Injury-induced BMP signaling negatively regulates *Drosophila* midgut homeostasis

Zheng Guo, Ian Driver, and Benjamin Ohlstein

1Department of Genetics and Development and 2Integrated Program in Cellular, Molecular and Biomedical Studies, Columbia University Medical Center, New York, NY 10032

Although much is known about injury-induced signals that increase rates of *Drosophila melanogaster* midgut intestinal stem cell (ISC) proliferation, it is largely unknown how ISC activity returns to quiescence after injury. In this paper, we show that the bone morphogenetic protein (BMP) signaling pathway has dual functions during midgut homeostasis. Constitutive BMP signaling pathway activation in the middle midgut mediated regional specification by promoting copper cell differentiation. In the anterior and posterior midgut, injury-induced BMP signaling acted autonomously in ISCs to limit proliferation and stem cell number after injury. Loss of BMP signaling pathway members in the midgut epithelium or loss of the BMP signaling ligand *decapentaplegic* from visceral muscle resulted in phenotypes similar to those described for juvenile polyposis syndrome, a human intestinal tumor caused by mutations in BMP signaling pathway components. Our data establish a new link between injury and hyperplasia and may provide insight into how BMP signaling mutations drive formation of human intestinal cancers.

Introduction

In *Drosophila melanogaster*, the adult midgut is maintained by multipotent intestinal stem cells (ISCs) that give rise to a daughter, the enteroblast (EB), which, depending on the level of Notch signaling, will differentiate into an enterocyte (EC) or enterondocrine cell (Micchelli and Perrimon, 2006; Ohlstein and Spradling, 2006, 2007). When ECs are injured by environmental stressors, JNK and Yki are activated in ECs, which results in expression of Janus kinase–signal transducer and activator of transcription (JAK-STAT) and EGF receptor (EGFR) signaling ligands (Biteau et al., 2008; Jiang et al., 2009, 2011; Buchon et al., 2010; Karpowicz et al., 2010; Shaw et al., 2010; Staley and Irvine, 2010; Biteau and Jasper, 2011). These ligands activate signaling in ISCs and EBs to promote ISC proliferation and EB differentiation (Jiang et al., 2009, 2011; Buchon et al., 2010; Lin et al., 2010; Biteau and Jasper, 2011; Xu et al., 2011). ISC divisions are also regulated by autocrine expression of JAK-STAT ligands (Jiang and Edgar, 2009; Karpowicz et al., 2010; Osman et al., 2012). Yki activation in ISCs (Karpowicz et al., 2010; Shaw et al., 2010), reactive oxygen species (Hochmuth et al., 2011), and activation of the insulin signaling pathway (Amcheslavsky et al., 2009; Choi et al., 2011) in ISC and EBs.

After acute compensation of injury-induced cell loss, stem cells need to rapidly return to preinjurious self-renewal rates to avoid tissue hyperplasia. Indeed, several observations confirm that ISC proliferation rates quickly decrease after removal of noxious stimuli (Jiang et al., 2009; Buchon et al., 2010). Yet, how this dynamic change of division rates is achieved is largely unknown.

Loss-of-function mutations in the bone morphogenetic protein (BMP) receptor type IA and SMAD4 (mother against decapentaplegic [Dpp] homologue 4) are present in a subset of juvenile polyposis (JP) patients (Houlston et al., 1998, 2001). Furthermore, inhibition of BMP signaling in mouse intestines results in phenotypes that resemble human JP syndrome (Haramis et al., 2004; He et al., 2004), suggesting that BMP signaling in the crypt acts to limit ISC proliferation. Given the similarities between vertebrate and *Drosophila* intestinal homeostasis (Lucchetta and Ohlstein, 2012), the BMP signaling...
pathway is an ideal candidate for exploring negative regulation of ISC proliferation.

As is the case with the vertebrate intestine, the *Drosophila* midgut varies along its length in function and cellular identity (Dubreuil, 2004). One of these regions, located in the middle of the midgut, is the copper cell region (CCR). The cells of the CCR are easily identified by their cup-shaped morphology (Filshie et al., 1971). Cells in this region secrete protons that maintain the CCR at low pH (Dubreuil, 2004; Strand and Michcelli, 2011) and are maintained by a population of relatively quiescent ISCs known as gastric stem cells (Strand and Michcelli, 2011). BMP signaling is required during development to establish epithelial expression of the homeotic gene *labial*, which is required for copper cell formation in embryos and larvae (Panganiban et al., 1990; Hoppler and Bienz, 1994; Staehling-Hampton and Hoffmann, 1994). Whether BMP signaling continues to play a role in establishing and maintaining regional identity in the adult midgut has not been established.

Here, we show that in the CCR of the adult *Drosophila* midgut, BMP signaling is constitutive and necessary for copper cell specification. In contrast, in the anterior and posterior midgut, injury-induced signaling regulates the expression of the BMP signaling ligand Dpp in visceral muscle (vm), which then activates the BMP signaling pathway in ISCs to negatively regulate their number and rate of division. Our data provide evidence for how coregulation of antagonistic signals mediate tissue homeostasis and how disconnect between these signals can lead to abnormal tissue homeostasis.

**Results**

**BMP signaling promotes adult *Drosophila* copper cell specification**

To determine the extent of active BMP signaling in the adult midgut, we examined the expression patterns of two markers of BMP signaling: (1) *Dad-lacZ* (Tsuneizumi et al., 1997) and (2) phosphorylated Mad (pMad; König et al., 2011). *Dad-lacZ* expression could be detected in the anterior and posterior midgut but was consistently expressed at high levels in and adjacent to the middle part of the midgut known as the CCR, a region delineated by expression of Labial, a CCR marker (Fig. 1 A; Chouinard and Kaufman, 1991; Strand and Michcelli, 2011). To determine in which cells of the CCR BMP signaling was active, we co-stained midguts for *Dad-lacZ* or pMad and markers of copper cells (Labial; Fig. 1 B and B’), gastric stem cells and EBs (*escargot* [esg]-GFP, a GFP protein trap in the *esg* gene; Fig. 1, C and C’; Michcelli and Perrimon, 2006; Buszczak et al., 2007), and enteroendocrine cells (*Prospero*; Fig. S1, A–A’; Michcelli and Perrimon, 2006; Ohlslein and Spradling, 2006). In all cases, *Dad-lacZ* or pMad was coexpressed with Labial, esg-GFP, and Prospero, demonstrating that BMP signaling is constitutively active in all epithelial cells of the CCR.

To identify a role for BMP signaling in the CCR, we made positively marked null mutant mosaic analysis with a repressible cell marker (MARC; Lee and Luo, 1999) clones of two members of the BMP signaling pathway, *thick veins* (*tkv*; *tkv*<sup>Δ</sup>) and Mad (*Mad*<sup>Δ</sup>). pMad staining was absent from *tkv*<sup>Δ</sup> clones in the CCR (Fig. 1, D and D’), whereas *Dad-lacZ* staining was absent from *Mad*<sup>Δ</sup> clones in the CCR (Fig. 1, E and E’), demonstrating that the pMad antibody and *Dad-lacZ* enhancer we used were specific reporters of BMP signaling in the CCR. Mutant nuclei were small and tightly packed, unlike wild-type (WT) copper cell nuclei, which are polyploid and regularly spaced (Fig. 1, E, E’, G, and G’; Hoppler and Bienz, 1994). Because the gene *labial* is necessary for copper cell identity in embryonic and larval midguts (Hoppler and Bienz, 1994), we stained the CCR for Labial. Although cells outside of the clone expressed Labial, Labial was undetectable within *tkv*<sup>Δ</sup> and *Mad*<sup>Δ</sup> clones (Fig. 1, F–G’). Expression of GFP by the temperature-inducible ISC–EB driver *esg*-Gal4 *tubulin* (*tub*)-Gal80<sup>Δ</sup> (*esg*<sup>Δ</sup>) had no effect on expression of Labial (Fig. S1 B). In contrast, knockdown of BMP signaling in the CCR using *esg*<sup>Δ</sup> to drive expression of *tkv* or *Mad* RNAi (Fig. S1 C and D) or the ISC driver Delta (Dl)-Gal4 (Fig. S1 E) to drive expression of *Mad* RNAi (Fig. S1 F) for 9 d at the permissive temperature (30°C) led to nearly complete loss of Labial expression.

