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ORIGINAL ARTICLE

Drosophila Myc integrates multiple signaling pathways to regulate intestinal stem cell proliferation during midgut regeneration

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Intestinal stem cells (ISCs) in the *Drosophila* adult midgut are essential for maintaining tissue homeostasis, and their proliferation and differentiation speed up in order to meet the demand for replenishing the lost cells in response to injury. Several signaling pathways including JAK-STAT, EGFR and Hippo (Hpo) pathways have been implicated in damage-induced ISC proliferation, but the mechanisms that integrate these pathways have remained elusive. Here, we demonstrate that the *Drosophila* homolog of the oncoprotein Myc (dMyc) functions downstream of these signaling pathways to mediate their effects on ISC proliferation. dMyc expression in precursor cells is stimulated in response to tissue damage, and dMyc is essential for accelerated ISC proliferation and midgut regeneration. We show that tissue damage caused by dextran sulfate sodium feeding stimulates dMyc expression via the Hpo pathway, whereas bleomycin feeding activates dMyc through the JAK-STAT and EGFR pathways. We provide evidence that dMyc expression is transcriptionally upregulated by multiple signaling pathways, which is required for optimal ISC proliferation in response to tissue damage. We have also obtained evidence that tissue damage can upregulate dMyc expression post-transcriptionally. Finally, we show that a basal level of dMyc expression is required for ISC maintenance, proliferation and lineage differentiation during normal tissue homeostasis.

***Keywords*:** Myc; ISC; Hpo; JAK-STAT; EGFR; regeneration

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Introduction

Adult stem cells play critical roles in tissue homeostasis throughout adult life. In response to injury, adult stem cells can adjust their proliferation and differentiation capacity to rapidly compensate for lost cells. The regulatory mechanisms that control stem cell proliferation and differentiation during normal tissue homeostasis or in response to tissue damage are not well understood.

Drosophila adult midgut has emerged as an attractive system to study stem cell biology in adult tissue homeostasis and regeneration not only because the cell lineage of this tissue is simple and well-defined, but also because it bears similarities to the mammalian intestine [1, 2]. *Drosophila* midgut contains self-renewing stem cells located adjacent to the basement membrane (BM) of the midgut epithelium [3, 4]. These intestinal stem cells (ISCs) undergo division and asymmetric cell fate decision, and each ISC produces a renewed ISC and an enteroblast (EB). The EB exits cell cycle and differentiates to either absorptive enterocyte (EC) or secretory enteroendocrine cell (EE) [3, 4]. *Drosophila* midguts undergo constant turnover and can regenerate in response to tissue damage [5]. Tissue damage induced by feeding flies with chemicals such as dextran sulfate sodium (DSS)

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or bleomycin, or by bacterial infection can stimulate ISC proliferation and mount a regeneration program in affected midguts [6, 7]. Several evolutionarily conserved signaling pathways, including insulin, JNK, JAK-STAT, EGFR, Wg/Wnt and Hippo (Hpo) pathways, have been implicated in the regulation of ISC proliferation during midgut homeostasis and regeneration [5, 6, 8-18]. All these pathways have been implicated in human cancers; therefore, investigating the mechanisms underlying the control of ISC proliferation in the *Drosophila* midgut may have important implications for human diseases.

Although a number of signaling pathways have been identified as important regulators of ISC proliferation in *Drosophila* midgut regeneration, the cell-intrinsic mechanisms that mediate the actions of these extrinsic signals have remained largely unknown. *Drosophila* Myc (dMyc), encoded by the *diminutive (dm)* gene, belongs to an evolutionally conserved family of transcription factors that controls multiple cellular processes including cell growth, cell cycle progression, DNA replication, cell survival and cell competition [19, 20]. Myc family members are frequently activated in cancer cells and have been implicated in stem cell biology [21, 22]; however, their precise roles in stem cell proliferation and maintenance in adult tissue homeostasis and regeneration have not been defined. In the course of identifying genes involved in *Drosophila* adult midgut regeneration in response to tissue damage, we identified dMyc as an essential regulator of ISC proliferation. Interestingly, we found that tissue damage upregulates dMyc expression in midgut precursor cells both transcriptionally and post-transcriptionally, and that dMyc is required for elevated ISC proliferation and gut regeneration in response to tissue damage. We demonstrate that tissue damage stimulates dMyc expression through Hpo, JAK-STAT and EGFR pathways, and that dMyc acts downstream of these pathways to mediate their effects on ISC proliferation. In addition, we provide evidence that dMyc is transcriptionally activated by these pathways in midgut precursor cells, and that transcriptional upregulation of dMyc is required for optimal ISC proliferation in response to tissue damage. Finally, we provide evidence that dMyc is required for ISC maintenance, proliferation and lineage differentiation during normal tissue homeostasis.

Results

dMyc is required for ISC proliferation and midgut regeneration in response to tissue damage

To identify genes that play essential roles in midgut regeneration, we performed an RNAi-based genetic screening using the *esg^{ts}F/O* system (*esg-Gal4 tubGal80^{ts} UAS-GFP; UAS-flp Act>CD2>Gal4*) that can monitor epithelial turnover in posterior midguts [5]. Tissue damage reagents including DSS and bleomycin were used to stimulate ISC proliferation and midgut regeneration [6]. Shifting *esg^{ts}F/O* adult flies to 29 °C resulted in excision of the Flp-out cassette to generate a ubiquitously expressed, heritable Gal4 driver (*act>Gal4*), so that GFP expression marked not only the precursor cells but also their progenies. Three days after temperature shift to 29 °C, *esg^{ts}F/O*-control flies or *esg^{ts}F/O* flies carrying individual *UAS-RNAi* contructs were fed with either sucrose (mock treatment), DSS or bleomycin for another 2 days, followed by immunostaining with a GFP antibody. Compared with flies with the mock treatment, *esg^{ts}F/O* flies fed with DSS or bleomycin exhibited increased number of GFP-positive cells in their midguts. In addition, DSS- or bleomycin-treated *esg^{ts}F/O* midguts contained many differentiating or differentiated GFP-positive cells with large nuclei, whereas mock-treated midguts contained GFP-positive cells that are mainly precursor cells with small nuclei (Figure 1A-1C), suggesting that DSS and bleomycin treatments accelerated midgut turnover as a consequence of tissue damage, which is in agreement with our previous finding [6]. By contrast, *esg^{ts}F/O* carrying a *UAS-dMyc-RNAi* contruct (VDRC #2948) contained much fewer GFP-positive cells (which contained small nuclei) after DSS or bleomycin treatment (Figure 1A'-1C'). Similar results were obtained with three other *UAS-dMyc-RNAi* lines (VDRC #2947; BL #25783; BL #25784). Therefore, dMyc is essential for midgut regeneration in response to tissue damage induced by these two chemicals. In addition, dMyc RNAi also affected midgut regeneration induced by infection with the pathogenic bacteria, *Pseudomonas entomorphia* (*Pe*) (Data not shown).

