

Cytokine/Jak/Stat Signaling Mediates Regeneration and Homeostasis in the *Drosophila* Midgut

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SUMMARY

Cells in intestinal epithelia turn over rapidly due to damage from digestion and toxins produced by the enteric microbiota. Gut homeostasis is maintained by intestinal stem cells (ISCs) that divide to replenish the intestinal epithelium, but little is known about how ISC division and differentiation are coordinated with epithelial cell loss. We show here that when enterocytes (ECs) in the *Drosophila* midgut are subjected to apoptosis, enteric infection, or JNK-mediated stress signaling, they produce cytokines (Upd, Upd2, and Upd3) that activate Jak/Stat signaling in ISCs, promoting their rapid division. Upd/Jak/Stat activity also promotes progenitor cell differentiation, in part by stimulating Delta/Notch signaling, and is required for differentiation in both normal and regenerating midguts. Hence, cytokine-mediated feedback enables stem cells to replace spent progeny as they are lost, thereby establishing gut homeostasis.

INTRODUCTION

Many animal tissues undergo homeostatic growth in which spent differentiated cells are replaced by the progeny of resident stem or progenitor cells. In the epithelial lining of animal intestines, high rates of cell turnover are presumed to vary according to changes in food composition and dietary exposures to toxins, pathogens, and chemical or mechanical injury. To maintain normal gut structure and function (i.e., homeostasis), intestinal stem cells (ISCs) likely respond to variations in cell loss with corresponding changes in rates of self-renewal and differentiation. How this occurs is not well understood. According to a prevalent view of the vertebrate intestine, stem and transient amplifying cell divisions in the crypts of Lieberkühn, promoted by WNT signaling, drive gut epithelial renewal in a “conveyor-belt” fashion, generating a constant supply of differentiated cells to the villi, where they are autonomously exfoliated (Gregorieff and Clevers, 2005; Sancho et al., 2004). In its simplest form, this model does not incorporate feedback from the differentiated

epithelium to progenitor cells and, therefore, lacks the means to maintain stasis when rates of epithelial cell loss vary. More sophisticated models that do incorporate feedback have been discussed; for instance, negative cross-talk between BMP signaling in the villi and WNT signaling in the crypts might allow true homeostasis (Crosnier et al., 2006; Gregorieff and Clevers, 2005). However, rigorous tests of the cross-regulatory interactions required have so far not been possible in a vertebrate. In this respect, the *Drosophila* midgut, which is simpler than its vertebrate counterparts but has similar cell types and signaling interactions, is technically advantageous.

The *Drosophila* adult midgut is maintained by ISCs that self-renew and also produce the two principal differentiated cell types of the intestinal epithelium: absorptive enterocytes (ECs) and secretory enteroendocrine (EE) cells (Micchelli and Perrimon, 2006; Ohlstein and Spradling, 2006). The midgut also maintains many nondividing, undifferentiated ISC daughters termed enteroblasts (EBs), which can differentiate directly. Differentiation requires Delta/Notch signaling from the ISC to its EB daughter, and, as in mammals, the fate decision taken (absorptive versus secretory) is thought to depend on the intensity of Notch signaling received by an EB (Ohlstein and Spradling, 2007). Lineage analysis suggests that, in well-fed flies, differentiated cells in the midgut epithelium turn over roughly weekly, as in mammals.

Studies of dissociated Lepidopteran midguts found that cell death caused by *Bacillus thuringiensis* endotoxin stimulated the division of a population of cells that were probably ISCs (Hakim et al., 2001; Loeb et al., 2001), and recent reports document mitoses in *Drosophila* midguts in response to ingested detergent (Amcheslavsky et al., 2009) or bacteria (Buchon et al., 2009). These findings suggest that the loss of damaged ECs stimulates ISC division. Since EB differentiation coincides with a reduction in their contact with a basement membrane, it has also been proposed that this membrane or underlying visceral muscle might provide a niche that promotes stemness and suppresses differentiation (Ohlstein and Spradling, 2007). Consistent with this, the WNT ligand *wg* is expressed in visceral muscle, and is important for ISC survival (Lin et al., 2008).

We show here that the *Drosophila* midgut can rapidly regenerate after enterocytes are ablated, or subjected to enteric infection or stress signaling. Damaged or stressed ECs produce the

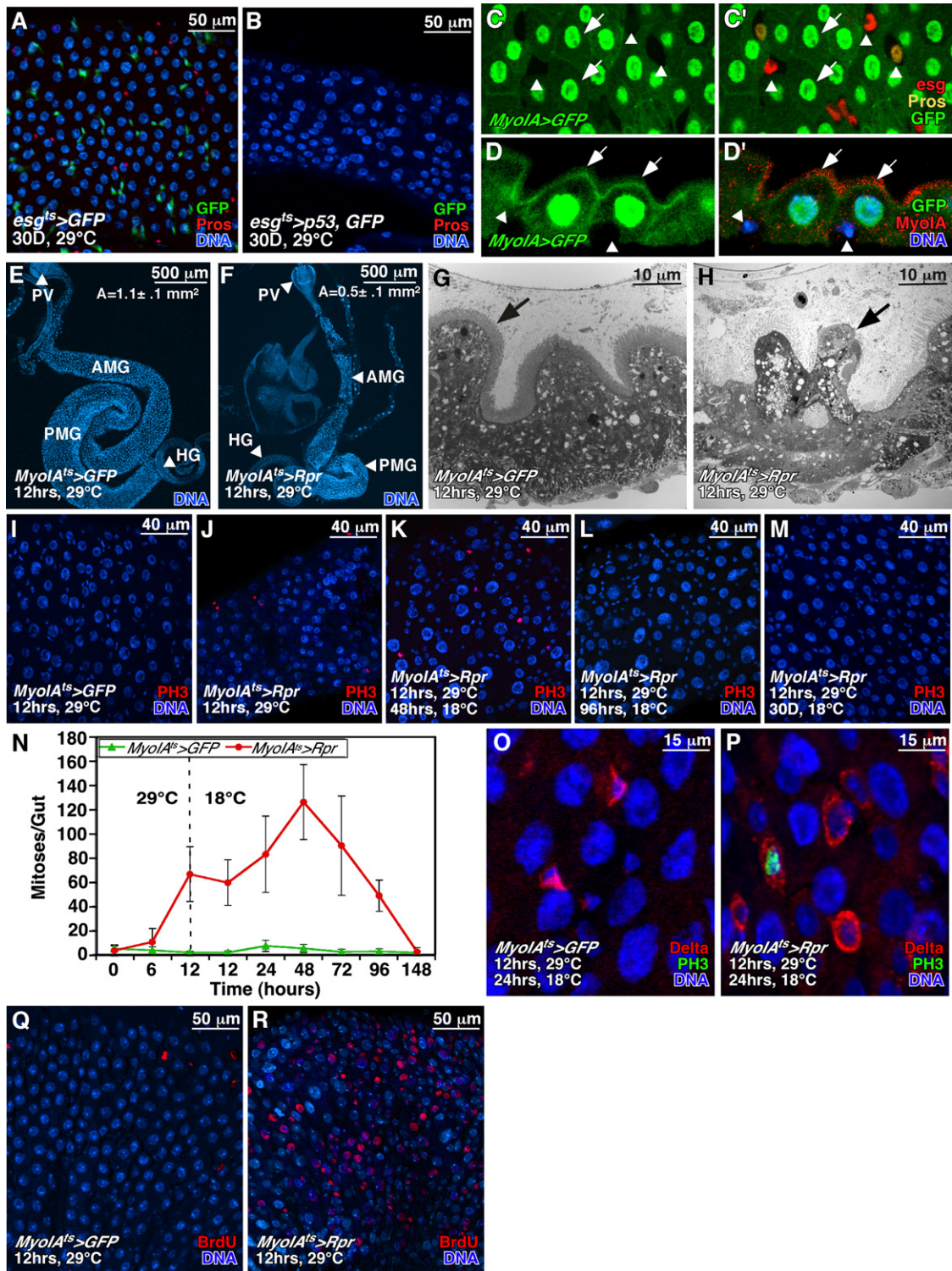


Figure 1. Midgut Cell Ablation and Renewal

(A and B) *Drosophila* p53 expressed with *esgGal4^{ts}* for 30 days ablated *esg⁺* progenitor cells (green), *pros⁺* EEs (red), and decreased ECs (blue; B). Controls over-expressed GFP alone (A).

(C–D') *MyoIAGal4*-driven *UAS-GFP* is expressed in ECs (arrows) but not in small basally located *esg/lacZ⁺* progenitors (red) or *pros⁺* EEs (yellow; arrowheads). *GFP⁺* ECs were positive for brush border Myosin IA (MyoIA is red in D').

