JAK/STAT signaling coordinates stem cell proliferation and multilineage differentiation in the Drosophila intestinal stem cell lineage

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Adult stem cells are the most primitive cells of a lineage and are distinguished by the properties of self-renewal and multipotency. Coordinated control of stem cell proliferation and multilineage differentiation is essential to ensure a steady output of differentiated daughter cells necessary to maintain tissue homeostasis. Here we investigate the role of the conserved JAK/STAT signaling pathway in the Drosophila intestinal stem cell (ISC) lineage. We show first, that JAK/STAT signaling is normally active in both ISCs and their newly formed daughters, but not in terminally differentiated enteroendocrine (ee) cells or enterocyte (EC) cells. Second, analysis of ISC lineages shows that JAK/STAT signaling is necessary but not sufficient for daughter cell differentiation, indicating that competence to undergo multilineage differentiation depends upon JAK/STAT. Finally, our analysis reveals JAK/STAT signaling to be a potent regulator of ISC proliferation, but not ISC self-renewal. On the basis of these findings, we suggest a model in which JAK/STAT signaling coordinates the processes of stem cell proliferation with the competence of daughter cells to undergo multilineage differentiation, ensuring a robust cellular output in the lineage.

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Introduction

Adult stem cell populations are present in a variety of tissues and function throughout the lifetime of an organism to maintain homeostasis. The dual characteristics of self-renewal and multipotency make stem cells ideally suited for this central role. In a variety of tissue systems, adult stem cells have been shown to reside in specialized microenvironments called niches, which regulate stem cell behavior at baseline homeostasis and dynamically respond to changing environmental stimuli by modulating lineage output (reviewed in Jones and Wagers, 2008). The coordinated control of stem cell proliferation and multilineage differentiation is essential to ensure a steady output of differentiated daughter cells at baseline homeostasis and in response to changing environmental conditions. However, little is currently known about the factors that coordinate these processes.

The ability to identify, mark and manipulate individual stem cell lineages has made Drosophila an excellent model system with which to dissect stem cell regulation. In the adult Drosophila midgut, for example, the stem cell compartment is comprised of individual intestinal stem cells (ISCs) that are dispersed throughout the entirety of the tissue (Figs. 1A–D; Micchelli and Perrimon, 2006; Ohištstein and Spradling, 2006). ISCs have a pyramidal morphology and are located in an epithelial niche distant from the midgut lumen and adjacent to both the basement membrane and surrounding visceral musculature of the midgut. ISCs are multipotent and give rise to a lineage that consists of two types of differentiated daughters, the enteroendocrine (ee) cells and the enterocyte (EC) cells. Together, these cell populations form a cellular monolayer lining the length of adult midgut.

The Jum Kriness/Signal Transducer and Activator of Transcription (JAK/STAT) pathway is a conserved signal transduction pathway that has been implicated in a number of distinct developmental and disease processes (reviewed in Arbusova and Zeidler, 2006). In Drosophila, the JAK/STAT pathway utilizes a set of core signaling components: a transmembrane receptor encoded by domeless (dome), a single JAK tyrosine kinase encoded by hopscotch (hop), the transcription factor stat92E, and unpaired (upd), as well as two related ligands encoded by upd2, upd3. Binding of Upd ligands to the Dome receptor leads to activation of Hop, a receptor-associated kinase, which has at least two substrates, Hop and Dome. Cytoplasmic Stat92E can bind to phosphorylated Dome/Hop complexes via SH2 domains. Once bound to the Dome/Hop complex, Stat molecules are also phosphorylated and form Stat dimers, which translocate to the nucleus and activate downstream transcriptional targets.

Phenotypic analysis in Drosophila has revealed that the JAK/STAT signaling pathway is a versatile regulator of stem cell populations and their cell lineages (reviewed in Fuller and Spradling, 2007; Gregory et al., 2008). Evidence suggests that JAK/STAT is necessary for the maintenance of germline and somatic stem cells and functions as a
powerful signal promoting stem cell proliferation (Kiger et al., 2001; Tulina and Matunis, 2001; Decotta and Spradling, 2005; Singh et al., 2007). JAK/STAT signaling is also required in the ovary for subsequent multilineage differentiation.

In this study, we investigate the role of the JAK/STAT pathway within the ISC lineage under conditions of baseline homeostasis. We show that JAK/STAT signaling is normally active in both ISCs and their newly formed daughters, but not in terminally differentiated enteroendocrine (ee) cells or enterocyte (EC) cells. We also show that cell autonomous loss of JAK/STAT signaling from individual ISC lineages results in a failure of ee and EC cell fate and their newly formed daughters, but not in terminally differentiated enteroendocrine (ee) cells (blue) and enterocytes (orange). (B) A model of ISC division; the ISC undergoes self-renewal and generates a daughter cell or enteroblast (EB), which can become either an ee or an EC cell. Specification of the EC cell fate requires N signaling.

