0.01  
60 represent a statistically significant difference. (E) Flies fed with EtOH (light blue) (*5966<sup>GS</sup>*  
61 *GAL4/+*, RU-, n=270) show no statistical difference in number of flies that have lost  
62 barrier function, when compared to those fed RU (blue) (*5966<sup>GS</sup> GAL4/+* fed, RU+,  
63 n=268). Fisher's exact test; two tailed. (F-G) EM images of SJ (arrowheads) between  
64 adjacent ECs in guts from 23do *5966<sup>GS</sup> GAL4/UAS-Gli<sup>RNAi</sup>* flies (RU+, n=12) (G)  
65 compared to control flies (F) *5966<sup>GS</sup> GAL4/UAS-Gli<sup>RNAi</sup>* (RU-, n=15). For each midgut  
66 n=9 EC/EC Septate Junctions were observed per experimental condition. Scale bars,  
67 0.1 $\mu$ m (H) Lifespan curves of *5966<sup>GS</sup> GAL4/UAS-Gli<sup>RNAi</sup>* [(RU+, red)(n=265) and RU-  
68 (grey)(n=240)] female flies. T50 was significantly lower in RU+ d30 compared with RU-

69 d37. Data analyzed with non parametric Log-Rank (Mantel-Cox) test; \*\*\* = P<0.001,  