Unpaired cytokines (*Upd*, *Upd2*, and *Upd3*). These ligands and their downstream effectors Domeless (*dome*, an IL-6R-like receptor), Hopscotch (*hop*, a Janus Kinase; Jak), and Stat92E (a STAT3-like transcription factor) have important roles in germ stem cell maintenance and the immune response in *Drosophila*. In the midgut, Upds produced by spent ECs trigger Jak/Stat signaling in ISCs and EBs, promoting their division and differentiation, respectively, and thereby driving renewal of the gut epithelium.

RESULTS

Progenitor Cells Are Required for Midgut Maintenance

To determine whether ISCs are required for midgut maintenance, we sought to ablate them. To express cell death effectors, we used *esgGal4* and the temperature-sensitive Gal4 repressor, *tubGal80^{ts}* (McGuire et al., 2004) (together referred to as *esg^{ts}*), to allow temporal activation of UAS-linked target genes in ISCs and EBs (Jiang and Edgar, 2009; Micchelli and Perrimon, 2006). Although induction of *reaper* (*rpr*, an inhibitor of *Drosophila* Inhibitor of Apoptosis-1; *DIAP-1*) had little effect on progenitor cells (i.e., ISCs and EBs), *ricin A* or *Drosophila p53* effectively ablated them (Figures 1A and 1B). Fifteen days of *p53* induction ablated nearly all *esg⁺* progenitor cells and reduced EE numbers, but the midguts were otherwise intact. After 30 days of *p53* induction, all ISCs, EBs, and EEs and many ECs were lost, and the midguts were shrunken (Figure 1B). Remaining ECs had grown in size, perhaps to compensate for the loss of absorptive surface area. This result concurs with clonal analyses (Ohlstein and Spradling, 2006) showing that the midgut epithelium turns over rapidly and must be constantly replenished by ISC progeny.

Midgut Regeneration from Stem Cells

To determine whether ISC division responds to epithelial cell loss, we sought to ablate ECs. To express genes in ECs we used the *Myo1AGal4* driver (*NP1-Gal4*), an enhancer trap in the gut-specific brush border *myosin IA* gene (Morgan et al., 1994), in combination with *tubGal80^{ts}*. UAS-GFP driven by *Myo1AGal4* was strongly expressed in all midgut ECs, identified by their large nuclei and expression of brush border Myosin IA. No expression was detected in ISCs, EBs, EEs, or visceral muscle (Figures 1C and 1D). We used the inducible *Myo1AGal4 tubGal80^{ts}* system (heretofore referred to as *Myo1A^{ts}*) to express the proapoptotic gene *reaper* (*rpr*), to trigger EC apoptosis. *Myo1AGal4 tubGal80^{ts} UAS-Rpr* (*Myo1A^{ts}>Rpr*) animals were raised to adults at 18°C (Gal4 “off”), shifted to 29°C (Gal4 “on”) for 12 hr, and then shifted to 18°C to extinguish *rpr* expression. Twelve hour induction of Rpr reduced midgut size due to widespread apoptosis (Figures 1F and 1J). Tissue sections showed the loss of EC brush borders

and apical extrusion (Figures 1G and 1H). Within days, however, the damaged midguts had regenerated substantially (Figures 1K–1M). We assayed the mitotic response of ISCs using antibodies to phospho-Ser10-histone 3 (PH3). PH3⁺ mitotic figures rose to >100/midgut by 48 hr after a 12 hr pulse of *reaper*, whereas controls maintained a mitotic index of 1–3 mitoses/midgut (Figures 1I–1K and 1N). Rpr-induced mitoses could be suppressed by coexpression of the caspase inhibitors p35 or DIAP1 (Figure 2K), indicating that apoptosis was required. Most (88%) PH3⁺ cells were positive for the ISC marker Delta, and all PH3⁺ cells were negative for the EE marker *prospero* (Figure 1P; see Table S1 available online). Delta⁺ cells in regenerating midguts were enlarged, consistent with increased growth, had higher Delta levels than in controls, and were often paired or clustered (Figures 1P).

Midgut mitoses declined after 2 days and reached basal levels within a week (Figures 1L–1N). Regenerating midguts regained their normal size by 60 hr of recovery, before the cessation of ISC proliferation or replenishment of the EC population. At this stage, the midgut epithelium consisted of fewer ECs than normal, but these ECs were larger and more polyploid than those in controls (Figures 1K and 1L). Following Rpr expression, extensive BrdU incorporation was rapidly induced not only in small cells (ISCs and EBs), but also in large polyploid ECs (Figure 1R). This finding suggests that existing ECs might respond directly to gut epithelial damage by compensatory EC growth and endoreplication. By one month of recovery, Rpr-damaged midguts had regained normal cellularity and EC size (Figure 1M). To summarize, the midgut can compensate for epithelial cell loss by increasing progenitor cell divisions and the consequent generation of new ECs.

JNK Signaling in ECs also Promotes ISC Division

To further investigate midgut regeneration, we tested the Jun N-terminal Kinase (JNK) pathway, a MAPK-type kinase cascade that is activated in response to cellular stress and that is involved in compensatory cell proliferation following injury in both insects and mammals (McEwen and Peifer, 2005; Nakagawa et al., 2008; Prieto, 2008; Ryoo et al., 2004). We activated JNK signaling in ECs by expressing RNAi directed against *puckered* (*puc*) using the *Myo1A^{ts}* system. *puc* encodes *Drosophila* Jun N-terminal kinase phosphatase. It is a potent suppressor of JNK activity and also a direct downstream target of JNK signaling (Martin-Blanco et al., 1998). Inducing *puc RNAi* in ECs for 2 days caused a large increase in ISC mitoses (Figure 2A). A similar but more rapid mitotic response was observed when an activated form of *hemipterous* (*Hep^{Act}*, *Drosophila* JNK kinase) was used to activate JNK in ECs (Figure 2B). We noted that *Hep^{Act}* induction

(E and F) Expression of Reaper (Rpr) with *Myo1A^{ts}* for 12 hr reduced midgut size (F). Control shown in (E). PV: proventriculus; AMG: anterior midgut; PMG: posterior midgut; HG: hindgut. Mean surface area (A) ± SD for 10 such midguts is indicated.

(G and H) TEM of midguts expressing Rpr (H) or GFP control (G). Rpr caused loss of brush border (arrows) and enterocyte.

(I and J) Expression of Rpr with *Myo1A^{ts}* for 12 hr ablated enterocytes and promoted mitoses (PH3⁺, red). Control in (I).

(K–N) Mitoses and midgut regeneration after enterocyte ablation. During recovery at 18°C, PH3⁺ mitotic cells (red) increased through 48–72 hr, but decreased as midguts regained their normal size by 96 hr. Midguts re-grew to their normal size, due partly to increases in enterocyte size (L). After 1 month, cell density and EC size returned to normal (M). Data in (N) represent means of all midguts evaluated (>25/time point) ± SD.

(O and P) Mitotic cells (PH3⁺, green) in regenerating midguts were positive for the ISC marker, Delta (red, P). ISCs in regenerating midguts (P) were larger than in controls (O), and had more Delta.

(Q and R) EC ablation by Rpr increased BrdU incorporation (red) in small progenitors and large ECs. Blue in all panels is DNA.