(C, D) All micrographs display superficial views of the midgut, except where indicated. (C) Low magnification view in cross section; esg-GFP (green) and phalldin (red). (D) High magnification view of midgut in cross section showing a newly divided ISC; vkg-GFP (green), esg-lacZ (red) and phalldin (blue). CM (circular muscle), LM (longitudinal muscle), BM (basement membrane).

The adult Drosophila midgut is maintained by a population of multipotent intestinal stem cells (ISCs). (A) Diagram of the adult midgut in cross section. ISCs (green) occupy an epithelial niche adjacent to the basement membrane and the visceral muscle (red). ISCs give rise to two types of differentiated daughters, enteroendocrine (ee) cells and enterocytes (ECs; orange).

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**Fig. 5 and S4:**
y, w; hsFLP, UAS-GFP / w; +; FRT82B / tubGal4, FRT82B tubGal80.
y, w; hsFLP, UAS-GFP / w; UAS-DI / +; FRT82B / tubGal4, FRT82B tubGal80.
y, w; hsFLP, UAS-GFP / w; UAS-NIR+/y, w; +; FRT82B / tubGal4, FRT82B tubGal80.
y, w; hsFLP, UAS-GFP / w; UAS-NIR+/y, w; +; FRT82B / tubGal4, FRT82B tubGal80.
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y, w; hsFLP, UAS-GFP / w; UAS-NIR+/y, w; +; FRT82B / tubGal4, FRT82B tubGal80.

**Mosaic analysis**

Positively marked ISC lineages were generated using the MARCM system. MARCM clones were induced by placing experimental fly vials in a 37 °C water bath for 35–45 min. Induction protocols consisted of 2–3 heat pulses within a 24-h period and were performed within the first 5–10 days of adulthood. Expression of UAS-upd using the Flip-out technique was performed by subjecting experimental flies to a single 30 min heat shock within the first 5–10 days of adulthood (Figs. 8C, D).

**Temperature shift experiments**

Two different temperature shift analyses were performed in this study (Figs. 3A, B and Figs. 8A, B). In both cases, we established and cultured crosses at 18 °C until collection of F1 progeny. In the stat92E73 analysis (Figs. 3A, B), we divided F1 progeny into two equal pools, maintaining controls at 18 °C and shifting the experimental group to 29 °C for 14 days. For the series of experiments shown in Figs. 8A and B, all F1 adult progeny were aged 4–7 days at 18 °C and were then shifted to 29 °C for 48 h prior to dissection.

**Histology**

Adult flies were dissected in 1 × PBS (Sigma, USA). The gastrointestinal tract was removed and fixed in a final solution of 0.5 × PBS (Sigma, USA) and 4% electron microscopy grade formaldehyde (Polysciences, USA) for a minimum of 30 min. Samples were washed in 1 × PBS + 0.1% Triton × 100 (PBST) for 2 h, then incubated with primary antibodies overnight. Samples were washed in PBST for 2 h then incubated with secondary antibodies for 3 h. Finally, samples were washed in PBST overnight. Mounting media containing DAPI (Vectashield, USA) was added and samples were allowed to clear for 1 h prior to mounting. All steps were completed at 4 °C with no mechanical agitation.

**BrdU incorporation experiments**

Flies were aged for 4 weeks on standard media following clone induction and then transferred to BrdU media for 1 week. BrdU was administered ad libitum in Drosophila food media (200ul of 6 mg/ml BrdU in 20% sucrose per fresh vial). Dissected samples were fixed for 30 min at room temperature and then washed for 30 min at room temperature. DNA was denatured by applying a 2.2N solution of HCl to samples for 30 min at room temperature followed by neutralization in Borax (100 mM) for 5 min and a final 30 min PBST wash. Samples were then stained as described above.

**Antisera**

**Primary antibodies**

Chicken anti-GFP (Abcam, USA) used at a dilution of 1:10,000; rabbit anti-β-Gal (Cappel, USA), 1:2000; mouse anti-β-Gal (Developmental Studies Hybridoma Bank; DSHB), 1:100; mouse anti-Pros (DSHB) 1:100; mouse anti-Dis (DSHB), 1:100; mouse anti-BrdU, 1:100; rabbit anti-Pdm-1 [gift of W. Chia (Yeo et al., 1995)] 1:1000; rabbit anti-phH3 (Upstate), 1:1000.

**Secondary antibodies**


**Mounting media**

Vectashield + DAPI mounting media (Vector, USA).

**Microscopy and imaging**

Samples were examined on a Leica DM5000 upright fluorescent microscope. Confocal images were collected using a Leica TCS SP5 confocal microscope system. Images were processed for brightness and contrast and assembled in Photoshop CS (Adobe, USA).