Copper cells secrete protons into the lumen of the CCR (Dubreuil et al., 1998; Dubreuil, 2004), lowering the local pH of the midgut. We knocked down BMP signaling in the CCR using *esg*<sup>Δ</sup> to drive expression of *Mad* or *tkv* RNAi for 9 d at 30°C and fed animals food containing bromophenol blue dye, a chemical indicator of midgut pH (Shanbhag and Tripathi, 2009). In control midguts, dye color was blue, pH > 4.6, in the anterior and posterior midgut and yellow, pH < 3.0, in the CCR (Fig. 1 H). In *esg*<sup>Δ</sup>*tkv* RNAi (Fig. 1 I) or *esg*<sup>Δ</sup>*Mad* RNAi midguts (Fig. S1 G), dye color was blue throughout the midgut, demonstrating that functional copper cells were not made. Thus, our data demonstrate that copper cells cannot be generated when BMP signaling is compromised in progenitor cells.

**Midgut injury up-regulates BMP signaling**

Comparing *Dad-lacZ* expression and pMad staining in multiple midguts, we found that in contrast to the CCR, BMP signaling in the anterior and posterior midgut varied greatly from midgut to midgut (Fig. 2 A, compare with Fig. 1 A and Fig. S2 A). We hypothesized that this variable pattern reflected the variation in local injury that the midgut is exposed to from bacteria and the digestive process (Buchon et al., 2009; Chatterjee and Ip, 2009; Jiang et al., 2009) and reasoned that extensive injury to the midgut should lead to a dramatic up-regulation of BMP signaling throughout the entire midgut. Because feeding bleomycin to animals results in widespread EC death (Amcheslavsky et al., 2009), we reared animals on control food or food containing bleomycin for 24 h and, immediately after, determined the expression of *Dad-lacZ*, pMad, and *upd3*-Gal4 upstream activating sequence (UAS)-GFP (*upd3*»GFP), a marker of damaged midgut cells (Jiang et al., 2009). In midguts from animals reared on control food, expression of *upd3*»GFP and *Dad-lacZ* was mostly limited to the CCR and part of the posterior midgut (Fig. S2, B and B’). In contrast, in midguts from animals fed bleomycin, the expression of all three reporters became broadly detectable (Fig. 2, B–C’). Similar results were obtained by feeding flies Ecc15 (*Erwinia carotovora* carotovora clonal driver).
To determine in which cells of the anterior and posterior midgut BMP signaling is active, we examined pMad and Dad-lacZ expression in esg-Gal4 UAS-GFP (esg>GFP) or esg-GFP midguts before (Fig. 2, E–G) and immediately after 24 h treatment with bleomycin (Fig. 2, H–J) and the reactive oxygen species–inducing drug paraquat (Fig. S2, C and C'); Biteau et al., 2008; Choi et al., 2008, 2011; Chatterjee and Ip, 2009; Hochmuth et al., 2011), demonstrating that feeding animals substances that injure the midgut results in up-regulation of BMP signaling.

To determine in which cells of the anterior and posterior midgut BMP signaling is active, we examined pMad and Dad-lacZ expression in esg-Gal4 UAS-GFP (esg>GFP) or esg-GFP midguts before (Fig. 2, E–G') and immediately after 24 h treatment with bleomycin (Fig. 2, H–J') and the reactive oxygen species–inducing drug paraquat (Fig. S2, C and C'). As expected, in the CCR, pMad and Dad-lacZ

Figure 1. BMP signaling promotes Drosophila copper cell specification. For all panels, left is anterior, and right is posterior. (A) Dad-lacZ expression in the adult midgut. Labial antibody staining marks the copper cell region (CCR). (B and B') Dad-lacZ marks Labial-positive copper cells (arrows). (C and C') pMad antibody stains esg-GFP-positive cells in the CCR (arrows). (D and D') tkv<sup>8</sup> clones 8 d after clone induction (ACI); pMad staining is absent from tkv<sup>8</sup> clones. (E and E') Mad<sup>12</sup> clones 8 d ACI. Dad-lacZ staining is absent from Mad<sup>12</sup> clones. (F and F') tkv<sup>8</sup> clones 11 d ACI. Labial is absent from tkv<sup>8</sup> clones. (G and G') Mad<sup>12</sup> clones 12 d ACI. Nuclei are small, tightly packed, and Labial negative. (H) Bromophenol blue dye staining of the esg<sup>ts</sup>-GFP gut from an animal reared at 30°C for 9 d. The anterior and posterior midgut is blue, pH > 4.6, whereas the CCR is yellow, pH < 3.0. (I) Bromophenol blue dye staining of the esg<sup>ts</sup>-tkv RNAi gut from an animal reared at 30°C for 9 d. CCR loss is indicated by absence of yellow. (D–G) Dashed lines mark clone boundaries. Bars: (A, H, and I) 100 µm; (B–G) 10 µm.
Figure 2. Injury up-regulates BMP signaling in the midgut. (A) Dad-lacZ–positive cells are present in the anterior and posterior midgut (also see Fig. 1 A). Insets represent close-up views of Dad-lacZ in selected regions. [B–C′] Dad-lacZ (B and B′), upd3-Gal4 (B and C), and pMad (C and C′) staining are broadly detectable after 24 h treatment with bleomycin. [D and D′] Dad-lacZ and upd3-Gal4 are broadly detectable after 24 h exposure to ECC15. (E–G′) pMad staining from animals reared on a bleomycin-free diet. (H–J′) pMad staining from animals fed bleomycin for 24 h. (E–J′) Arrows show esg-GFP–positive cells. Arrowheads show ECs. Bars: (A–D) 100 µm; (E–J) 10 µm.

were expressed in all cells (Fig. 2, F, F′, I, and I′; and Fig. S2, E, E′, H, and H′). In contrast, before bleomycin treatment, the anterior and posterior midgut showed weak pMad and Dad-lacZ expression in ECs and much weaker staining in ISCs and EBs (esg-GFP- and esg-GFP–positive cells; Fig. 2, E, E′, G, and G′; and Fig. S2, D, D′, F, and F′). In the anterior and posterior midguts from animals fed bleomycin, pMad and Dad-lacZ expression became intensively elevated in all progenitor cells
Injury to the midgut results in activation of the INK, JAK-STAT, Yki, EGFR, Pvr (PDGF and VEGF receptor related), and Wnt signaling pathways (Lucchetta and Ohlstein, 2012). These signaling pathways act as part of a positive feedback loop that increases ISC proliferation and daughter differentiation so as to produce new daughters that replace cells lost as a result of injury. Because midgut injury also results in increased BMP signaling, we wondered whether BMP signaling acts in concert with these pathways to promote ISC proliferation and daughter differentiation. Conversely, we considered that BMP signaling acts as part of a negative feedback loop to antagonize ISC activity after injury.

To distinguish between these two possibilities, WT and mutant BMP signaling clones were generated and analyzed in the female posterior midgut. If BMP signaling acts as part of a positive feedback loop, the mean number of cells in mutant ISC clones over time should be less than that of age-matched WT clones. Alternatively, if BMP signaling acts as part of a negative feedback loop, the mean number of cells in mutant ISC clones over time should be greater than that of age-matched WT clones. pMad and Dad–lacZ stainings were absent from tkv	extsuperscript{ts} and Mad	extsuperscript{ts} clones in the anterior and posterior midgut (Fig. S3, A–D), demonstrating that the mutants we used efficiently block BMP signaling. 8 d after clone induction (ACI), the number of cells in tkv	extsuperscript{ts}, tkv	extsuperscript{ts}, and Mad	extsuperscript{ts} clones was significantly higher than in WT clones (Fig. 3, A–B’ and E), demonstrating that BMP signaling acts as part of a negative feedback loop to restrict the number of cells in a clone.

Because BMP signaling is up-regulated in response to injury, we hypothesized that BMP signaling mutant clones contained more cells because they were located in a region exposed to local injury during the course of the experiment. We next asked whether widespread injury caused by bleomycin would lead to further increases in the number of cells in mutant clones over an 8-d time course. We induced WT and mutant clones as before, injured the gut by feeding flies bleomycin for 24 h between 4 and 5 d ACI, and determined the number of cells per clone 3 d later (8 d ACI). As expected, the number of cells per WT clone increased; however, mutant clones (Fig. 3, D–E) had more cells than both WT clones (Fig. 3, C, C’ and E) and mutant clones before injury (Fig. 3, B, B’, and E).

We next used various cell markers to determine whether ISC daughter fate was affected in mutant clones. By 5 d ACI, WT clones typically contain one DIl-positive stem cell and at least one EC (Fig. 3 F). At 5 d ACI, tkv	extsuperscript{ts} clones contained a stem cell (DI antibody), enterocendocrine cells (Propero antibody), and ECs (polyploid nuclei; Fig. 3 G), and Mad	extsuperscript{ts} clones contained cells positive for the EC marker Pdm-1 (Fig. 3 H; Lee et al., 2009), suggesting that BMP signaling is not required for midgut epithelial cell differentiation. tkv	extsuperscript{ts} and Mad	extsuperscript{ts} clones also occasionally contained TUNEL-positive cells (Fig. 3 I and Fig. S3 E), suggesting that BMP signaling is not required for apoptosis.