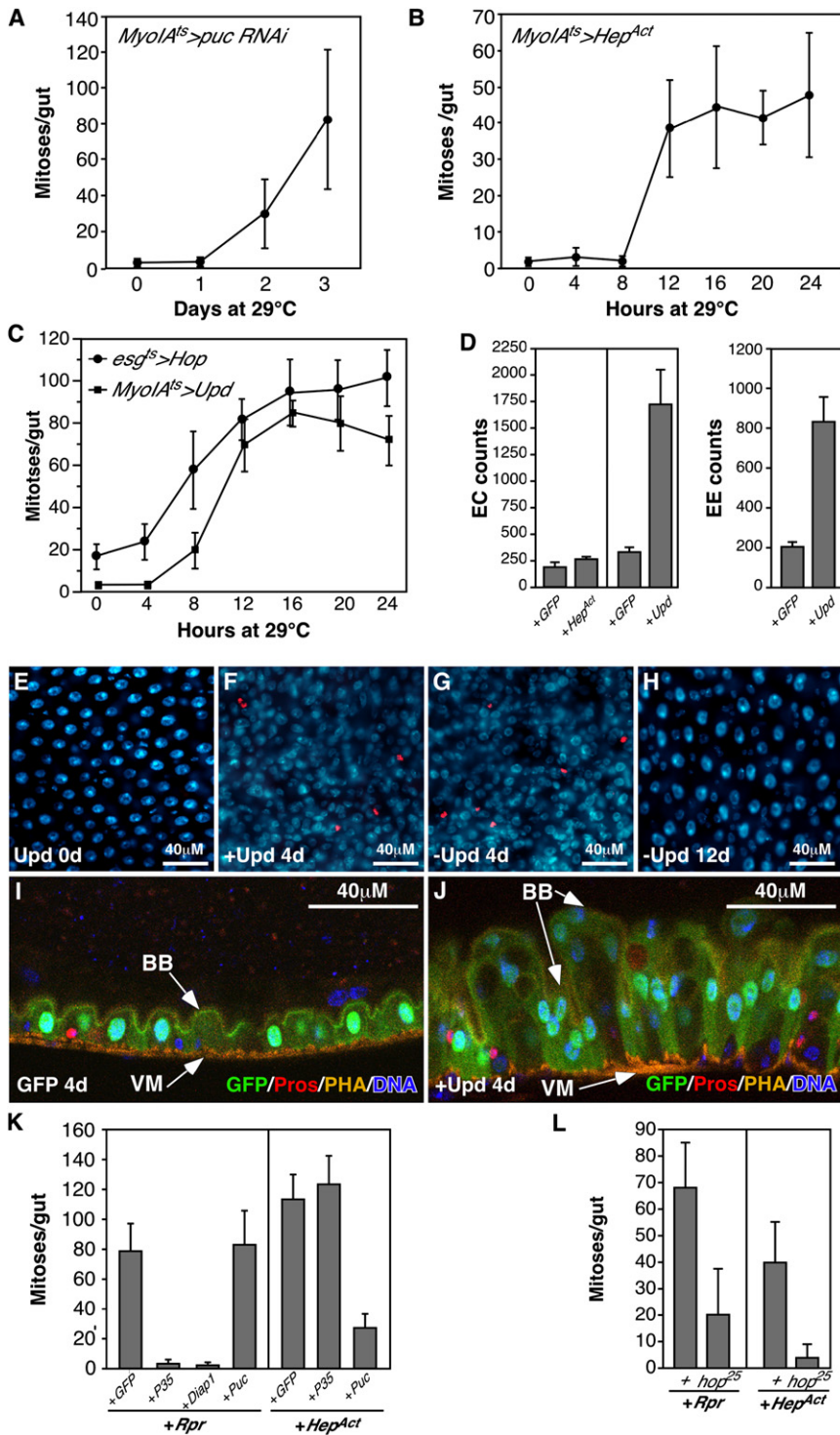


Figure 2. JNK and Cytokine/Jak/Stat Signaling Induce ISC Mitosis

(A and B) ISC mitoses, quantified by PH3⁺ mitotic figures/midgut. JNK activation in ECs was achieved by depleting puckered (*puc*) with *RNAi* (A), or expressing activated Hemipterous (*Hep^{Act}*, B) using the *MyoIA^{ts}* system.

(C) Expression of *Upd* using *MyoIA^{ts}*, or Hopscotch (Jak) using *esg^{ts}* induced ISC mitosis.

(D) Quantification of gut hyperplasia (ECs and EEs) induced by JNK (*MyoIA^{ts} > Hep^{Act}* 4 days, +*Hep^{Act}*) or *Upd* (*MyoIA^{ts} > Upd* 4 days, +*Upd*). *Pros⁺* cells were scored as EEs. Large polyploid *Pros⁻ DI⁻* cells were scored as ECs.

(E–H) *Upd*-induced midgut hyperplasia and recovery. *Upd* induction in ECs increased PH3⁺ mitotic figures (red) and midgut cell density (blue: DNA). Midguts recovered normal morphology within 12 days of silencing ectopic *Upd* by temperature shift (H).

(I and J) Sagittal view of the midgut hyperplasia induced by *Upd*. *Upd* induction using *MyoIA^{ts}* increased ECs in the epithelium (J). One side of the midgut epithelium is shown. Actin is stained with phalloidin (PHA, yellow), marks the visceral muscle and brush border, EEs are marked by *Pros* (red). ECs are marked by GFP (green). DNA is in blue. BB, brush border; VM, visceral muscle. (K) Epistasis of *Rpr*- and JNK-induced mitoses. Midgut mitotic indices 24 hr after transgene activation. *Rpr*-induced proliferation was suppressed by coexpressed P35 or DIAP1, but not by coexpressed *Puc*. *Hep*-induced proliferation was suppressed by *Puc* but not P35.

(L) Epistasis showing that *Rpr* and JNK-induced proliferation require *hop* (Jak). Midgut mitotic figures were quantified after expressing either *Rpr* or *Hep^{Act}* for 12 hr in ECs in *hop²⁵* males or control males (+).

increased the number and density of small Delta⁺ cells, suggesting that JNK activation elevated the numbers of ISC-like progenitors (Figures 5B, 5D, and S1B). As observed in other contexts (Kanda and Miura, 2004; McEwen and Peifer, 2005), prolonged JNK activation caused significant cell death (data not shown), but the onset of mitoses commenced long before EC apoptosis

was observed. Moreover, coexpression of the caspase inhibitor *p35* with *Hep^{Act}* did not prevent JNK-mediated mitoses (Figure 2K). Thus, apoptosis appeared not to be responsible for JNK-induced ISC divisions. Control experiments showed that coexpressed *puc* significantly inhibited ISC mitoses induced by *Hep^{Act}* (Figure 2K), but interestingly, *puc* or another JNK inhibitor, *Bsk^{DN}* (Adachi-Yamada et al., 1999), did not suppress ISC divisions induced by *Rpr* (Figure 2K and data not shown). This indicates that stem cell divisions can be triggered by at least two independent pathways: a caspase-independent relay involving JNK signaling, and a caspase-dependent relay.

Upd/Jak/Stat Signaling Drives Midgut Renewal

Because cytokine signaling has been implicated in several models of regeneration (Lin and Karin, 2007; Prieto, 2008), we

investigated its role in ISC proliferation. *Drosophila* has three lepin-like (IL-6 family) cytokines called Unpaireds (*Upd*, *Upd2*, and *Upd3*). These cytokines bind an IL-6R type receptor, Domeless (*dome*), that activates a Janus kinase (Jak) called Hopscotch (*hop*) and thereby promotes the translocation of a STAT3-like transcription factor (*STAT92E*) to the nucleus. Transcriptional targets of *STAT92E* include the receptor, *Dome*, and a repressor of receptor/Jak complexes, *Socs36E*. We first tested this pathway's effect on ISCs by overexpressing *UAS-Upd* either in ECs using *MyoIA^{ts}*, or in ISCs and EBs using *esg^{ts}*. Expression of *Upd* in either cell type induced ISC mitosis (Figures 2C, 2F, and 2G) and resulted in dramatic gut hyperplasia with large increases in numbers of *MyoIA⁺* ECs (Figures 2D and 2J), *pros⁺* EEs (Figures 2D and 2J), and small *Delta⁺* ISCs (Figures 5B and 5F). Ectopic expression of *Upd2* had similar effects (data not shown). We also observed increased midgut mitoses after expressing *Hop* (JAK) in progenitor cells using *esg^{ts}* (Figure 2C), and in *hop* gain-of-function mutants (*hop^{Uml}*; data not shown). Thus, Upd/Jak/Stat signaling is a potent ISC mitogen, but does not block differentiation. Compared with other signals reported to cause midgut hyperplasia (Wnt signaling (Lin et al., 2008) and loss of Notch signaling (Micchelli and Perrimon, 2006)), *Upd* or *Hop* caused a much more rapid, dramatic increase in ISC mitoses and midgut cell numbers. Remarkably, hyperplastic midguts generated by *Upd* induction returned to normal size, morphology, and cellularity within 2 weeks of silencing the *UAS-Upd* transgene (Figure 2H). Similarly, JNK-induced hyperplasia was also reversible (Figures S1B–S1D).

Upd/Jak/Stat Mediates Apoptosis- and JNK-Dependent ISC Activation

Reverse transcriptase quantitative PCR (RT-qPCR) assays showed that all three *Upd* mRNAs were strongly up-regulated after EC apoptosis was triggered by *Rpr*, or after JNK was activated by *Hep^{Act}* or *Puc RNAi* (Figure 3A). *Upd3* was the most induced, to nearly 200 fold. A reporter for *Upd* transcription (*UpdlacZ*) (Chao et al., 2004) was also induced after JNK activation or EC ablation (Figures 3B–3D), mainly in small progenitor cells and larger *MyoIA⁻* cells (GFP⁻), which we believe are early, partially differentiated ECs. Levels of the STAT target, *Socs36E*, were also profoundly increased by either JNK signaling or EC apoptosis (Figure 3A).

Epistasis tests showed that ISC mitoses induced by either *Hep^{Act}* or *Rpr* were strongly reduced in *hop^{25/Y}* mutant animals (Figure 2L), which have reduced JAK activity. Control *hop^{25/Y}* mutants had normal numbers of *esg⁺* progenitor cells (data not shown), and thus the reduction in induced mitoses was unlikely to be due to decreased ISC numbers. These results indicate that Upd/Jak/Stat signaling is both sufficient and required for triggering ISC mitoses during regeneration.

