Hippo signaling regulates *Drosophila* intestine stem cell proliferation through multiple pathways

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Intestinal stem cells (ISCs) in the *Drosophila* adult midgut are essential for maintaining tissue homeostasis and replenishing lost cells in response to tissue damage. Here we demonstrate that the Hippo (Hpo) signaling pathway, an evolutionarily conserved pathway implicated in organ size control and tumorigenesis, plays an essential role in regulating ISC proliferation. Loss of Hpo signaling in either midgut precursor cells or epithelial cells stimulates ISC proliferation. We provide evidence that loss of Hpo signaling in epithelial cells increases the production of cytokines of the Upd family and multiple EGFR ligands that activate JAK-STAT and EGFR signaling pathways in ISCs to stimulate their proliferation, thus revealing a unique non–cell-autonomous role of Hpo signaling in blocking ISC proliferation. Finally, we show that the Hpo pathway mediator Yorkie (Yki) is also required in precursor cells for injury-induced ISC proliferation in response to tissue-damaging reagent DSS.

Yap | Wts | regeneration

Adult stem cells play critical roles in maintaining tissue homeostasis throughout adult life. Proliferation of adult stem cells and differentiation of their progenies are under tight control to achieve the normal balance between removing dead cells and producing new cells, and disruption of the regulatory mechanisms could result in excessive proliferation of stem cells/progenitor cells, which eventually leads to tumor growth.

The *Drosophila* adult midgut has emerged as an attractive model to investigate how stem cell proliferation and differentiation are regulated not only because the cell lineage in this tissue is simple and well characterized but also because it bears similarities to the mammalian intestine (1). The *Drosophila* adult midgut contains intestine stem cells (ISCs) that are located adjacent to the basement membrane of the midgut epithelium (2, 3). The asymmetric division of an ISC produces a renewed ISC and an enteroblast (EB) that can undergo differentiation to become either absorptive enterocyte (EC) or secretory enteroendocrine cell (EE) (2, 3). Several conserved signaling pathways including Notch, Wingless (Wg)/Wnt, and JAK-STAT pathways regulate the maintenance, proliferation, and differentiation of ISCs (3–8). Moreover, tissue damage can induce ISC proliferation and differentiation to replenish damaged cells, and the JAK-STAT and Insulin pathways are the critical mediators of damage-induced ISC proliferation (6, 9–12). However, our understanding of ISC regulation is still incomplete, and it is likely that additional pathways may participate in the regulation of ISC proliferation and differentiation.

Initially discovered in *Drosophila*, the Hippo (Hpo) signaling pathway has emerged as an evolutionarily conserved pathway that controls cell growth, proliferation, and survival, and its abnormal activity has been linked to several types of cancer (13–15). The Hpo pathway acts through a kinase cascade consisting of an upstream kinase Hpo and a downstream kinase Warts (Wts) to restrict the activity of the transcriptional coactivator Yorkie (Yki) (13, 14, 16, 17). In the absence of Hpo signaling activity, Yki enters the nucleus and forms a complex with the TEAD family of transcription factor Scalloped (Sd) to activate genes involved in cell proliferation, cell growth, and apoptosis (18–20).

Although the Hpo pathway has been extensively studied in the *Drosophila* appendage development at the larval stage, its role in stem cell proliferation and adult tissue homeostasis has not been investigated. In this study, we demonstrate that the Hpo pathway restricts ISC proliferation in the adult midgut. We uncover a non–cell-autonomous mechanism by which the Hpo pathway negatively regulates stem cell proliferation by restricting the production of cytokines and mitogens that activate the JAK-STAT and EGFR pathways. In addition, we demonstrate that Yki is required in the precursor cells for dextran sulfate sodium (DSS)-induced ISC proliferation.

Results

Loss of Hpo Signaling in Precursor Cells Stimulates ISC Proliferation. As an initial step to explore the role of Hpo signaling in midgut homeostasis, we expressed a Wts transgenic RNAi line (*UAS-Wts-RNAi*) along with *UAS-GFP* using *esg-Gal4*, which is specifically expressed in adult ISCs and EBs (collectively referred to as precursor cells; Fig. 1*J*). Wts knockdown clearly increased the number of esg-GFP$^+$ cells (compare Fig. 1*B* with Fig. 1*A*), suggesting an expansion of the precursor cell population. Coexpression of *UAS-Yki-RNAi* or *UAS-Sd-RNAi* transgene with *UAS-Wts-RNAi* suppressed the increase in the number of esg-GFP$^+$ cells (Fig.1 *C* and *D*), suggesting that Wts restricts the precursor cell population through inhibiting Yki/Sd. Immunostaining with an antibody against Phospho-Histone3 (PH3), a specific marker for mitotic cells, indicates that Wts RNAi guts contained increased PH3$^+$ cells, and such increase was suppressed by knockdown of Yki or Sd (Fig. 1 *B'–D'* and *H*). Overexpression of Yki also led to an increase in the number of esg-GFP$^+$ and PH3$^+$ cells (Fig. 1 *E, E',* and *H*), which was suppressed by Sd RNAi (Fig. 1*H*). Previous studies indicate that ISCs are the only cells that can undergo cell division in the midgut (2, 3). Double labeling with Dl antibody, which specifically marks the ISCs (21), confirmed that PH3$^+$ cells in Wts RNAi or Yki overexpression guts are ISCs (Fig. 1 *F* and *G*). Taken together, these results demonstrate that Wts restricts ISC proliferation by limiting the activity of Yki/Sd in precursor cells.