**Measurements, cell counts and statistical analysis**

Maximum nuclear diameter was measured in both anterior and posterior midgut frames at 40× magnification (A2.5 and P2.5; see Lee et al., 2009 for additional information on midgut nomenclature) using the Leica Application Suite (LAS; Fig. 3E). We scored the number of Pros+ or Pdm1+ cells in both anterior and posterior midgut frames 5 days following clone induction (Figs. 4D, F; 5D; 6D; 54D). The number of GFP+ cells per frame was quantified in both the anterior and posterior midgut at 5, 10 and 20 days following clone induction (Fig. 7G, Supplemental Fig. 5E). In the experiments described above, we counted cells on the top surface of the midgut and excluded cells along the side and bottom surfaces. Combined data for anterior and posterior regions are displayed (Figs. 3E, 7G and Supplemental Fig. 5E). To determine the number of dividing cells following UAS transgene activation, we counted the number of GFP+ phH3+ cells along the entire length of the midgut 48 h after temperature shift (Fig. 8B). The number of GFP+, Pros+ small cells was quantified in both anterior and posterior 40× frames and combined (Supplemental Figs. 5E, B). The number of GFP+, phH3+ small cells was counted for the entire length of the midgut (Supplemental Fig. 2C). All t-tests were performed using Prism (GraphPad Software, USA); significance was tested at the 95% CI.
Results

JAK/STAT signaling is dynamically regulated in the midgut

JAK/STAT activation in the midgut was examined using a transcriptional reporter for pathway activation (Bach et al., 2007). The reporter, 10XSTAT92E-GFP, is composed of multimerized Stat92E consensus binding sites fused to GFP, which is expressed in a manner that recapitulates the tissue specific distribution of Stat92E protein. Moreover, JAK/STAT signaling is both necessary and sufficient for the activation of this reporter (Bach et al., 2007). Thus, 10XSTAT92E-GFP functions as a high fidelity reporter for JAK/STAT activity in vivo.

Examination of the adult midgut revealed the presence of numerous 10XSTAT92E-GFP positive cells with small nuclei distributed along the entire anterior-posterior axis of the tissue (52/52 midguts analyzed). Previous analysis has shown that esg marks both individual ISCs and their undifferentiated EB daughters, which can often be detected as pairs of cells or “doublets” in the midgut (Micchelli and Perrimon, 2006). Thus, esg distinguishes the undifferentiated cells of the ISC lineage from differentiated ee and EC cells. To determine the precise identity of the cells expressing the JAK/STAT reporter, we double labeled the midgut to reveal the distribution of esg-lacZ and the 10XSTAT92E-GFP reporter. Double staining showed complete coexpression, suggesting that the JAK/STAT signal is transduced in both the ISC and EB (Fig. 2A). It has also been shown that elevated DI protein levels mark a subset of midgut ISCs (Ohlstein and Spradling, 2007). Consistently, double staining of these reporters in midgut ISCs (Fig. 2B). The observation that 10XSTAT92E-GFP reporter coexpresses with esg-lacZ in the midgut (Fig. 2A) and that expression of the reporter is dependent on JAK/STAT activation in the midgut ISCs (Fig. 2B) suggests a role for JAK/STAT signaling in the ISC lineage.

Fig. 2. JAK/STAT signaling is dynamically regulated in midgut ISCs and EBs. (A–F) Characterizing transcriptional reporters of JAK/STAT activity in the midgut (green); nuclei are counterstained with DAPI (blue), except in D. (A–D) 10XSTAT92E-GFP reporter with stable GFP. (A) ISC and EB cells (esg-lacZ; red) express the 10XSTAT92E-GFP reporter. (B) ISCs (Dl-lacZ; red) express the 10XSTAT92E-GFP reporter. (C) Differentiated ee cells are marked with anti-Pros (red). (D) Differentiated EC cells are marked with anti-Pdm1 (red). (E, F) 10XSTAT92E-GFP reporter adjacent to ISCs with destabilized reporter. (F) EB cells expressing the destabilized reporter adjacent to ISCs with no detectable expression. High magnification, inset. Scale bar: 50 μm.