Dome and Stat Are Required for EC Differentiation

Upd/Dome/Hop signaling drives the nuclear translocation of Stat92E, the sole *Drosophila* STAT homolog. In normally fed wild-type midguts, nuclear Stat92E was observed in *esg⁺* progenitors (ISCs and EBs; Figure 4A), but not in ECs or EEs. STAT activity was also assayed using three transcriptional reporters, *10XStat-DGFP* (Bach et al., 2007), (*Gas*)*3lacZ* (Gilbert et al., 2005), and an

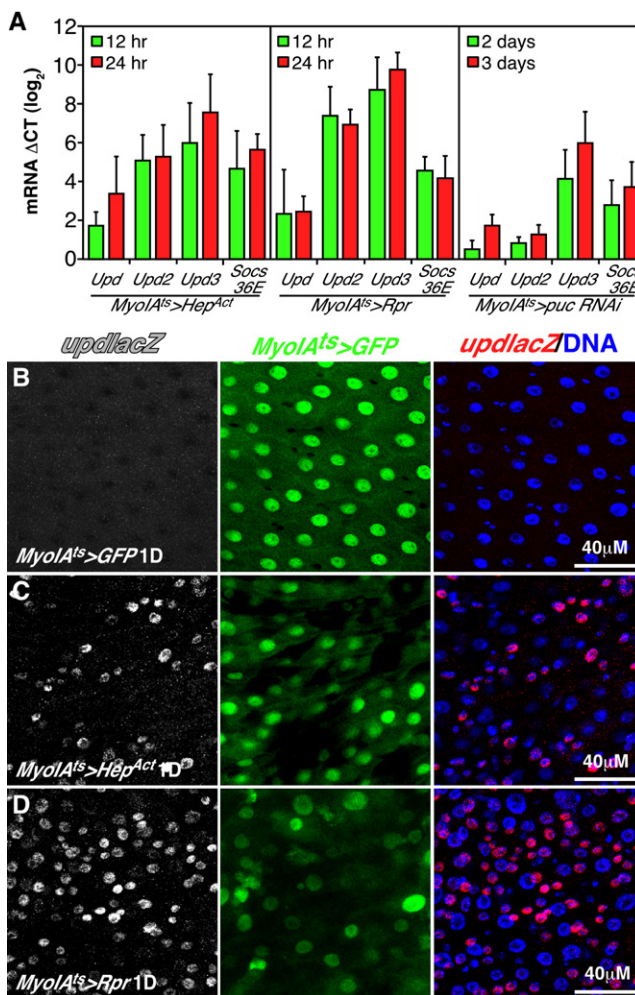


Figure 3. Apoptosis and JNK Activation Trigger Cytokine Production

(A) RT-qPCR of whole midgut cDNA after activating EC apoptosis (*MyoIA^{ts}*>*Rpr*) or JNK signaling (*MyoIA^{ts}*>*Hep^{Act}* or *puc RNAi*). *Upd*, *Upd2*, *Upd3*, and *Socs36E* were induced within 12 hr of transgene activation. Δ CT is the difference in calculated normalized Ct (threshold cycle) values between experimental and 0 hr control samples ($\sim\log_2$ scale).

(B–D) Expression of the *upd* reporter, *UpdlacZ*, in control midguts (B), and following expression of *Rpr* (C) or *Hep^{Act}* (D) for 24 hr by *MyoIA^{ts}*. GFP marks *Gal4*-expressing ECs.

enhancer trap at the *domeless* locus, *domeGal4*. In normal midguts, each Stat reporter was also expressed only in *esg⁺* progenitor cells (Figures 4B and 4C; data not shown). Thus, Stat signaling is normally active in ISCs and EBs, but not in ECs or EEs.

To further test the function of Jak/Stat signaling, we generated ISC clones mutant for strong loss-of-function alleles of *Stat92E*, *Stat^{85C9}*, or *Stat³⁹⁷* (Bach et al., 2007). While control clones comprised both small diploid progenitors and large polyploid ECs positive for the differentiation marker, *MyoIA^{ts}lacZ*, (Figures 4D–4D'), all cells in *Stat^{85C9}* mutant clones had small nuclei and lacked *MyoIA^{ts}lacZ* expression (Figures 4E–4E'). Most *Stat^{85C9}* mutant cells lacked Pros and Delta (data not shown),

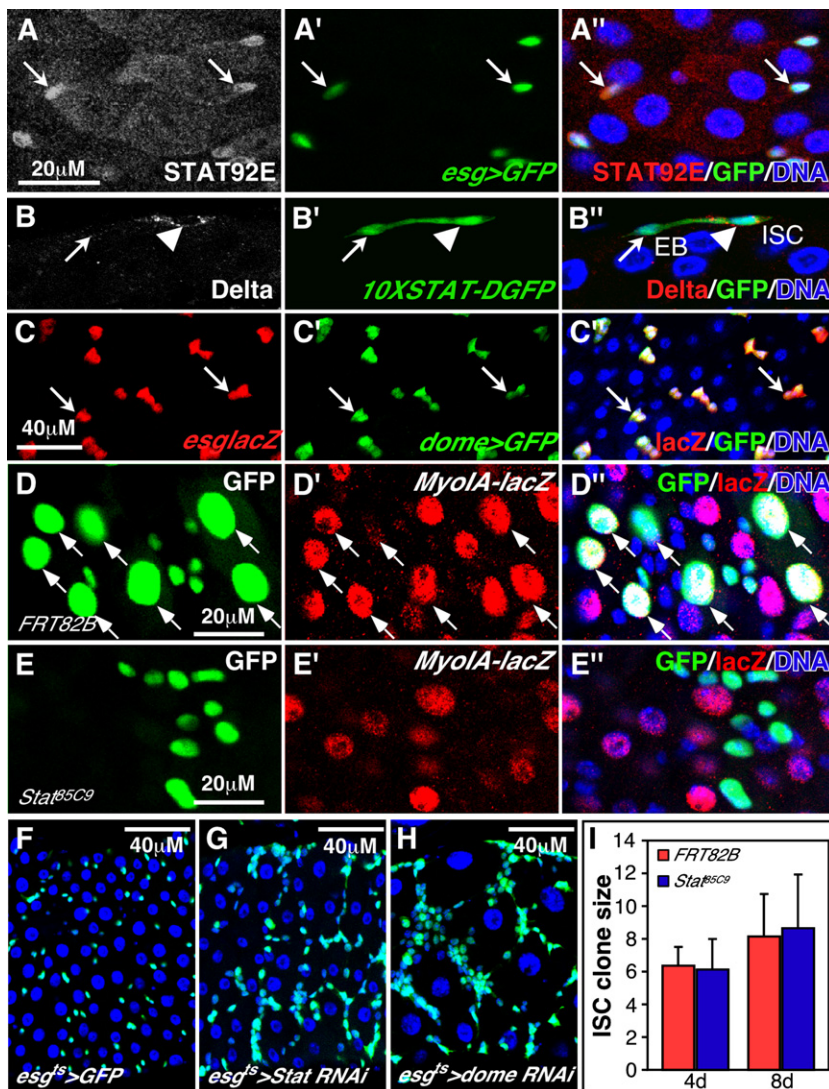


Figure 4. Cytokine/Jak/Stat Signaling Is Required for EB Differentiation

(A) Nuclear STAT92E in *esg*⁺ progenitor cells.

(B) *10XSTAT-DGFP* is active in progenitor cells including *DI*⁺ ISCs (arrowhead) and *DI*⁻ EBs (arrow).

(C) *domeGal4* drives UAS-GFP in *esg*Gal4⁺ progenitor cells.

(D and E) MARCM clones showing that *stat*^{85C9} mutant cells fail to enlarge or express the EC differentiation marker *Myo1A-lacZ* (red, E). A control clone (D) has many *lacZ*⁺ ECs (red, arrows). GFP-marked clones were analyzed 8 days after induction.

(F–H) Expression of *Stat92E RNAi* (G) or *Dome RNAi* (H) using the *esg*^{ts} system for 8 days resulted in progenitor cell accumulation and EC depletion.

(I) Cell numbers in control and *stat*^{85C9} mutant MARCM clones. For each sample, >100 clones from 10 midguts were counted; data are presented as mean ± SD (error bars).

Delta⁺ cells were observed, suggesting that the pool of functional stem cells was expanded (Figures 5B and 5F). These results suggested that Jak/Stat signaling might promote differentiation by increasing Delta expression and stimulating Notch receptor activity. This notion was supported by RT-qPCR showing that *E(spl)*-complex genes, which are Notch targets, were up-regulated by expressing *Hep*^{Act} in ECs and down-regulated when Stat was depleted in progenitor cells (Figure 5G). Consistently, *Hep*^{Act} expression caused widespread activation of a Notch activity reporter, *Gbe-Su(H)lacZ* (Furriols and Bray, 2001) (Figures 5H and 5I). However, overexpressing *Delta* in *Stat*^{85C9} mutant ISCs, or in progenitor cells expressing *Stat RNAi* or *Domeless RNAi*, did not restore the ability of these cells to differentiate (Figure S3F). Thus, Stat targets, in addition to *Delta*, are required for EC differentiation. The dual function of Upd/Jak/Stat signaling as a mitogen for ISCs and a differentiation factor for EBs may serve to couple these processes.