Because BMP signaling reporters can be detected after injury in ISCs and ECs, we considered two possibilities to explain in which cell type BMP signaling acts to regulate the number of cells per ISC clone: (1) BMP signaling is autonomously required in ISCs to limit ISC activity, and (2) BMP signaling in ECs regulates the production of secreted factors that then nonautonomously regulate ISC activity. To determine between these two possibilities, we used cell-specific Gal4 drivers to express tkv and Mad RNAi and examined the effect on PH3	extsuperscript{*} number in the posterior midgut. RNAi knockdown of Mad or tkv in ISCs and EBs using esg	extsuperscript{ts} (Fig. 3, J–L) or the ISC driver Di-Gal4 (Fig. 3 L and Fig. S3, F–F’) at 30°C for 8 d led to a dramatic increase in PH3-positive cell number in the posterior midgut compared with sibling controls (Fig. 3 L), demonstrating that BMP signaling acts autonomously in ISCs to regulate midgut homeostasis. Strikingly, although we found that RNAi knockdown of Mad or tkv in ECs by Myo1A	extsuperscript{ts} (Jiang et al., 2009) decreased Dad–LacZ and pMad expression in ECs after 8 d (Fig. S3, G–H’ and J–K’), it had no effect on PH3	extsuperscript{*} number (Fig. 3 L and Fig. S3 L), further demonstrating that BMP signaling acts autonomously in ISCs to regulate their proliferation rate.
Figure 3. BMP signaling negatively regulates midgut homeostasis. (A and B) Control and tkv\(^{8}\) clones 8 d after clone induction (ACI), in the posterior midgut. (C and D) WT and tkv\(^{8}\) clones 8 d ACI, in the posterior midgut from animals fed bleomycin for 24 h between 4 and 5 d ACI. (E) Quantification of ISC clone size (number of cells per ISC clone) in posterior midguts of indicated genotypes. The data shown are from a single representative experiment out of four repeats. Each dot corresponds to one ISC clone. 8 d ACI, \(n = 82\) (40A, five guts), 82 (tkv\(^{8}\), six guts), 108 (tkv\(^{4}\), seven guts), and 74 (Mad\(^{12}\), six guts). 8 d ACI, 3 d after bleomycin, \(n = 67\) (40A, four guts), 111 (tkv\(^{8}\), six guts), 113 (tkv\(^{4}\), eight guts), and 127 (Mad\(^{12}\), five guts). The black bars indicate mean value. ***, \(P < 0.0001\), by one-way ANOVA test. (F) At 5 d ACI, a control MARCM clone contains one stem cell (DI staining, arrow) and at least one EC (arrowhead). (G) A tkv\(^{8}\) clone contains a stem cell (DI staining, arrow), enteroendocrine (ee) cells (Prospero staining), and ECs (arrowheads). (H) Mad\(^{12}\)
bleomycin, Dad^{212} clones had fewer cells than WT clones (Fig. 4, J–L and R, green dots) 24, 48, and 72 h after bleomycin treatment. UAS-tkv^{CA} clones also had fewer cells than WT clones (Fig. 4, O, P, and R, purple dots) 48 and 72 h after bleomycin treatment. Together, these gain-of-function experiments suggest that activation of the BMP signaling pathway antagonizes the response of ISCs to injury.

BMP signaling regulates both ISC number and the rate of ISC division

Increased cell number in BMP signaling mutant ISC clones could be caused by an increase in stem cell number per clone and/or an increased rate of stem cell division. The only known cell to divide in the midgut is the ISC. Therefore, a PH3-positive cell should indicate an ISC in mitosis. However, because mitosis
represents a small window of the cell cycle, it is difficult to identify clones that contain a PH3+ cell. We therefore used colcemid, a microtubule-depolymerizing drug that disrupts the mitotic spindle, thereby arresting dividing cells in metaphase. ISC divisions. We generated WT, tkvΔ, and Mad12 clones, fed animals between 8 and 9 d ACI, and counted PH3+ cell number per clone 9 d ACI (Fig. 5, A–C). WT clones contained on average 0.53 PH3+ cells per clone, whereas tkvΔ and Mad12 clones contained on average 1.83 and 1.84 PH3+ cells per clone, respectively (Fig. 5 D), suggesting that mutant clones contain more than one ISC.

In further support of our observation that mutant clones contained more than one ISC, we generated WT, tkvΔ, and Mad12 clones and counted the number of ISCs and the total number of cells in each clone. At 3 d ACI, 67 of 69 WT clones contained one stem cell (Fig. 5, E–E″), whereas 11 of 45 tkvΔ already contained more than one stem cell (Fig. 5 H). At 9 d ACI, mean stem cell number per clone was 1.03:1.29:1.33 (WT/tkvΔ/Mad12). At 9 d ACI, mean stem cell number per clone was 1.06:1.76:1.98 (WT/tkvΔ/Mad12). (I) Quantification of clone size in WT, tkvΔ, and Mad12 clones at 3 d ACI or 9 d ACI. The black bars indicate mean values. (E–E‴) A WT clone with one ISC, 3 d ACI. (F–F‴) tkvΔ clones at 3 d ACI with one (F) or two ISCs (G). Arrows show ISCs; arrowheads show EBs. (H) Quantification of stem cell number in WT, tkvΔ, and Mad12 clones 3 d ACI or 9 d ACI. At 3 d ACI, mean stem cell number per clone was 2.68:4.06:3.79 (WT/tkvΔ/Mad12), and at 9 d ACI, it was 7.81:10.03:13.94 (WT/tkvΔ/Mad12). Data are represented as means ± SEM. **, P < 0.001; ***, P < 0.0001, by one-way ANOVA test. Bars, 10 µm.
of 42 Mad\textsuperscript{12} clones contained more than one stem cell (Fig. 5 H), whereas 32 of 34 WT clones still contained one stem cell (Fig. 5 H), demonstrating that loss of BMP signaling results in a gradual increase in ISC number over time.

To determine whether ISCs divide more often in mutant clones than in WT clones, we compared the total number of cells per clone from one-stem-cell WT, one-stem-cell tk\textsuperscript{v}, and one-stem-cell Mad\textsuperscript{12} clones. One-stem-cell tk\textsuperscript{v} clones (n = 34, 3 d ACI; or n = 32, 9 d ACI) and one-stem-cell Mad\textsuperscript{12} clones (n = 28, 3 d ACI; or n = 16, 9 d ACI) contained more cells than WT clones (n = 67, 3 d ACI; or n = 32, 9 d ACI; Fig. 5 I), indicating that BMP mutant ISCs divide more often than WT ISCs. Thus, our data suggest that mutant clone growth results from increases in ISC proliferation and ISC number.

**Loss of BMP signaling-induced ISC proliferation requires EGFR signaling**

JAK-STAT and EGFR signaling are activated in ISCs after tissue injury or bacterial infection (Buchon et al., 2009; Jiang et al., 2009, 2011; Li et al., 2013), we found that ectopic expression of UAS-JAK-STAT signaling is not required for loss of BMP signaling (Fig. 6, J). Similarly results were obtained for both dpp-RNAi and mad\textsuperscript{12}; UAS-Ras\textsuperscript{DN} clones after bleomycin treatment (Fig. 6 J). Therefore, active EGFR signaling is required for loss of BMP signaling–induced ISC proliferation.

To block JAK-STAT signaling, we made MARCM clones expressing an RNAi against Stat92E. Consistent with studies that JAK-STAT signaling is required for EC differentiation (Jiang et al., 2009; Beebe et al., 2010), expression of Stat92E RNAi in MARCM clones resulted in clones lacking ECs (Fig. 6, A and D). At 8 d ACI, Stat92E RNAi (Fig. 6 A) clones were significantly smaller than BMP signaling mutant clones (Fig. 6, B and J). However, although ectopic expression of Stat92E RNAi in Mad\textsuperscript{12} ISC MARCM clones (Fig. 6 C) blocked EC differentiation, it had no effect on clone cell number (Fig. 6 J), suggesting that increases in cell number seen with BMP signaling mutant clones do not require JAK-STAT signaling. Because JAK-STAT signaling is sufficient but not required for ISC proliferation (Jiang et al., 2009; Beebe et al., 2010), we next examined the effect of bleomycin treatment, a potent inducer of JAK-STAT signaling (Jiang et al., 2009; Beebe et al., 2010), on the growth of BMP signaling and JAK-STAT signaling double mutant clones. At 8 d ACI, 3 d after bleomycin, both Mad\textsuperscript{12} and Mad\textsuperscript{12}; UAS-Stat92E RNAi clones (Fig. 6, E, F, and J) were larger than Stat92E RNAi clones (Fig. 6, D and J). These epistasis experiments demonstrate that JAK-STAT signaling is not required for loss of BMP signaling–induced ISC proliferation.

