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JAK–STAT Is Restrained by Notch to Control Cell Proliferation of the *Drosophila* Intestinal Stem Cells

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ABSTRACT

The *Drosophila* midgut epithelium undergoes continuous regeneration that is sustained by multipotent intestinal stem cells (ISCs) underneath. Notch signaling has dual functions to control ISC behavior: it slows down the ISC proliferation and drives the activated ISCs into different differentiation pathways at a dose-dependent manner. Here we identified a molecular mechanism to unite these two contradictory functions. We found JAK–STAT signaling controls ISC proliferation and this ability is negatively regulated by Notch at least through a transcriptional control of the JAK–STAT signaling ligand, *unpaired* (*upd*). This study provides insight into how stem cells, under steady conditions, balance the processes of proliferation and differentiation to maintain the stable cellular composition of a healthy tissue. J. Cell. Biochem. 109: 992–999, 2010.

KEY WORDS: JAK–STAT; STEM CELL; *DROSOPHILA*; INTESTINE

The *Drosophila* intestinal stem cell (ISC) is emerging as an excellent system to investigate stem cell behaviors due to its simple and well-characterized lineage [Micchelli and Perrimon, 2006; Ohlstein and Spradling, 2006]. ISCs are aligned on the base membrane enclosing the digestive duct. Following one round of asymmetrical cell division, one ISC produces a new ISC and an immature daughter cell, enteroblast (EB), which will eventually differentiate into an enterocyte (EC) or an enteroendocrine (ee) cell (Fig. 1a). ISCs are characterized by expressing high levels of *Delta* (*Dl*) on a cell's surface, which triggers Notch signaling in neighboring EBs. A Notch reporter of *Su(H)GBE-lacZ* can be used as a EB cell marker [Micchelli and Perrimon, 2006]. The two differentiated cell types, including EC and ee-cells, are more apically localized toward the lumen: ee-cells express the transcription factor of Prospero (*pros*) in the nucleus and mature ECs can be unambiguously distinguished from other cell types by their polyploid nuclei and large cell bodies.

Notch (*N*) was known to promote the tissue homeostasis by initiating ISC differentiation and specifying the terminal cell fates of EBs [Ohlstein and Spradling, 2007]. It was also noticed previously that Notch suppresses the default proliferation state of ISCs, which slows down their turnover speed [Micchelli and Perrimon, 2006]. Thus, an interesting question arises of how Notch is able to exert these two seemingly contradictory functions within a single stem cell system.

The *Drosophila* JAK–STAT signal transduction pathway regulates cell proliferation in several different stem cell systems [Decotto and Spradling, 2005; Singh et al., 2007; Sheng et al., 2009]. The signaling is initiated by the glycosylated unpaired proteins (*upd*, *upd2*, *upd3*) binding to a transmembrane receptor, *domeless* (*dome*), which signals through the only *Drosophila* JAK kinase homologue, *hopscotch* (*hop*), and activates the only *Drosophila* STAT homologue, *stat92E*. The activated Stat92Es dimerize and enter into the nucleus to turn on the transcription of the target genes, including *stat92E* itself [Arbouzova and Zeidler, 2006].

We reported here that the canonical JAK–STAT signaling promotes ISC proliferation, allowing activated ISCs to go through either self-renewal or differentiation. Under normal conditions, this function is suppressed by Notch at least through a transcriptional repression of the signaling ligand, *unpaired* (*upd*). Our work revealed that Notch, working as a differentiation signal, provides a negative feedback to the ISC activation process. As a result, a stable cellular architecture of the gut epithelium is maintained, which is important for its proper physiological functions.

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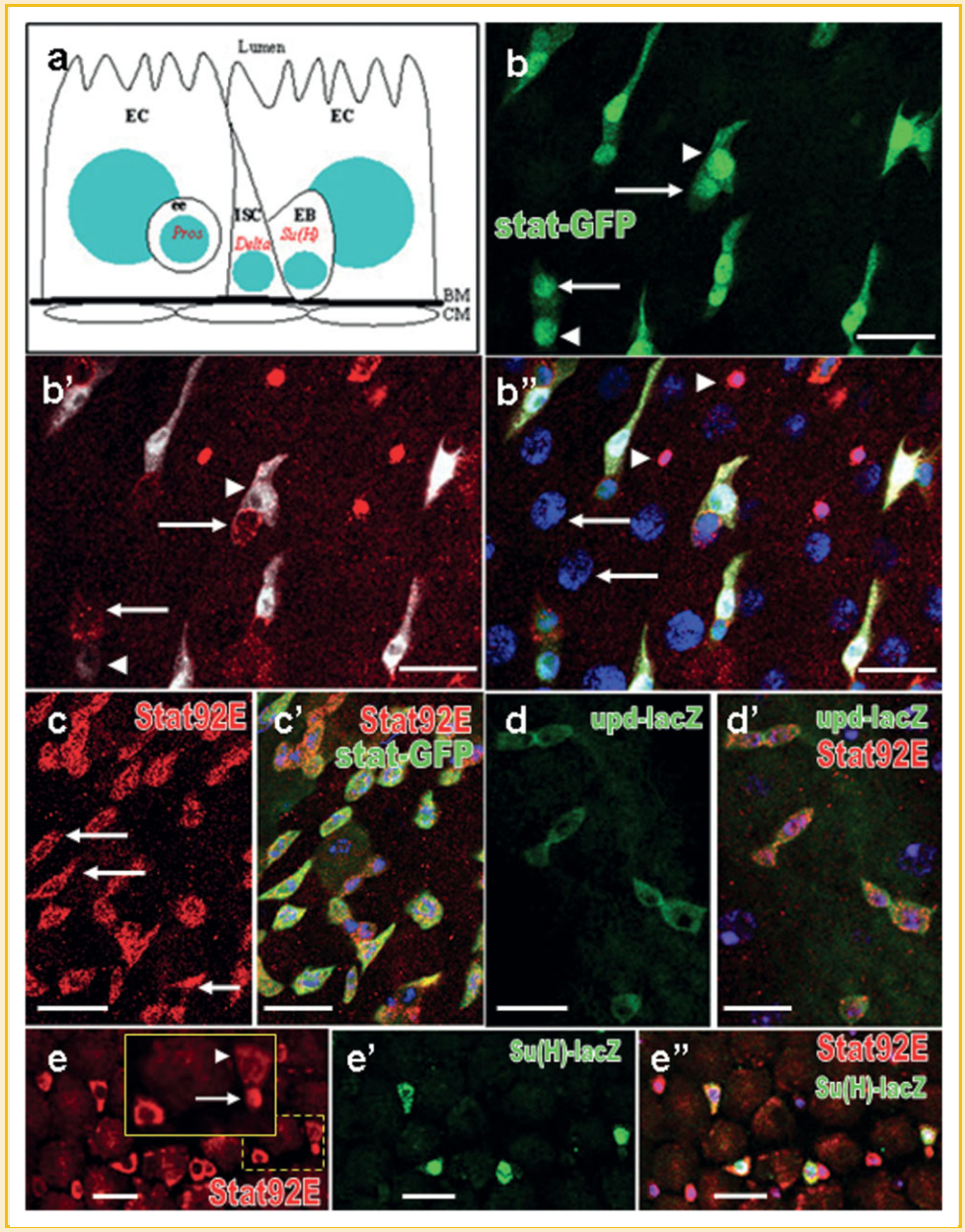