70 represent a statistically significant difference.

71

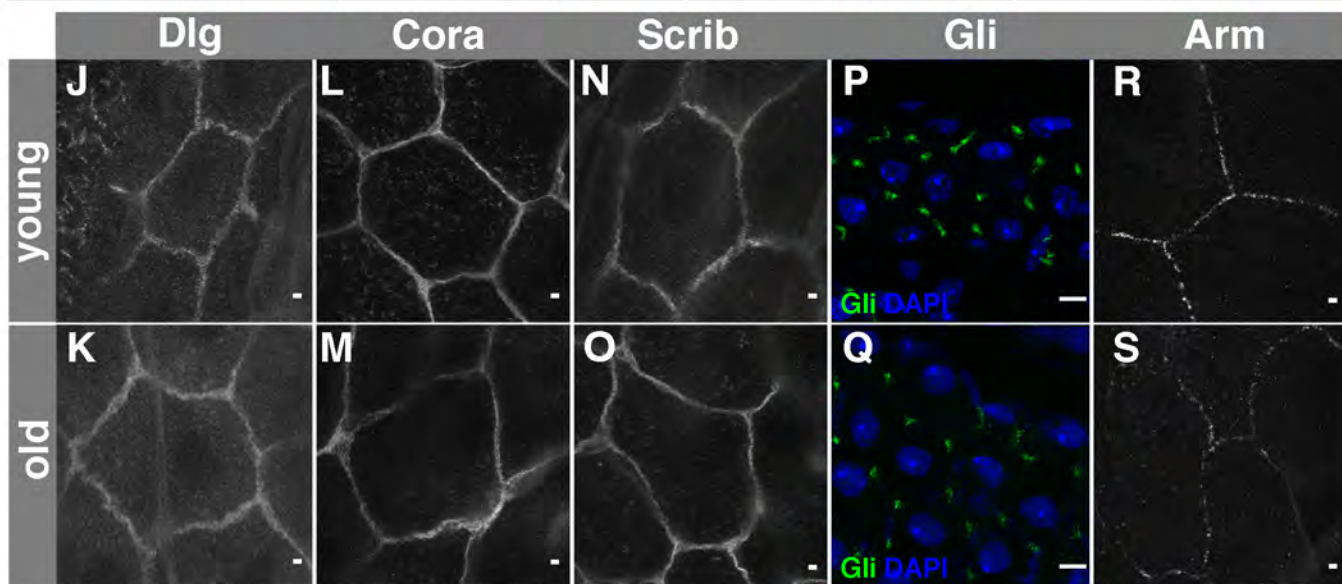
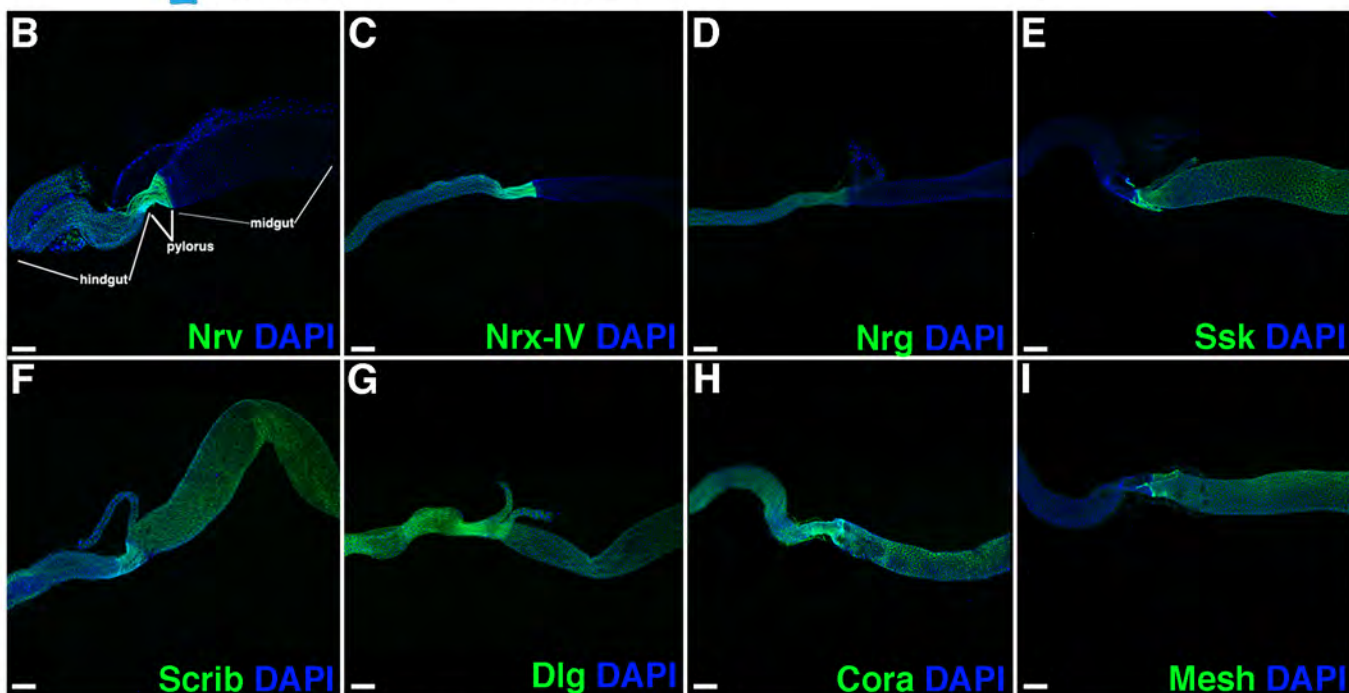
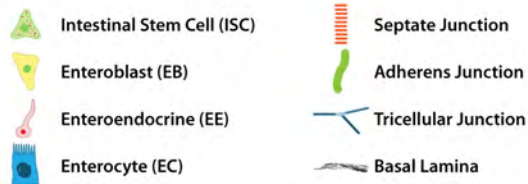
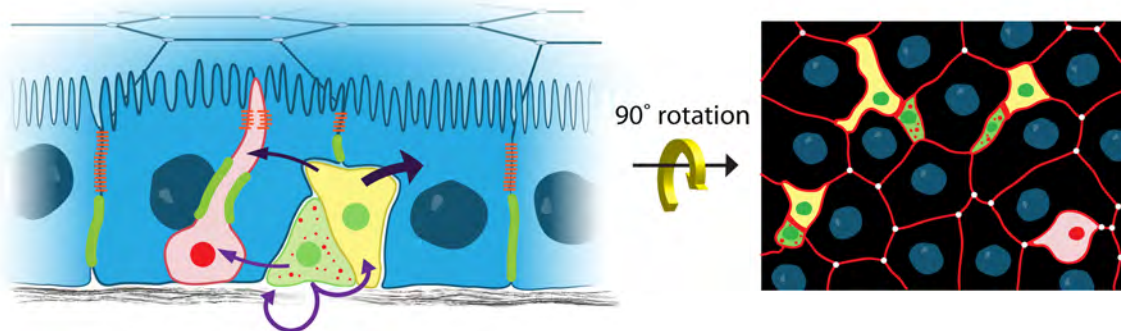
72 **FIG. 3:** Effects of Gli depletion in the posterior midgut. (A-F) *5966<sup>GS</sup> GAL4; esg-GFP* flies  
73 (RU+ or RU-) crossed with RNAi lines targeting Gli or Dlg. (A-D) Multiple *Gli<sup>RNAi</sup>* lines  
74 induce an increase of ISC/EB cells, when expressed (RU+) (B, n=12; D, n=11)  
75 compared to RU- controls (A, n=11; C, n=9), similar to results presented in Fig.4D. (E-F)  
76 Depletion of Dlg (*dlg<sup>RNAi</sup>*, RU+) (E, n=15) induces an increase in ISC/EBs compared to  
77 controls (RU-) (F, n=14). (G-K) Changes in ISC/EB number after *Gli* depletion are  
78 reversible (*5966<sup>GS</sup> GAL4; esg-GFP UAS-Gli<sup>RNAi</sup>* RU+, RU-) (G) Graphical summary  