We next examined the requirement of dMyc in ISC proliferation upon dMyc knockdown in the precursor cells only. Adult flies expressing GFP alone or together with dMyc-RNAi under the control of *esg-Gal4/tub-Gal80^{ts}* (*esg^{ts}*) were fed with sucrose, DSS or bleomycin for 2 days. Compared with sucrose treatment, DSS or bleomycin treatment led to increased number of GFP- and phospho-Histone3 (PH3, a specific marker for mitotic cells)-positive cells in midguts (Figure 1D-1G), indicating elevated ISC proliferation. Knockdown of dMyc in precursor cells suppressed DSS- or bleomycin-induced ISC proliferation (Figure 1D'-1F', 1G). Knockdown of dMyc in ISCs using *Dl-Gal4* driver also attenuated DSS- or bleomycin-induced ISC proliferation (Supplementary information, Figure S1), revealing a cell-autonomous role of dMyc in the regulation of ISC proliferation.

dMyc expression is upregulated in response to injury

Immunostaining with a dMyc antibody revealed that

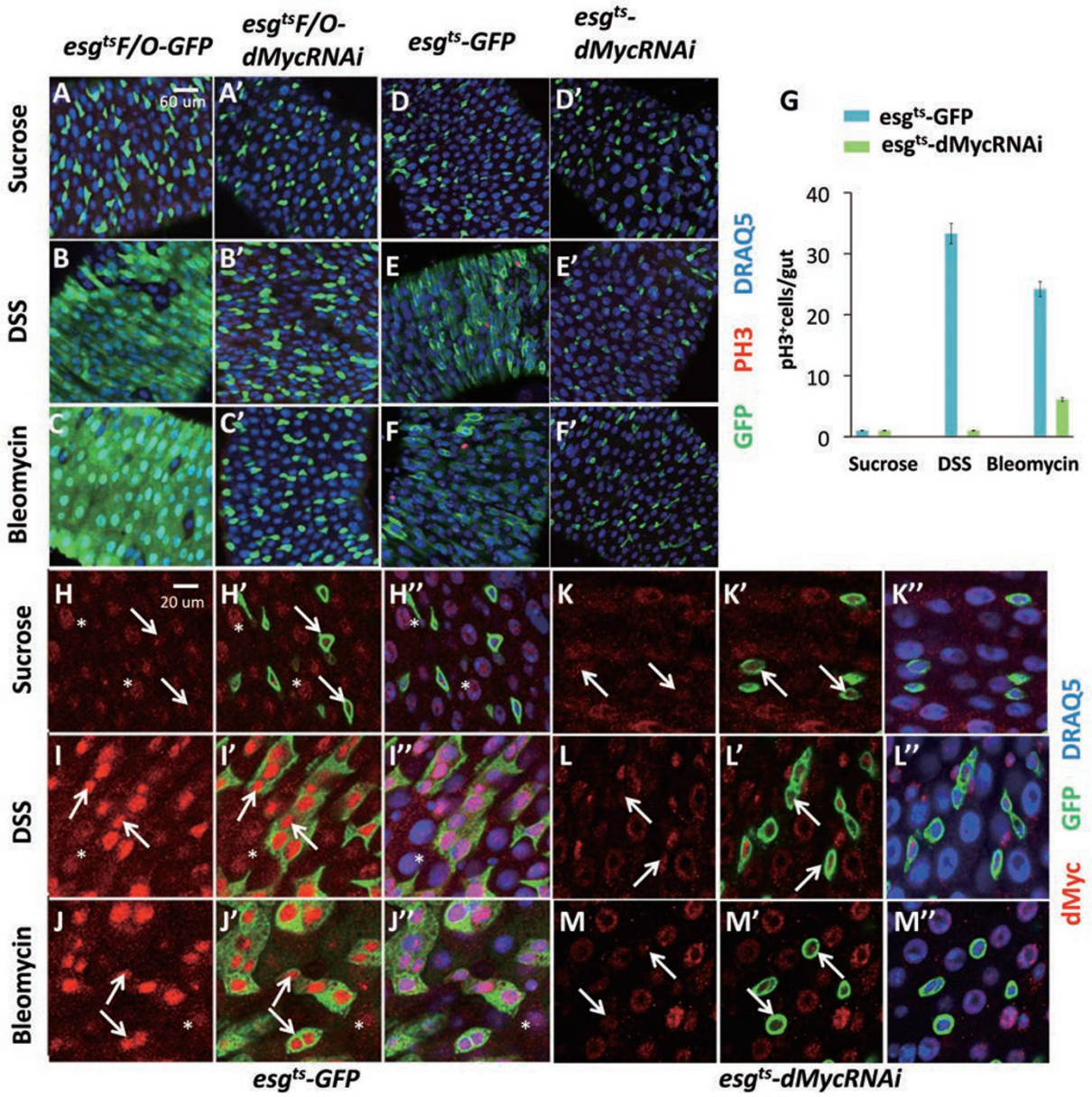

Figure 1 dMyc is required for ISC proliferation and midgut regeneration in response to tissue damage. **(A-C')** Adult midguts expressing *esg^{ts}F/O* without **(A-C)** or with *UAS-dMyc-RNAi* **(A'-C')** were treated with sucrose **(A-A')**, DSS **(B-B')**, or bleomycin **(C-C')**, and immunostained with a GFP (green) antibody and a nuclear dye DRAQ5 (blue). **(D-F')** Adult midguts expressing *UAS-GFP* without **(D-F)** or with *UAS-dMyc-RNAi* **(D'-F')** using the *esg^{ts}* system were treated with sucrose **(D-D')**, DSS **(E-E')**, or bleomycin **(F-F')**, and immunostained with GFP (green), PH3 (red) antibodies, and a nuclear dye DRAQ5 (blue). Adult flies were shifted to non-permissive temperature (29 °C) for 3-5 days and then fed with different reagents for another 2 days before dissection. The scale bar shown in **A** is also applied to **A-F'**. **(G)** Quantification of PH3^{+} cells in midguts of the indicated genotypes (*n* = 15 for each genotype). **(H-M")** Adult midguts expressing *esg^{ts}-GFP* **(H-J")** or *esg^{ts}-GFP* + *dMycRNAi* **(K-M")** were treated with sucrose **(H-H"**, **K-K")**, DSS **(I-I"**, **L-L")**, or bleomycin **(J-J"**, **M-M")**, and immunostained with GFP (green) and dMyc (red) antibodies, and a nuclear dye DRAQ5 (blue). Arrows indicate precursor cells whereas asterisks indicate ECs. The scale bar shown in **H** is also applied to **H-M"**.

dMyc is expressed at low levels in precursor cells (arrows in Figure 1H-1H') and at modest levels in ECs (indicated by asterisks in Figure 1H-1H") in homeostatic posterior midguts from 5- to 10-day-old adult females. Both DSS and bleomycin treatments upregulated dMyc expression levels in precursor cells (arrows in Figure 1I-1J'), but did

not cause a significant change of dMyc expression in ECs (asterisks in Figure 1I-1J"). In addition, DSS- or bleomycin-induced elevation in dMyc staining was suppressed in the precursor cells expressing *esg^{ts}-dMyc-RNAi* (Figure 1L-1M'). Infection with the pathogenic bacteria *Pe* also induced upregulation of dMyc expression in precursor cells (Supplementary information, Figure S2).