esg-Gal4 is also expressed in adult midgut progenitor cells (AMPs) that proliferate during larval development (22). To confirm that perturbation of Hpo signaling at the adult stage is sufficient to affect ISC proliferation, we used *tublin-Gal80^{ts}* to temporarily control *esg-Gal4* target gene expression (*esg^{ts}*) (23). After shifting adult flies to 29 °C for 14 d, *esg^{ts}-Wts-RNAi* and *esg^{ts}-Yki* adult midguts exhibited a dramatic increase in the number of esg-GFP$^+$ and PH3$^+$ cells (Fig. 1*I* and Fig. S1). Furthermore, Yki RNAi reversed the phenotypes caused by Wts RNAi (Fig. 1*I* and Fig. S1). Taken together, these results suggest that Hpo signaling is required in the adult midgut precursor cells to inhibit ISC proliferation.
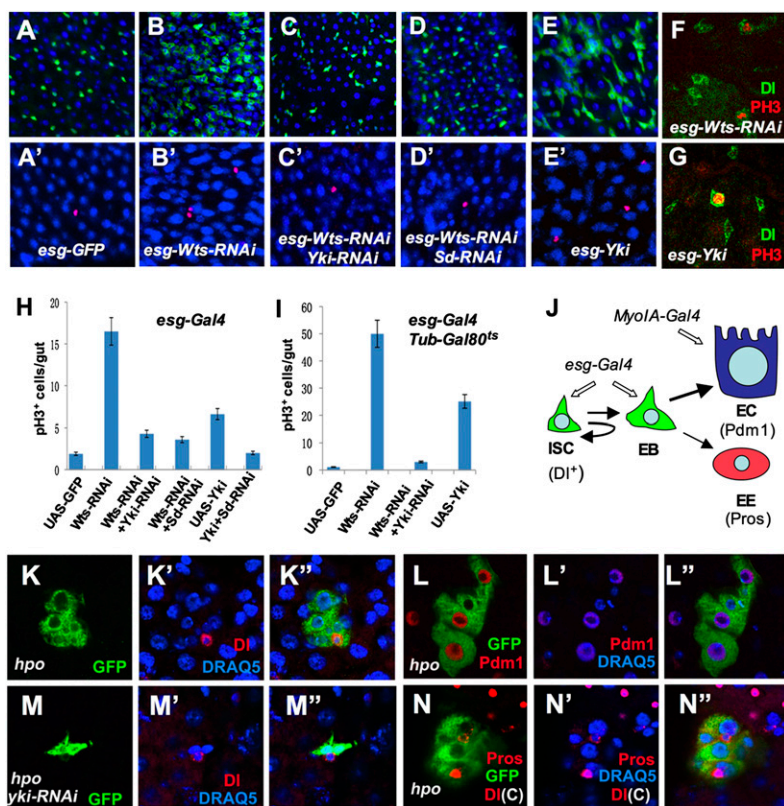
Fig. 1. Hpo signaling restricts ISC proliferation by inhibiting Yki/Sd. (*A–E'*) Adult fly midguts, 3–5 d old, expressing *esg-Gal4/UAS-GFP* without (*A* and *A'*) or with *UAS-Wts-RNAi* (*B* and *B'*), *UAS-Wts-RNAi + UAS-Yki-RNAi* (*C* and *C'*), *UAS-Wts-RNAi + UAS-Sd-RNAi* (*D* and *D'*), or *UAS-Yki* (*E* and *E'*) were immunostained with GFP (green) and PH3 (red) antibodies, and a nuclear dye DRAQ5 (blue). (*F* and *G*). Adult midguts expressing *esg-Wts-RNAi* (*F*) or *esg -Yki* (*G*) were immunostained with Dl (green) and PH3 (red) antibodies. (*H* and *I*) Quantification of PH3$^+$ cells in midguts from adults of the indicated genotypes (mean ± SD, $n \geq 15$ for each genotype). (*J*) ISC lineage in *Drosophila* adult midguts. EB, enteroblast; EC, enterocyte; EE, enteroendocrine cell; ISC, intestine stem cell. (*K–N''*) Adult midguts containing GFP-labeled *hpo* clones (*K–L''* and *N–N''*) or *hpo* clones expressing *Yki-RNAi* (M-M'') were immunostained to show the expression of GFP (green), Dl (cytoplasmic red in *K–K''* and *M–N''*), Pdm1 (red in *L–L''*), Pros (nuclear red in *N–N''*), and DRAQ5 (blue). Guts were dissected out from adult flies grown at 18 °C for 5 d after clone induction.

Hpo Pathway Mutant Clones Exhibit Elevated Proliferation and Produce Differentiated Cells. To further explore the role of Hpo signaling in adult midguts, we generated GFP positively marked clones mutant for various *hpo* pathway components in the ISC cell lineage using the mosaic analysis with a repressible cell marker (MARCM) system (24). We then compared the clone size (indicated by the number of GFP$^+$ cells per clone) between the control clones and mutant clones for *hpo* pathway components. The adult flies were grown at 18 °C for 5 d after clone induction (*Experimental Procedures*). Under such condition, the control clones contained 2–3 cells per clone (Fig. S2). In contrast, *hpo* or *wts* mutant clones contained 5–7 cells per clone (Fig. 1 *K–K''* and Fig. S2). GFP$^+$ clones mutant for *fat* (*ft*), which encodes a protocadherin that functions as a Hpo pathway receptor (25–27), also contained 5–7 cells per clone under the same condition (Fig. S2). Expressing *Yki-RNAi* in Hpo pathway mutant clones reduced their clone size (Fig. 1 *M–M''* and Fig. S2). The increased clone size associated with Hpo pathway mutant clones suggests that Hpo signaling is required to restrict ISC growth and proliferation during adult midgut homeostasis.