Fig. 3. JAK/STAT signaling is required in the ISC lineage. (A, B) stat92E temperature shift analysis, nuclei (DAPI, grayscale). (A) stat92E, unshifted controls. (B) stat92E, shifted to non-permissive temperature for 14 days. Global reduction of stat92E leads to the formation of clusters of small cells in the midgut. (C–F) The MARCM system was used to positively identify ISC lineages with GFP 5 days after induction (anti-GFP, green; DAPI, blue). (C) Wild type ISC lineages. (D) ISC lineages lacking stat92E. Loss of stat92E leads to generation of cells with abnormal clonal morphology and reduced nuclear size. (E) Quantification of nuclear size. Histogram displays the distribution of nuclear size in wild-type (white) and stat92E lineages (black). stat92E mutant lineages fail to generate cells of the larger nuclear size classes. The average nuclear size of wild type EB cells (Su(H)GBe-lacZ) is indicated by a circle; the average nuclear size of wild type EC cells (anti-Pdm1) is indicated by a square. (F) ISC lineages lacking hop resemble those lacking stat92E. We note that many of the cells detected in stat92E and hop mutant lineages exhibited the cytoplasmic foot characteristic of EB cells. Scale bar: 50 μm.

We next asked if JAK/STAT pathway activation could be detected in the differentiated cells of the ISC lineage. Staining for Pros protein, a marker for differentiated ee cells, revealed an inverse correlation with the 10XSTAT92E-GFP reporter (Fig. 2C). Similarly, EC cells characterized by large polyploid nuclei and positive staining for Pdm1 also did not detectably express 10XSTAT92E-GFP (Fig. 2D). Together these expression studies suggest that JAK/STAT signaling is transduced primarily in the ISCs and EB cells.

The observation that 10XSTAT92E-GFP is expressed at elevated levels in both the ISCs and EB cells raised the possibility that the EB signal detected is due to GFP perdurance. To investigate this possibility, we examined a more sensitive reporter of JAK/STAT
activity, 10XSTAT92E-GFP\textsuperscript{destabilized}(D) (Bach et al., 2007). Previous analysis has suggested that Su(H)\textit{GBE-lacZ} marks nascent EB cells (Michelli and Perrimon, 2006, Ohlstein and Spradling, 2007); EBs can often be identified morphologically by the presence of a flared cytoplasmic “foot” (e.g. Fig. 2A). To determine if 10XSTAT92E-GFP\textsuperscript{destabilized} is detectable in EB cells we crossed the reporter into a genetic background containing Su(H)\textit{GBE-lacZ}. Double labeling revealed that 10XSTAT92E-GFP\textsuperscript{destabilized} and Su(H)\textit{GBE-lacZ} are both expressed at elevated levels in EB cells (Figs. 2E, F). Importantly, detectable levels of 10XSTAT92E-GFP\textsuperscript{destabilized} could also be observed in Su(H)\textit{GBE-lacZ} cells adjacent to ISCs with no detectable expression (Fig. 2F, inset). These findings suggest that the JAK/STAT reporter is active in the EB cell.

In marked contrast to the stable reporter line, lower levels of expression and a great degree of heterogeneity existed in ISC/EB pairs (Supplemental Figs. 1A, B; 50/50 midguts analyzed). In ISC/EB pairs that both expressed the reporter, ISC expression levels were often detectably lower than in the EB (Supplemental Figs. 1C, D). These patterns of activity differed among individual midguts analyzed and were observed with two independent destabilized transgenic strains indicating that variation was not due to transgene insertion site. Consistent with these findings, examination of \textit{upd1} and \textit{upd3} reporters revealed detectable levels of both JAK/STAT ligands distributed throughout the midgut, although the extent of expression varied widely even among age-matched samples (Supplemental Figs. 1E–H). Together, these observations suggest that at baseline homeostasis, JAK/STAT signaling is dynamically regulated in the midgut at both the regional and cellular level.

\textit{JAK/STAT} signaling is required in the ISC lineage

Characterization of 10XSTAT92E-GFP transcriptional reporters suggested that the JAK/STAT pathway might have a function in the midgut. To directly test the functional requirement of JAK/STAT, the effects of partially reducing JAK/STAT signaling were examined using a temperature sensitive, hypomorphic allelic combination of \textit{stat92E\textsuperscript{TS}} and \textit{Dl\textsuperscript{WT}} (referred to subsequently as \textit{stat92E\textsuperscript{TS}/Dl\textsuperscript{WT}}). The distribution of cell nuclei in \textit{stat92E\textsuperscript{TS}/Dl\textsuperscript{WT}} adults was examined in DAPI stained samples following a 14-day shift to the non-permissive temperature. In contrast to unshifted controls, \textit{stat92E\textsuperscript{TS}/Dl\textsuperscript{WT}} adults exhibited an aberrant midgut organization characterized by the presence of