To block EGFR signaling, we made clones expressing UAS-Ras\textsuperscript{DN}. Consistent with previous studies (Biteau and Jasper, 2011; Jiang et al., 2011), we found that ectopic expression of Ras\textsuperscript{DN} (Ras\textsuperscript{DN}) dramatically inhibited the clone growth (Fig. 6 G). Expression of Ras\textsuperscript{DN} in tk\textsuperscript{v} or Mad\textsuperscript{12} clones also dramatically decreased BMP signaling mutant clone sizes (Fig. 6, H, I, and J). Similar results were obtained for both tk\textsuperscript{v}; UAS-Ras\textsuperscript{DN} and Mad\textsuperscript{12}; UAS-Ras\textsuperscript{DN} clones before bleomycin treatment (Fig. 6 J). Thus, active EGFR signaling is required for loss of BMP signaling–induced ISC proliferation.

**Injury-induced Drosophila midgut homeostasis**

In Drosophila, three ligands, dpp, screw, and glass bottom boat, are known to activate the BMP signaling pathway (Wharton and Derynck, 2009). Real-time quantitative PCR (RT-qPCR) from total midgut RNA revealed that dpp is expressed in the midgut and is up-regulated along with Dad (Tsuneizumi et al., 1997), a target of BMP signaling, and upd\textsuperscript{3}, a marker of damaged cells (Jiang et al., 2009), by bleomycin treatment, suggesting that it might promote injury-induced midgut BMP signaling (Fig. 7 A). Recently, by in situ hybridization analysis and lacZ staining of a P element enhancer trap in the dpp gene (dpp\textsuperscript{10638}; Jiang and Struhl, 1995), Li et al. (2013) reported that dpp is expressed in the trachea of the midgut, where it acts to regulate EC viability and midgut homeostasis. Because dpp levels increase after injury (Fig. 7 A), we examined the expression of lacZ in dpp\textsuperscript{10638} flies fed paraquat. Consistent with Li et al. (2013), we found that lacZ was strongly expressed in midgut trachea. However, as a new finding, lacZ was detected in circular muscle (Fig. S5, A and A′). In addition, we were able to identify by in situ hybridization the expression of dpp in circular muscle (Fig. S5 B), suggesting that midgut vm might be a source of functionally relevant dpp.

To gain further insight into the relevant midgut expression pattern of dpp, we screened a collection of 12 putative dpp enhancer-Gal4 lines available from the Bloomington Stock Center (FlyBase; Pfeiffer et al., 2008). Two of these Gal4 lines containing ~1-kb overlapping DNA sequences (Fig. S5 C) drove UAS-GFP expression in midgut circular muscle (Fig. 7 B), whereas the remaining 10 Gal4 lines failed to drive expression (not depicted). Of the two lines, one has a stronger expression pattern, which we refer to as dpp-Gal4\textsuperscript{49a} (dpp\textsuperscript{em}). Both Gal4 lines drive expression of UAS-GFP in terminal filament and cap cells in the Drosophila germarium (Fig. S5 D). The terminal filament and cap cells are the proposed cellular source of Dpp that maintains female germline stem cells (Xie and Spradling, 1998; Guo and Wang, 2009), suggesting that these Gal4 lines are driven by dpp enhancer sequences. Several other pieces of data indicate that dpp-Gal4\textsuperscript{49a} UAS-GFP (dpp\textsuperscript{em}>GFP) represents part of the endogenous midgut dpp expression pattern: dpp\textsuperscript{em}>GFP expression is strong in circular muscle of the CCR yet highly variable in the circular muscle of the anterior and posterior midgut (Fig. 7 B). dpp\textsuperscript{em}>GFP expression becomes broadly expanded in circular muscle along the midgut after 24 h of bleomycin treatment (Fig. 7 C), consistent with increases in dpp RNA levels seen after bleomycin treatment (Fig. 7 A).

To identify which cellular source of Dpp is functionally relevant, we used cell-specific drivers and UAS-dpp RNAi to knockdown dpp expression and examined the effect on Labial and pMad expression. Expression of dpp-RNAi by the temperature-inducible vm-specific driver how-Gal4 UAS-GFP; tub-Gal80\textsuperscript{o} for 8 d at the permissive temperature eliminated pMad staining in the CCR and anterior midgut (Fig. S5 E). However, in contrast to claims that how-Gal4 is muscle specific in the adult (Jiang et al., 2009, 2011; Li et al., 2013), we found that how-Gal4 UAS-GFP is expressed in both vm and trachea (Fig. S5 F), an expression pattern identical to that reported in the larval midgut.
Given the lack of specific vm expression by how-Gal4, we used *dpp*-Gal4 to express *dpp* RNAi in vm. Expression of *dpp*-RNAi by *dpp*-Gal4 dramatically decreased pMad and Labial staining in the CCR (Fig. 7, D–E); and Fig. S5, G and H). In a screen of a collection of Gal4 lines generated by Pfeiffer et al. (2008), we identified another vm driver, *vm*-Gal4 (Fig. S5 I). Expression of *dpp*-RNAi by *dpp*-Gal4 also dramatically decreased pMad (Fig. 7, F and F’); and Labial (Fig. S5 J) staining in the midgut, further demonstrating that Dpp is required in vm to initiate and maintain BMP signaling in the intestine.

By heat shock–inducible flippase induction (See Materials and methods), conversion of *tub*-flippase recognition target (FRT)-Gal80-FRT, *vm*-Gal4 to *tub*-FRT, *vm*-Gal4 results in Gal4 expression in circular muscle in a mosaic pattern. We used this convertible *tub*-FRT-Gal80-FRT, *vm*-Gal4 system (referred to as *vm*-flp) to express UAS-GFP and UAS-*dpp* RNAi (*vm*-flp > *dpp* RNAi; Fig. 7 G) in subsets of vm. Only in epithelial regions adjacent to *vm*-flp-positive circular muscle was pMad staining absent (Fig. 7, H–H’). In contrast to claims by Li et al. (2013), expression of *dpp* RNAi in trachea using *btl*-Gal4 (Fig. S5, K–L’) or 14D03-Gal4 (Fig. S5 M) had no effect on
Figure 7. **Dpp is required in vm to regulate midgut homeostasis.** (A) RT-qPCR demonstrates that bleomycin-induced injury increases levels of upd3, dpp, and Dad mRNA in the midgut. Data are represented as means ± SEM. *, P < 0.01, by Student’s t test. (B) dpp-Gal4,UAS-GFP expression is strongest in circular muscle associated with the copper cell region (CCR); however, regions of weaker expression are present in anterior and posterior midgut circular muscle. (C) 24 h after treatment with bleomycin, dpp-Gal4,UAS-GFP becomes strongly expressed in circular muscle throughout most of the midgut. (D and D’) pMad staining in the CCR of dpp-Gal4,UAS-GFP. (E and E’) Expression of dpp RNAi in circular muscle by dpp-Gal4 results in decreased pMad staining in the CCR. (F and F’) Expression of dpp RNAi in midgut vm by vm-Gal4 results in decreased pMad staining in the CCR. (G) Diagram describing generation of mosaic expression of dpp RNAi in vm. (H–H’) 8 d after heat shock and 24 h after bleomycin treatment, GFP and dpp RNAi are expressed in a subset of circular muscle bands in the posterior midgut. Note that pMad staining is absent in regions adjacent to GFP-positive vm (brackets). (I) Quantification of PH3+ cells in posterior midguts from the indicated genotypes. dpp RNAi was driven at 30°C for 8 d. The data shown are from a single representative experiment out of three repeats. Each dot corresponds to one midgut. The number of guts from left to right are as follows: 10, 9, 11, 10, and 18. **, P < 0.001, by one-way ANOVA test. Bars: (B and C) 100 µm; (D–F and H) 10 µm.
pMad staining or PH3+ number (Fig. 7 I). Similarly, expression of dpp RNAi in ECs using Myo1A-Gal4 (Fig. S5 N) or in midgut progenitors using esg-Gal4 (Fig. S5 O) had no effect on pMad staining or PH3+ number (Fig. 7 I). Knockdown of dpp by vm-Gal4 for 8 d, however, resulted in a dramatic increase in PH3+ cell number as compared with vm-Gal4 driving expression of GFP only (Fig. 7 I). Together, our data demonstrate that muscle-derived Dpp is the functionally relevant source of ligand that acts locally to initiate and maintain BMP signaling.