Enteric Infection Induces Upd/Jak/Stat Signaling and ISC Mitoses

To investigate the physiological relevance of the regenerative responses described above, we searched for natural environmental challenges that might stimulate ISC proliferation in *Drosophila*. Ingestion and enteric infection with *Pseudomonas entomophila*, a gram-negative bacterium, has been reported to kill ECs and activate JNK signaling (Vodovar et al., 2005). Feeding flies *P. entomophila* for 2 days induced a strong mitotic response in the midgut (Figure 6A), and RT-qPCR showed that this coincided with the induction of the JNK target *puc*, all three *Upd* cytokines, the Stat target *Socs36E*, and *Delta* (Figure 6B). Temporal analysis (data not shown) indicated that these genes were appreciably induced by 2 hr after infection and had plateaued by 8 hr,

suggesting that they were EBs that failed to differentiate, rather than ISC-like cells defective in *Notch* signaling. *Stat*³⁹⁷ mutant clones showed a similar inability to differentiate into ECs (Figure S2E), and this could be rescued by *Gal4*-driven *Stat92E* (Figure S2F). Similar differentiation defects were observed when *Stat92E* or the Upd receptor, *dome*, were depleted with RNAi either clonally (Figure S2) or in progenitors using *esgGal4*^{ts} (Figures 4G and 4H). Cells homozygous for *Stat*^{85C9} or *Stat*³⁹⁷ or expressing RNAi against *Stat92E* or *dome* appeared to divide at rates comparable to WT cells (Figures 4I and S2). Thus, Jak/Stat signaling is required for EC differentiation, though it may not be required for basal rates of ISC division.

Next, we applied assays of Delta/Notch signaling, which is essential for differentiation of EBs to the EC fate (Micchelli and Perrimon, 2006; Ohlstein and Spradling, 2006, 2007). *Delta* mRNA was reduced when *Stat92E* or *dome* was depleted in progenitor cells (Figure 5A). Conversely, *Delta* mRNA and protein were increased following induction of Upd, Rpr, or *Hep*^{Act} in ECs (Figures 5A and 5F). In these cases, increased numbers of small

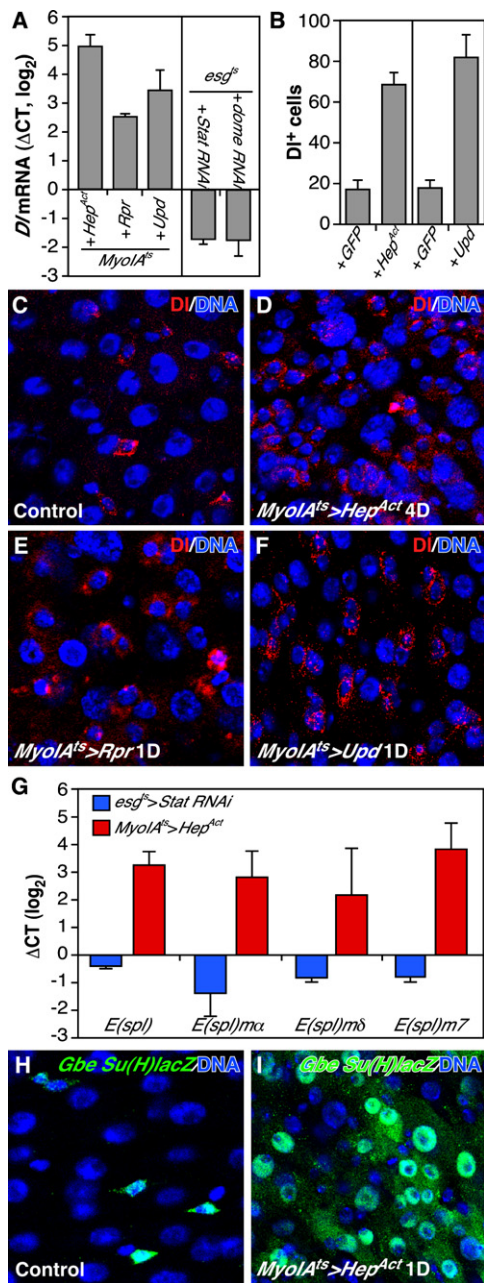


Figure 5. Rpr, JNK, and Upd Signaling Upregulated Delta/Notch Signaling

(A) Midgut *Di* RNA expression measured by RT-qPCR. *UAS-Hep^{Act}* or *UAS-Upd* were induced for 24 hr, whereas *UAS-Rpr* was induced for 12 hr, in ECs using the *MyoIA^{ts}* system. *UAS-Stat RNAi* or *UAS-Dome RNAi* were induced in progenitor cells for 4 days using the *esg^{ts}* system. Data were normalized to GFP only controls (*MyoIA^{ts}>GFP* or *esg^{ts}>GFP*, respectively). Three biological replicates were assayed and data is presented as mean \pm SD (error bars).

(B) Quantification of *D1⁺* progenitor cells in midguts expressing GFP (control), *Hep^{Act}* or *Upd* using the *MyoIA^{ts}* system.

(C–F) Delta protein (red) after induction of *Hep^{Act}* (D), *Rpr* (E), or *Upd* (F) in ECs. (G) Notch activity as measured by *E(spl)*-C RNAs using RT-qPCR. *UAS-Hep^{Act}* was induced for 24 hr with *MyoIA^{ts}* and *UAS-Stat RNAi* was induced for 4 days using *esg^{ts}*. The data were normalized as in (A).

and that the mitotic response began within 4 hr. The locations of JNK activation and cytokine induction were assessed using reporter genes. The JNK reporter, *puclacZ^{E69}*, was expressed at low levels in scattered ECs prior to infection and was induced to high levels in most ECs after infection (Figures 6E and 6F). *Upd^{lacZ}* was not detected prior to infection and was induced in small *esg⁺* progenitor cells (ISCs/EBs) and slightly larger early ECs (pre-ECs) after infection (Figures 6G and 6H; see also Figures 3B–3D). *Upd3Gal4*-driven GFP (Agaisse et al., 2003) was found in a few scattered ECs in controls but was highly induced in almost all ECs after infection (Figures 6I and 6J). The *10XSTAT-DGFP Stat* reporter was heavily induced by *P. entomophila*, in both small and large cell types (Figures 6K and 6L). Since these cells turned over rapidly (Figure 7), however, some or all of the DGFP observed in ECs could have been inherited from progenitors.

As in the other cases of midgut regeneration described above, *Delta* expression and Notch signaling were increased by *P. entomophila* (Figures 6B and S4), and there were small increases in the numbers of *MyoIA⁺* ECs, *pros⁺* EEs, and *Delta⁺* progenitors (Figure 6D). The relative proportions of these cell types remained essentially normal (Figure 6D; Table S2). To determine the identity of mitotic cells following *P. entomophila* infection we scored *PH3⁺* mitotic cells for the ISC marker *Delta*, the EE marker *prospero*, and the Notch reporter *GbeSu(H)lacZ*, an early marker of EC differentiation (Table S2). Most (85%) mitotic cells expressed high levels of *Delta*, just as in WT (Ohlstein and Spradling, 2007), and all *PH3⁺* cells were negative for *GbeSu(H)lacZ* and *pros* (Table S2). This finding suggests that EE and EB cells do not de-differentiate and re-enter the cell cycle. The expression of *GbeSu(H)lacZ* and *Delta* were also mutually exclusive (Table S2), indicating normal *Delta/Notch* signaling. Clonal analysis showed that, after infection, there were generally only one or two *Delta⁺* cells/clone, as in controls (Figure S6). Newly generated EEs and ECs occurred at the normal ratio of \sim 1:9 (Table S2). These observations all indicate that the ISC lineage and differentiation program are normal in midguts regenerating from *P. entomophila* infection.

To test whether ISC mitoses induced by *P. entomophila* required Jak/Stat signaling, we expressed RNAi against either *stat92E* or *Dome* in progenitor cells using *esg^{ts}*, and then fed the flies *P. entomophila*. The mitotic response to infection was entirely suppressed in these animals (Figures 6A and 7H'), indicating that Jak/Stat signaling is required. *P. entomophila* did, however, induce mitosis in JNK-defective *hep¹* mutants. Consistently, suppressing JNK in ECs, using *MyoIA^{ts}* to drive *Puc* or *Bsk^{DN}*, also had no detectable effect on ISC mitoses induced by *P. entomophila* (Figure 6A; data not shown), or on the induction of the Upds (Figure 6C). We infer that JNK signaling is not required for ISC activation in response to *P. entomophila* but that Jak/Stat signaling is.