79 showing the statistical significance in changes in ISC/EB number depicted in H-K. (H)  
80 Intestine from *5966<sup>GS</sup> GAL4; esg-GFP UAS-Gli<sup>RNAi</sup>* (fed RU+) for 9 days. (I) Flies were  
81 shifted onto food containing EtOH (RU-) to re-initiate Gli expression for 11 days. Re-  
82 expression of Gli resulted in resumption of normal ISC behavior and morphology  
83 compared to guts from 20do RU- *5966<sup>GS</sup> GAL4; esg-GFP UAS-Gli<sup>RNAi</sup>* controls (J) or guts  
84 from 20do *5966<sup>GS</sup> GAL4; esg-GFP Gli<sup>RNAi</sup>* flies fed RU+ (K). Scale bars 10µm. Each data  
85 point is an average proportion calculated from 4 independent images per midgut and  
86 bars are the mean +/- S.E.M of those averages (ONE-way ANOVA/Tukey's multiple  
87 comparisons test). \*\*\*\* = P< 0.0001, \*\*\* = P<0.001, \*\* = P<0.01 represent a statistically  
88 significant difference. (H) n=60 (4 images taken per midgut n=15); (I) n=76 (4 images  
89 taken per midgut n=19); (J) n=84 (4 images taken per midgut n=21); (K) n=80 (4 images  
90 taken per midgut n=20) (L) Graphical summary showing that depletion of Gli from EEs  
91 using *Rab3-GAL4, tubGAL80<sup>TS</sup>* for 6d at 29°C (red) did not result in any changes in ISC  
92 behavior, compared to control flies (*y,w<sup>1118</sup>*; grey). Each data point is an average  
93 proportion calculated from 4 independent images per midgut and bars are the median  
94 with interquartile range of those averages (Kruskal-Wallis/Dunn multiple comparisons  
95 test). (M-N) No significant differences were detected in AMP expression, after 2, 5, 9 and  
96 21 days post GliRNAi expression. \*\* = P<0.01, represent statistically significant  
97 difference. (M) Drosomycin, (N) Dipteracin. n=6 replicates of 5. Boxplots display the first  
98 and third quartile, with horizontal bar at the median and whiskers showing the most  