**JAK-STAT signaling can induce BMP signaling in midgut epithelium**

Because midgut injury induces JAK-STAT ligand expression in ECs (Jiang et al., 2009) and induces pMad and Dad-lacZ expression in midgut epithelia, we asked whether ectopic activation of the JAK-STAT signaling pathway alone could induce BMP signaling in midgut epithelium. Driving expression of UAS-unpaired (upd) in ECs using Myo1Ats (Jiang et al., 2009) led to a dramatic increase in the expression of pMad in the midgut, suggesting that the injury-induced JAK-STAT signaling activates BMP ligand expression (Fig. 8, A and A'). Consistent with this model, ectopic activation of JAK-STAT signaling in circular muscle by dpp>hopTum (a constitutively active form of the Drosophila JAK; Yan et al., 1996) resulted in pMad staining in midgut epithelium (Fig. 8 B).

Because ectopic expression of upd results in increased ISC proliferation, we considered the possibility that dividing ISCs were the source of signals that induce Dpp in the muscle after injury. Recently, it has been shown that upd is required for ISC maintenance (Osman et al., 2012). We reasoned that if ISCs were required for the induction of Dpp, we would fail to see activation of BMP signaling in the midgut after bleomycin treatment in intestines lacking ISCs. Although expressing upd RNAi in ISCs and EBs, using esgts, led to ISC loss in the anterior midgut, it had no effect on pMad staining in ECs after injury (Fig. 8, C and C'), suggesting that ECs, and not ISCs, are the source of signals that induce Dpp in vm (Fig. 8 D).
Discussion

To understand the active to quiescent transition of ISC proliferation after injury, we examined the role of the BMP signaling pathway in the *Drosophila* adult midgut. Midgut injury induces expression of JAK-STAT signaling ligands and EGFR signaling ligands in ECs. These ligands act directly on ISCs to increase their rate of proliferation and on vm to up-regulate Vein expression, which also acts on ISCs to promote stem cell division (Lucchetta and Ohlstein, 2012). Concomitantly, injury induces expression of Dpp in vm. Dpp constrains stem cell number and stem cell proliferation rate by directly activating BMP signaling in ISCs (Fig. 8 D). Thus, to meet the local needs of the midgut, local injury-induced BMP signaling, in conjunction with mitotic signals, modulates the ISC response to ensure the proper balance of signals to achieve an optimal response to injury.

Recently, Li et al. (2013) characterized the role of BMP signaling in the *Drosophila* adult midgut and reported that loss of BMP signaling in ECs results in widespread cell death, secretion of JAK-STAT and EGFR signaling ligands by dying cells, and a subsequent nonautonomous increase in ISC proliferation. However, using the same *M. Markstein*, University of Massachusetts, Amherst, MA), *Su(H) binding sites from the E(spl) gene are fused upstream of a minimal enhancer region-GAL4 fusion and a UAS-GFP reporter; obtained from N. Perrimon, Harvard Institute of Biological Science, Beijing, China; Tsuneizumi et al., 1997; Zhao et al., 2006), *NRE-lacZ* [a lacZ transgene inserted into the *Drosophila* midgut homeostasis and increased cellular proliferation (Roth and Helwig, 1963; Haramis et al., 2004). Although BMP signaling ligands suppress sporadic colorectal cancer growth (Hardwick et al., 2004; Beck et al., 2006; Loh et al., 2008), and 70% of colorectal cancers fail to stain positive for various pSMads (Kodach et al., 2008), mutants in BMP signaling components have not been implicated in the initiation of intestinal adenomas (Hardwick et al., 2008). Based on our data, we would argue that BMP signaling acts as a modulator of the injury response. In states of high injury, BMP signaling loss would lead to an increase in stem cells, resulting in de novo crypt formation and hamartomas. As ISC number increases, so would the number of target cells available to acquire mutations in genes implicated in intestinal cancer, such as *APC* (Adenomatous polyposis coli) and *Kras*, thereby hastening the development of hamartomas into adenomas (Hardwick et al., 2008).

Materials and methods

Fly genetics

All *Drosophila* experimental stocks and crosses (except for RNAi ectopic expression) were cultured with daily changes of cornmeal food (referred to as control food) without live yeast at 23–25°C. Information regarding nomenclature and other relevant information on stocks used can be found at FlyBase.

Mutant and WT controls

Mutant and WT controls were *UAS-GFP* (an amorphic allele with amino acid replacement C144@; the predicted product terminates immediately N-terminal to the conserved cysteine cluster in the extracellular domain; obtained from A. Spradling, Carnegie Institution of Washington, Washington, DC; Xie and Spradling, 1998), *tkv* FRT 40A (an amorphic allele with amino acid replacement W476@; obtained from R. Mann, Columbia University, New York, NY; Nellen et al., 1994), *Mad* FRT 40A (an amorphic allele with amino acid replacement Q417@; obtained from G. Struhl, Columbia University, New York, NY; Xie and Spradling, 1998), *FRT82B pun* FRT 40A (amorphic allele replacement A376f; the point mutation is within the kinase domain; obtained from T. Xie, Stowers Institute for Medical Research, Kansas City, MO; Xie and Spradling, 1998), *FRT82B Dpp* FRT 40A (a genetic null allele, in which an imprecise excision has generated a deletion that has removed three exons from the *Dad* gene, corresponding to amino acids 1–391; obtained from T. Tabata, University of Tokyo, Japan; Ogiso et al., 2011), *FRT82B ry* FRT 40A (Bloomington Stock Center [Bloomington, IN], and *FRT40A* (BL#1835).

Reporters and Gal4 drivers

Reporters and drivers used were *DadlacZ* [a lacZ transgene inserted into *Dad* that acts as a reporter of Dad expression; obtained from R. Xi, National Institute of Biological Science, Beijing, China; Tsuneizumi et al., 1997; Zhao et al., 2008], *upd3-Gal4 UAS-GFP*; *CyO* (a upd3 enhancer region-GAL4 fusion and a UAS-GFP reporter; obtained from N. Perrimon, Harvard Medical School, Boston, MA; Agassie et al., 2003), *Gbe Su(H)lacZ* [a putative *D. melanogaster* enhancer-GAL4 fusion; the point mutation is within the kinase domain; obtained from T. Xie, Stowers Institute for Medical Research, Kansas City, MO; Xie and Spradling, 1998], *FRT82B Dad* FRT 40A (a genetic null allele, in which an imprecise excision has generated a deletion that has removed three exons from the *Dad* gene, corresponding to amino acids 1–391; obtained from T. Tabata, University of Tokyo, Japan; Ogiso et al., 2011), *FRT82B ry* FRT 40A (Bloomington Stock Center [Bloomington, IN], and *FRT40A* (BL#1835).

Discussion

To understand the active to quiescent transition of ISC proliferation after injury, we examined the role of the BMP signaling pathway in the *Drosophila* adult intestine. Midgut injury induces expression of JAK-STAT signaling ligands and EGFR signaling ligands in ECs. These ligands act directly on ISCs to increase their rate of proliferation and on vm to up-regulate Vein expression, which also acts on ISCs to promote stem cell division (Lucchetta and Ohlstein, 2012). Concomitantly, injury induces expression of Dpp in vm. Dpp constrains stem cell number and stem cell proliferation rate by directly activating BMP signaling in ISCs (Fig. 8 D). Thus, to meet the local needs of the midgut, local injury-induced BMP signaling, in conjunction with mitotic signals, modulates the ISC response to ensure the proper balance of signals to achieve an optimal response to injury.

Recently, Li et al. (2013) characterized the role of BMP signaling in the *Drosophila* adult midgut and reported that loss of BMP signaling in ECs results in widespread cell death, secretion of JAK-STAT and EGFR signaling ligands by dying cells, and a subsequent nonautonomous increase in ISC proliferation. However, using the same *M. Markstein*, University of Massachusetts, Amherst, MA), *Su(H) binding sites from the E(spl) gene are fused upstream of a minimal enhancer region-GAL4 fusion and a UAS-GFP reporter; obtained from N. Perrimon, Harvard Institute of Biological Science, Beijing, China; Tsuneizumi et al., 1997; Zhao et al., 2006), *NRE-lacZ* [a lacZ transgene inserted into the *Drosophila* midgut homeostasis and increased cellular proliferation (Roth and Helwig, 1963; Haramis et al., 2004). Although BMP signaling ligands suppress sporadic colorectal cancer growth (Hardwick et al., 2004; Beck et al., 2006; Loh et al., 2008), and 70% of colorectal cancers fail to stain positive for various pSMads (Kodach et al., 2008), mutants in BMP signaling components have not been implicated in the initiation of intestinal adenomas (Hardwick et al., 2008). Based on our data, we would argue that BMP signaling acts as a modulator of the injury response. In states of high injury, BMP signaling loss would lead to an increase in stem cells, resulting in de novo crypt formation and hamartomas. As ISC number increases, so would the number of target cells available to acquire mutations in genes implicated in intestinal cancer, such as *APC* (Adenomatous polyposis coli) and *Kras*, thereby hastening the development of hamartomas into adenomas (Hardwick et al., 2008).