Fig. 1. JAK–STAT is present in ISCs and EBs of the adult *Drosophila* midgut. a: Illustration of the ISC lineage and molecular markers in the *Drosophila* midgut, CM, circular muscle; BM, base membrane. b–b′′: Adult midgut of stat92E-GFP/Su(H)-lacZ flies stained with the indicated markers. Red: Dl (on cell surface) and Pros (in nucleus); white: Su(H)-LacZ; green: stat92E-GFP; blue: DAPI (nucleus). Note stat92E-GFP (green) was expressed in both ISCs (Dl+, arrows in b′) and EBs [Su(H)-lacZ+, arrowheads in b′], but not in ee-cells (pros+, arrowheads in b′′) or ECs (polyploid nuclei and large cell bodies, arrows in b′′). c,c′: Stat92E (red) co-localizes with stat92E-GFP (green). Note in most expressing cells, Stat92E protein is concentrated in the cytoplasm (arrows). d,d′: *upd* is expressed in the progenitor cells. An enhancer trap line (upd-lacZ, green) indicates upd is transcribed in the same cells expressing stat92E (red in d′). e–e′′: Adult midgut of *Su(H)-lacZ* flies was stained with Stat92E (red) and LacZ (green). Most ISCs (Stat92E+, LacZ-) and EBs (LacZ+) have cytoplasmic Stat92E. An ISCs coupled with a newly formed EB (arrowhead in inset of e) has nuclear-enriched Stat92E (arrow in inset of e), suggesting this ISC has just finished cell division. Scale bars: 20 μm.

MATERIALS AND METHODS

FLY STOCKS

Fly stocks used in this study, described either in FlyBase or as otherwise specified, were as follows: *esg-Gal4* (a kind gift from S. Hyashi); FRT^{82B} $Stat92E^{06346}$; FRT^{82B} $Stat92E^{j6C8}$; hop^{C111} FRT^{19A}; $Notch^{55e11}$ FRT^{19A} (kind gifts from K. Irvine); $Su(H)^{1B115}$ FRT^{40A}, $UAS\text{-}N^{act}$ and $UAS\text{-}N^{DN}$ (kind gifts from M. Fortini); FRT^{82B} $neur^{11}$ (a kind gift from B. Ohlstein and A. Spradling); *stat-GFP* (a kind gift from G. Baeg); *upd-lacZ* (a kind gift from H. Sun); *Su(H)GBE-lacZ* (a kind gift from S. Bray); $UAS\text{-}dTCF^{\Delta N}$, and $tub\text{-}GAL80^{ts}$. Homologous recombination was used to generate double mutants, including $N^{55e11}hop^{C111}$ FRT^{19A} and FRT^{82B} $neur^{11}$ $Stat92E^{06346}$.

All flies were cultured on a standard medium in either a 25°C incubator or at room temperature (about 23°C), unless otherwise indicated.

MARCM CLONE ASSAY

To induce MARCM clones of genes on the X-chromosome, including the mutations hop^{C111}, N^{55e11}, and double mutants of hop^{C111} and N^{55e11}, we generated the following flies: *tub-Gal80* FRT^{19A}/*mutant-*FRT^{19A}; *SM6B*, *hsflp*/+; *act>Gal4*, *UAS-GFP*/+. To induce MARCM clones of *Su(H)*, we generated the following flies: $hsflp^{122}$/+; *tub-Gal80* FRT^{40A}/$Su(H)^{1B115}$ FRT^{40A}; *act>Gal4*, *UAS-GFP*/+. To induce MARCM clones of $Stat92E^{06346}$, $neur^{11}$ and their double mutant, we generated the following flies: $hsflp^{122}$/+; *act>Gal4*, *UAS-GFP*/+; FRT^{82B} *tub-Gal80/FRT82B mutant-*. One- or 2-day-old adult female flies were heat-shocked at 37°C for 60 min twice a day with an interval of 8 h. The flies were transferred to fresh food daily after the final heat shock, and their midguts were processed for analysis at the indicated times.