99 extreme data point, which is no more than 1.5 times the interquartile range from the box.

100

101 **FIG 4:** Neither changes in bacteria nor death of ECs induces changes in ISC behavior  
102 downstream of Gli. (A-F) Epistatic analysis between Gli and apoptosis inhibitors. (A)

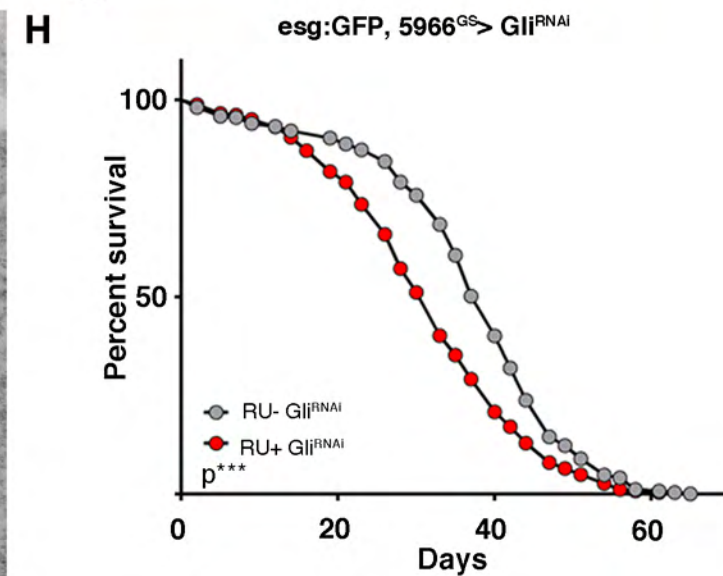
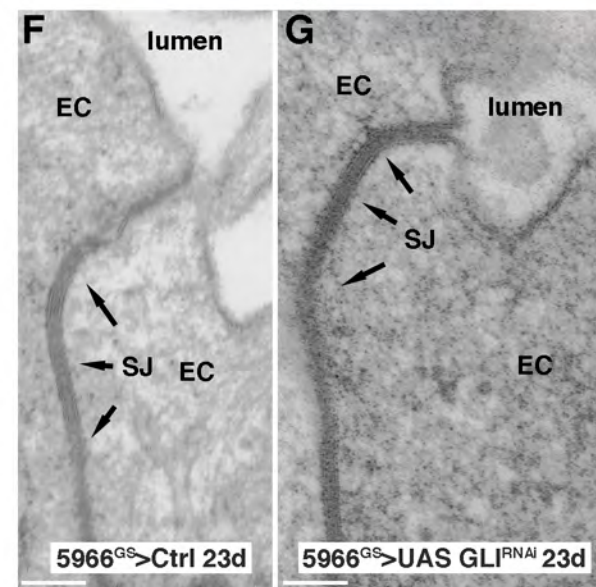
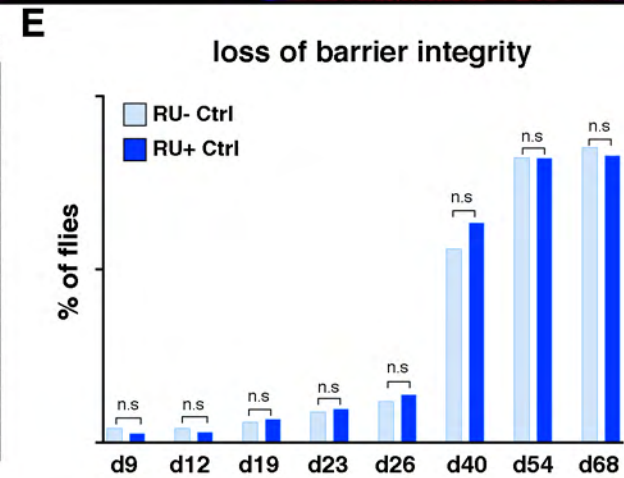
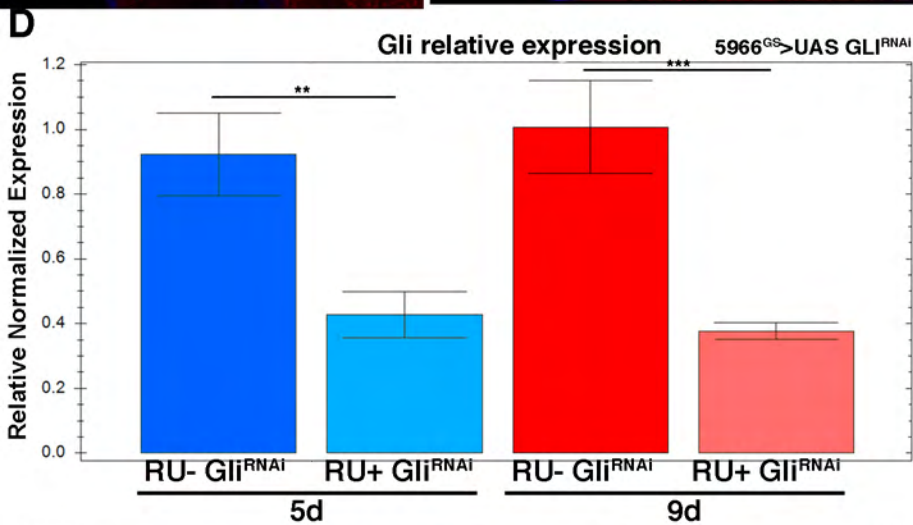
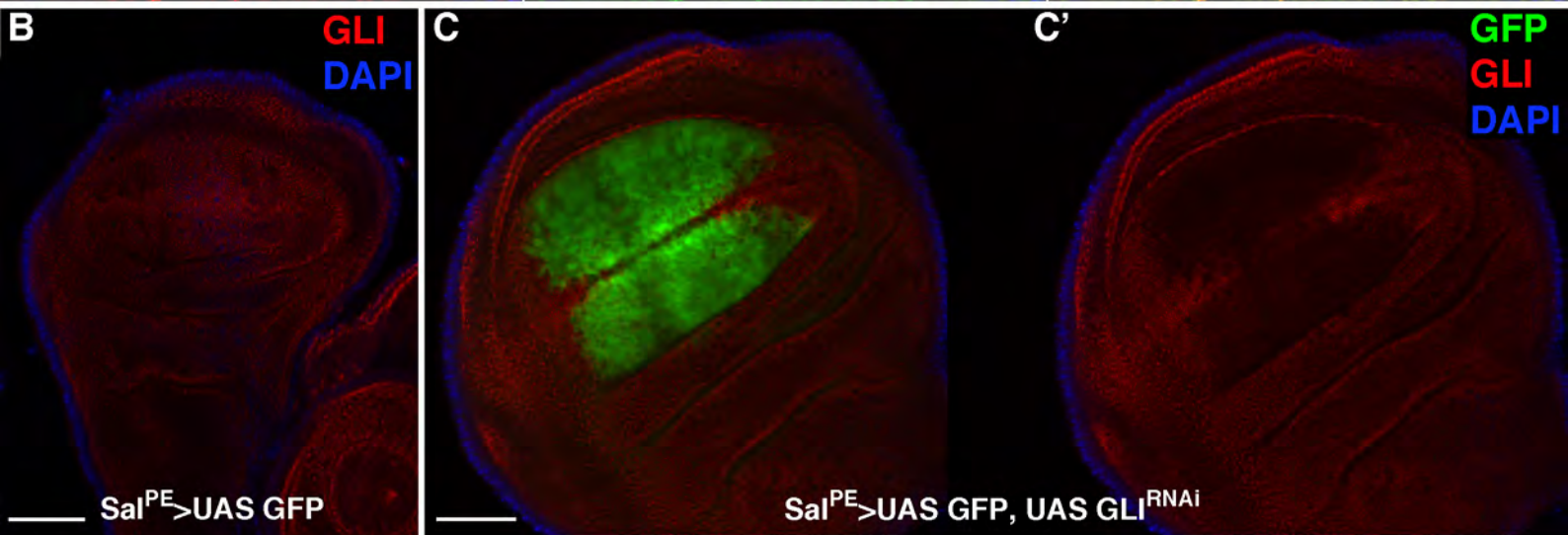
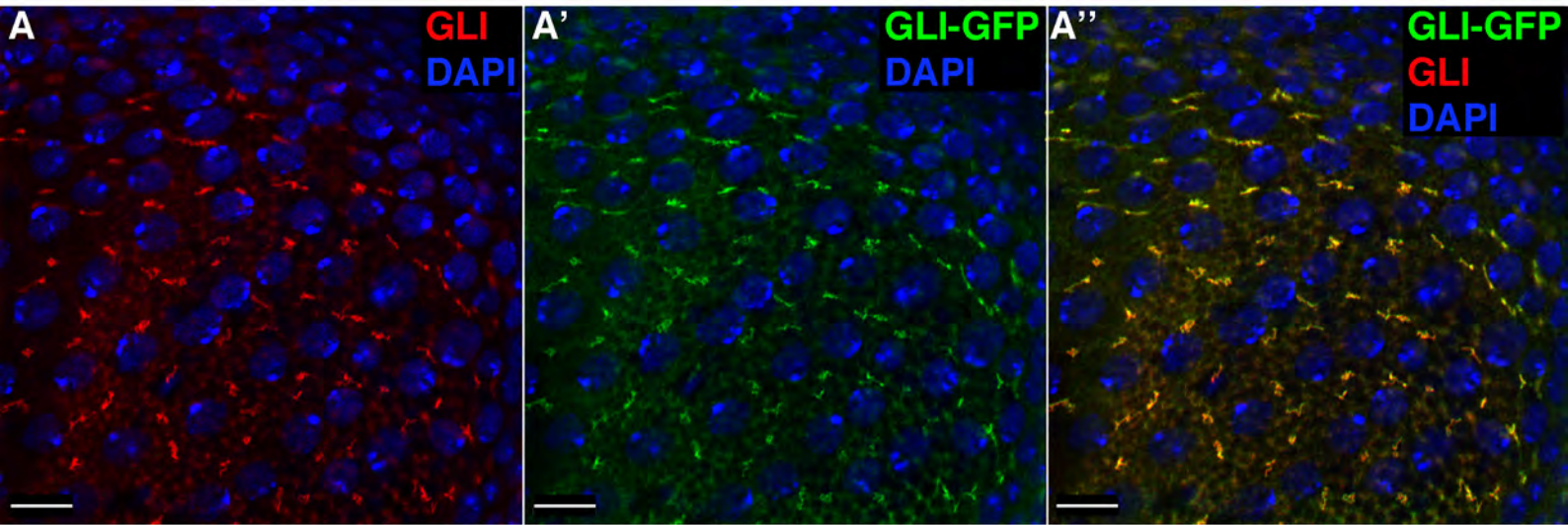


103 ISC/EB counts in flies of indicated genotypes. Each data point is an average proportion  
104 calculated from 4 independent images per midgut and bars are the mean +/- S.E.M  
105 (ONE-way ANOVA/Tukey's multiple comparisons test). (B) Mitosis counts in midguts  
106 from flies of indicated genotypes. Each data point is an average proportion calculated  
107 from 4 independent images per midgut and bars are the median with interquartile range  