Materials and methods

Fly genetics

All *Drosophila* experimental stocks and crosses (except for RNAi ectopic expression) were cultured with daily changes of cornmeal food (referred to as control food) without live yeast at 23–25°C. Information regarding nomenclature and other relevant information on stocks used can be found at FlyBase.

Mutant and WT controls

Mutant and WT controls were *UAS-GFP* (an amorphic allele with amino acid replacement C144@; the predicted product terminates immediately N-terminal to the conserved cysteine cluster in the extracellular domain; obtained from A. Spradling, Carnegie Institution of Washington, Washington, DC; Xie and Spradling, 1998), *tkv* FRT 40A (an amorphic allele with amino acid replacement W476@; obtained from R. Mann, Columbia University, New York, NY; Nellen et al., 1994), *Mad* FRT 40A (an amorphic allele with amino acid replacement Q417@; obtained from G. Struhl, Columbia University, New York, NY; Xie and Spradling, 1998), *FRT82B pun* FRT 40A (amorphic allele replacement A376f; the point mutation is within the kinase domain; obtained from T. Xie, Stowers Institute for Medical Research, Kansas City, MO; Xie and Spradling, 1998), *FRT82B Dpp* FRT 40A (a genetic null allele, in which an imprecise excision has generated a deletion that has removed three exons from the *Dad* gene, corresponding to amino acids 1–391; obtained from T. Tabata, University of Tokyo, Japan; Ogiso et al., 2011), *FRT82B ry* FRT 40A (Bloomington Stock Center [Bloomington, IN], and *FRT40A* (BL#1835).

Reporters and Gal4 drivers

Reporters and drivers used were *DadlacZ* [a lacZ transgene inserted into *Dad* that acts as a reporter of Dad expression; obtained from R. Xi, National Institute of Biological Science, Beijing, China; Tsuneizumi et al., 1997; Zhao et al., 2008], *upd3-Gal4 UAS-GFP*; *CyO* (a upd3 enhancer region-GAL4 fusion and a UAS-GFP reporter; obtained from N. Perrimon, Harvard Medical School, Boston, MA; Agassie et al., 2003), *Gbe Su(H)lacZ* [a putative *D. melanogaster* enhancer-GAL4 fusion; the point mutation is within the kinase domain; obtained from T. Xie, Stowers Institute for Medical Research, Kansas City, MO; Xie and Spradling, 1998], *FRT82B Dad* FRT 40A (a genetic null allele, in which an imprecise excision has generated a deletion that has removed three exons from the *Dad* gene, corresponding to amino acids 1–391; obtained from T. Tabata, University of Tokyo, Japan; Ogiso et al., 2011), *FRT82B ry* FRT 40A (Bloomington Stock Center [Bloomington, IN], and *FRT40A* (BL#1835).
bf-Gal4 UAS-GFP (a btf promoter region-Gal4 fusion and a UAS-GFP reporter), vm-Gal4 (a putative hairy enhancer Gal4; BL#48547; tub-gal80-STOP obtained from K. Scott, University of California, Berkeley, CA), tub-gal80-STOP; UAS-GFP, vm-Gal4, dpp-Gal4 

5' - primers forward, 5' A 924-bp fragment of the

To induce ISC MARCM clones, 3-day-old adult female flies were heat shocked and emasculated at 37°C for 40 min and then transferred daily to new cornmeal food without live yeast. GFP- or RFP-labeled mutants were present on a chromosome containing FRT82B, to knockdown BMP signaling during embryo or larval stage usually causes lethality before fly eclosion. Midgut drivers combined with temperature-inducible tub-Gal80° (Jiang et al., 2009) were used to selectively ectopically express Mad, tkv, and dpp RNAi in Drosophila adults as described in Fig. 1 (H and I), Fig. 3 (J-L), Fig. 8 (A and C), Fig. 10 (B-D and G), Fig. 13 (G–K), and Fig. S5 (F and K–O). Flies were cultured at 18°C during development, and then adult female flies were shifted to 30°C to induce ectopic expression of RNAi transgenes. To eliminate copper cells as described in Fig. 1, flies were cultured at room temperature, and then, female adult flies were shifted to 30°C to induce ectopic expression of RNAi transgenes. Mosaic guts were analyzed at various time points after temperature shifts as described in the Results or figure legends.

We used the following criteria to identify stem cells: Dl-positive, NRE-lacZ-negative, and Prospero-negative diploid cells. NRE-lacZ expression (Choi et al., 2011) was used to identify EBs. Prospero was used to identify enteroendocrine cells (Mitchell and Perrimon, 2006; Ohi stein and Spradling, 2006), and nuclear size (Ohi stein and Spradling, 2006) was used to identify ECs.

Bromophenol blue dye feeding

A 1-mL solution of 0.15% bromophenol blue was added to 0.5 g of dry yeast resulting in a yeast paste. Animals were fed this yeast paste for 5–8 h, and midguts were dissected and analyzed as described in Fig. 1 (H and I) and Fig. S1 G.

Bleomycin, pararaport treatment, and ECC15 infection

Knocking down BMP signaling during embryonic or larval stage usually causes lethality before fly eclosion. Midgut drivers combined with temperature-inducible tub-Gal80° (Jiang et al., 2009) were used to selectively ectopically express Mad, tkv, and dpp RNAi in Drosophila adults as described in Fig. 1 (H and I), Fig. 3 (J-L), Fig. 8 (A and C), Fig. 10 (B-D and G), Fig. 13 (G–K), and Fig. S5 (F and K–O). Flies were cultured at 18°C during development, and then adult female flies were shifted to 30°C to induce ectopic expression of RNAi transgenes. To eliminate copper cells as described in Fig. 1, flies were cultured at room temperature, and then, female adult flies were shifted to 30°C to induce ectopic expression of RNAi transgenes. Mosaic guts were analyzed at various time points after temperature shifts as described in the Results or figure legends.

Cell identification in mutant clones

We used the following criteria to identify stem cells: Dl-positive, NRE-lacZ-negative, and Prospero-negative diploid cells. NRE-lacZ expression (Choi et al., 2011) was used to identify EBs. Prospero was used to identify enteroendocrine cells (Mitchell and Perrimon, 2006; Ohi stein and Spradling, 2006), and nuclear size (Ohi stein and Spradling, 2006) was used to identify ECs.

Colcemid treatment

Bromophenol blue dye feeding

A 1-mL solution of 0.15% bromophenol blue was added to 0.5 g of dry yeast resulting in a yeast paste. Animals were fed this yeast paste for 5–8 h, and midguts were dissected and analyzed as described in Fig. 1 (H and I) and Fig. S1 G.

Bleomycin, pararaport treatment, and ECC15 infection

Knocking down BMP signaling during embryonic or larval stage usually causes lethality before fly eclosion. Midgut drivers combined with temperature-inducible tub-Gal80° (Jiang et al., 2009) were used to selectively ectopically express Mad, tkv, and dpp RNAi in Drosophila adults as described in Fig. 1 (H and I), Fig. 3 (J-L), Fig. 8 (A and C), Fig. S1 (B-D and G), Fig. S3 (G–K), and Fig. S5 (F and K–O). Flies were cultured at 18°C during development, and then, female adult flies were shifted to 30°C to induce ectopic expression of RNAi transgenes. To eliminate copper cells as described in Fig. 1, flies were cultured at room temperature, and then, female adult flies were shifted to 30°C to induce ectopic expression of RNAi transgenes. Mosaic guts were analyzed at various time points after temperature shifts as described in the Results or figure legends.

Ablation of ISCs in the anterior midgut

Cells excised in a subset of muscle cells, leading to mosaic expression of UAS-GFP, vm-Gal4/ FRT40A MARCM flies were transferred into vials with new cornmeal food. 48 h after treatment, flies were dissected and stained with anti–phospho-histone H3 antibody.

