The Hippo tumor suppressor pathway regulates intestinal stem cell regeneration

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Supplemental Information

Fig. S1. Yki overexpression and RNAi controls. (**A**) Confocal images showing that Yki antibody signal is reduced, although not completely absent, in *yki RNAi-1* clones (*yki RNAi-1* is *hsFlp*/+*; UAS-GFP, act>CD2>Gal4*/+*; yki RNAi-1*/+). Arrows show where Yki(+) signal is low in a Dl(+) ISC, when compared with an adjacent wild-type Dl(+) ISC (arrowhead), whose signal is higher. (**B**) Confocal micrographs of wild type (left panels are *esg>GFP*: w^{1118}/+*; esg-Gal4, UAS-GFP, Tub-Gal80*^{TS}/+), RNAi against *yki* (middle panels are *esg>yki RNAi-1*: *esg-Gal4, UAS-GFP, Tub-Gal80*^{TS}/+*; yki RNAi-1*/+) and overexpression of *yki* (right panels are *esg>yki*: *esg-Gal4, UAS-GFP, Tub-Gal80*^{TS}/+*; UAS-yki*/+) stained for Yki protein. RNAi against *yki* decreases but does not completely abolish signal (compare arrowheads in *esg>GFP* with *esg>yki RNAi-1*), whereas overexpression of *yki* increases signal. Even when *yki* is overexpressed in ISCs (arrows), the predominant localization is still cytoplasmic and not nuclear. These results are consistent with the predominantly cytoplasmic localization observed during Yki-GFP overexpression in developing imaginal discs (Oh and Irvine, 2008). (**C**) Proliferation is increased 18 hours after *yki* or *ykiS168A* overexpression using the *esg-Gal4* driver, as well as after *yki* overexpression using the *myo1A-Gal4* driver, or combination of both *esg-Gal4* and *myo1A-Gal4* drivers.

Fig. S2. Characterization of Yki overexpression and Yki/Hpo RNAi by clonal analysis. (**A**) Similar to wild-type clones (w^{1118}), *hpo RNAi* clones contain Dl(+) cells, and in some cases clones show an expansion in Dl(+) cell number. Clones overexpressing yki^{S168A} (a constitutively activated allele) or depleted of *hpo* show the ability to produce differentiated progeny. Similar to wild-type clones (see Fig. 1F and Fig. 2E), these clones contain large polyploid EC nuclei and (Pros+) ee cells. (**B**) The same mosaic analysis that was carried out in Fig. 2, was carried out using the flip out system to generate overexpression and RNAi clones. An expansion in clone number was observed in *hpo RNAi-1* (*hpo RNAi-1* is *hsFlp*/+*; UAS-GFP, act>CD2>Gal4*/+*; hpo RNAi-1*/+) and *yki* (*yki* is *hsFlp*/+*; UAS-GFP, act>CD2>Gal4*/+*; UAS-yki*/+) clones, but not in *yki RNAi-1* clones (*yki RNAi-1* is *hsFlp*/+*; UAS-GFP, act>CD2>Gal4*/+*; yki RNAi-1*/+), which are similar to wild type (*w1118 (Ctrl)* is *hsFlp*/+*; UAS-GFP, act>CD2>Gal4*/+). Frequencies are shown as percentage normalized to the 4-day timepoint when each gut contained 2-10 clones in total (n=8-10 guts examined at each timepoint). Error bars indicate s.e.m. (P<0.05) (**C**) Histograms show that large clones are more frequent among *yki* and *hpo RNAi-1* clones than in wild type or *yki RNAi-1* (compare with Fig. 2F).