To determine whether the basal Yki activity is essential for ISC proliferation, we generated GFP positively marked *yki* mutant clones. Twenty days after clone induction, the clone size for *yki* mutant clones was comparable to that of control clones (Fig. S3). Thus, the basal Yki activity is not critical for ISC proliferation under normal gut homeostasis.

Dl staining revealed that isolated *hpo*, *wts*, or *ft* mutant clones often contained one Dl$^+$ cell per clone (Fig. 1*K–K''* and Fig. S2), implying that Hpo pathway mutant ISCs can self-renew and produce differentiated cells. Immunostaining with anti-Pdm1 and anti-Prospero (Pro) antibodies, which label EC and EE, respectively (Fig. 1*J*) (2–4), confirmed that *hpo*, *wts*, or *ft* mutant clones contained differentiated EC and EE cells (Fig. 1 *L–L''* and *N–N''* and Fig. S4). Thus, although loss of Hpo signaling activity increases ISC proliferation, it does not block ISC lineage differentiation.

hpo Mutant Clones Can Induce ISC Proliferation Nonautonomously. PH3 staining of *Drosophila* midguts carrying control or *hpo* mutant clones revealed an increase in the number of mitotic cells within the *hpo* mutant clones compared with control clones (Fig. 2 *A*, *B*, and *B'*; quantification in Fig. 4*F*), which is expected if Hpo signaling acts cell autonomously to restrict ISC proliferation. Unexpectedly, we also observed an increase in the number of PH3$^+$ cells located outside of the *hpo* mutant clones (arrows in Fig. 2 *B* and *B'*). These observations imply that *hpo* mutant cells may produce a secreted factor(s) that acts in a paracrine fashion to stimulate the proliferation of neighboring WT ISCs.

The implicated secreted factor(s) associated with *hpo* mutant clones could be produced by precursor cells, differentiated cells or both. To determine whether loss of Hpo signaling in differentiated cells could also stimulate ISC proliferation, we used an EC specific Gal4 driver, *Myo1A-Gal4*, in conjunction with *tub-Gal80^{ts}* (*Myo1A^{ts}*) to express *Wts-RNAi* or *UAS-Yki* in adult midgut epithelia. Strikingly, knockdown of Wts or overexpression of Yki in ECs induced a dramatic increase in ISC proliferation, as indicated by the increased number of PH3$^+$ cells that are also Dl positive (Fig. 2 *C*, *D''*, and *E*). Coexpression of *Yki-RNAi* with *Wts-RNAi* or *SD-RNAi* with *UAS-Yki* suppressed the stimulated effect on ISC proliferation (Fig. 2*E*). Taken together, these results suggest that the Hpo-Yki pathway may act in the ECs to regulate the production of a secreted factor(s) that controls ISC proliferation in a paracrine fashion.

Inactivation of Hpo Signaling Increases Production of Upds and EGFR Ligands. To identify the secreted factor(s) deregulated by loss of Hpo signaling, we examined the expression of ligands for pathways implicated in ISC proliferation, including the Wg/Wnt, JAK-STAT, and EGFR pathways (5, 6, 11, 12). We expressed *Wts-RNAi* or *UAS-Yki* in either ECs or ISC/EBs and measured the expression of Wg, the JAK-STAT pathway ligands: Unpaired (Upd), Upd2, and Upd3 (28), and the EGFR ligands: Vein (Vn), Keren (Krn), and Spitz (Spi) (29), by reverse transcriptase quantitative PCR (RT-qPCR). Wts RNAi or Yki overexpression in ECs or ISC/EBs did not cause a significant increase in *wg* mRNA levels (Fig. 3*A*). On the other hand, Wts RNAi or Yki
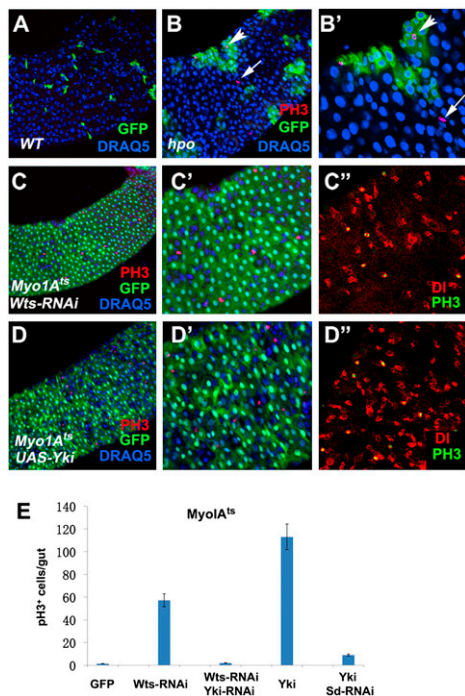
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Fig. 2. Loss of Hpo signaling can induce ISC proliferation nonautonomously. (*A–B′*) Adult guts containing WT (*WT*) control clones (*A*) or *hpo*^BF33^ clones (*B* and *B′*; *B′* is an enlarged view of *B*) were immunostained to show the expression of GFP (green), PH3 (red), and DRAQ5 (blue). Control and mutant clones were generated using the MARCM system and marked by GFP expression. *hpo* Mutant clones stimulated cell division of neighboring WT ISCs (arrows in *B* and *B′*). Arrowheads indicate dividing cells within *hpo* mutant clones. (*C–D″*) Adult midguts that expressed *UAS-Wts-RNAi* (*C–C″*) or *UAS-Yki* (*D–D″*) together with *UAS-GFP* using *MyoIA*^ts^ were immunostained to show the expression of PH3 (red in *C* and *C′* and *D* and *D′*; green in *C″* and *D″*), Dl (red in *C″* and *D″*), GFP (green in *C, C′, D,* and *D′*) and DRAQ5 (blue). GFP marked ECs. Guts were dissected from adult flies grown at 29 °C for 5 d. (*E*) Quantification of PH3⁺ cells in midguts of the indicated genotypes (mean ± SD, *n* ≥ 15).