TEMPERATURE-SHIFT EXPERIMENT

Flies carrying transgenes of *esg-Gal4*, *UAS-GFP*; $tub\text{-}Gal80^{ts}$ alone or together with the respective UAS-line were raised at 18°C and shifted to 30°C (restrictive temperature) to turn on the Gal4 transcriptional activity. The following UAS-lines were used: $UAS\text{-}N^{intra}$, $UAS\text{-}N^{DN}$, $UAS\text{-}dTCF^{\Delta N}$, and *UAS-upd*.

JAK2 INHIBITOR TREATMENT

The JAK2 inhibitor of AG490 (T-9142, LC Laboratories) was dissolved into DMSO (25 mg/ml) and 100 μl (or 200 μl) was added directly on the surface of fly vial food to reach a final concentration of 250 ng/ml (or 500 ng/ml). Two-day-old adult flies ($UAS\text{-}N^{DN}$/+; *esg-Gal4*, *UAS-GFP*/+; $tub\text{-}Gal80^{ts}$/+) were used for experiments. They were transferred from 18 to 30°C and fed with normal or AG490 added food. Twelve days later, guts were processed for analysis. Fly food was replaced every 2 days.

APOPTOSIS ASSAY

Apoptosis was analyzed using the ApopTag Red in situ apoptosis detection kit (Millipore, Cat# S7165).

HISTOLOGY AND IMAGE CAPTURE

The fly intestines were dissected in PBS and fixed in PBS containing 4% formaldehyde for 30 min. After four times 15-min rinses with PBT (PBS + 0.1% Triton X-100), the samples were incubated with the primary antibody at room temperature for 2 h or overnight at 4°C. The tissues were then incubated with the fluorescence-conjugated secondary antibody for 2 h at room temperature. Samples were mounted in 90% glycerol. We used the following antibodies: rabbit polyclonal anti-Stat92E [1:500; Chen et al., 2002]; rabbit polyclonal anti-β-Gal (1:1,000; Cappel); mouse anti-β-Gal (1:100, Promega); mouse anti-Dl (1:50; DSHB); mouse monoclonal anti-Pros (1:50; DSHB); rabbit polyclonal anti-GFP (1:500; Molecular Probes); mouse monoclonal anti-GFP (1:500; Molecular Probes); and rabbit anti-phospho-histone H3 (Upstate Biotechnology, 1:1,000). Secondary antibodies used were goat anti-mouse and goat anti-rabbit IgG conjugated to Alexa 488 or Alexa 568 (1:400; Molecular Probes). DAPI (Sigma) was used to stain DNA. Images were captured with the Zeiss LSM 510 confocal system and processed with LSM Image Browser and Adobe Photoshop.

RESULTS

JAK–STAT IS EXPRESSED IN THE PROGENITORS OF THE *DROSOPHILA* MIDGUT

In an effort to dissect signalings controlling ISC behavior, we found a broad JAK–STAT expression in the adult *Drosophila* midgut. First, a JAK–STAT reporter line [*stat-GFP*; Bach et al., 2007] revealed the signaling is in both ISCs (Dl^+, arrows in Fig. 1b′) and EBs [Su(H)GBE-$lacZ^+$, arrowheads in Fig. 1b′], but is mostly absent from ECs (polyploid nuclei, arrows in Fig. 1b″) and ee-cells ($pros^+$ in nucleus, arrowheads in Fig. 1b″). Consistent with this discovery, the Stat92E protein (red in Fig. 1c) completely co-localizes with the GFP reporter (Fig. 1c′). Moreover, a transcriptional reporter of the signaling ligand [*upd-lacZ*; Sun et al., 1995, green in Fig. 1d] indicates *upd* is produced in the same stat92E-expressing cells (Fig. 1d′). Taken together, we confirmed the signaling ligand, the nuclear effector, and the signaling output in the two undifferentiated cell types of the *Drosophila* midgut epithelium.

Interestingly, we noticed that the Stat92E protein was mainly concentrated in the cytoplasm of most ISCs and EBs (Fig. 1e–e″), but a few ISCs [$Stat92E^+$, Su(H)GBE-$lacZ^-$; Fig. 1e–e″] coupled with EBs [Su(H)GBE-$lacZ^+$; Fig. 1e′] had strong Stat92E in the nucleus (arrow in Fig. 1e). It is known that the translocation of STATs into the nucleus is a hallmark of strong JAK–STAT signaling [reviewed in Arbouzova and Zeidler, 2006]. We speculate that the cells with nuclear accumulation of Stat92E represent a group of activated ISCs. A strong JAK–STAT signaling might function in these activated ISCs.

JAK–STAT IS REQUIRED FOR ISC PROLIFERATION

To examine if and how JAK–STAT functions in the homeostasis of the midgut, we generated JAK–STAT mutant clones using a repressible cell marker technique [MARCM; Lee and Luo, 1999]. $stat92E^{06346}$ represents a loss-of-function allele [Hou et al., 1996]. Two days after clone induction (ACI), we could detect a similar number of clones in wild-type and *stat92E* mutant samples (Fig. 2a,b), indicating comparable clone induction efficiency. Both samples contained several types of GFP-positive cells, including ECs (marked by their large nuclei and cell bodies), ee-cells ($pros^+$) and ISCs (Dl^+) (Fig. 2a,c). Because of the short clone-chasing time, the $stat92E^-$ ECs and ee-cells probably originated from transient clones [Ohlstein and Spradling, 2006]. We speculate that either the Stat92E protein has not been completely turned over yet or that JAK–STAT plays a smaller role in specifying the ISC daughter cell fates.