Fig. S3. Perturbation of Hippo signaling does not impair differentiation. (**A**) Similarly, wts^{X1} mutant clones (hsFlp, Tub-Gal4, UAS-nlsGFP/+; wts^{X1}, FRT82B/Gal80^{TS}, FRT82B) and ex^{e1} mutant clones (hsFlp, Tub-Gal4, UAS-nlsGFP/+; ex^{e1}, FRT40A/Gal80^{TS}, FRT40A) do not show obvious differentiation defects. (**B**) Both yki and hpo mutant clones show the ability to produce Pros(+) ee cells and large polyploid EC cells. (**C**) Quantification of the different cell types in MARCM clones confirms that differentiation is not affected by the loss of Hpo or Yki. A small increase in Dl(+) cell frequency is noted in hpo mutant clones. Graph shows the frequency of each cell type per genotype examined 14 days following clone induction.

Fig. S4. Characterization of Hippo signaling in the midgut. (**A**) Quantification of Esg(+) cell frequency in one field of view in the posterior region of the midgut at 12 hours following temperature shift. At this timepoint, little difference is apparent between any of the lines described. Error bars show ±s.d. We examined whether division was increased at these timepoints and whether this increase was reversible. A 1-day temperature shift induced a 18.4±2.6 times increase (relative to controls) in the number of phosphorylated-Histone3(+) cells when *yki* was overexpressed, and, similarly, a 3-day temperature shift induced 6.8±1.0 times increase using *hpo RNAi-1*. When these same shifted flies were returned to 18°C to terminate transgene expression, relative to controls, *yki* overexpressing/shifted flies returned to 1.3±0.3 times and *hpo* RNAi-1 overexpressing/shifted

flies returned to 0.8±0.2 times, respectively. (**B**) Quantification of Esg(+) cell frequency in one field of view in the posterior region of the midgut 6 days after temperature shift. At this timepoint, differences appear between all Hippo pathway components and wild-type (Ctrl), except *ds* RNAi which is equivalent to wild-type at all timepoints examined. Error bars show ±s.d. (**C**) Quantification of the frequency of Dl(+) and Pros(+) cells in the posterior midgut epithelium. A slight increase in Dl(+) frequency is observed upon Yki overexpression in the ISC/Eb cells. In all lines examined, no obvious differentiation effects were noted. Large polyploid EC nuclei and Pros(+) ee cells are present even at 11 days following temperature shift. As above, this suggests that Hippo signaling does not overly affect differentiation processes, unlike Notch (Micchelli and Perrimon, 2006; Ohlstein and Spradling, 2007), and that Eb cells undergoing knockdown are able to differentiate normally. Error bars show ±s.d. (**D**) Confocal images show Pdm1 antibody staining in the midgut of wild-type (*esg>GFP*: w^{1118}/+; *esg-Gal4, UAS-GFP, Tub-Gal80*^{TS}/+), *yki* (*esg>yki*: *esg-Gal4, UAS-GFP, Tub-Gal80*^{TS}/+; *UAS-yki*/+) and *hpo RNAi-1* (*esg>hpo RNAi-1*: *esg-Gal4, UAS-GFP, Tub-Gal80*^{TS}/+; *hpo RNAi-1*/+). Esg(+) cells are usually negative for Pdm1 but larger Esg(+), possibly those differentiating into ECs, at times show some Pdm1(+) signal. This may reflect some perdurance of the GFP protein. However, we note that at 11 days temperature shift, most of the surrounding mature, Pdm1(+), polyploid ECs are GFP(−), even though these are the progeny of the Esg(+)/Pdm1(−) ISC/Ebs. This suggests that the rapid proliferation of ISCs observed in *yki* and *hpo RNAi-1* midguts results in a minority of Esg(+)/Pdm1(+) cells that still retain some GFP from their time as Ebs, but before their differentiation is complete. Such a minority is even lower in wild-type cells, the division of which is slower − allowing GFP to disappear as Pdm1 is expressed.