overexpression in ECs up-regulated the mRNA levels of the three *Upd* genes as well as the three EGFR ligands, with *Upd3* up-regulated by ~40-fold and *Vn* by ~8-fold with Yki overexpression (Fig. 3*A*). However, Wts RNAi or Yki overexpression in ISCs/EBs only slightly increased the expression of Upds and EGFR ligands (Fig. 3*A*). In midguts containing GFP-labeled *hpo* mutant clones, the expression of an *upd* reporter gene, *upd-lacZ*, was increased in *hpo* mutant cells, most notably in mutant ECs (arrows in Fig. 3 *B–B″*). Taken together, these results reveal that loss of Hpo signaling, mainly in ECs, results in ectopic expression of multiple ligands of the JAK-STAT and EGFR pathways.

Excessive Yki Activity in ECs Activates JAK-STAT and EGFR Signaling in ISCs. The increased expression of Upds and EGFR ligands predicted that loss of Hpo signaling or excessive Yki activity might result in elevated JAK-STAT and EGFR signaling activity. Indeed, RT-qPCR revealed that Wts RNAi or Yki overexpression in ECs induced a ~10-fold increase in the expression of *Socs36E*, a JAK-STAT pathway target gene, whereas inactivation of Wts or overexpression of Yki in ISCs/EBs induced a much lesser increase (<3-fold) (Fig. 3*A*). To monitor the JAK-STAT pathway activity more closely, we examined the expression of a pathway reporter gene, *10XSTAT-dGFP* (30). In line with previous studies (6–8), *10XSTAT-dGFP* was expressed in midgut precursor cells, with ISCs exhibiting weaker expression than EBs (arrows in Fig. 3 *C–C″*). We found that overexpression of Yki in ECs but not in ISCs/EBs resulted in a clear increase in the level of *10XSTAT-dGFP* expression in precursor cells as well as ectopic expression in large differentiating cells (arrows in Fig. 3 *D–D″* and Fig. S5*A*).

To monitor the EGFR pathway activity, we examined the expression of phosphorylated *Drosophila* ERK (dpERK, an EGFR pathway activity readout) by immunostaining with a phospho-specific antibody (31). In control guts, dpERK signals were weakly detected in ISCs and EBs (arrows in Fig. 3 *E* and *E′*). Acute activation of Yki in ECs (1 d after temperature shift of *MyoIA*^ts^-*Yki* guts) resulted in a clear increase in the levels of dpERK signal in both ISCs and associated EBs (arrows Fig. 3 *F* and *F′*); however, prolonged Yki activation in ECs (3 d after temperature shift of *MyoIA*^ts^-*Yki* guts) induced ectopic dpERK signals mainly in large differentiated cells (Fig. S6). In contrast, overexpression of Yki in ISCs/EBs induced little, if any, increase in the levels of dpERK signal (Fig. S5*B*). Taken together, these results demonstrate that excessive Yki activation in midgut epithelia increased the production of Upds and EGFR ligands, which in turn activate JAK-STAT and EGFR signal pathways in ISCs.

JAK-STAT and EGFR Pathway Activities Are Required for Elevated ISC Proliferation Induced by Loss of Hpo Signaling. To determine whether JAK-STAT and EGFR pathway activities are required for the elevated ISC proliferation due to deregulation of Hpo signaling, we carried out genetic epistasis experiments. We generated *hpo* mutant clones in which JAK-STAT or EGFR signaling was inactivated by knockdown of Dome (*Dome-RNAi*), the receptor for the JAK-STAT pathway (28), or EGFR/TOP (*EGFR-RNAi*), the receptor for the EGFR pathway (29), using the MACRM system (*Experimental Procedures*). Compared with *hpo* mutant clones, *hpo* mutant clones expressing *Dome-RNAi* (referred to as *hpo⁻Dome⁻*) or *EGFR-RNAi* (referred to as *hpo⁻EGFR⁻*) exhibited reduced clone size (Fig. 4 *A–C′*). Furthermore, PH3 staining indicated that Dome or EGFR RNAi suppressed the elevated mitosis within but not outside the expression domain of the MARCM clones (Fig. 4 *A–C′* and *F*). Thus, blocking the JAK-STAT or EGFR pathway suppressed ISC proliferation induced by loss of Hpo signaling. The sustained, elevated mitosis outside *hpo⁻Dome⁻* or *hpo⁻ EGFR⁻* clones suggests that these mutant clones may still produce excessive ligands for the JAK-STAT/EGFR pathways that can stimulate the proliferation of neighboring WT ISC in a paracrine fashion.