DSS induces dMyc upregulation through the Hpo pathway

Our previous study showed that DSS stimulates ISC proliferation through a cell-autonomous role of the Hpo pathway transcriptional effector Yki in precursor cells [23]. We then asked whether damage-induced dMyc upregulation depends on Yki activity. Indeed, inactivation of Yki by RNAi in precursor cells blocked DSS-induced dMyc upregulation (Figure 2C-2C" compared with Figure 2A-2B"). By contrast, inactivation of Yki alone in precursor cells did not block bleomycin-induced dMyc upregulation (Figure 2E-2E" compared with Figure 2A-

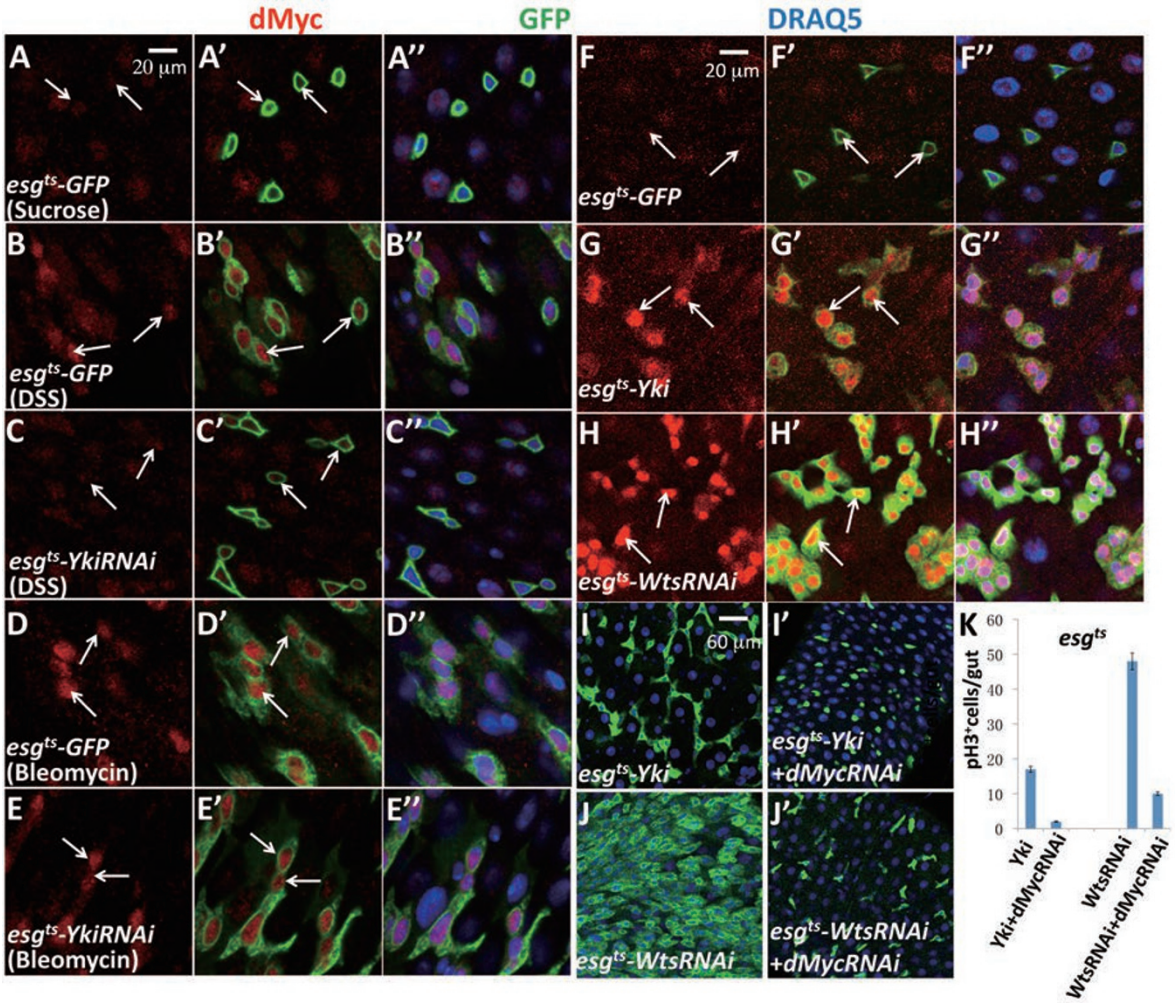

Figure 2 dMyc functions downstream of Hpo signaling pathway. **(A-E")** Adult midguts expressing *UAS-GFP* without **(A-A"**, **B-B"**, **D-D")** or with *UAS-Yki-RNAi* **(C-C"**, **E-E")** using the *esg-Gal4^{ts}* system were treated with sucrose **(A-A")**, DSS **(B-B"**, **C-C")**, or bleomycin **(D-D"**, **E-E")**, and immunostained with GFP (green) and dMyc (red) antibodies, and a nuclear dye DRAQ5 (blue). Arrows indicate precursor cells. The scale bar shown in **A** is also applied to **A-E"**. **(F-H")** Adult midguts expressing *UAS-GFP* without **(F-F")**, or with *UAS-Yki* **(G-G")** or *UAS-Wts-RNAi* **(H-H")** using *esg^{ts}* were immunostained with GFP (green) and dMyc (red) antibodies, and a nuclear dye DRAQ5 (blue). Arrows indicate precursor cells. The scale bar shown in **F** is also applied to **F-H"**. **(I-J')** Adult midguts expressing *UAS-GFP* with *UAS-Yki* **(I)**, *UAS-Yki* + *dMyc-RNAi* **(I')**, *UAS-Wts-RNAi* **(J)**, or *UAS-Wts-RNAi* + *dMyc-RNAi* **(J')** using *esg^{ts}* were immunostained with a GFP (green) antibody and a nuclear dye DRAQ5 (blue). The scale bar shown in **I** is also applied to **I-J'**. **(K)** Quantification of PH3$^+$ cells in midguts of the indicated genotypes (*n* = 15 for each genotype).

2A" and 2D-2D"), which is consistent with previous findings that bleomycin stimulates ISC proliferation through a non-autonomous mechanism emanating from ECs [9, 18].

We next determined whether dMyc acts downstream of Hpo signaling to mediate its effect on ISC proliferation. To modulate Hpo signaling, we either overexpressed Yki or knocked down an upstream kinase Wts in precursor cells; both of these conditions could promote cell proliferation [9, 14, 16]. We found that overexpression of *UAS-Yki* or *UAS-Wts-RNAi* using *esg*^{ts} upregulated dMyc expression in precursor cells (Figure 2G-2H" compared with Figure 2F-2F"). Furthermore, knockdown of dMyc in precursor cells suppressed the elevated ISC proliferation induced by excessive Yki or loss of Wts, as indicated by the reduction of both GFP- and PH3-positive cells (Figure 2I-2K). These results suggest that dMyc in precursor cells functions as a downstream mediator of Hpo signaling in the regulation of ISC proliferation.

Bleomycin induces dMyc expression through JAK-STAT and EGFR signaling pathways

We next investigated how dMyc is upregulated in precursor cells in response to bleomycin treatment. Tissue damage induced by bleomycin feeding promotes ISC proliferation through activation of the JAK-STAT and EGFR pathways (Supplementary information, Figure S3) [9], raising a possibility that bleomycin treatment may regulate dMyc expression through JAK-STAT and/or EGFR pathways. Indeed, inactivation of either the JAK-STAT pathway by expressing *UAS-Dome-RNAi* or the EGFR pathway by expressing *UAS-EGFR-RNAi* in precursor cells reduced, whereas their combined inactivation nearly abolished, bleomycin-induced dMyc upregulation in these cells (Figure 3A-3F). Hence, the JAK-STAT and EGFR pathways act additively to mediate dMyc upregulation in response to tissue damage induced by bleomycin.