It takes about one week for the transient clone turnover [Ohlstein and Spradling, 2006]. Two weeks ACI, we found that most wild-type ISCs had finished at least one cell cycle and stayed with their progenies in big clusters (Fig. 2b). In contrast, most $stat92E^{06346}$ clones were composed of ISC-like cells (Dl^+, esg/GFP) or a small number of isolated EC- and ee-like cells (weak GFP, big nuclei or $pros^+$; Fig. 2d). As a result, the ISC-like cells occupy a large portion of the total GFP-positive clones in *stat92E* mutants (Fig. 2j). We

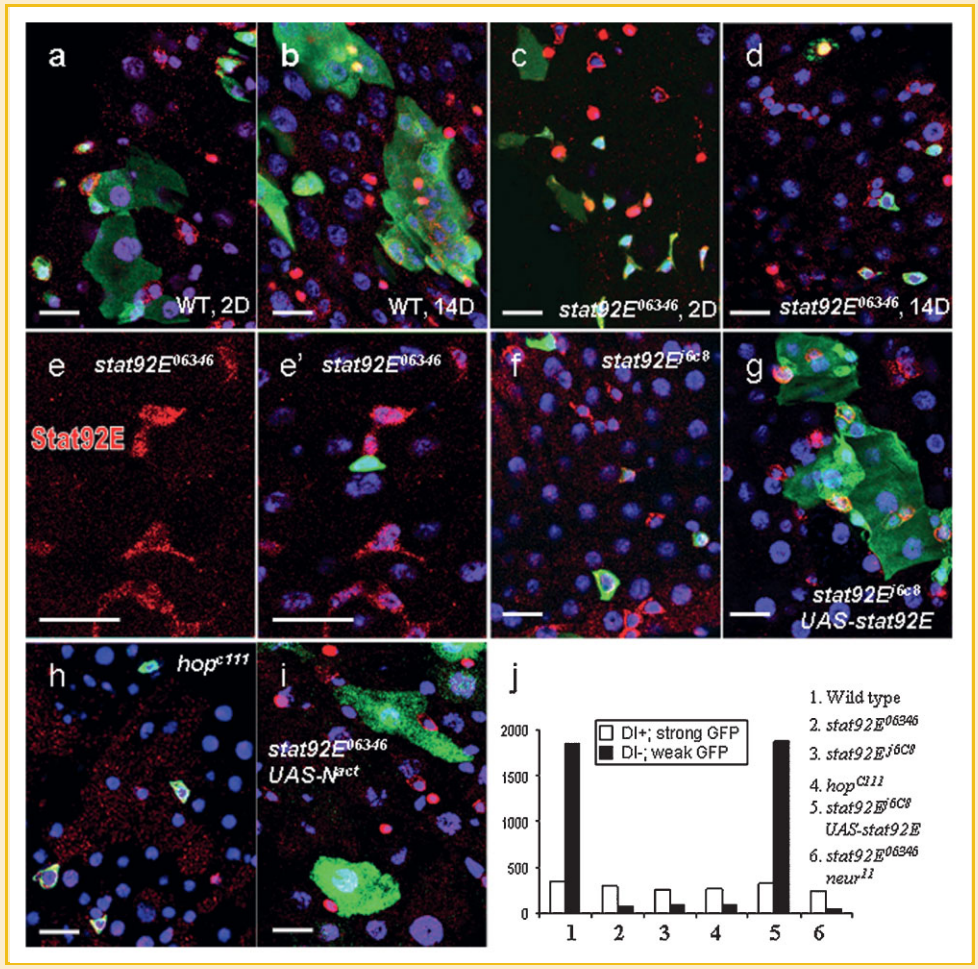

Fig. 2. JAK–STAT is required for ISC proliferation. MARCM clones (GFP+) were generated in the posterior midguts of adult flies with background of: a,b, Wild-type controls of *FRT82B Pim*; c–e′, *stat92E*^{06346}; f, *stat92E*^{j6C8}; g, *stat92E*^{j6C8}, *UAS-stat92E*; h, *hop*^{c111}; i, *stat92E*^{06346}, *UAS-N*^{intra}. Green: GFP; red: Dl and Pros; blue: DAPI. a,c, 2 days ACI. The clone induction efficiency is comparable in different backgrounds and all cell types can be labeled in both control (a) and *stat92E* mutants (c). b,d–h: 14 days ACI. Wild-type ISC clones have grown into big clusters containing different kinds of cells (b); while most stat92E mutant clones (d–f,h) have only single ISC-like cells and a few of EC and ee-cells. e and e′: the phenotype shown in c and d was confirmed to be associated with loss of *stat92E* by antibody staining (Stat92E in red). g: The *stat92E* mutant phenotype can be rescued by supplying a wild-type allele (*stat92E*^{j6C8}, *UAS-stat92E*). i: Forced expression of active Notch (*UAS-N*^{intra}) is sufficient to drive JAK/STAT deficient ISCs into differentiation. j: Quantitative analysis shows a significant decrease of the differentiated cells within JAK/STAT mutant clones. Ten complete guts for each genotype (14-day ACI) were picked and the total number of ICS-like cells (Dl+, strong esg/GFP) and non-ISC cells (Dl-, weak GFP) were counted respectively. Scale bars: 20 μm.

confirmed the phenotype was associated with the loss of *stat92E* by staining the Stat92E protein (Fig. 2e,e′).