Mosaic expression of dpp RNAi in muscle

Flies with the genotype tub-FRT-Gal80/FRT; hs-flp; UAS-GFP; vm-Gal4/ UAS-dpp RNAi were dissected and reared on cornmeal food at 25°C. The expression of Gal80 by the tub promoter prevents expression of the vm-Gal4 driver, which is expressed in vm. Exposure of flies to heat shock temperatures leads to excision of the Gal80 cassette, which results in vm-Gal4 expression. Depending on the length of heat shock, the Gal80 cassette will excise in a subset of muscle cells, leading to mosaic expression of UAS-dpp RNAi. 3 d after eclosion, mosaic expression was induced by a 30-min heat shock at 37°C in a water bath. Flies were then transferred to cornmeal food and reared at 25°C. 24 h after induction, GFP-labeled mosaic ectopic expression of dpp RNAi in muscle was analyzed by staining for pMad and GFP.

dpp mRNA in situ by FISH

48 single-labeled oligonucleotides designed to selectively bind to dpp mRNA transcripts were purchased from Biosearch Technologies (Custom
Flies were used as RT-qPCR sample flies. For each RT-qPCR, 20 newly eclosed female flies were collected for 2 days, aged for an additional 3 days, and then equally split into two groups. One group of flies was fed 25 mg/ml bleomycin, and the other group was transferred into commercial food as a control. After 24 h of treatment, midguts were dissected from flies and kept in Schneider’s media with 10% FBS before RNA extraction.

RT-qPCR

esg-Gal4 UAS-GFP flies were used as RT-qPCR sample flies. For each RT-qPCR, 60 newly eclosed female flies were collected for 2 days, aged for an additional 3 days, and then equally split into two groups. One group of flies was fed 25 mg/ml bleomycin, and the other group was transferred into commercial food as a control. After 24 h of treatment, midguts were dissected from flies and kept in Schneider’s media with 10% FBS before RNA extraction.

RNA preparation was repeated three times. u03d3, dpp, and Dad mRNA expression levels were normalized against mRNA levels of Rp49 for each cDNA sample. mRNA expression levels were normalized against mRNA levels of Rp49 for each cDNA sample.

Statistical analysis

MARCM clone sizes and Ph3+ cell number were plotted as individual values. Error bars in Fig. 5 (H and I) and Fig. 7 A are SEM. Statistics were performed using one-way analysis of variance (ANOVA) test on Prism (GraphPad Software). Significance was accepted at *, P < 0.01; **, P < 0.001; and ***, P < 0.0001.

Online supplemental material

Fig. S1 shows coexpression of Dad-lacZ and the endorecodominant marker prospero and that BMP signaling is required in progenitors for copper cell differentiation. Fig. S2 shows up-regulation of Dad-lacZ expression after paraquat and bleomycin treatment. Fig. S3 shows loss of pMad and Dad-lacZ expression in BMP mutant clones in the anterior and posterior midgut, TUNEL labeling in Mad mutant clones, DsRed4 expression the CCR, and the loss of pMad and Dad-lacZ expression after knockdown of BMP signaling in ECs. Fig. S4 shows up-regulation of pMad expression in cells in which BMP signaling is constitutively active and a diagram outlining the experimental approach to determine the effect of loss of BMP signaling and activation of BMP signaling on clone growth before and after injury. Fig. S5 shows that Dpp is required in vm to regulate midgut homeostasis. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.201302049/DC1. Additional data are available in the JCB Data Viewer at http://dx.doi.org/10.1083/jcb.201302049.dv.

The authors would like to thank the following for providing reagents: A. Spradling, E. Laufer, R. Mann, G. Struhl, T. Tabata, R. Xi, N. Perrimon, B. Edgar, T. Xie, M. Markstein, K. Irvine, and K. Scott.

I. Driver was supported in part by 5T32HD051565-01. This work was supported by National Institutes of Health grant RO1 DK082456-01 to B. Ohlstein.

Submitted: 11 February 2013
Accepted: 29 April 2013

References

Adachi-Yamada, T., M. Nakamura, K. Irie, Y. Tomoyasu, Y. Sano, E. Mori, S. Goto, N. Ueno, Y. Nishida, and K. Matsumoto. 1999. p38 mitogen-activated protein kinase can be involved in transforming growth factor beta superfamily signal transduction in Drosophila wing morphogenesis. Mol. Cell. Biol. 19:2322–2329.

Agassie, H., U.M. Petersen, M. Boutros, B. Mathey-Prevot, and N. Perrimon. 2003. Signaling role of hemocytes in Drosophila JAK/STAT-dependent response to septic injury. Dev. Cell. 5:541–540. http://dx.doi.org/10.1016/S1547-7459(03)00162-0

Amcheslavsky, A. J., Jiang, and Y.T. Ip. 2009. Tissue damage-induced intestinal stem cell division in Drosophila. Cell Stem Cell. 4:49–61. http://dx.doi.org/10.1016/j.stem.2008.10.016

Basset, A., R.S. Khush, A. Braun, L. Gardan, F. Bocard, J.A. Hoffmann, and B. Lemaire. 2000. The phytophathogenic bacteria Erwinia carotovora infects Drosophila and activates an immune response. Proc. Natl. Acad. Sci. USA. 97:3376–3381. http://dx.doi.org/10.1073/pnas.97.7.3376

Beck, S.E., B.H. Jung, A. Fiorino, J. Gomez, E.D. Rosario, B.S. Huang, J.Y. Chow, and J.M. Carethers. 2006. Bone morphogenic protein signaling and growth suppression in colon cancer. Am. J. Physiol. Gastrointest. Liver Physiol. 291:G135–G145. http://dx.doi.org/10.1152/ajpgi.00482.2005

Beeke, K., W.C. Lee, and C.A. Miccilli. 2010. JAK/STAT signaling coordinates stem cell proliferation and multineage differentiation in the Drosophila intestinal stem cell lineage. Dev. Biol. 338:28–37. http://dx.doi.org/10.1016/j.ydbio.2010.04.015

Biteau, B., and H. Jasper. 2011. EGFR signaling regulates the proliferation of intestinal stem cells in Drosophila. Development. 138:1045–1055. http://dx.doi.org/10.1242/dev.056671

Biteau, B., C.E. Hochmuth, and H. Jasper. 2008. JNK activity in somatic stem cells causes loss of tissue homeostasis in the aging Drosophila gut. Cell Stem Cell. 3:422–455. http://dx.doi.org/10.1016/j.stem.2008.07.024

Buchon, N., N.A. Broderick, S. Chakrabarti, and B. Lemaire. 2009. Invasive and indigenous microbiota impact intestinal stem cell activity through multiple pathways in Drosophila. Genes Dev. 23:2333–2344. http://dx.doi.org/10.1101/gad.182709

Buszczak, M., S. Paterno, D. Lighthouse, J. Bachman, J. Planck, S. Owen, A.D. Skora, T.G. Nystul, B. Ohlstein, A. Allen, et al. 2007. The carnegigene protein trap library: a versatile tool for Drosophila developmental studies. Genetics. 175:1505–1531. http://dx.doi.org/10.1534/genetics.106.065961

Chatterjee, M., and Y.T. Ip. 2009. Pathogenic stimulation of intestinal stem cell response in Drosophila. J. Cell. Physiol. 220:664–671. http://dx.doi.org/10.1002/jcp.21808

Choi, N.H., J.G. Kim, D.J. Yang, Y.S. Kim, and M.A. Yoo. 2008. Age-related changes in Drosophila midgut are associated with PVF2, a PDGF/VEGF-like growth factor. Aging Cell. 7:318–334. http://dx.doi.org/10.1111/j.1474-9726.2008.00380.x

Choi, N.H., E. Luccchetta, and B. Ohlstein. 2011. Nonautonomous regulation of Drosophila midgut stem cell proliferation by the insulin-signaling pathway. Proc. Natl. Acad. Sci. USA. 108:18702–18707. http://dx.doi.org/10.1073/pnas.1109348108

Chourouard, S., and T.C. Kaufman. 1991. Control of expression of the homeotic labial (lab) locus of Drosophila melanogaster: evidence for both positive and negative autoregulatory function. Development. 113:1267–1280.

Dubreuil, R.R. 2004. Copper cells and stomach acid secretion in the Drosophila midgut. Int. J. Biochem. Cell Biol. 36:742–752. http://dx.doi.org/10.1016/j.biocel.2003.07.004

Dubreuil, R.R., J. FrankeI, P. Wang, J. Howrylak, M. Kappil, and T.A. Grushko. 1998. Mutations of alpha spectrin and labial block cuprophilic cell differentiation in Drosophila midgut. Int. J. Biochem. Cell Biol. 30:152–157.