To determine whether JAK-STAT or EGFR pathway hyperactivation suffices to induce dMyc upregulation, we overexpressed a ligand of the JAK-STAT pathway (*UAS-Upd*) or a constitutively active form of EGFR (*UAS-EGFR*^{A887T}) using *esg*^{ts}. We found that *esg*^{ts}*-Upd* and *esg*^{ts}*-EGFR*^{A887T} flies exhibited elevated dMyc expression levels in precursor cells after shifting to 29 °C for 2 days (Figure 3G-3I"). To determine whether dMyc acts downstream of JAK-STAT and EGFR pathways to mediate their effects on ISC proliferation, *UAS-dMyc-RNAi* was coexpressed with *UAS-Upd* or *UAS-EGFR*^{A887T} in precursor cells using *esg*^{ts} at 29 °C for 2 days. As shown in Figure 3J-3K', coexpression of *UAS-dMyc-RNAi* suppressed ISC proliferation induced by overexpression of Upd or EGFR^{A887T}, which is indicated by the reduction in the number of GFP-positive cells (compare Figure 3J, 3K with 3J', 3K') and PH3-positive cells (Figure 3L). Therefore, dMyc is a common downstream mediator of Hpo, JAK-STAT and EGFR signaling pathways to increase ISC proliferation. Consistent with our findings, a recent study showed that overexpression of Upd in *Drosophila* precursor cells induced dMyc upregulation and that knockdown of dMyc suppressed Upd-driven ISC proliferation [24].

dMyc is transcriptionally regulated by Hpo, JAK-STAT and EGFR pathways

We next asked whether *dMyc* is regulated at the transcriptional level by various pathways. Two independent *dMyc-lacZ* enhancer trap lines that express *lacZ* from the *dm* locus were used to monitor *dMyc* transcription in midguts, and similar results were obtained. In homeostatic wild-type (WT) midguts from 5- to 10-day-old adult females, *dMyc-lacZ* expression was weakly detected in precursor cells (Figure 4A-4A"). Inactivation of Wts or overexpression of Yki, Upd or EGFR^{A887T} in precursor cells markedly increased *dMyc-lacZ* expression in these cells (Figure 4B-4E"). Further, we found that overexpression of an active form of Stat (StatΔNΔC) [25], or that of Point2 (Pnt2-Vp16) [26], a transcription factor of the EGFR pathway, also activated *dMyc-lacZ* expression (Figure 4F-4G"). The quantification of *dMyc-lacZ* expression is shown in Figure 4H. These results suggest that *dMyc* is transcriptionally regulated by Hpo, JAK-STAT and EGFR signaling pathways. Consistently, tissue damage induced by feeding with DSS or bleomycin or infection with *Pe* also stimulated *dMyc-lacZ* expression in precursor cells (Supplementary information, Figure S4). Thus, dMyc transcription is stimulated in response to tissue injury through multiple signaling pathways.

dMyc is a transcriptional target of Hpo and JAK-STAT pathways

The observation that dMyc is regulated by Hpo, JAK-STAT and EGFR pathways at the transcriptional level led us to ask whether dMyc is a direct transcriptional target of these pathways. We searched the *dm* locus for binding sites of Scalloped (Sd), Stat and Pnt, transcription factors of Hpo, JAK-STAT and EGFR pathways, respectively [27-29]. Several potential binding sites for each of these transcription factors were found to be located in a ~1.0 kb region that comprises the first exon and part of the first intron (Figure 5A). We then generated a *luciferase* reporter gene construct (*dMyc1.0-luc*) containing this region and examined its response to the activation of Hpo, JAK-STAT or EGFR pathway in S2 cells. The basal activity of *dMyc1.0-luc* was low, but it could be significantly activated by coexpression of Yki plus Sd or StatΔNΔC