Similar phenotypes were obtained using a different *stat92E* allele (*stat92E*^{j6C8}, Fig. 2f,j), which could be rescued by supplying wild-type Stat92E proteins (*UAS-stat92E*; Fig. 2g). We also checked *hop*^{C111}, a loss of function alleles of *Drosophila* JAK [Binari and Perrimon, 1994], and observed the same results (Fig. 2h).

The significant loss of differentiated cells in the JAK–STAT mutant clones could be explained by two mechanisms: excess cell death or poor ISC proliferation. Four days ACI, an abundance of ECs and ee-cells remained in the JAK–STAT mutant clones. In addition, we did not find induced apoptosis (Apoptag analysis, data not shown), thus cell death could not account for the reduction of differentiated cells. We also counted the ISC-like cells of 30-day-old mutant clones and found only a slight decrease compared with 14-day-old samples, reflecting a slower ISC proliferation (22/gut vs. 29/gut). We concluded that without JAK–STAT, ISCs stay in quiescent state and cannot go through the cell cycle to generate new differentiated daughter cells or make self-renewal. The small number of remaining ECs and ee-cells in old JAK–STAT mutant clones might come from transient clones. They either represent some slow turning-over cells, or it is due to the leaky of FLP recombinase production (*hsp-flp*). Interestingly, forced expression of a constitutive form of *N* (*N*^{act}) is still able to transform the quiescent ISC-like cells in JAK–STAT mutant clones into the EC-like cells [Fig. 2i; Ohlstein and Spradling, 2007]; suggesting that JAK–STAT does not interfere with the differentiation pathway specified by Notch.

ELEVATED JAK–STAT ACCELERATES ISC PROLIFERATION

Since the loss of JAK–STAT leads to poor ISC proliferation, we wondered if elevated signaling would be able to accelerate this process. We increased JAK–STAT signaling by over-expressing Upd

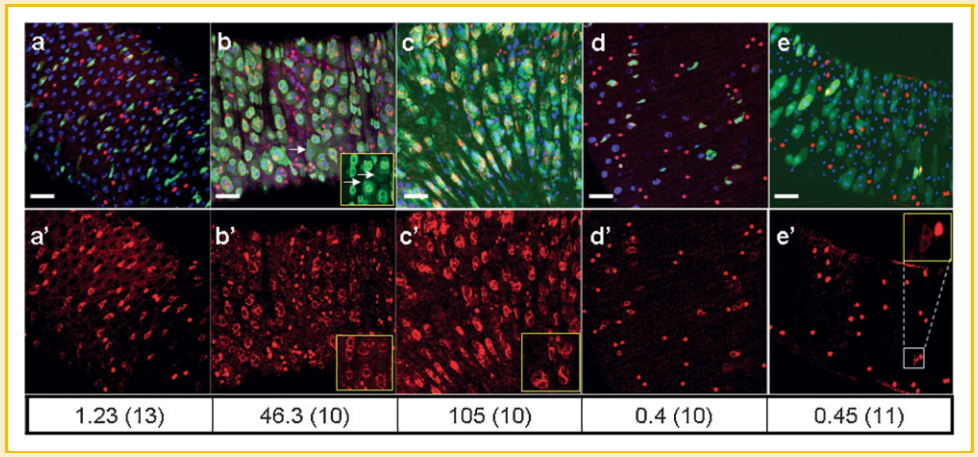

Fig. 3. Elevated JAK–STAT accelerates ISC proliferation. a,a′: Control flies were shifted to 30°C for 5 days (*esg-gal4, UAS-GFP/CyO; tub-Gal80ts/+*); b–c′, over-expression of *upd* for 2 days (b,b′) and 5 days (c,c′) (*esg-gal4, UAS-GFP/UAS-upd; tub-Gal80ts/+*, shifted to 30°C). We focused on the surface of the tissue to highlight progenitor cells; there are also a lot of young ECs marked with large nuclei and weak GFP underneath (arrows in b). Note there are many paired ISCs (insets in b′ and c′), probably they are newly formed ISCs right after mitosis. d,d′: Over-expression of $dTCF^{\Delta N}$ to block wg and ISC self-renewal for 5 days. There is a clear reduction of ISCs. e,e′: Co-expression of $dTCF^{\Delta N}$ and *upd* leads to a fast exhaustion of ISCs. A majority of GFP+ cells are young ECs (e, marked by with weak GFP and big nucleus) and only few of paired ISCs left (inset in e′). In all panels, red: Dl and Pros; green: GFP, blue: DAPI. Scale bars: 20 μm. The mean number of Ph3+ cells per complete gut was shown under each genotype; total number of samples counted was shown in the parenthesis.

using the following flies: *esg-Gal4*, *UAS-GFP/UAS-upd*; *tub-Gal80*^{ts}/+. *esg-Gal4* is able to drive the expression of *GFP* and *upd* in both ISCs and EBs [Fig. 3a; Micchelli and Perrimon, 2006], which is otherwise suppressed by Gal80^{ts} at 18°C. Two days after shifting to the restrictive temperature of 30°C to inactivate Gal80^{ts}, we found an increased number of ISC-like cells (Dl^{+}, strong esg/GFP, Fig. 3b,c) and young ECs underneath (arrows in Fig. 3b and inset, marked with big cell bodies and weak GFP due to the stable GFP protein). Staining with a mitotic marker of phosphorylated-H3 (Ph3) revealed a significant increase of the mitotic index (Ph3+ cells/gut; Fig. 3, numbers under each genotype). Interestingly, we also found many paired ISC-like cells (insets of Fig. 3b′,c′). We believe either they are two newly formed ISCs following self-renewal or it is because the differentiation pathway has not started yet. Five days after shifting to 30°C, flies became inactive and started to die. We dissected these flies and found a stronger over-proliferation phenotype. All of them developed gut hyperplasia (n = 12, Fig. 3c). The gut wall was much thicker than controls (composing more than 3 cell layers vs. 1–2 in controls) and it was mixed with excessive ISC-like cells (Fig. 3c′) and a large amount of young daughter cells (*Dl*-, weak GFP, large nuclei, toward the lumen). As a result, the percentage of mature ECs (counted as cells with nuclei at least four times bigger than the diploid cells: 8n vs. 2n) within the total cell pool was significantly decreased (about 20% vs. 70% in controls).