To further probe the relationship between the Hpo and the JAK-STAT/EGFR pathways in the regulation of ISC proliferation, we ectopically activated JAK-STAT or EGFR signaling by overexpressing Upd or an activated EGFR (EGFR^A887T^) (32) in ISCs/EBs and simultaneously inactivated Yki by coexpressing *Yki-RNAi* using the *esg*^ts^ system. Consistent with JAK-STAT and EGFR pathways acting downstream of the Hpo pathway to control ISCs proliferation, we found that inactivation of Yki did not affect ISC proliferation induced by Upd overexpression or EGFR activation (Fig. 4 *D, E,* and *G* and Fig. S7).

Yki Is Required in Precursor Cells for DSS-Induced ISC Proliferation. Tissue damage caused by feeding flies with DSS or bleomycin, or by bacterial infection (such as *Pseudomonas entomophila* or *PE*) stimulates ISC proliferation (6, 9–12). We found that bleomycin and *PE* but not DSS activated the JAK/STAT pathway in ISCs (Fig. S8), suggesting that different tissue damaging reagents may stimulate ISC proliferation via distinct mechanisms. To determine whether the Hpo pathway participates in the regulation of tissue damage-induced ISC proliferation, flies expressing *Yki-RNAi* in either ISCs/EBs or ECs were treated with various tissue damaging reagents. Yki RNAi in ISCs/EBs but not in ECs suppressed DSS-induced ISC proliferation, as indicated by the reduction in the number of esg-GFP⁺ and PH3⁺ cells (Fig. 5 *A–C*). These observations suggest that Yki is required in ISCs/EBs for DSS-stimulated ISC proliferation. In contrast, bleomycin or *PE*-induced ISC proliferation was not significantly affected by Yki inactivation in either ISCs/EBs or ECs (Fig. 5 *A–C*).

If DSS stimulates proliferation through the Hpo pathway in ISCs, it should not further stimulate cell proliferation when Hpo signaling is inactivated in ISCs. Indeed, DSS treatment did not further increase ISC proliferation in *esg-Wts-RNAi* guts compared
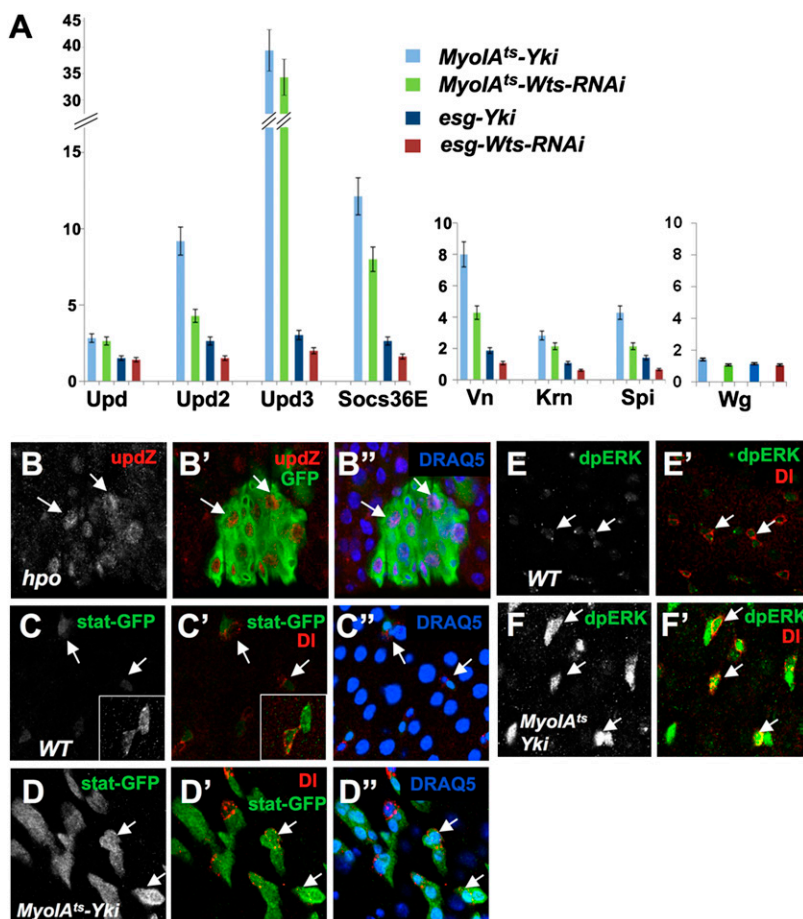
Fig. 3. Hpo signaling regulates the JAK-STAT and EGFR pathways. (*A*) Relative mRNA levels of Upds, Socs36E, EGFR ligands, and Wg in whole midguts of the indicated genotypes measured by RT-qPCR. Numbers indicate fold of activation over the control guts. Wts RNAi or Yki overexpression in ECs (*MyoIA^{ts}*) and, to a much lesser extent, in precursor cells (*esg-Gal4*), induced elevated mRNA levels of the JAK-STAT and EGFR pathway ligands as well as the JAK-STAT pathway target Socs36E. (*B–B′′*) Expression of *upd-lacZ* in midguts carrying *hpo* mutant clones induced by the MARCM system. *upd-lacZ* was ectopically expressed in *hpo* mutant clones as well as some WT cells adjacent to mutant clones. (*C–D′′*) Expression of the JAK-STAT reporter, *10XSTAT-dGFP* (STAT-GFP) in WT (*C–C′′*) or *MyoIA^{ts}*-Yki (*D–D′′*) guts. *Insets* in *C* and *C′* show images obtained by scanning at increased gain. *10XSTAT-dGFP* is expressed in the precursors with lower levels in ISCs. Yki overexpression in ECs induced increased expression of *10XSTAT-dGFP* in ISCs (arrows in *D* and *D′′*) as well as associated EBs and differentiating cells. (*E* and *F′*) Expression of phosphorylated ERK (dpERK) in WT (*E* and *E′*) and *MyoIA^{ts}*-Yki (*F* and *F′′*) guts. *MyoIA^{ts}*-Yki flies were shifted to 29 °C for 1 d before dissection. Low levels of dpERK signal were detected in ISCs and associated EBs in control guts (*E* and *E′*). Yki overexpression in ECs increased the levels of the dpERK signal in ISCs (arrows) and associated EBs (*F* and *F′*).