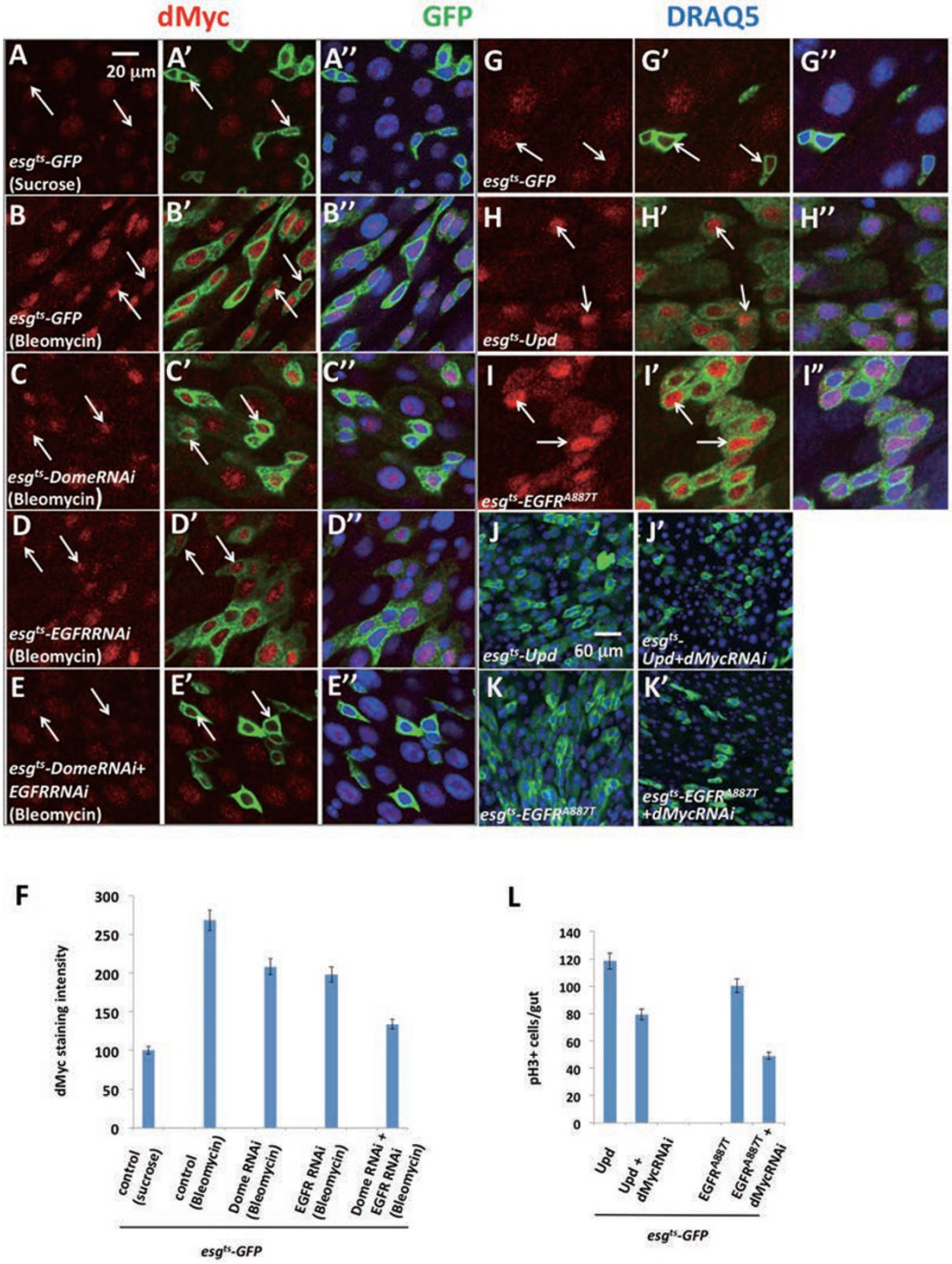

Figure 3 dMyc functions downstream of both JAK-STAT and EGFR pathways. **(A-E")** Adult midguts expressing *UAS-GFP* without **(A-B")**, or with *UAS-Dome-RNAi* **(C-C")**, *UAS-EGFR-RNAi* **(D-D")**, or *UAS-Dome-RNAi* plus *UAS-EGFR-RNAi* **(E-E")** using *esg*^{ts} were treated with sucrose **(A-A")** or bleomycin **(B-E")** and immunostained with GFP (green) and dMyc (red) antibodies, and a nuclear dye DRAQ5 (blue). Arrows indicate precursor cells. **(F)** Quantification of dMyc staining in precursor cells shown in **A-E** (n > 40 for each genotype). **(G-I")** Adult midguts expressing *UAS-GFP* without **(G-G")**, or with *UAS-Upd* **(H-H")** or *UAS- EGFR*^{A887T} **(I-I")** using *esg*^{ts} were immunostained with GFP (green) and dMyc (red) antibodies, and a nuclear dye DRAQ5 (blue). Arrows indicate precursor cells. **(J-K')** Adult midguts expressing *UAS-GFP* together with *UAS-Upd* **(J)**, *UAS-Upd + dMyc-RNAi* **(J')**, *UAS-EGFR*^{A887T} **(K)**, or *UAS-EGFR*^{A887T} *+ dMyc-RNAi* **(K')** using the *esg*^{ts} system were immunostained with GFP (green) antibody and a nuclear dye DRAQ5 (blue). 3- to 5-day-old females were shifted to 29 °C for 2 days before dissection and immunostaining. **(L)** Quantification of PH3^{+} cells in midguts of the indicated genotypes (n = 15 for each genotype).

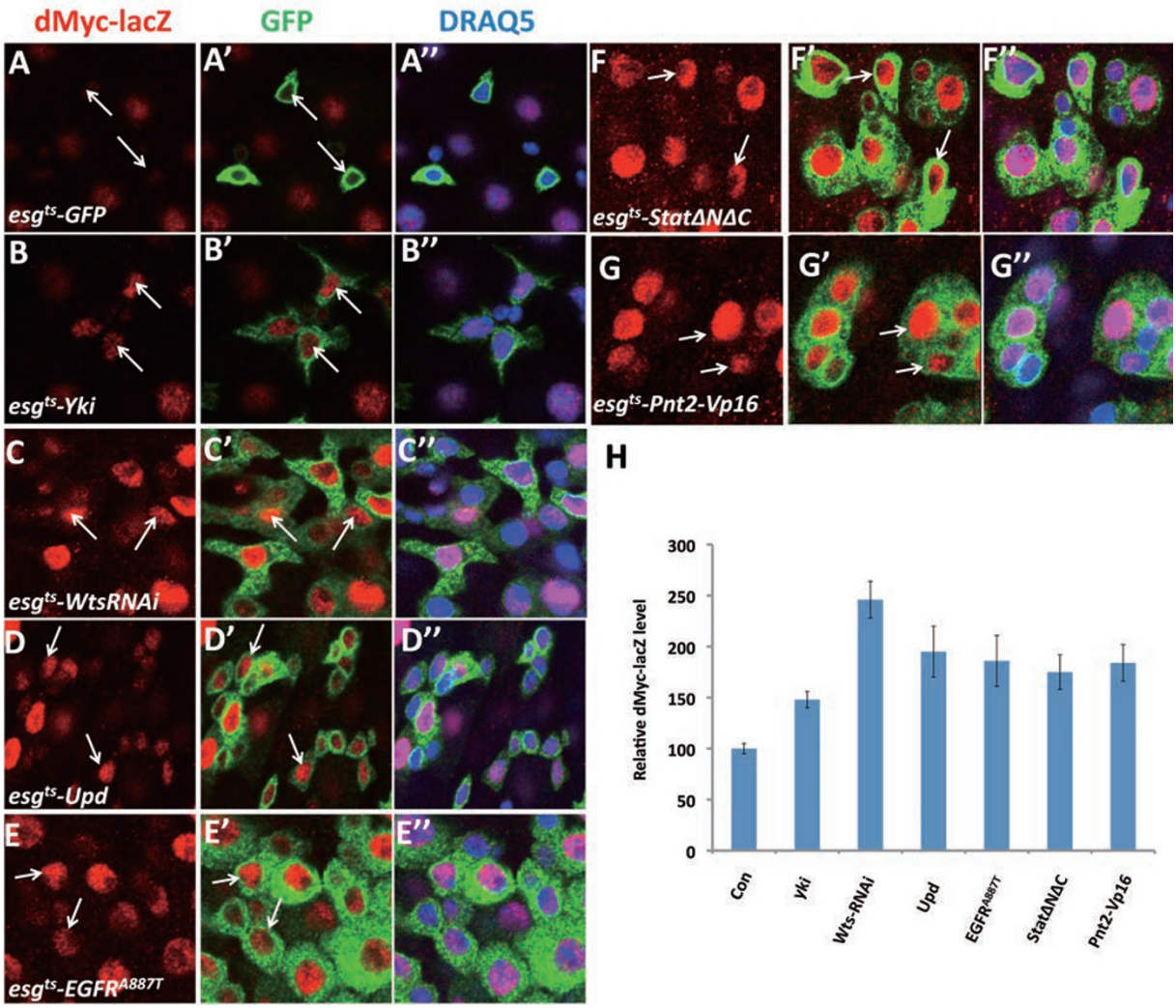

Figure 4 dMyc is regulated at transcriptional level by multiple signaling pathways. **(A-G")** Adult midguts expressing *dMyc-lacZ* and *UAS-GFP* without **(A-A")**, or with *UAS-Yki* **(B-B")**, *UAS-Wts-RNAi* **(C-C")**, *UAS-Upd* **(D-D")**, *UAS- EGFR^{A887T}* **(E-E")**, *UAS-StatΔNΔC* **(F-F")**, or *UAS-Pnt2-Vp16* **(G-G")** using the *esg^{ts}* system were immunostained with GFP (green) and lacZ (red) antibodies and a nuclear dye DRAQ5 (blue). Arrows indicate precursor cells. **(H)** Quantification of *dMyc-LacZ* expression in precursor cells shown in **A-G** (n > 50 for each genotype).

plus Hopscotch (Hop, the *Drosophila* Janus Kinase, Jak) (Figure 5B). Furthermore, mutating all the consensus Stat-binding sites in the *dMyc1.0* region (*dMyc1.0m-luc*) blocked StatΔNΔC + Hop-induced but not Yki + Sd-induced luciferase activity (Figure 5B). We found that *dMyc1.0-luc* was not activated by Pnt2-Vp16 (data not shown). It is possible that additional regions of the *dm* locus are required for the EGFR pathway to activate *dMyc*. Alternatively, the EGFR pathway may indirectly regulate *dMyc* transcription through other mechanisms.