To further investigate if both the self-renewal and differentiation were accelerated following stimulated JAK–STAT, we tried to block one of the two pathways. *wingless* (*wg*)/APC signaling was known to promote ISC self-renewal without interfering with its differentiation pathway [Lin et al., 2008; Lee et al., 2009]. We expressed a dominant-negative form of *dTCF* (*esg-Gal4/UAS-*$dTCF^{\Delta N}$) to block *wg* and ISC self-renewal. Five days after shifting to 30°C, there was a significant reduction of ISCs in $dTCF^{\Delta N}$-over-expressed samples (Fig. 3d,d′; esg/GFP, Dl+). This is consistent with previous work, indicating the exhausted ISCs could not be replenished through self-renewal. The remaining ISCs might be some quiescent stem cells that were not activated during the experiment. Interestingly, when we co-expressed $dTCF^{\Delta N}$ and *upd*, the ISCs were lost much more quickly (compare Fig. 3d,e). Most esg/GFP+ cells were only weakly labeled with GFP and showed large nuclei and body size, representing young ECs (Fig. 3e). We speculate the ISC self-renewal and differentiation pathways were not coupled when the overall ISC proliferation was accelerated by JAK–STAT; the over-expression of $dTCF^{\Delta N}$ specifically impaired ISC self-renewal but not its differentiation. It also indicates that elevated JAK–STAT pushed most (if not all) ISCs into activated states. Once the self-renewal pathway was blocked, the ISCs had to go through the only differentiation pathway sooner or later, which results their quick exhaustion. Taken together, we concluded that JAK–STAT positively controls ISC proliferation, a prerequisite for downstream self-renewal and differentiation pathways.

NOTCH SUPPRESSES JAK–STAT THROUGH A TRANSCRIPTIONAL CONTROL OF UPD

The over-proliferation phenotype upon elevated JAK–STAT reminds us of the similar consequences in the Notch mutant background. It has been shown before that the loss of Notch is sufficient to block ISCs differentiation and induce cell cycle at the same time, causing ISC-like tumors [Micchelli and Perrimon, 2006; Ohlstein and Spradling, 2006, 2007; Fig. 4a]. To investigate if JAK–STAT and Notch have any crosstalk, we checked the expression of *stat92E* in Notch mutant clones. It turned out that Stat92E was predominantly localized within the nuclei of N^{-} cells (Fig. 4a,a′), suggesting a strong induction of JAK–STAT signaling. Similar results were obtained when we used a loss-of-function allele of *Su(H)*, a signal transducer of *N* in the nucleus (data not shown). We propose that a

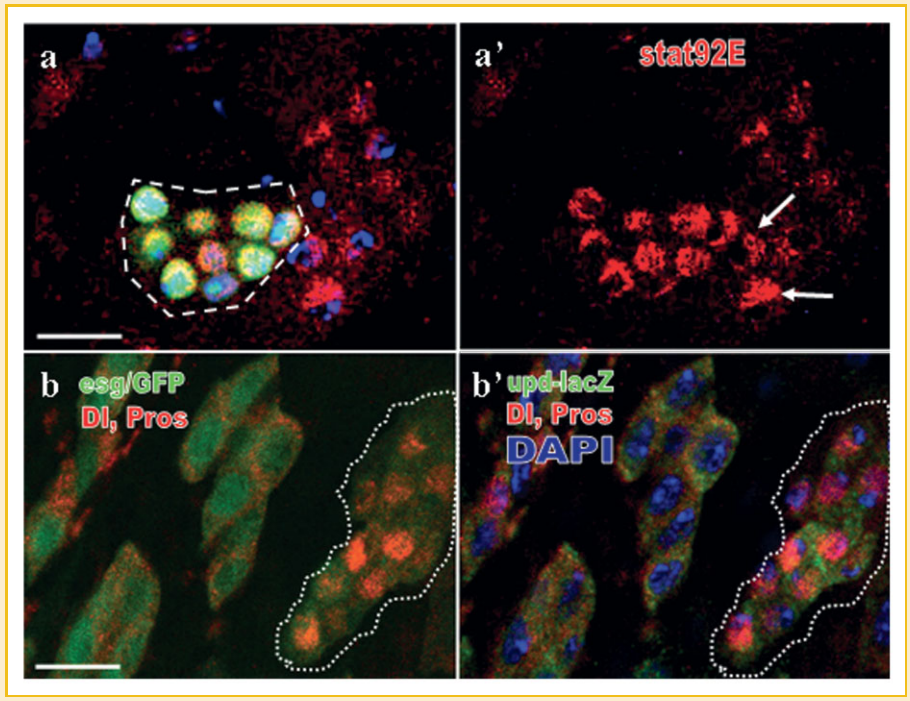

Fig. 4. Notch suppresses JAK–STAT through a transcriptional control of UPD. a,a′: JAK–STAT is stimulated upon loss of Notch. Red, Stat92E; green, GFP. Note the nuclear-enriched Stat92E within and adjacent (arrows in a′) to the clones, indicating a non-cell autonomous induction. b,b′: The transcription of *upd* (monitored by *upd-lacZ*) is induced cell-autonomously within notch mutant clones. Flies (*UAS-N^{DN}/upd-lacZ; esg-Gal4, UAS-GFP/+; tub-Gal80ts/+*) were raised at 18°C and then shifted to 30°C for 5 days before dissection. Red, Delta and Prospero; green in b, GFP; green in b′, LacZ. Note upd-lacZ (green in b′) is ectopically induced within the ee-clusters. Scale bars: 20 μm.