Fig. S5. ISCs express Fat/pros-Gal4 expression analysis. (**A**) Confocal micrograph shows Fat staining (red) in wild-type ISCs (*esg>GFP*: w^{1118}/+; *esg-Gal4, UAS-GFP, Tub-Gal80*^{TS}/+), which positive for both Dl (magenta) and Esg (green). Doublets of Esg(+)/Fat(+) cells show that Fat expression is present in both ISCs and Eb cells. (**B**) Micrographs showing the *myo1A-Gal4* driver line after shift to 29°C (*Tub-Gal80*^{TS}/+; *myo1A-Gal4, UAS-GFP*/+). Images show slightly different section through the pseudostratified epithelium as ECs and ISCs are at slightly different positions in the tissue. GFP expression is generally limited to cells stained for the Pdm1 antibody which marks differentiated ECs (left panels), though some large polyploid GFP(+) cells were observed that do not stain for Pdm1 (not shown). These may be ECs during early stages of differentiation from Ebs, as most Pdm1(+) cells colocalize with GFP(+) and are recognizable ECs by their large nucleus and morphology. It is possible that the expression of *pdm1* and *myo1A*, though both present in mature ECs, is not completely coincident during differentiation and *myo1A* expression precedes *pdm1* expression. As shown, Pros(+) cells (middle panels) and Dl(+) (right panels) cells are never GFP(+). Thus, the *myo1A-Gal4* driver is restricted to ECs in this tissue.

Fig. S6. Yki localizes to the nucleus following damage or Hpo knockdown. (**A**) Yki antibody staining shows that its localization changes in some of the wild-type Esg(+) ISCs and progenitors upon injury with DSS. Confocal micrograph shows Yki staining, Esg(+) and DAPI labeling of the same cells. Arrows indicate nuclei where Yki signal is now nuclear and cytoplasmic rather than exclusively cytoplasmic (compare with Fig. 1B.). (**B**) Confocal image showing that Yki expression is altered also in Esg(+) ISCs or Eb cells where Hpo is depleted by RNAi (*esg>hpo RNAi-1*: *esg-Gal4, UAS-GFP, Tub-Gal80*^{TS}/+; *hpo RNAi-1*/+). Arrows indicate nuclei where Yki signal is nuclear and cytoplasmic, rather than just cytoplasmic (compare with Fig. 1B and Fig. S1). (**C**) Confocal images showing when JAK/STAT is activated by overexpressing either *upd* (*esg>upd*: *esg-Gal4, UAS-GFP, Tub-Gal80*^{TS}/*UAS-upd*) or constitutively active *hop*^{TUML} (*esg> hop*^{TUML}: *esg-Gal4, UAS-GFP, Tub-Gal80*^{TS}/*UAS- hop*^{TUML}), Esg(+) cells still show a mostly cytoplasmic signal resembling that in wild-type cells (Fig. 1), rather than when *yki* is overexpressed (Fig. S1) or when *hpo* is depleted (see above). Arrows indicate nuclei where Yki signal is chiefly cytoplasmic.

Fig. S7. No increase in TUNEL labeling is observed in Yki mutant cells. Confocal images show *yki*^{B5} mutant cells, 14 days after three heat shocks to induce polyclones composed of multiple fused ISC clones. TUNEL-positive cells (arrows) were not common, and were observed in both wild-type (GFP−) and *yki*^{B5} (GFP+) cells. These results suggest that *yki*^{B5} clones are not reduced in frequency, or size, owing to an increase in cell death.