108 (Kruskal-Wallis/Dunn multiple comparisons test) \*\*\*\* =  $P < 0.0001$ , represent statistically  
109 significant difference. (C-F) IF images of midguts from flies of indicated genotypes  
110 stained with DAPI (nuclei, blue), GFP (*esg*<sup>+</sup> cells, green) and PH3 (mitotic cells, red,  
111 arrowheads) following 9 days of incubation in RU+ or RU-. Co-expression of *Gli*<sup>RNAi</sup> with  
112 *diAP* (*5966*<sup>GS</sup> *GAL4/UAS-Gli*<sup>RNAi</sup>; *UAS-diAP*, RU+) or *P35* (*5966*<sup>GS</sup> *GAL4/UAS-Gli*<sup>RNAi</sup>;  
113 *UAS-P35*, RU+ (A-B; E-F) does not rescue the effect on ISCs produced by depletion of  
114 *Gli* (*5966*<sup>GS</sup> *GAL4/UAS-Gli*<sup>RNAi</sup> *UAS-LacZ*, RU+)(A-B, D). Compare to control flies fed  
115 EtOH (RU-) (*5966*<sup>GS</sup> *GAL4/UAS-Gli*<sup>RNAi</sup> *UAS-LacZ*, RU-)(A-B, C). (C) n=80 (4 images  
116 taken per midgut n=33); (D) n=132 (4 images taken per midgut n=33); (E) n=132 (4  