To determine whether Sd and Stat occupy the *dMyc* enhancer *in vivo*, we carried out chromatin immunoprecipitation (ChIP) experiments. We selected two segments in the *dMyc1.0* region (Amplicon 1 and 2), each of which is ~150 bp in length and contains several potential Sd- and Stat-binding sites (Figure 5A). A segment 2 kb upstream of the *dMyc1.0* region, which does not contain any Sd- or Stat-binding sites, was used as a control. As precursor cells only constitute a small portion of the total cell mass in midguts, we knocked down Notch (N) in precursor cells to expand the ISC population. *UAS-HA-Sd +Myc-Yki* or *UAS-HA-StatΔNΔC* was expressed together with *UAS-N-RNAi* in precursor cells under the control of *esg^{ts}*, and midguts were dissected for ChIP analysis. We found that both HA-Sd and HA-StatΔNΔC bound to *dMyc1.0* Amplicon 1 and 2, but not to the up-

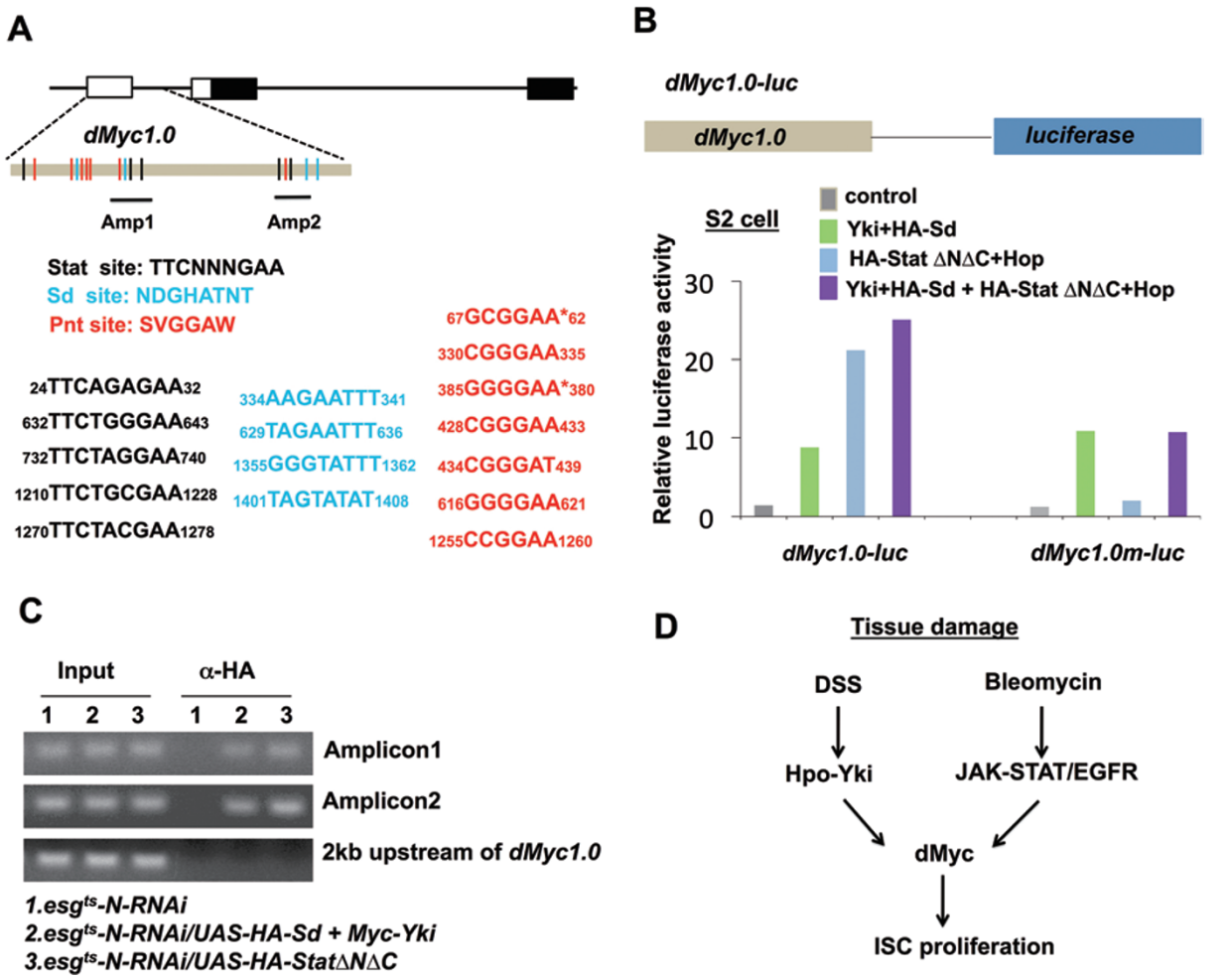

Figure 5 *dMyc* promoter/enhancer region contains consensus Sd-, Stat- and Pnt-binding sites. **(A)** Schematic representation of the *dm* locus showing the first three exons. Black rectangles represent coding regions, white rectangles represent noncoding regions; lines denote introns. The blowup indicates the 1.0 kb enhancer region with black bars indicating consensus Stat-binding sites, blue bars indicating consensus Sd-binding sites and red bars indicating consensus Pnt-binding sites, respectively. W: A or T; N: any nucleotide; D: G, A or T; H: A, C or T; S: C or G; V: A, C or G. The DNA sequences for individual putative binding sites are listed. **(B)** (Top) Diagram of the *dMyc1.0-luc* reporter gene. The *dMyc1.0* enhancer region was placed upstream of the heat shock basal promoter followed by the luciferase-coding sequence. (Bottom) S2 cells were transfected by the indicated expression constructs plus the luciferase reporter gene, and the cell lysates were subject to the dual-luciferase reporter assay. *dMyc1.0m-luc* has all the Stat-binding sites mutated. **(C)** ChIP experiment to detect the direct binding of exogenously expressed HA-Sd or HA-Stat to the *dMyc1.0* enhancer region. Transgenic flies expressing *UAS-N-RNAi* alone or together with *UAS-HA-Sd* plus *UAS-Myc-Yki* or *UAS-HA-StatΔNΔC* with esg^{ts} were subject to ChIP experiment using an anti-HA antibody. The enhancer regions encompassed by different primers were indicated in **A**. **(D)** dMyc integrates multiple signaling pathways to drive ISC proliferation in response to tissue damage.

stream fragment (Figure 5C), suggesting that Sd and Stat can be directly recruited to *dMyc* enhancer regions in precursor cells.

When fused to a GFP reporter (*dMyc1.0-GFP*), the 1 kb *dMyc* enhancer failed to drive GFP expression in midgut precursor cells in response to DSS or bleomycin treatment (data not shown), suggesting that other regulatory regions of the *dMyc* locus are required for damage-induced dMyc upregulation.

Transcriptional upregulation of dMyc is required for optimal ISC proliferation in response to tissue damage

To determine whether transcriptional upregulation of dMyc is important for injury-induced ISC proliferation, we examined adult flies that are homozygous for a *dMyc*-null mutation, dm^4, but carry a *dMyc* transgene under the

control of the ubiquitous tubulin promoter (*tub-dMyc*). dm^4/dm^4*; tub-dMyc* adult flies exhibited slightly smaller body size than WT flies or dm^4 heterozygous flies (dm^4*/+; tub-dMyc*). 5- to 6-day-old dm^4/dm^4*; tub-dMyc* and dm^4*/+; tub-dMyc* adult females were fed with sucrose, DSS or bleomycin for 2 days, followed by immunostaining with a PH3 antibody. As expected, midguts from dm^4*/+; tub-dMyc* flies exhibited elevated PH3 staining after DSS or bleomycin treatment (Figure 6A). Intriguingly, midguts from dm^4/dm^4*; tub-dMyc* flies also exhibited elevated PH3 staining in response to tissue damage (Figure 6A). However, the increase in the mitotic index (indicated by PH3 staining) was significantly lower in dm^4 homozygous flies than that in dm^4 heterozygous flies (Figure 6A).