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**Fig. 4.** \textit{stat92E} is required for multilineage differentiation. (A–F) The MARCM system was used to positively identify ISC lineages with GFP 5 days following induction. (anti-GFP, green; DAPI, blue). (A) Wild type ISC lineages contain \textit{esg-lacZ} positive and negative cells (anti-\textit{fgal}, red). (B) ISC lineages lacking \textit{stat92E} have an increased number of \textit{esg-lacZ}+ cells and a decreased number of \textit{esg-lacZ}− cells (anti-\textit{fgal}, red). (C, D) \textit{stat92E\textsuperscript{TS/TS}} mutant lineages have a significantly reduced number of \textit{Pros}+ cells in both the anterior (A) and posterior (P) midgut. (C) Representative micrograph. (D) Quantification (n = 8; anti-\textit{Pros}, red). (E, F) \textit{stat92E\textsuperscript{TS/TS}} mutant lineages have a significantly reduced number of \textit{Pdm1}+ cells in both the anterior (A) and posterior (P) midgut. (E) Representative micrograph. (F) Quantification (n = 8; anti-\textit{Pdm1}, red). Error bars denote s.e.m. Scale bar: 50 μm.

**Fig. 5.** \textit{stat92E} is epistatic to Dl/N signaling for EC specification. (A–F) The MARCM system was used to positively identify ISC cell lineages with GFP 5 days following induction (anti-GFP, green; DAPI, blue). (A) Wild type ISC lineages generate EC cells distributed throughout the midgut, although the extent of expression varied widely even among age-matched samples (Supplemental Figs. 1E–H). Together, these observations suggest that at baseline homeostasis, JAK/STAT signaling is dynamically regulated in the midgut at both the regional and cellular level.

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distinct multicellular clusters most prominent in the posterior midgut (Figs. 3A, B; 6/8 midguts analyzed). Moreover, cells within the cluster typically displayed a small nuclear morphology unlike that of differentiated EC cells. Such clusters appeared to be the product of individual ISCs, although this could not be verified due to the absence of a genetic lineage marker.

To examine the consequence of reducing JAK/STAT signaling from individual ISC lineages we conducted a mosaic analysis of the stat92E. Positively marked ISC lineages were generated in the adult midgut using the MARCM system (Lee and Luo, 1999) and identified on the basis of GFP expression 5 days after induction (Figs. 3C–E). In contrast to wild type cell lineages, ISCs lacking stat92E produced lineages consisting of smaller cells with the clones themselves often appearing fragmented (compare Figs. 3C, D). Measurements of nuclear diameter indicated that daughter cells failed to attain the maximal nuclear size detected in wild type lineages (Fig. 3E). ISC lineages lacking hop produced a similar phenotype (Fig. 3F). Together, this analysis suggests that JAK/STAT signaling is required for normal differentiation in the ISC lineage.

In our mosaic analysis, we occasionally observed that the overall organization of the midgut epithelium was disrupted. For example, regions of increased small cell number could be detected in heterozygous tissue (e.g. GFP− cells in Fig. 3D) raising the possibility that an increased number of small cells exist in the genetic background used to generate clones. To address this possibility we quantified the number of small cells in uninduced wild type and stat92EESC9 midguts used in our mosaic analysis however, no significant difference was observed (Supplemental Fig. 2A). A second possible explanation is that stat92EESC9 clones can exert a non-autonomous effect on the surrounding tissue. To examine this possibility we quantified the number of both GFP− small cells and pH3+ cells in induced wild type and stat92EESC9 midguts and observed a significant increase in each measure (Supplemental Figs. 2B, C).

Thus, the variation in the midgut epithelium we observe is not a result of genetic background but is consistent with a non-autonomous effect of stat92E clone induction.

**JAK/STAT signaling is required for differentiation in the ISC lineage**

Clone morphology suggested that ISC lineages unable to transduce the JAK/STAT signal fail to undergo normal multilineage differentiation. To more rigorously analyze cell fate in JAK/STAT mutant lineages, we examined a panel of molecular markers. In wild type lineages esg is expressed in ISCs and EBs, but not in either of the two differentiated cell types of the midgut, the ee or EC cells (Fig. 4A;
Dominant negative construct leads to a further increase in pH3+ cell number. (C, D) were rarely detectable within. To test this directly, we examined the expression of UAS-GFP, tubGal80TS. Differentiation does not occur in the absence of JAK/STAT, however the level of expression often could be detected, suggesting that ISCs are present if ISCs were present in lineages lacking JAK/STAT signaling we revealed a reduction or absence of Pdm1 staining (Figs. 4E, F; 21/21 midguts examined; wild type, Dlwt, n = 8). Taken together, these observations suggest that there are significant regional differences affecting cell fate in the ISC lineage.