default function of Notch is to suppress JAK–STAT in the *Drosophila* midgut epithelium. We also noticed some cells next to the N^- clones had nuclear-concentrated Stat92E, implying non-cell autonomous induction (arrow in Fig. 4b′). In *Drosophila*, JAK–STAT is triggered by Upd proteins, which can secrete into the cell matrix to work on adjacent cells [Arbouzova and Zeidler, 2006]. We examined *upd-lacZ* to monitor the transcription of *upd* in cells that expressed a dominant-negative form of *N* (*upd-lacZ/UAS-N^{DN}; esg-Gal4, UAS-GFP/+; tub-Gal80^{ts}/+*). After shifting to 30°C for 5 days, all these flies developed ISC- and ee-like tumors, a typical loss of Notch phenotype (Fig. 4b, b′). *upd-lacZ* is normally expressed only in ISCs and EBs (Fig. 1d′), but it could be cell-autonomously induced in both the ISC- and ee-like clusters where Notch was down-regulated (green in Fig. 4b′). These data suggest that Notch could directly suppress the transcription of *upd* to inhibit JAK–STAT in the *Drosophila* midgut.

Next, we asked if the elevated JAK–STAT signaling is a cause of the N^- tumors. *Neuralized* (*neur*) positively regulates Notch by stabilizing *Dl* on the cell surface. The loss of *neur* developed the same N^- phenotype in midgut, including ISC- and ee-like tumors [Ohlstein and Spradling, 2007; Fig. 5a]. Interestingly, the double mutant clones of *neur* and *stat92E* gave rise to a similar phenotype of *stat92E*$^-$ alone (Fig. 4b). Moreover, we blocked Notch by expressing N^{DN} and fed these flies with a mammalian JAK2/STAT3 inhibitor [AG490, Beckles et al., 2006]. After culturing at 30°C for 12 days, all the flies fed with normal food developed a strong loss-of-Notch phenotype (Fig. 5c). In contrast, such a phenotype could be mildly suppressed by AG490 (8 out of 10 samples; Fig. 5d). The suppression was more evident as the inhibitor dose was increased (250 and 500 ng/ml of fly food were tested). These results further support that Notch functions through JAK–STAT to negatively regulate ISC proliferation and JAK–STAT seems to be an important signaling to mediate the tumor phenotype in the Notch mutant background.

DISCUSSION

In this work, we characterized JAK–STAT as an important signaling to control ISC proliferation. First, we found differential Stat92E subcellular localizations: a small number of ISCs (often coupled with newly formed EBs) have strong JAK–STAT signaling manifested by nuclear-accumulated Stat92E (Fig. 1e), while most other ISCs and EBs have cytoplasmic concentration. Second, we observed a slight reduction of the ISC-like cells (Dl^+) and an obvious loss of differentiated cells in JAK–STAT mutant clones (Fig. 1j). These results suggested that ISC proliferation was compromised when JAK–STAT was blocked and we speculate the small number of ISCs with strong JAK–STAT represents the activated stem cells undergoing proliferation (Fig. 1e).

Recently, two groups reported that insulin receptor (InR) and EGFR signaling pathways control ISC proliferation [Amcheslavsky et al., 2009; Jiang and Edgar, 2009a]. Mutant analysis of either pathway revealed the loss of large ISC lineages, which is similar to the JAK–STAT mutant phenotype (Fig. 2). We speculate that different cell growth factors and cytokines might work coordinately