To correlate the change in mitotic index with that of dMyc expression, midguts from WT (*wt*) control flies, dm^4 homozygous and heterozygous flies were immunostained with a dMyc antibody in the presence or absence of tissue damage. As expected, *wt* and dm^4 heterozygous

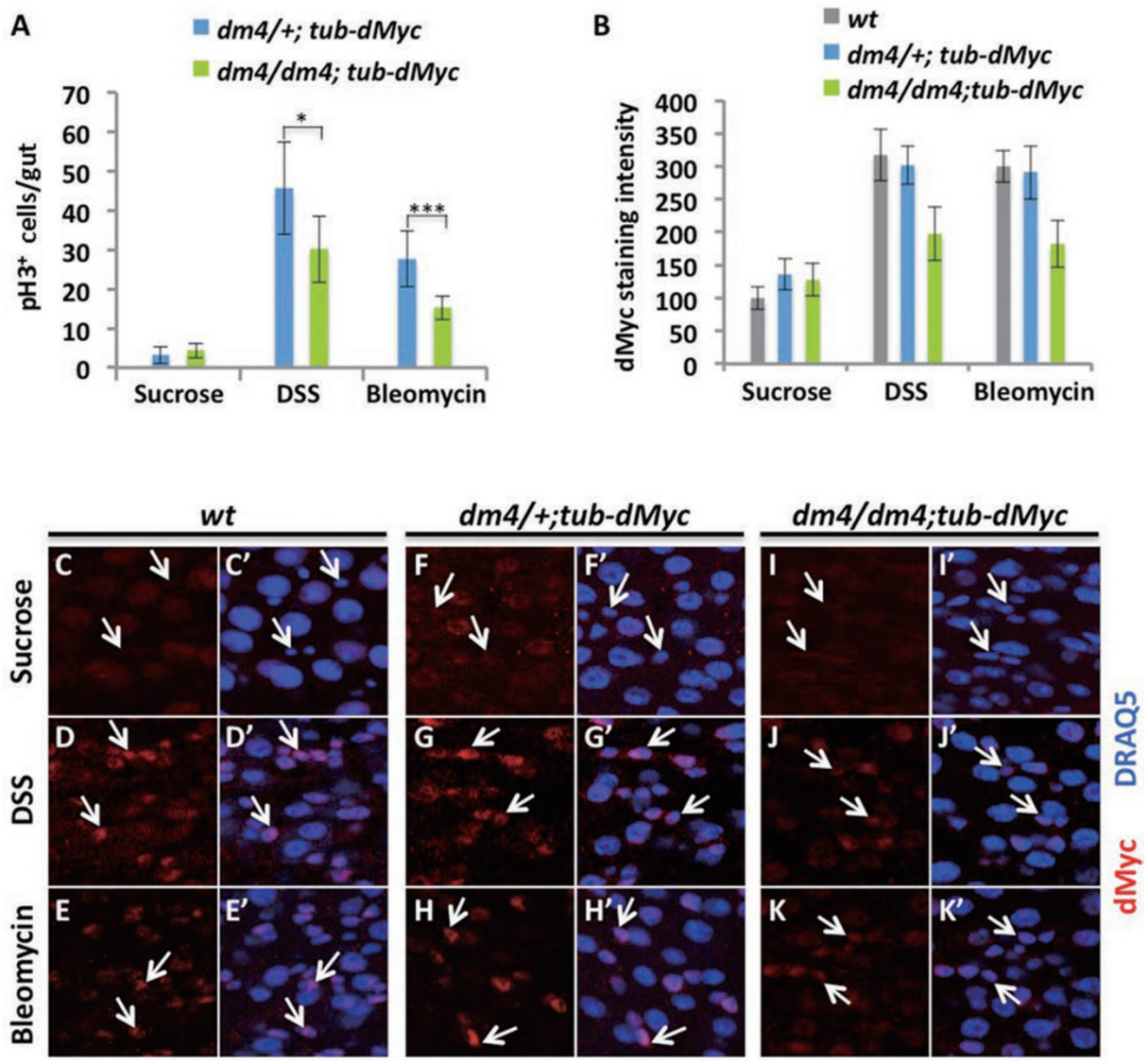

Figure 6 Transcriptional upregulation of dMyc is required for optimal ISC proliferation in response to injury. **(A)** Quantification of $PH3^+$ cells in midguts of dm^4*/+; tub-dMyc* or dm^4/dm^4*; tub-dMyc* adult flies fed with sucrose, DSS or bleomycin (n = 20 for each genotype). Statistical significance was determined by Student's t test (* $P < 0.05$, *** $P < 0.001$). **(B)** Quantification of dMyc staining in precursor cells shown in **C**-**K** ($n > 50$ for each genotype). **(C**-**K')** Adult midguts with indicated genotype were treated with sucrose **(C**-**C'**, **F**-**F'**, **I**-**I')**, DSS **(D**-**D'**, **G**-**G'**, **J**-**J')** or bleomycin **(E**-**E'**, **H**-**H'**, **K**-**K')** and immunostained with a rabbit anti-dMyc (red) antibody and a nuclear dye DRAQ5 (blue). Arrows indicate precursor cells marked by small nuclear size.