To quantify the effect of Dlwt expression in ISC lineages we scored the number of Pros+ cells in the lineage. In contrast to wild type, ISC lineages expressing Dlwt resulted in a significant decrease in the number of clones containing Pros+ cells within the lineage (Supplemental Figs. 4C, D; wild type, n = 12; Dlwt, n = 12). In addition, staining with the EC marker Pdm1 suggested that ISCs expressing Dlwt produce lineages consisting almost entirely of ECs (Figs. 5A, B, Supplemental Fig. 4E). These effects on cell fate were also observed in Dpmt expressing clones analyzed 10 days after induction, suggesting that the reduction in Pros+ cells was not simply due to a delay in ee cell differentiation (Supplemental Fig. 4F). We note that at later time points, ISCs expressing Dlwt also produced large clones that appeared to contain many undifferentiated cells, as they were negative for both Pros and Pdm1 (Supplemental Fig. 4F). Such cells appeared to most closely resemble ectopic ISCs or EBs, raising the possibility that Dl has an additional role in regulating ISC proliferation. Taken together, these findings demonstrate that Dlwt is sufficient to specify EC cell fate at the expense of ee cells and supports a model in which ISCs signal non-autonomously to nascent EB cells to specify the EC cell fate (Micchelli and Perrimon, 2006; Ohlstien and Spradling, 2007).

**JAK/STAT is epistatic to Dl/N signaling in multilineage differentiation**

To determine if stat92E is required for Dl-mediated EC cell fate specification we conducted a mosaic analysis of ISC lineages expressing Dpmt but lacking stat92E85C9 function. In contrast to Dlwt expressing lineages alone, Dpmt, stat92E85C9 lineages appeared to lack differentiated EC cells based on the reduced number of Pdm1+ cells and were virtually indistinguishable from stat92E85C9 mutants (compare Figs. 5B, C and Fig. 4E). Similar results were also obtained by analyzing a second allele, stat92E06346 (Supplemental Fig. 5C).

A quantitative analysis was performed to determine the effect of stat92E85C9 loss on Dpmt expressing lineages by comparing the number of Pdm1+ cells present in Dpmt lineages with the number of Pdm1+ cells present in Dlwt, stat92E85C9 lineages. This analysis revealed a significant decrease in the number of Pdm1+ GFP+ cells in Dpmt, stat92E85C9 lineages compared to Dlwt alone (Fig. 5D; Dpmt, n = 12; Dlwt, stat92E85C9, n = 12). Consistent with this finding, we observe that differentiated ECs induced by cell autonomous N activation were also blocked by the loss of stat92E (Figs. 5F, E; 29/29 midguts analyzed). Thus, although stat92E is required in the ISC for normal levels of Dl induction (Fig. 3), our epistasis analysis shows that stat92E is also necessary for EC differentiation in the presence of Dl/N signaling.

We next sought to extend the epistasis analysis to determine the requirement for JAK/STAT in differentiation of ee cells. Previous analysis has demonstrated that ISC lineages lacking N

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**Fig. 8.** JAK/STAT activation promotes ISC proliferation. (A–D) Expression of upd leads to an increase in ISC proliferation. (A, B) Conditional expression of upd using the esgGal4, UAS-GFP, tubGal80TS driver (anti-GFP, green; anti-pH3, grayscale; DAPl, blue). (A) Expression of upd is sufficient to increase the number of pH3+ cells 48 h after induction. (B) Quantification. Note that simultaneous reduction of N activity using a dominant negative construct leads to a further increase in pH3+ cell number. (C, D) Expression of upd using the Flip-out (F/O) cassette (anti-GFP, green; anti-pH3, grayscale; DAPl, blue). (C) Cells marked in Flip-out experiments were associated with increased numbers of pH3+ cells. (D) Many marked cells rapidly delaminate from the midgut, as seen here in cross section. (E) ISC lineages expressing the activated form of hop (hopTum) are rapidly lost from the midgut (anti-GFP, green; anti-Pros, red; DAPI, blue). (F) hopTum lineages 5 days after induction. (F) hopTum lineages 10 days after induction. Error bars denote s.e.m. Scale bar: 50 μm.
generate two phenotypic classes, clones that appear to be comprised of ectopic ISCs and clones that appear to be comprised of ectopic ee cells (Fig. 6A; Ohlstein and Spradling, 2006). To test whether JAK/STAT signaling is required for ee differentiation, we asked if the production of Pros+ ee cells present in N mutant lineages depends on the JAK/STAT signaling pathway. To test this requirement, genetic mosaics were created in which both N and stat92E functions were reduced. In contrast to NRNAi mosaic analysis of NRNAi; stat92EESC5 clones revealed a reduction in ee cells (Figs. 6B, C; 30/30 midguts examined). Similar results were obtained by analyzing a second hypomorphic allele, stat92E66346 (Supplemental Fig. 5D).

A quantitative analysis was performed to determine the effect of stat92EESC9 loss on NRNAi expressing lineages. This analysis revealed a significant decrease in the number of Pros+ GFP+ cells in NRNAi; stat92EESC5 lineages compared to NRNAi alone (Fig. 6D; NRNAi, n = 8; NRNAi; stat92EESC5, n = 8). Thus, the transcription factor encoded by stat92E is required downstream or in parallel to N, suggesting that stat92E is also required in EB cells for ee differentiation.