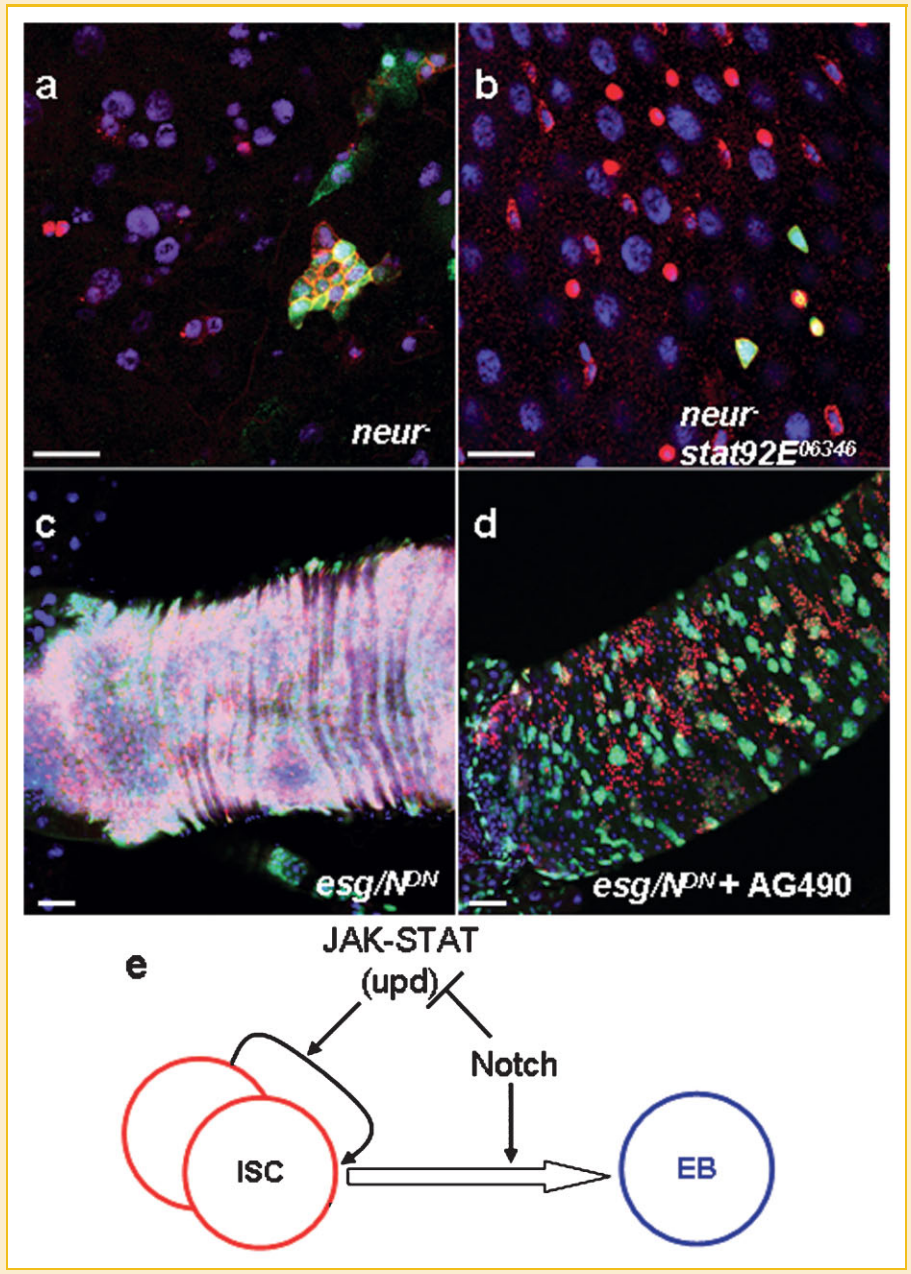

Fig. 5. JAK–STAT signaling is required for the Notch mutant tumor formation. GFP-marked MARCM clones (14 days ACI) were generated in the posterior midguts with the indicated genotypes: a, *neur*^{11}; b, *stat92E*^{06346}, *neur*^{11}. c: Over-expressing N^{DN} caused over-proliferation phenotype (esg/N^{DN} at 30°C for 12 days), which can be suppressed by a JAK inhibitor of AG490 (d). Samples of c and d were focused on the same areas of the posterior midgut. In all panels, red: Dl and Pros; green, GFP, blue, DAPI. e: Proposed model in which Notch balances the differentiation and proliferation of ISCs. Cell proliferation is a prerequisite for ISC to make self-renew and generate differentiated daughter cells. Notch promotes the ISC differentiation and tends to produce more mature cells. But this process is slowed down by suppression of JAK–STAT. Eventually, the numbers of both mature cells and stem cells are kept at a steady level. Scale bars: 20 μm.

to regulate the ISC proliferation. It would be worth investigating if the absence of two or more of these growth signals would bring about even faster ISC loss or cell death. Several groups have characterized that the proliferation of ISCs could be stimulated by various damage treatments, and the compensatory reaction was mediated through induced JAK–STAT signaling [Amcheslavsky et al., 2009; Buchon et al., 2009; Cronin et al., 2009; Jiang et al., 2009b; Chatterjee and Ip, 2009]. This is quite consistent with our conclusion, although we only focused on its role under normal conditions. In particular, during the preparation of this manuscript, one of these works [Jiang et al., 2009b] demonstrated that there were transient EB cells formed in JAK–STAT mutants and hence they concluded JAK–STAT might not interfere with basal ISC proliferation. The different observations regarding the EBs could be explained by the low JAK–STAT requirement in normal tissue homeostasis. We found a rather weak JAK–STAT signaling in the

epithelium (revealed by weak *upd-lacZ* and cytoplasmic Stat92E in most cells) is sufficient to maintain normal tissue homeostasis. The RNA interference experiment in their work and the available JAK–STAT mutants may not be sufficient enough to completely block the signaling. We speculate a longer chasing time after JAK–STAT mutant clone induction might help to reveal the defects (14 days or 30 days in our work vs. 8 days in their report).

It was observed before that Notch is able to promote ISCs differentiation and also restrict their proliferation, but the molecular mechanism remains unknown. We demonstrated here that *N* has at least two functions to control the ISC behavior. First, it promotes the differentiation of ISC. Second, it down-regulates JAK–STAT to slowdown ISC proliferation at least through a transcriptional control of *upd*. It should be pointed out that Notch was normally turned on in EB cells, but we could not detect a clear difference of the *upd-lacZ* pattern between ISCs and EBs due to its general weak expression (Fig. 1d). Thus, it is possible that Notch also has post-transcriptional controls of *upd* or genes other than Notch are involved in the above process.

To perform their physiological functions properly, many adult tissues need to retain a stable cellular architecture, which is maintained by a fixed ratio of progenitors versus mature daughter cells. Using the adult *Drosophila* ISCs as a model, we uncovered an interesting mechanism in which a differentiation signal negatively feeds back to the stem cell proliferation pathway to stabilize the mature daughter cell numbers (Fig. 5e). The Notch activity oscillates within a narrow threshold to control the ISC behavior. High Notch promotes ISC differentiation to generate more daughter cells; on the other hand, it also slows down the ISC proliferation speed. In turn, low Notch tends to reduce the differentiated cells; but the repression of the stem cell proliferation is also released. Eventually, the numbers of stem cells and mature differentiated cells are kept at stable levels.

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