with mock treatment (Fig. 5D). However, DSS treatment further increased ISC proliferation in *Myo1A^{ts}-Wts-RNAi* guts (Fig. 5E), suggesting that DSS acts through an Yki-dependent mechanism in ISCs that parallels the non–cell-autonomous mechanism elicited by loss of Hpo signaling in ECs. Both bleomycin treatment and *PE* infection had an additive effect with Wts RNAi either in ISCs/EBs or ECs to drive ISC proliferation (Fig. 5 *D* and *E*). Taken together, these observations suggest that DSS stimulates ISC proliferation likely through a cell autonomous role of Yki in the progenitors, whereas bleomycin and *PE* can stimulate ISC proliferation through an Yki-independent mechanism.

Although Yki RNAi did not significantly affect bleomycin or *PE*-induced ISC proliferation, it did not rule out the possibility that bleomycin or *PE*-induced tissue damage could induce Yki activation. Indeed, treatment with bleomycin and *PE* but not DSS increased the expression of an Hpo pathway responsive gene (*ex-lacZ*) in ECs of the posterior midguts (Fig. S9) (33). Recently, Staley et al. showed that a more complete inactivation of Yki in ECs by the combined expression of *UAS-Yki-RNAi* and *UAS-dicer 2* partially blocked bleomycin-induced ISC proliferation (33). Thus, it is possible that bleomycin and *PE* may stimulate ISC proliferation through both Yki-dependent and Yki-independent mechanisms.

Discussion

The Hpo signaling pathway controls organ size in both *Drosophila* and mammals (13, 14, 34, 35); however, its role in stem cell regulation has not been explored until very recently. Using the *Drosophila* adult midgut as a model, we provide evidence that Hpo signaling plays a critical role in restricting adult stem cell proliferation. In addition, we provide evidence that Hpo signaling can regulate cell proliferation through a non–cell-autonomous mechanism.

The prevailing view regarding the mechanism of Hpo action is that Hpo signaling acts cell autonomously to control cell growth,

proliferation, and survival by regulating genes involved in these processes (13, 14). Our finding that loss of Hpo signaling or overexpression of Yki in the precursor cells can stimulate ISC proliferation is in line with this view. Unexpectedly, however, we found that *hpo* mutant clones can stimulate the proliferation of neighboring WT ISCs. Strikingly, we found that loss of Hpo signaling or overexpression of Yki in enterocytes also stimulated ISC proliferation, thus demonstrating a non–cell-autonomous mechanism. Furthermore, we provided evidence that loss of Hpo signaling in enterocytes leads to increased production of cytokines and mitogens that activate the JAK-STAT and EGFR pathways in ISCs to drive their proliferation. The nonautonomous mechanism demonstrated here is likely to be conserved in mammals, as a recent study showed that activation of the mammalian ortholog of Yki, Yap, in cultured breast epithelial cells activates an EGFR ligand that can drive cell proliferation nonautonomously in vitro (36).

While our manuscript was under review, Stanley et al. reported that Wts inactivation or Yki overexpression in ECs induced ISC proliferation nonautonomously (33), which is in line with our findings here. However, they did not observe a cell-autonomous role for Hpo signaling in the regulation of ISC proliferation. The reason for such a discrepancy is not clear but could be due to difference in experimental conditions used by different laboratories.