Little evidence that JAK/STAT signaling is required for ISC self-renewal

Analysis of the 10XSTAT92E-GFP transcriptional reporters revealed detectable expression in ISCs (Figs. 2A, B), suggesting that JAK/STAT signaling might also have a function in the ISC. Studies of Drosophila germline stem cells first employed a genetic lineage-tracing assay to measure stem cell self-renewal (Margolis and Spradling, 1995). A pulse/chase design was used to determine the number of marked stem cell lineages retained in the midgut at defined times following induction. To determine if JAK/STAT signaling is required for ISC self-renewal we generated labeled ISCs lacking components of the JAK/STAT signaling pathway and analyzed the lineages at 5, 10, and 20 days after induction. In controls, wild type lineages could be detected in the midgut throughout the chase interval. Similarly, in hopC111 and stat92EESC9 mutants, marked lineages were detectable in the midgut 20 days after induction (Figs. 7A–F).

A quantitative analysis was performed to examine the effect of JAK/STAT loss on ISC self-renewal. As noted above, loss of JAK/STAT signaling often results in a distinct clonal morphology and the inability to unambiguously identify individual clones (e.g. Figs. 3D, F). As such, clone number is not a suitable measure of ISC number in the JAK/STAT mutants we examined. Therefore, to approximate the number of ISCs we scored the number of GFP+ daughter cells in defined regions of each sample. Quantification of the pulse/chase studies showed that reductions in hopC111; stat92EESC9, or stat92E66346 were associated with the retention of 67%, 22% or 31% of labeled cells, respectively, at 20 days after induction (n = 12; Fig. 7G, Supplemental Fig. 5E). In addition, we observed that hopC111 and stat92EESC9 lineages could be identified at late time points that stained positively for both pH3 and BrdU, suggesting that JAK/STAT is not absolutely required for ISC proliferation (Figs. 7B, D, F and Supplemental Fig. 6D, E). We also note that hyperplastic stat92E clones could be detected at late time points suggesting that JAK/STAT may be a necessary modulator of ISC proliferation (Supplemental Figs. 6A–C). Taken together these data suggest that ISC self-renewal is not grossly disrupted in the absence of JAK/STAT signaling.

The JAK/STAT pathway promotes ISC proliferation

To determine if the levels of JAK/STAT signaling affect the ISC lineage, we examined the consequences of pathway activation in the midgut. Using conditional Gal4 induction in esg+ cells (esgG3), we first examined the effect of expressing the JAK/STAT ligand upd on the number of pH3+ cells 2 days after induction. Quantification revealed a significant increase in the number pH3+ cells following upd expression, compared to both wild type controls and the expression of a dominant negative form of N (n = 18; Figs. 8A, B). The effect of upd expression on proliferation was enhanced by simultaneous expression of a dominant negative form of N, as would be predicted if Upd acts directly on ISCs to stimulate proliferation (n = 18; Fig. 8B). Similar increases in pH3+ were obtained when upd was expressed using the FLP-out (F/O) technique (27/27 midguts examined; Fig. 8C). However, in addition to the increase in pH3+ cells, we also observed many cells that appeared to be in the process of delaminating from the midgut epithelium (Fig. 8D). Finally, using the MARCM system we generated ISC lineages expressing constitutively active hopTam(Harrison et al., 1995); such clones were rapidly lost from the midgut (7/7 midguts examined; Figs. 8E, F). Thus, activation of JAK/STAT signaling promotes ISC proliferation in the midgut, although this response may depend on the precise level and cell types in which the pathway is activated.

Discussion

Stem cells, JAK/STAT and self-renewal

A series of studies have addressed the requirement of JAK/STAT signaling for stem cell self-renewal in distinct Drosophila stem cell populations. For instance, male germline stem cells (GSCs) are arrayed in rosette like pattern around a focus of “hub” cells situated at the apical tip of the testis. Hub cells are a source of Upd ligand; GSCs lacking the ability to transduce the JAK/STAT signal are detectably reduced by 5 days and completely lost after 9 days. In contrast, misexpression of the Upd ligand increases the number of GSC like cells in the testis and mitigates GSC loss at the hub (Kiger et al., 2001; Tulina and Matunis, 2001; Decotta and Spradling, 2005; Singh et al., 2007). Thus, JAK/STAT functions as a signal for GSC self-renewal. The rapid failure of stem cell self-renewal and concomitant depletion of the stem cell compartment following loss of JAK/STAT has subsequently been reported for female GSCs, cyst progenitor cells (CPCs), follicle stem cells (FSCs), and renal stem cells (RNSCs) (Kiger et al., 2001; Tulina and Matunis, 2001; Boyle et al., 2007). Thus, the theme that has emerged is that JAK/STAT signaling is generally required for Drosophila stem cell self-renewal.