Fig. S8. Yki targets are activated following injury. (**A**) Confocal micrographs showing the posterior midguts of control flies (fed sucrose) versus those fed DSS or *Pseudomonas*. All samples were prepared identically and images were taken using the same parameters. Genotypes are as indicated; *esg>Gal4* refers to the *esg-Gal4, UAS-GFP, Tub-Gal80^{TS}*/+ genotype. Insets confirm the increase in Esg(+) cell frequency as an outcome of damage. An increased number of *lacZ*(+)/Esg(+) cells is seen for *CycE* and, more weakly, for the *ex* reporter. *DIAP1* signal is increased primarily in EC cells following damage. When Hpo is depleted using RNAi, an increase in *ex-lacZ* expression is observed, similar to the RT-qPCR results shown in Fig. 6B. When *yki* is depleted by RNAi, and the midgut is injured using DSS, an increase in *ex-lacZ* is not observed, suggesting that this Yki target gene remains at baseline levels. (**B**) Similar micrographs show the expression of the microRNA *bantam*, another Yki target, in response to damage. The *bantam* sensor is a GFP construct, the downregulation of which is caused by increases in the *bantam* microRNA (Brennecke et al., 2003). The decrease in GFP expression following injury demonstrates that this Yki target is upregulated relative to controls, in which GFP is more uniformly distributed among the cells present.

Additional reference

Brennecke, J., Hipfner, D. R., Stark, A., Russell, R. B. and Cohen, S. M. (2003). *bantam* encodes a developmentally regulated microRNA that controls cell proliferation and regulates the proapoptotic gene hid in Drosophila. *Cell* **113**, 25-36.

Fig. S9. Yki targets are activated in ISCs following injury. (**A**) Confocal micrographs showing increased magnification of ISCs from the same analyses shown in **Fig. S8.** Genotypes are as indicated; *esg>Gal4* refers to the *esg-Gal4, UAS-GFP, Tub-Gal80^{TS}*/+ genotype. ISCs are labeled using Dl antibody (red). An increased in *lacZ*(+) reporter (purple) is seen in ISCs for *CycE, DIAP1* and, as above, more weakly for the *ex* reporter (arrows).

Fig. S10. JAK/STAT pathway knockdown. (**A**) When Stat92E was depleted in conjunction with Hpo (*esg>hpo RNAi-1, stat92E RNAi*: *esg-Gal4, UAS-GFP, Tub-Gal80^{TS}*/+*; hpo RNAi-1*/*stat92E RNAi*), 11/43 guts examined contained one hyperplasia as shown. The simultaneous knockdown of Hop and Hpo also produced one hyperplasia per gut in two out of 32 samples. Localized overgrowths were also observed upon simultaneous overexpression of *yki* and knockdown of either *stat92E* (seven out of 19 hyperplasia per gut) or *hop* (two out of 17 hyperplasia per gut). This effect appears to be a result of JAK/STAT pathway perturbation, as knockdown of *stat92E* alone produced localized hyperplasia in eight out of 15 guts examined. (**B**) Quantification of the frequency of Esg(+) cells in one field of view in the posterior region of the midgut (top), as well as total phosphorylated-Histone3(+) mitoses scored in the entire midgut (bottom) when *yki* is overexpressed. The depletion of *hop* and *stat92E* suppress the overproliferation in *yki* overexpressing midgets, similar to when *hpo* is depleted (see Fig. 5A,B), error bars indicate s.e.m. (P<0.05). Simultaneous overexpression of *yki* and *hpo* slightly reduces overproliferation from the overexpression of *yki* alone but is insufficient to completely prevent overgrowth. This may be due to differences in the expression levels of these randomly inserted transgenes.

Fig. S11. Similar integration of Hippo and JAK/STAT signaling following injury. The same effects are observed during injury with DSS as under normal homeostatic conditions. (A-C) The quantification of total phosphorylated-Histone3(+) mitoses scored in the entire midgut (at left) and the frequency of Esg(+) cells in one field of view in the posterior region of the midgut (at right). (**A**) The depletion of *hop* and *stat92E* suppress the overproliferation observed due to *hpo* depletion alone. Error bars indicate s.e.m. (P<0.05). (**B**) Similar effects are observed when *yki* is overexpressed, error bars indicate s.e.m. (P<0.05). (**C**) When both *yki* and *hop*, or *yki* and *stat92E* are depleted, no significant differences are observed from *yki* depletion alone. Error bars indicate s.e.m. (P<0.05).