We found that Wts RNAi or Yki overexpression with *esg-Gal4* did not significantly increase either the JAK-STAT or EGFR pathway activity in ISCs. Thus, the increased ISC proliferation observed in *esg-Wts-RNAi* and *esg-Yki* midguts may reflect a cell-autonomous role of Hpo signaling in the regulation of ISC proliferation. Interestingly, Yki is specifically required in ISCs/EBs for DSS to stimulate ISC proliferation. Furthermore, loss of Hpo signaling in ECs but not in ISCs/EBs can cooperate with DSS to stimulate ISC proliferation. A likely explanation is that DSS deregulates Hpo signaling in ISCs and promotes the cell-
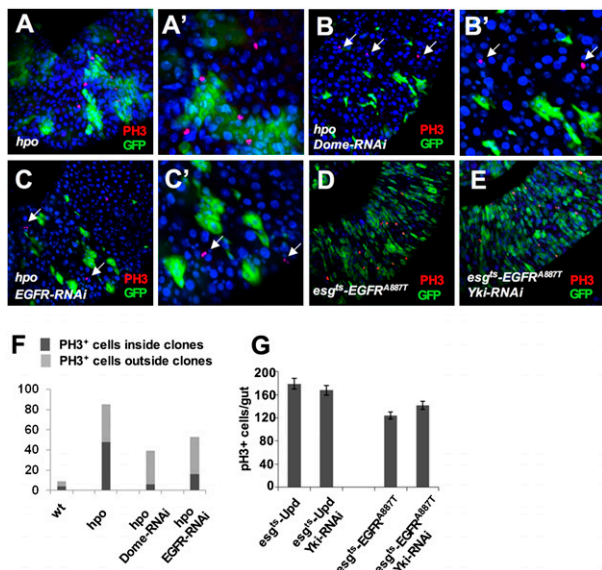
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Fig. 4. Inactivation of JAK-STAT or EGFR signaling in *hpo* mutant ISCs suppressed their proliferation. (*A–C'*) Low-magnification (*A–C*) and high-magnification (*A'–C'*) images of adult midguts carrying *hpo* mutant clones (*A* and *A'*), *hpo* mutant clones expressing *Dome-RNAi* (*B* and *B'*), or *hpo* mutant clones expressing *EGFR-RNAi* (*C* and *C'*) and stained with anti-PH3 (red) and anti-GFP (green) antibodies. Mutant clones were generated by the MARCM system and the guts were dissected out from flies grown at 25 °C for 5 d after clone induction. *hpo* Mutant clones expressing *Dome-RNAi* or *EGFR-RNAi* exhibited reduced clone size and contained less PH3⁺ cells within the clones; however, ectopic PH3 signals can be readily detected in WT cells near the mutant clones (arrows in *B–C'*). (*D* and *E*) esg-GFP (green) and PH3 (red) expression in adult midguts expressing an active form of EGFR, EGFR^A887T, in the absence (*D*) or presence (*E*) of *Yki-RNAi* transgene using the *esg^ts* system. Adult flies were shifted to 29 °C for 6 d before dissection. (*F*) Quantification of PH3 positive cells inside or outside the control or mutant clones of the indicated genotypes. A total of 12 guts were counted for each genotype. (*G*) Quantification of PH3 positive cells from guts of the indicated genotypes (mean ± SD, *n* ≥ 5).

autonomous function of Yki to drive ISC proliferation (Fig. 6). The mechanism by which DSS regulates Hpo signaling is not clear, but our previous study reveals that DSS disrupts the

basement membrane organization whereas bleomycin causes damage of midgut epithelia (12). As ISCs contact the basement membrane, our finding thus raises an interesting possibility that the Hpo pathway may sense ISC interaction with the basement membrane to restrict ISC proliferation, and that disruption of basement membrane organization may alleviate Hpo pathway-mediated contact inhibition of ISC proliferation.

Our finding has important implications regarding tumorigenesis. Deregulation of Hpo signaling has been implicated in several types of human cancer (37–40). Indeed, liver specific over-expression of Yap or knockout of MST1/2 (the mammalian ortholog of Hpo) caused hepatocellular carcinomas (34, 41–44). Furthermore, MST1/2 mutant livers contain proliferative oval cells that are facultative stem cells contributing to both the hepatocyte and biliary lineages, implying that Hpo signaling may repress adult liver stem cell activation (42). Thus, it would be important to determine whether Hpo signaling is more widely involved in adult stem cell regulation and whether Hpo pathway activity is deregulated in response to tissue injury that is thought to contribute to tumorigenesis (45). Our finding that Hpo signaling can regulate stem cell proliferation through non–cell-autonomous mechanisms implies that deregulation of Hpo signaling in tumor microenvironments may also contribute to tumorigenesis.

Experimental Procedures

***Drosophila* Stocks and Genetics.** The following fly stains were used for this study: hpo^BF33 (46); wts^X1 (47); yki^B5 (16); ft^G-rv (48); esg-Gal4 (2); MyoIA-Gal4, UAS-Dome-RNAi, UAS-Upd, and upd-lacZ (6); UAS-Wts-RNAi (VDRC #106174); UAS-Yki-RNAi, UAS-Sd-RNAi, and UAS-Yki (18); UAS-EGFR-RNAi (VDRC #43267); UAS-EGFR^A887T (BL #9534). Mutant clones were generated using the MARCM system (24). Fly stocks were crossed and cultured at 18 °C. F1 adult flies, 5 d old, with the appropriate genotypes were subjected to heat shock at 37 °C for 1 h. After clone induction, flies were raised at 18 °C or 25 °C for the indicated period before dissection. For experiments involving tubGal80^ts, crosses were set up and cultured at 18 °C to restrict Gal4 activity. F1 adult flies were then shifted to 29 °C to inactivate Gal80^ts. The genotypes for making mutant clones are described in *SI Experimental Procedures*.