Our studies have revealed an exception to this generalization. Here, we examined the consequences of JAK/STAT loss in the midgut up to 20 days following induction (~40% of the fly lifetime), at which point marked ISC lineages were still detected. Consistently, BrdU and pH3 staining suggested that ISCs were still capable of dividing up to 5 weeks in the absence of JAK/STAT signaling. Finally, very large stat92E clones could occasionally be detected in the midgut. Taken together, these findings are not consistent with a requirement for JAK/STAT in ISC self-renewal. We note that our studies differed from the aforementioned in one respect, experimental limitations prevented us from unambiguously identifying individual ISC lineages. Therefore the number of stem cell progeny at each time point was scored, to infer the presence of stem cells. While these experiments do not rule out a requirement for JAK/STAT in long term ISC maintenance, they provide little evidence that JAK/STAT signaling is required for ISC self-renewal.

JAK/STAT coordinates competence to undergo multilineage differentiation and ISC proliferation

Two lines of evidence support a role for JAK/STAT signaling in multilineage differentiation of ISC daughters. First, a synthetic JAK/STAT transcriptional reporter is expressed throughout the midgut; coexpression studies demonstrate that JAK/STAT signaling occurs in Su(H)GBe-lacZ+ cells, a marker for undifferentiated EB daughter cells. In addition, we observe JAK/STAT reporter expression in daughters adjacent to ISCs, which have no detectable JAK/STAT reporter expression themselves, suggesting this is not due to GFP
and associated JAK/STAT activity that we report here is a direct manifestation of midgut stress, albeit to a lesser extent in unchallenged animals, or if there is a distinct regulation of Upd ligands under baseline homeostasis. Previous studies indicate that apoptosis and stress signaling are both detectable in the adult midgut at baseline homeostasis (Ohlstein and Spradling, 2006; Biteau et al., 2008). Studies of antimicrobial peptide reporters, which serve as markers of infection, indicate a limited signal in the adult midgut (Tzou et al., 2000). Nonetheless, a degree of infection undetectable by such reporters under conditions of laboratory culture is possible. If activation of Upd ligands is exclusively regulated in response to exogenous factors this would provide an efficient means of coupling the ISC proliferative response to the magnitude, duration and location of the stimulus. However, in this view one might predict that the additive effects of locally generated stress in the midgut under normal circumstances would, over time, lead to a distortion of tissue architecture. On the other hand, if there is also a distinct regulation of Upd ligands under baseline homeostasis, this might contribute to a stereotyped mode of growth control and tissue homeostasis, in conjunction with other signals known to regulate ISC proliferation (Lin et al., 2008; Lee et al., 2009). Such models are not mutually exclusive. Future experiments, which clarify the precise mechanisms of Upd ligand regulation will provide important insights into the control of the ISC lineage under a broad range of biological conditions.

Acknowledgments


Appendix A. Supplementary data

Supplementary data associated with this article can be found in the online version, at doi:10.1016/j.ydbio.2009.10.045.

References


N and JAK/STAT signaling pathways interact to regulate the ISC lineage

We have conducted genetic epistasis experiments to determine the nature of the interaction between DI/N signaling and the JAK/STAT signaling pathway in the ISC lineage. Our experiments show that while JAK/STAT is required for wild type levels of DI expression in the ISC, the requirement for JAK/STAT in EB differentiation is independent of DI/N signaling. This observation suggests that JAK/STAT functions downstream or in parallel with DI to establish competence of EBs to undergo differentiation. Recent studies, however, suggest a different relationship. Studies of midgut regeneration show that DI ligand is strongly induced in ISCs following Upd ligand expression, directed epithelial cell ablation or bacterial infection (Jiang et al., 2009; Buchon et al., 2009). These studies suggest that under conditions of adaptive homeostasis, Upd ligands are produced in gut epithelial cells and activate DI in ISCs to promote new cell production. Consistent with this model, we observe that expression of DI leads to an increase in the size of ISC lineages, suggesting a potential role for DI in promoting ISC proliferation. Thus, there is evidence that JAK/STAT signaling functions both upstream and downstream of DI/N in the ISC lineage.

Regulation of Upd ligands at baseline and adaptive homeostasis

At baseline homeostasis, the most distinctive feature of Upd ligand expression that we observe is heterogeneity among age-matched samples. Prior studies show that Upd ligands are strongly induced following bacterial infection, directed cell ablation, or stress signaling (Buchon et al., 2009; Jiang et al., 2009; Cronin et al., 2009). It remains an open question as to whether Upd ligand expression...