Enteric Infection Drives Rapid Gut Epithelial Turnover

We expected the combination of increased EC death and ISC division following *P. entomophila* infection to result in faster turnover

(H and I) The Notch activity reporter, *GbeSu(H)lacZ* was observed in more gut epithelial cells after induction of *Hep^{Act}* in ECs. Since *LacZ* is stable its presence in ECs may be due to perdurance.

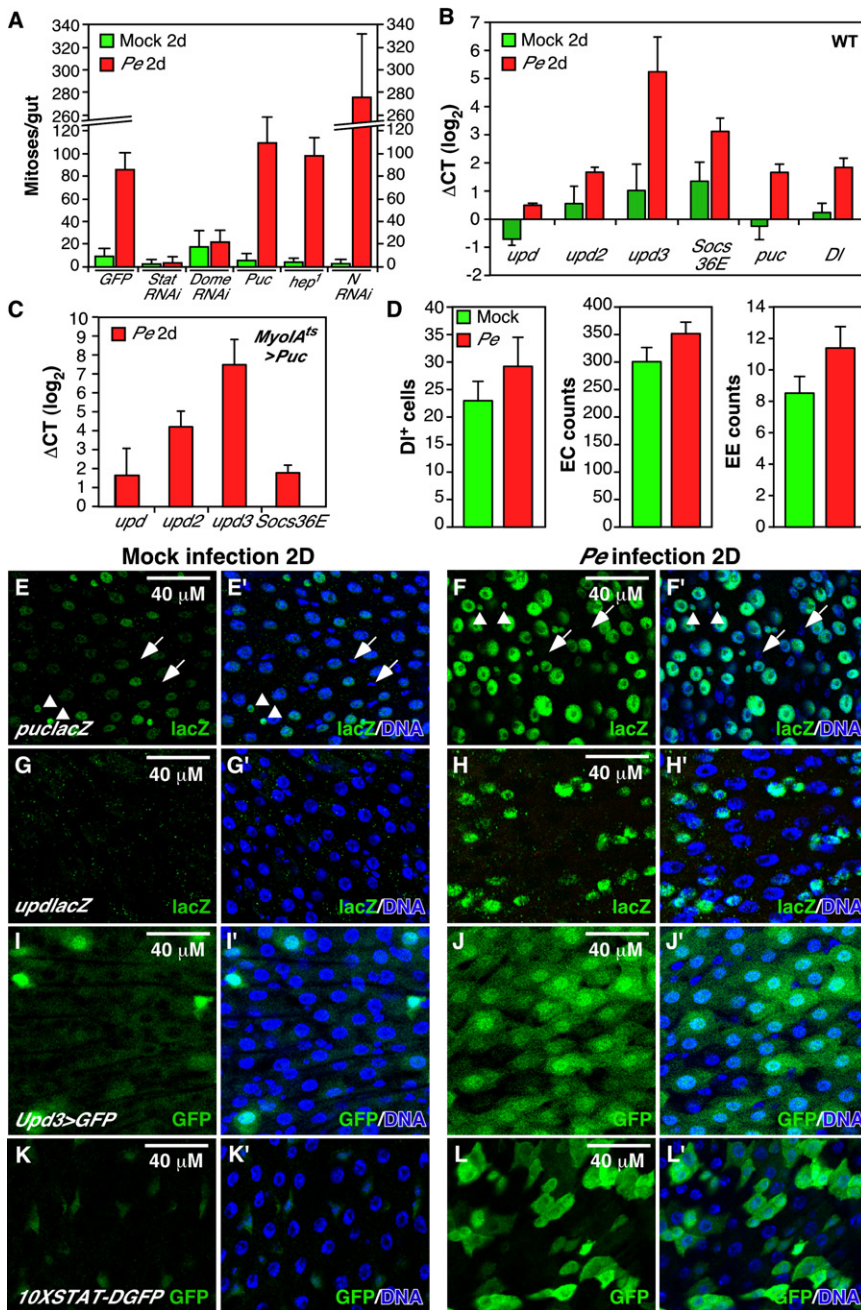


Figure 6. *Pseudomonas entomophila* Ingestion Induces ISC Proliferation

(A) Stat92E, Dome, or Notch was depleted from progenitor cells by UAS-RNAi using *esg^{ts}*, 2 days prior to *P. entomophila* infection. *hep¹* is a hypomorphic JNKK allele. JNK signaling was also down-regulated in ECs by expressing UAS-Puc using *MyoIA^{ts}*. Midguts were scored for PH3⁺ mitotic figures 2 days after infection (green). “Mock” indicates control animals shifted to food without *P. entomophila*.

(B) Midgut mRNAs measured in WT by RT-qPCR, 2 days after *P. entomophila* infection (red) or in controls (green). The data were normalized to mock infected samples.

(C) Midgut mRNAs measured in *MyoIA^{ts}>Puc* animals by RT-qPCR, 2 days after *P. entomophila* infection. The data were normalized to mock infected controls.

(D) Quantification of Delta⁺ ISCs, *MyoIA-GFP⁺* ECs and *pros⁺* EEs during gut regeneration induced by *Pe*.

(E–L) Reporter expression in midguts 2 days after mock (E, G, I, and K) or *P. entomophila* (F, H, J, and L) infection.

(E and F) A low level of the JNK reporter, *puclacZ*, was observed in ECs and EEs (usually paired, arrowheads) in controls (E). Widespread *puclacZ* after *P. entomophila* infection (F). *puclacZ* was not expressed in progenitor cells (arrows).

(G and H) *updlacZ* was not expressed in controls (G), but was induced in progenitor cells or pre-ECs after *P. entomophila* infection (H).

(I and J) *upd3Gal4*-driven GFP in controls (I), or after *P. entomophila* infection (J).

(K and L) The Stat reporter, *10XSTAT-DGFP*, was normally confined to progenitor cells (K), but was present in many cells after *P. entomophila* infection (L). Blue: DNA in all panels.

of the gut epithelium. To test this, we devised a method to mark all progenitor cells at a specific time point with a heritable marker. In this method, which we refer to as *esg^{ts} Flp-Out* (*esg^{ts}F/O*), UAS-Flp recombinase is induced in progenitor cells by temperature shift using *esgGal4^{ts}*. Flp excises the CD2 cassette from *Act>CD2>Gal4* (or *tub>CD2>Gal4*), converting it to the ubiquitously expressed heritable driver, *ActGal4* (or *tubGal4*). This marks ISCs and their progeny with Gal4-driven GFP and absence of CD2. The *esg^{ts}F/O* system proved to be reliable for measuring epithelial turnover in the posterior (but not anterior) midgut. In normally fed adult females, the posterior midgut epithelium renewed

itself within about 12 days of temperature shift (Figures S5A–S5C). In males, significant numbers of newborn GFP⁺ cells were not observed until ~3 weeks after inducing Flp (data not shown). Using the *esg^{ts}F/O* system, in males we found that gut renewal was greatly accelerated in the gain-of-function Jak mutant, *hop^{TumL}* (Figures S5D and S5E). Similarly, inducing UAS-*Hop^{TumL}* using the *esg^{ts}F/O* system generated many new epithelial cells within 2 days, causing hyperplasia (Figure 7B). Consistent with the role of Notch in differentiation, inducing a transcriptionally active intracellular form of Notch (*N^{intra}*) with *esg^{ts}F/O* promoted the rapid differentiation progenitor cells into ECs, depleting the gut of progenitor cells (Figure 7D). We also used *esg^{ts}F/O* to overexpress the E2F/DP transcription factor, which specifically promotes cell cycle progression. E2F greatly increased the number of small progenitor cells but did not increase new, GFP-marked ECs (Figure 7C). Thus, rates of ISC proliferation and EB differentiation are separable parameters that are likely to be independently regulated.

We further tested the function of Jak/Stat signaling in midgut turnover by combining the *esg^{tsF/O}* system with *P. entomophila* infection. First, *Stat92E* was depleted using RNAi expressed in progenitor cells and their progeny for 2 days, and then the flies (males) were fed *P. entomophila* for 2 days to generate an enteric infection. These flies were then transferred to food lacking *P. entomophila* and containing antibiotics (which rescued them from death by infection) for another 2 days. While the midgut epithelium in mock-infected controls did not turn over significantly during this 6-day experiment (Figure 7E), *P. entomophila* infection induced a virtually complete midgut renewal (Figure 7F). In midguts depleted of Stat92E, however, there was little if any renewal (Figures 7G and 7H). Instead, the midgut lost most of its resident ECs and shrank to a small disorganized structure composed mostly of small, nondividing cells (Figures 7H, 7H', and 6A). Similarly, *P. entomophila* infection failed to induce gut renewal in *hop²⁵* mutants (Figures S5F and S5G). Moreover, controls infected with *P. entomophila* and then cured with antibiotics survived, whereas transient infection was lethal to flies expressing *Stat92E* RNAi (Figure 7K). Thus Stat signaling is essential for midgut regeneration in response to infection.