Fisch, B.K., D.F. Poulsen, and D.F. Waterhouse. 1971. Ultrastructure of the copper-accumulating region of the Drosophila larval midgut. Tissue Cell. 3:77–102. http://dx.doi.org/10.1016/0040-4186(71)80033-2

Guo, Z., and Z. Wang. 2009. The gypican Dally is required in the niche for the maintenance of germline stem cells and short-range BMP signaling in the Drosophila ovary. Development. 136:3627–3635. http://dx.doi.org/10.1242/dev.036939

Haramis, A.P., H. Begthel, M. van den Born, J. van Es, S. Jonkheer, G.J. Offerhaus, and H. Clevvers. 2004. De novo crypt formation and juvenile polyposis on BMP inhibition in mouse intestine. Science. 303:1684–1686. http://dx.doi.org/10.1126/science.1093587

Injury-induced Drosophila midgut homeostasis • Guo et al.
Lin, G., N. Xu, and R. Xi. 2010. Paracrine unpaired signaling through the JAK/STAT pathway controls self-renewal and lineage differentiation of Drosophila intestinal stem cells. J. Mol. Cell Biol. 2:37–49. http://dx.doi.org/10.1010/jcb.200910028

Loh, K., J.A. Chia, S. Greco, S.J. Cozzi, R.L. Buttenhews, C.E. Bond, L.A. Simms, T. Pike, J.P. Young, J.K. Jass, et al. 2008. Bone morphogenic protein 3 inactivation is an early and frequent event in colorectal cancer development. Genes Chromosomes Cancer. 47:449–460. http://dx.doi.org/10.1002/gcc.20552

Lucchetta, E.M., and B. Ohlstein. 2012. The Drosophila midgut: a model for stem cell driven tissue regeneration. WIREs Dev. Biol. 1:781–788. http://dx.doi.org/10.1002/wdev.51

Miccelli, C.A., and N. Perrimon. 2006. Evidence that stem cells reside in the adult Drosophila midgut epithelium. Nature. 439:475–479. http://dx.doi.org/10.1038/nature04371

Nahmad, M., and A. Stathopoulos. 2009. Dynamic interpretation of hedgehog signaling in the Drosophila wing disc. PLoS Biol. 7:e1000202. http://dx.doi.org/10.1371/journal.pbio.1000202

Nellen, D., M. Affolter, and K. Bailer. 1994. Receptor serine/threonine kinases implicated in the control of Drosophila body patterning by decapentaplegic. Cell. 78:225–237. http://dx.doi.org/10.1016/0092-8674(94)90293-3

Ogiso, Y., K. Tsunezumi, N. Masuda, M. Sato, and T. Tabata. 2011. Robustness of the Dpp morphogen activity gradient depends on negative feedback regulation by the inhibitory Smad. Dev. Differ. Growth. 63:668–678. http://dx.doi.org/10.1111/j.1440-169X.2011.01274.x

Ohlstein, B., and A. Spradling. 2006. The adult Drosophila posterior midgut is maintained by pluripotent stem cells. Nature. 439:470–474. http://dx.doi.org/10.1038/nature04333

Ohlstein, B., and A. Spradling. 2007. Multitropism Drosophila intestinal stem cells specify daughter cell fates by differential notch signaling. Science. 315:988–992. http://dx.doi.org/10.1126/science.1136606

Osman, D., N. Buchon, S. Chakrabarti, Y.T. Huang, W.C. Su, M. Prive, Y.C. Tsai, and B. Lematte. 2012. Autocrine and paracrine unpaired signaling regulate intestinal stem cell maintenance and division. J. Cell Sci. 125:5944–5949. http://dx.doi.org/10.1242/jcs.113100

Panganiban, G., P. Scott, and F.M. Hoffmann. 1990. A Drosophila growth factor homolog, decapentaplegic, regulates homeotic gene expression within and across germ layers during midgut morphogenesis. Development. 104:1041–1051.

Pfeiffer, B.D., A. Jenett, A.S. Hammonds, T.T. Ngo, S. Misra, C. Murphy, A. Scully, J.W. Carlson, K.H. Wan, T.R. Laverty, et al. 2008. Tools for neuroanatomy and neurogenetics in Drosophila. Proc. Natl. Acad. Sci. USA. 105:9715–9720. http://dx.doi.org/10.1073/pnas.0803697105

Roberts, D.J., R.L. Johnson, A.C. Burke, C.E. Nelson, B.A. Morgan, and C. Tabin. 1995. Sonic hedgehog is an endodermal signal inducing Bmp-4 and Hox genes during induction and regionalization of the chick hindgut. Development. 121:3163–3174.

Roth, S.L., and E.B. Helwig. 1963. Juvenile polyps of the colon and rectum. Cancer. 16:468–479. http://dx.doi.org/10.1002/1097-0142(196304)16:4<468::AID-CNR2820160408>3.0.CO;2-F

Shanbhag, S., and S. Tripathi. 2009. Epithelial ultrastructure and cellular mechanisms of acid and base transport in the Drosophila midgut. J. Exp. Biol. 212:1731–1744. http://dx.doi.org/10.1242/jeb.092930

Shaw, R.L., A. Kohlmaier, C. Polcelslo, C. Veelken, B.A. Edgar, and N. Tapon. 2010. The Hippo pathway regulates intestinal stem cell proliferation during Drosophila adult midgut regeneration. Development. 137:4147–4158. http://dx.doi.org/10.1242/dev.052506

Silver, D.L., E.R. Geisbrecht, and D.J. Montell. 2005. Requirement for JAK/STAT signaling throughout border cell migration in Drosophila. Development. 132:3439–3442. http://dx.doi.org/10.1242/dev.01910

Staehling-Hampton, K., and F.M. Hoffmann. 1994. Ectopic decapentaplegic in the Drosophila midgut alters the expression of five homeotic genes, dpp, and wingless, causing specific morphological defects. Dev. Biol. 164:502–512. http://dx.doi.org/10.1006/dbio.1994.1219

Staley, B.K., and K.D. Irvine. 2010. Warts and Yorkie mediate intestinal regeneration by influencing stem cell proliferation. Curr. Biol. 20:1580–1587. http://dx.doi.org/10.1016/j.cub.2010.07.041

Strand, M., and C.A. Micchelli. 2011. Quiescent gastric stem cells maintain the adult Drosophila stomach. Proc. Natl. Acad. Sci. USA. 108:17696–17701. http://dx.doi.org/10.1073/pnas.1109794108

Tsunezumi, K., T. Nakayama, Y. Kamoshida, T.B. Kornberg, J.L. Christian, and T. Tabata. 1997. Daughters against dpp modulates dpp organizing activity in Drosophila wing development. Nature. 389:627–631. http://dx.doi.org/10.1038/353962

Tulina, N., and E. Matunis. 2001. Control of stem cell self-renewal in Drosophila spermatogenesis by JAK-STAT signaling. Science. 294:2546–2549. http://dx.doi.org/10.1126/science.1066700

Wharton, K., and R. Derynck. 2009. TGFbeta family signaling: novel insights into developmental and disease processes. Development. 136:3691–3697. http://dx.doi.org/10.1242/dev.040855

Xie, T., and A.C. Spradling. 1998. decapentaplegic is essential for the maintenance and division of germ line stem cells in the Drosophila ovary. Cell. 94:251–260. http://dx.doi.org/10.1016/S0092-8674(00)81424-5

Xu, N., S.Q. Wang, D. Tan, Y. Gao, G. Lin, and R. Xi. 2011. EGFR, Wingless and JAK/STAT signaling cooperatively maintain Drosophila intestinal stem cells. Dev. Biol. 354:31–43. http://dx.doi.org/10.1016/j.ydbio.2011.03.018
Yan, R., H. Luo, J.E. Darnell Jr., and C.R. Dearolf. 1996. A JAK-STAT pathway regulates wing vein formation in *Drosophila*. Proc. Natl. Acad. Sci. USA. 93:5842–5847. http://dx.doi.org/10.1073/pnas.93.12.5842

Zeng, X., C. Chauhan, and S.X. Hou. 2010. Characterization of midgut stem cell- and enteroblast-specific Gal4 lines in *Drosophila*. *Genesis*. 48:607–611. http://dx.doi.org/10.1002/dvg.20661

Zhao, R., Y. Xuan, X. Li, and R. Xi. 2008. Age-related changes of germ-line stem cell activity, niche signaling activity and egg production in *Drosophila*. *Aging Cell*. 7:344–354. http://dx.doi.org/10.1111/j.1474-9726.2008.00379.x