117 images were taken per midgut n=19); (F) n=68 (4 images taken per midgut n=17). (G-H')  
118 Under axenic conditions, reduction of *Gli* expression in ECs still initiates JNK pathway  
119 activation, as reported by *puc-lacZ* expression (G, H: red or G', H': grey) in ECs 2 days  
120 after reducing *Gli* expression (H-H', n=13), compared to RU- controls (G-G', n=11).  
121 ISC/EBs marked by *esg*-GFP (green), cell nuclei marked by DAPI (blue). (I) MRS plates  
122 showing absence of bacterial colonies from cultured fly homogenates of axenic flies at  
123 7do. (J) RT-qPCR of posterior midguts from *5966*<sup>GS</sup> *GAL4/UAS-Gli*<sup>RNAi</sup> flies (RU+ and  
124 RU-) showing an increase of *puc* expression after 2, 5, 9 days post *Gli*<sup>RNAi</sup> expression.  
125 n= 75 females posterior midguts per condition. Bars are the mean +/- S.E.M (two tailed,  
126 unpaired Student's t-test). \*\*\* =  $P < 0.001$ , \*\* =  $P < 0.01$ , \* =  $P < 0.05$  represent statistically  
127 significant difference. (K) Lifespan curves of *5966*<sup>GS</sup> *GAL4* RU+ female flies crossed with  
128 *yw*<sup>118</sup> (grey, n=290), *UAS-Bsk*<sup>DN</sup> (green, n=277), *UAS-Gli*<sup>RNAi</sup> flies (red, n=259) and  
129 *Gli*<sup>RNAi</sup> *UAS-Bsk*<sup>DN</sup> (blue, n=264). ●●● $P < 0.001$ , non parametric Log-rank (Mantel-Cox)  
130 test. Scale bars, 10  $\mu$ m

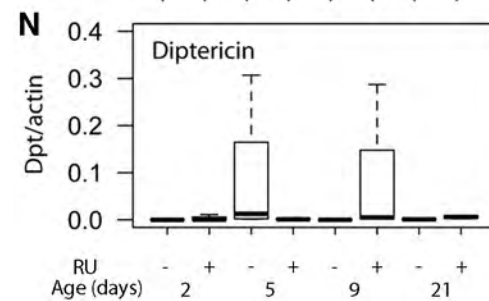
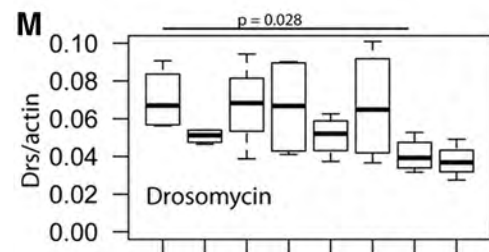
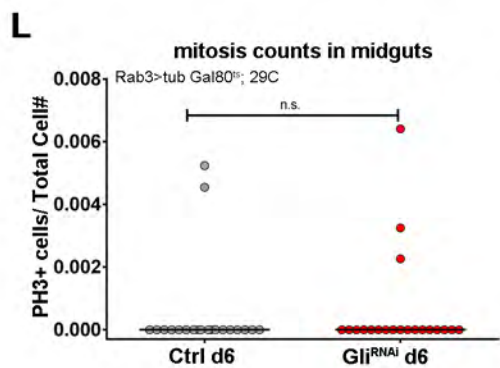
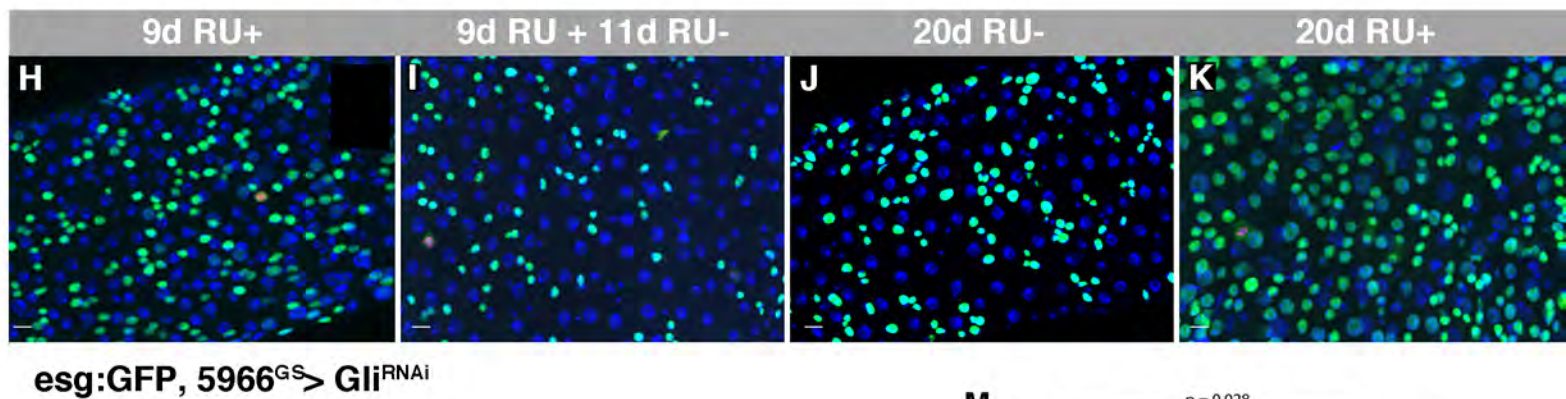
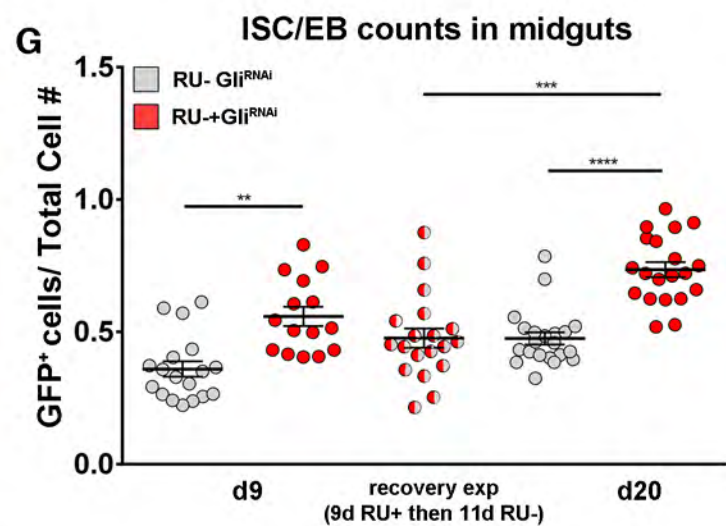
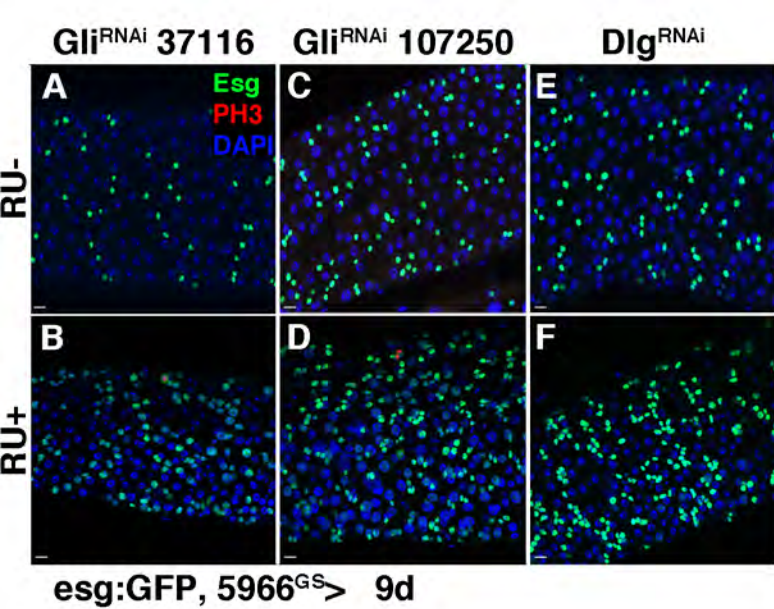
**A**

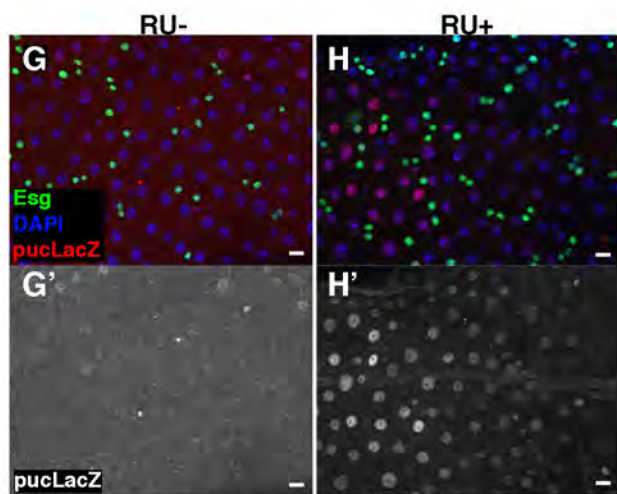
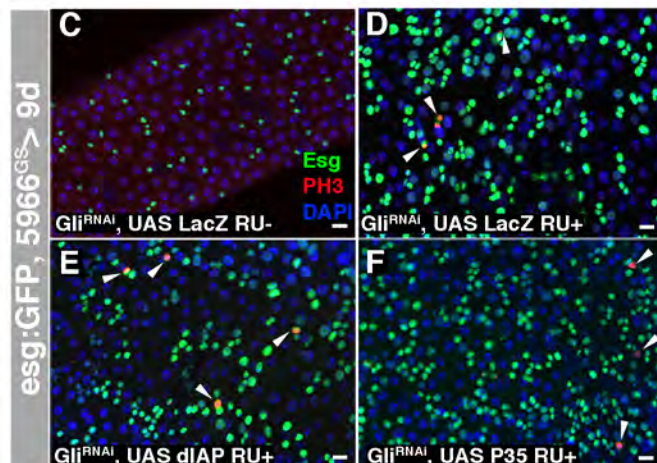
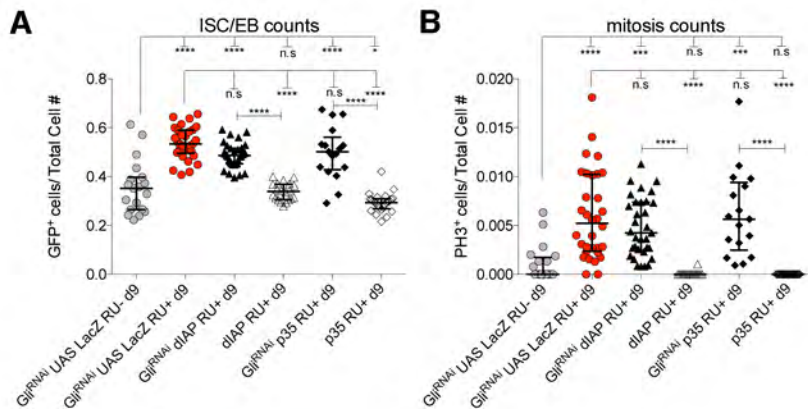
Resnik-Docampo *et al.* Sup. Fig. 1











*esg:GFP 5966<sup>GS</sup> > Gli<sup>RNAi</sup>; puLacZ 2d, Axenic*