Feeding Experiments. Female adult flies, 5–10 d old, were used for feeding experiments. Flies were cultured in an empty vial containing a piece of 2.5 × 3.75-cm chromatography paper (Fisher) wet with 5% sucrose solution as feeding medium. Flies were fed with 3% of dextran sulfate sodium (MP Biomedicals) or 25 μg/mL bleomycin (Sigma) dissolved in 5% sucrose for 3 d at 29 °C. *PE* infection was carried out as previously described (6).
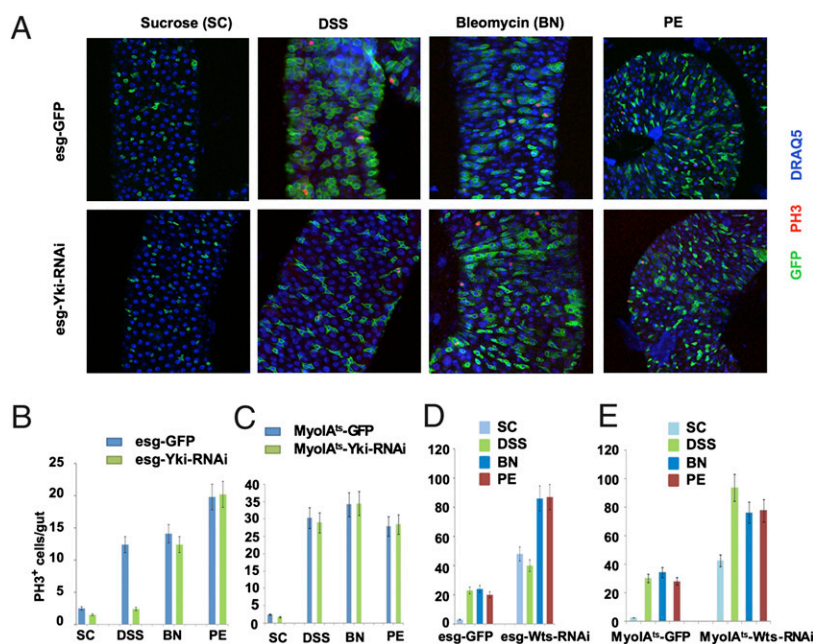

Fig. 5. Yki is required in the precursor cells for DSS-stimulated ISC proliferation. (*A*) Adult flies expressing *esg-Gal4/UAS-GFP* (*Top*) or *esg-Gal4/UAS-GFP + UAS-Yki-RNAi* (*Bottom*) were treated with sucrose (SC), DSS, bleomycin (BN), or *PE* for 3 d before midguts were dissected out and immunostained with GFP (green) and PH3 (red) antibodies and DRAQ5 (blue). (*B–E*) Quantification of PH3⁺ cells in control adult midguts or adult midguts expressing *esg-Yki-RNAi* (*B*), *MyoIA^ts-Yki-RNAi* (*C*), *esg-Wts-RNAi* (*D*), or *MyoIA^ts-Wts-RNAi* (*E*) treated with sucrose, DSS, bleomycin, or *PE* (mean ± SD, *n* ≥ 10 for each genotype).
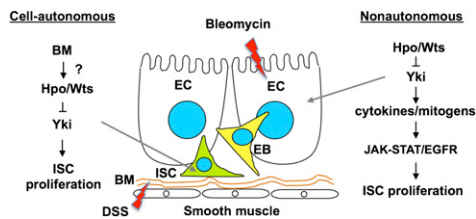
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Fig. 6. Hpo signaling regulates ISC proliferation through both cell-autonomous and non–cell-autonomous mechanisms. Hpo/Wts restricts the activity of Yki in the precursor cells to inhibit ISC proliferation. This cell-autonomous mechanism could be regulated by contact between ISC and basement membrane (BM), which is disrupted by DSS. Hpo signaling also acts in the ECs to restrict the production of ligands for the JAK-STAT and EGFR pathways, thereby inhibiting ISC proliferation by limiting the activities of these two pathways. Bleomycin and possibly *PE* cause damage of ECs and induce ISC proliferation through Yki-dependent and Yki-independent mechanisms. Of note, each arrow does not necessarily mean direct regulation.

RT-qPCR. RNA was extracted from 10 female midguts using RNAeasy Mini kit (Qiagen), and cDNA was synthesized using the iScript cDNA synthesis kit (Bio-Rad). RT-qPCR was performed using iQ5 System (Bio-Rad). Primer sequences are listed in *SI Experimental Procedures*. RT-qPCR was performed in duplicate on each of three independent biological replicates. RpL11 was used as a normalization control.

Immunostaining. Female flies were used for gut immunostaining in all experiments. The entire gastrointestinal tract was taken and fixed in 1 X PBS plus 4% EM grade formaldehyde (Polysciences) for 3 h, except for Delta staining, for which the fixation time was 30 min. Samples were washed and incubated with primary and secondary antibodies in a solution containing 1 X PBS, 0.5% BSA, and 0.1% Triton X-100. The following primary antibodies were used: mouse anti-Delta (DSHB), 1:100; mouse anti-Prospero (DSHB), 1:50; rabbit anti-PH3 (Upstate Biotechnology), 1:2,000; rabbit anti-GFP (Santa Cruz), 1:500; rabbit anti-Pdm1 (gift from Xiaohang Yang, Institute of Molecular and Cell Biology, Singapore), 1:2,000; 1:1,000; rabbit anti-dpERK (Cell Signaling Technology), and 1:500; DRAQ5 (Cell Signaling Technology).

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