We used the same strategy to assess the role of Notch signaling in midgut renewal after *P. entomophila* infection. When *Notch* RNAi was expressed in progenitor cells and the flies were infected with *P. entomophila*, mitotic indices were much higher than in controls (Figure 6A), and the midgut became populated almost entirely with small proliferative progenitor cells (Figure 7J). Thus, Notch signaling appears not to be required for ISC mitoses in response to infection, though it is still required for differentiation. As with Stat depletion, animals depleted of Notch in progenitor cells failed to survive after *P. entomophila* (Figure 7K).

DISCUSSION

The *Drosophila* Midgut Is Homeostatic

Rates of cell turnover in the intestine are likely to be in constant flux in response to varying stress from digestive acids and enzymes, chemical and mechanical damage, and toxins produced by both commensal and infectious enteric microbiota. As we show here, feedback from differentiated cells in the gut epithelium to stem and progenitor cells is a key feature of this system. Genetically directed enterocyte ablation, JNK-mediated stress signaling, or enteric infection with *P. entomophila* all disrupt the *Drosophila* midgut epithelium and induce compensatory ISC division and differentiation, allowing a compromised intestine to rapidly regenerate. Other recent reports note a similar regenerative response following three additional types of stress: detergent (DSS)-induced damage (Amcheslavsky et al., 2009), oxidative stress by paraquat (Biteau et al., 2008), and enteric infection with another less pathogenic bacterium, *Erwinia carotovora* (Buchon et al., 2009). Remarkably, the fly midgut can recover not only from damage, but also from severe induced hyperplasia, such as that caused by ectopic cytokine (Upd) production (Figures 2F–2H). Thus, this system is robustly homeostatic.

Each of the three stress conditions we studied induced all three Upd cytokines, and genetic tests showed that Upd/Jak/Stat signaling was both required and sufficient for compensatory

ISC division and gut renewal (Figures 2L, 6A, 7H, and S5F). Although JNK signaling was also activated in each instance, it was not required for the stem cell response to either EC apoptosis or infection (Figures 2K, 6A, and 6C), implying that other mechanisms can sense EC loss and trigger the cytokine and proliferative responses. JNK signaling may be important in specific contexts that we did not test, such as following oxidative stress, which occurs during some infections (Ha et al., 2005), activates JNK, and stimulates midgut DNA replication (Biteau et al., 2008).

The Stem Cell Lineage during Regeneration

Following *P. entomophila* infection, virtually the entire midgut epithelium could be renewed in just 2–3 days (Figure 7), whereas comparable renewal took more than 3 weeks in healthy flies. Despite this radical acceleration of cell turnover, the relative proportions of the different gut cell types generated (ISC, EB, EE, and EC; Figures 6D and S6; Table S2) remained similar to those in midguts undergoing slow, basal turnover. Our data (Table S2) suggested that de-differentiation did not occur, and we obtained little evidence of symmetric stem divisions (stem cell duplication) induced by enteric infection (Figure S6). Hence, we suggest that asymmetric stem cell divisions as described for healthy animals (Ohlstein and Spradling, 2006), together with normal Delta/Notch-mediated differentiation, remain the rule during infection-induced regeneration. The results we obtained using Reaper to ablate ECs are also consistent with this conclusion, as are those from detergent-induced midgut regeneration (Amcheslavsky et al., 2009).

Unlike infection, direct genetic activation of JNK or Jak/Stat signaling promoted large increases not only in midgut mitoses, but also in the pool of cells expressing the stem cell marker *Delta* (Figures 2 and 5B). Cell type marker analysis discounted de-differentiation of EEs or ECs as the source of the new stem cells, but the reactivation of EBs as stem cells seems possible. For technical reasons, we did not test whether stem cell duplications occur in response to Jak/Stat or JNK signaling, and this also remains possible. The ability of hyperplastic midguts to recover to normal following the silencing of cytokine expression (Figures 2F–2H) suggests that excess stem cells are just as readily eliminated as they are generated. Further studies are required to understand how midgut stem cell pools can be expanded and contracted according to need.

How Are the Upd Cytokines Induced?

How the *Upds* are induced in the midgut by JNK, apoptosis, or infection remains an open question. Paradoxically, ISC divisions triggered by Reaper required EC apoptosis but not JNK activity, whereas ISC divisions triggered by JNK did not require apoptosis (Figure 2K), and ISC divisions triggered by infection required neither apoptosis (data not shown) nor JNK activity (Figures 6A and 6C). These incongruent results suggest that different varieties of gut epithelial stress may induce Upd cytokine expression via distinct mechanisms. In the case of EC ablation, physical loss of cells from the epithelium might drive the cytokine response. In the case of infection, we expected the critical inputs to be the Toll and/or IMD innate immunity pathways, which signal via NF- κ B transcription factors (Ryu et al., 2008).

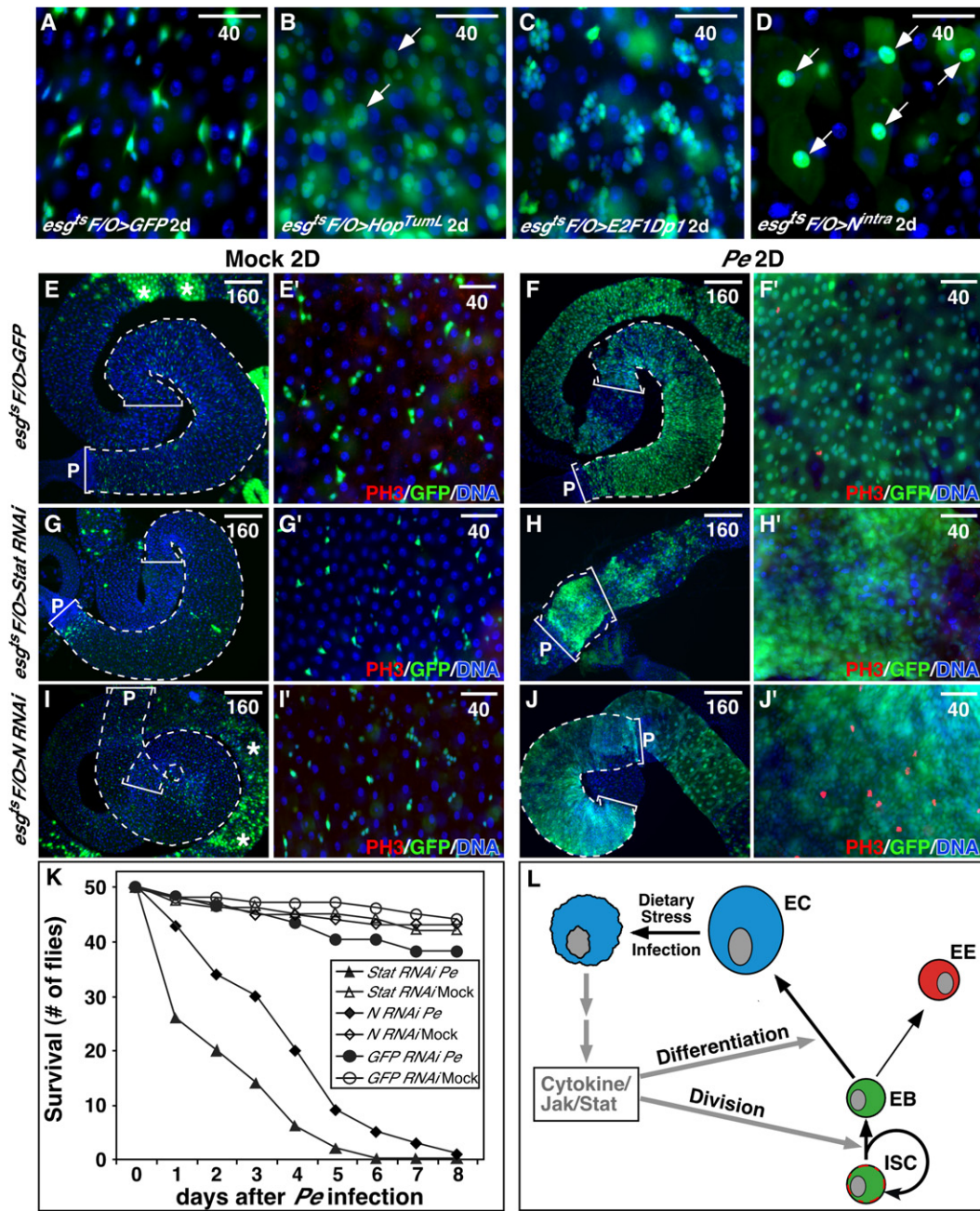


Figure 7. Midgut Renewal after *P. entomophila* Infection

Midgut turnover in 3- to 10-day-old adult males, measured using the *esg^{ts}F/O* system.

(A) Gut turnover in a control after 2 days. Progenitor cells and their newborn progeny are marked with GFP.

(B) UAS-*Hop^{TumL}* expressed with the *esg^{ts}F/O* system in progenitor cells for 2 days. These midguts contained many GFP⁺ cells, including large newborn ECs (arrows).

(C) E2F expressed with the *esg^{ts}F/O* system caused progenitor cell accumulation, but not EC hyperplasia.

(D) Activated Notch (*N^{intra}*) promoted the rapid differentiation of progenitor cells into ECs (arrows), depleting the midgut of progenitors.

(E–J) Males of the indicated genotypes were shifted to 29°C for 2 days, fed *P. entomophila* for 2 days, and treated with antibiotics for 2 days. “Mock” infected controls received no *P. entomophila*, but were aged identically and received antibiotics. Brackets and dotted lines indicate the posterior midgut. P indicates the posterior end. Asterisks (E and I) indicate induction-independent GFP in the anterior midgut. Midguts were stained for PH3 (red). *P. entomophila* infection caused almost complete midgut renewal (F), whereas few new ECs were generated in controls (E). *P. entomophila*-induced cell turnover was inhibited by *Stat92E RNAi* expressed under *esg^{ts}F/O* control (G and H). These midguts (H) were shrunken by EC depletion and condensation of the remaining nonmitotic progenitor cells (H’). When *Notch RNAi* was expressed, *P. entomophila* infection resulted in midguts composed mostly of small nondifferentiating, proliferative progenitor cells (J and J’).

Functional tests, however, indicated that the Toll and IMD pathways are required for neither Upd/Jak/Stat induction nor compensatory ISC mitoses following enteric infection by gram-negative bacteria (data not shown and Buchon et al., 2009). Hence, other unknown inputs likely trigger the Upd cytokine response to infection.

Is the cytokine response to infection relevant to normal midgut homeostasis? This seems likely. We observed low levels of *Upd3* expression and Stat signaling in healthy animals (Figures 6I and 6K), and midgut homeostasis required the IL-6R-like receptor *Dome* and *Stat92E* even without infection (Figure 4). Wild *Drosophila* subsist on a diet of rotting fruit, which is a good source of protein because it is teeming with bacteria and fungi. Given such a diet it seems likely that midgut cytokine signaling is constantly modulated by ever-present factors that impose dietary stress—food composition and commensal microbiota—even in healthy animals.

Jak/Stat in Mammalian Intestinal Homeostasis and Cancer

Although studies in mammals have yet to unravel the details of a feedback mechanism underlying gut homeostasis, experimental evidence implies that such a mechanism exists and involves Cytokine/Jak/Stat signaling. As in *Drosophila*, damage to the mouse intestinal epithelium caused by detergents or infection can stimulate cell proliferation in the crypts, where stem and transient amplifying cells reside (McConnell et al., 2008; Rigby et al., 2007). In a mouse model of detergent (DSS)-induced colitis (Karin, 2008), colon epithelial damage caused by DSS allows exposure to commensal microbes, activating NF- κ B signaling in resident macrophage-like Dendritic cells. These cells respond by expressing inflammation-associated cytokines, one of which (IL-6) activates Stat3 and is believed to promote cell proliferation and regeneration. Consistent with a functional role for Jak/Stat, disruption of the Stat inhibitor SOCS3 in the mouse gut increased the proliferative response to DSS and also increased DSS-associated colon tumorigenesis (Rigby et al., 2007). Also pertinent is the presence of high levels of phospho-Stat3 in a majority of colon cancers, where it correlates with adverse outcome (Kusaba et al., 2005), and the observation that IL-6 can promote the growth of colon cancer cells, which are thought to derive from ISCs or transient amplifying cells (Becker et al., 2005; Schneider et al., 2000). Increased colon cancer incidence is associated with gut inflammatory syndromes, such as inflammatory bowel disease (IBD) and Crohn's disease (Freeman, 2008), which are likely to involve enhanced cytokine signaling. Whether cytokines mediate gut epithelial turnover in healthy people or only during inflammation is presently unclear, but it nevertheless seems likely that the mitogenic role of IL-6-like cytokines and Jak/Stat signaling in the intestine is conserved from insects to humans.

The connection to inflammation suggests that our findings may also be relevant to the activity of nonsteroidal anti-inflam-

matory drugs (NSAIDs), such as aspirin, ibuprofen, and celecoxib, as suppressors of colorectal carcinogenesis. These drugs target the cyclooxygenase activity of prostaglandin H synthases (PGHS, COX), which are rate-limiting for production of prostaglandin E₂, a short-range lipid signal that promotes inflammation, wound healing, cell invasion, angiogenesis, and proliferation (Cha and DuBois, 2007; Gupta and Dubois, 2001). Notably, COX-2 has been characterized as an immediate early gene that can be induced by signals associated with infection and inflammation, including the proinflammatory cytokines IL-1 β and IL-6, which activate NF- κ B and STAT3, respectively (Chun and Surh, 2004; Rummel et al., 2006; Xuan et al., 2003). Whether prostaglandins mediate the effects of Jak/Stat signaling in the fly midgut remains to be tested, but insects do produce prostaglandins, and *Drosophila* has a functional COX homolog, *pxt*, whose activity can be suppressed by NSAIDs (Tootle and Spradling, 2008).

EXPERIMENTAL PROCEDURES

Genetics

See Supplemental Experimental Procedures for details.

Histology

After dissection and fixation, midguts were stained with mouse monoclonal anti-Delta (1:100) or anti-Prospero (1:100) (Developmental Studies Hybridoma Bank); rabbit polyclonal anti-phosphoSer10-histone 3 (1:4000) (Upstate Biotechnology), or rabbit polyclonal anti- β -galactosidase (1:2000) (Cappel), and anti-STAT92E (1:250, S. Hou). For BrdU incorporation, midguts were dissected in Ringer's solution and incubated with 100 μ g/ml BrdU for 30 min in Schneider's medium. Midguts were then fixed, treated with 3M HCl, and stained with anti-BrdU (1:100, Becton Dickinson). Samples were analyzed on a Nikon Eclipse Ti or a Zeiss LSM510 confocal microscope.

Cell Counts

Mitotic indices were quantified by counting PH3⁺ cells in >10 midguts of the proper genotype, time point, or condition (5 females and 5 males unless noted otherwise), and are presented as means \pm standard deviation (SD). For quantification of gut hyperplasia induced by JNK, Jak/Stat, or *P. entomophila*, cells were counted in a defined posterior midgut region between the hindgut and the copper cells, and the values were corrected for changes in the area of this region. These data were also collected from 10 midguts/genotype or condition, and are presented as mean cell numbers with SD (error bars).

Gut Turnover Analysis

UAS-transgenes were crossed to an *esg^{ts}F/O* tester: *w; esgGal4 tubGal80^{ts} UAS-GFP*; and *UAS-flp Act>CD2>Gal4*. Three- to ten-day-old male progeny (raised at 18°C) were shifted to 29°C for 2 days, and then midguts were dissected and analyzed. For RNAi experiments, 3- to 10-day-old adult males (raised at 18°C) were shifted to 29°C for 2 days before being transferred to fly food laced with either 0.5 ml of 10 \times concentrated overnight *P. entomophila* culture or 0.5 ml of 5% sucrose (mock infection). After 2 days, the flies were transferred to food containing antibiotics (100 μ g/ml ampicillin, 50 μ g/ml vancomycin, 100 μ g/ml neomycin, and 100 μ g/ml metronidazole) for an additional 2 days before being dissected and analyzed. See Supplemental Experimental Procedures for additional details.

(K) Survival after *P. entomophila* infection. Fifty flies (25 males, 25 females) were scored for survival after 2 days of *P. entomophila* infection (day 0) followed by another 2 days of antibiotic treatment. Flies were then transferred to normal food. Controls (Mock or *GFP RNAi* with *P. entomophila* infection) survived after antibiotic treatment, but flies depleted of N or Stat92E died rapidly.

(L) Model for midgut homeostasis.

RT-qPCR

RNA was extracted from 10 midguts (5 males/5 females) using TRIzol (Invitrogen). RNA was cleaned using RNeasy (QIAGEN), and cDNA was synthesized using the iScript cDNA synthesis kit (Bio-Rad). qPCR was performed using the iScript one step RT-PCR SYBR green kit (Bio-Rad). Data were acquired using an iQ5 System (Bio-Rad). Primer sequences are listed in [Supplemental Experimental Procedures](#). RT-qPCR was performed in duplicate on each of 3 independent biological replicates. All results are presented as means \pm SD of the biological replicates. We used RpL11 as a normalization control.

SUPPLEMENTAL DATA

Supplemental Data include five figures, two tables, and Supplemental Experimental Procedures and can be found with this article online at [http://www.cell.com/supplemental/S0092-8674\(09\)00571-6](http://www.cell.com/supplemental/S0092-8674(09)00571-6).

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