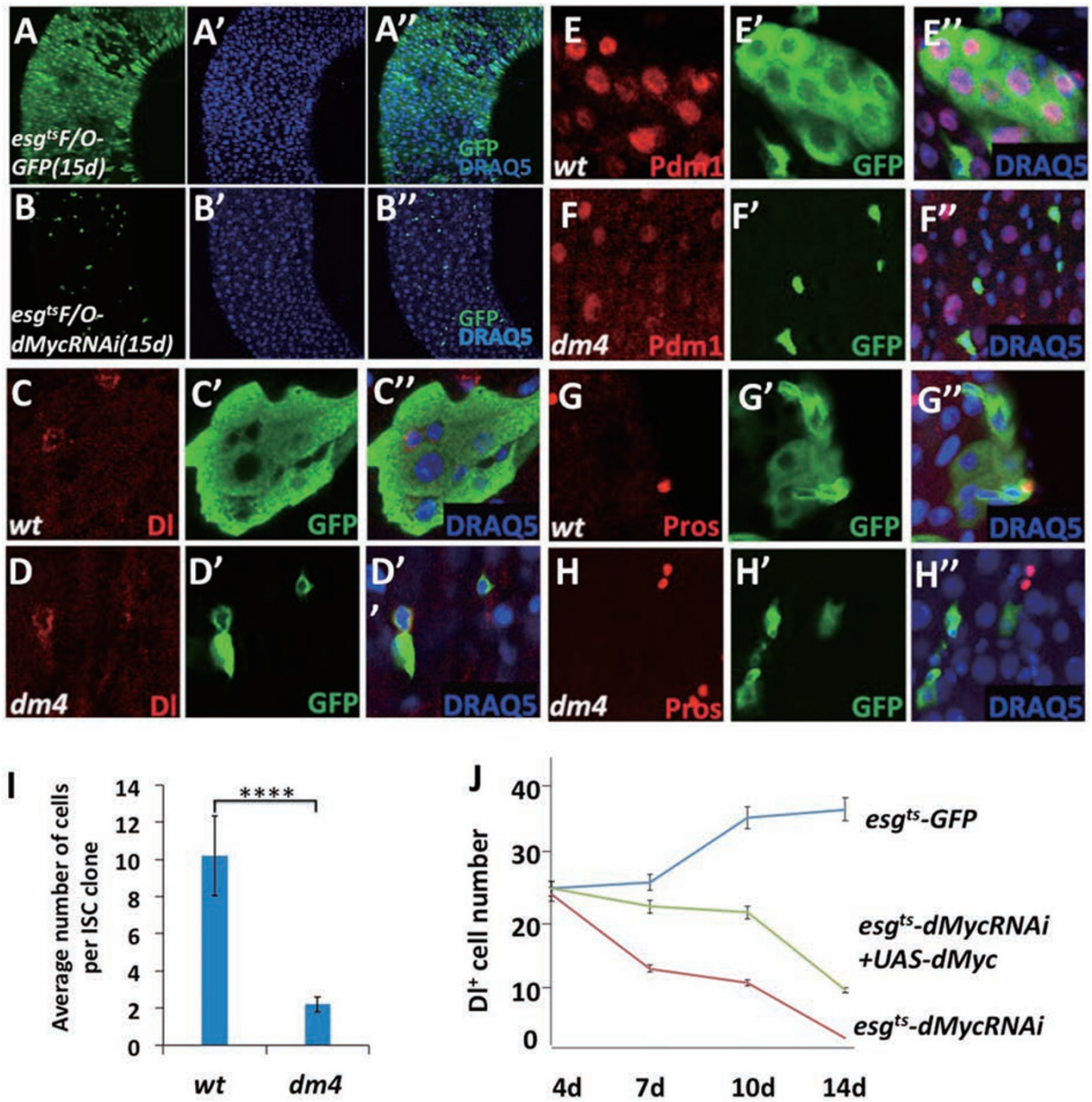

Figure 7 dMyc is essential for midgut homeostasis.**(A-B")** Adult midguts expressing *esg^{ts}F/O* without **(A-A")** or with *UAS-dMyc-RNAi* **(B-B")** were cultured at non-permissive temperature (29 ^{0}C) for 15 days, followed by immunostaining with a GFP (green) antibody and a nuclear dye DRAQ5 (blue). **(C-H")** Adult midguts containing GFP-positive WT (*wt*) clones **(C-C"**, **E-E"**, **G-G")**, or *dm^{4}* clones **(D-D"**, **F-F"**, **H-H")** were immunostained to show the expression of GFP (green), Dl (red in **C-D"**), Pdm1 (red in **E-F")**, Pros (red in **G-H"**), and DRAQ5 (blue). *dm^{4}* mutant clones grew slower and failed to differentiate into ECs (Pdm1^{+}) or EEs (Pros^{+}). The control and mutant clones were generated using the MARCM system. Guts were dissected out from adult flies grown at 18 °C for 20 days after clone induction. **(I)** Quantification of ISC lineage clone size for the control (*wt*) and *dm^{4}* clones (n > 50 for each genotype). **** P < 0.0001. **(J)** 2- to 5-day-old adult females expressing *UAS-GFP* without (blue curve) or with *UAS-dMyc-RNAi* (red curve) or with *UAS-dMyc-RNAi* + *UAS-dMyc* (green curve) using the *esg^{ts}* system were cultured at non-permissive temperature (29 °C) for various periods of time. Their midguts were dissected at the indicated time points after temperature shift and immunostained with Dl and GFP antibodies. The number of ISCs in the posterior midguts was quantified for different time points.

midguts exhibited elevated dMyc staining in their precursor cells in response to tissue damage, consistent with a transcriptional upregulation from the endogenous *dMyc* locus (Figure 6B, 6C-6H'). Surprisingly, *dm^{4}/dm^{4}; tub-*

dMyc guts also exhibited elevated dMyc staining in their precursor cells albeit at lower magnitude compared with *wt* or dm^4 heterozygous guts (Figure 6B, 6I-6K'), suggesting that dMyc might be regulated at a post-transcriptional level in addition to the transcriptional level. Indeed, previous studies suggest that both mammalian and *Drosophila* Myc can be regulated at the level of protein stability [30]. Taken together, these results suggest that transcriptional upregulation of dMyc is required for optimal ISC activation in response to injury; in its absence, post-transcriptional upregulation can support damage-induced ISC proliferation albeit less effectively.

We also examined whether overexpression of dMyc alone could stimulate ISC proliferation. We found that overexpression of either a WT dMyc or stabilized dMyc variants, $dMyc^{AB}$ or $dMyc^{PV}$ [31], in precursor cells failed to drive ISC overproliferation (Supplementary information, Figure S5). These observations suggest that upregulation of additional factors might be required for the accelerated ISC proliferation in response to tissue damage.

dMyc is required for ISC proliferation, differentiation and maintenance during normal homeostasis

The above experiments only address the regulation and function of dMyc during the midgut regeneration in response to tissue damage. We also wanted to determine whether dMyc plays any essential role during normal tissue homeostasis. Under normal homeostatic situation, the posterior midguts of WT female adults turn over once in more than 2 weeks [5]. Using the $esg^{ts}F/O$ system to monitor posterior midgut self-renewal, we found that 15 days after temperature shift to 29 °C, most ECs in the posterior region of $esg^{ts}F/O$ midguts were GFP^+ (Figure 7A-7A"), indicating that the posterior midgut had undergone extensive renewal. However, posterior midgut of $esg^{ts}F/O$-*dMyc-RNAi* flies contained very few GFP^+ cells, all of which contained small nuclei (Figure 7B-7B"), suggesting that they were arrested at precursor cell stage. Thus, dMyc is required for the midgut renewal during normal tissue homeostasis.

To further determine whether the basal dMyc activity is essential for ISC proliferation and differentiation during normal midgut homeostasis, we generated GFP-marked dm^4 mutant clones in the ISC cell lineage using the MARCM system [32]. The adult flies were grown at 18 °C for 20 days after clone induction. We found that the average clone size of dm^4 mutant clones was greatly reduced compared with that of WT control clones (Figure 7C-7H"). Most of the clones derived from dm^4 ISCs contained two cells, whereas the WT ISC-lineage clones contained ~10 cells (Figure 7I). dm^4 mutant clones did not contain ECs ($Pdm1^+$) or EEs ($Pros^+$) cells (Figure 7F-7H"). These results suggest that dMyc is also required for ISC proliferation and lineage differentiation during normal tissue homeostasis. We also confirmed that dm^4 mutation blocked ISC overproliferation induced by tissue damage (Supplementary information, Figure S6).

We observed a gradual loss of ISC in adult midguts expressing esg^{ts}-*dMyc-RNAi* over time, as indicated by the reduced number of Delta $(Dl)^+$ cells in these midguts, and that the ISC loss phenotype was partially rescued by coexpression of *UAS-dMyc* (Figure 7J and Supplementary information, Figure S7), suggesting that dMyc is also required for stem cell maintenance in adult midguts.

Discussion

ISC proliferation is under tight control during adult homeostasis. Evolutionarily conserved signaling pathways including Hpo, JAK-STAT and EGFR pathways have been shown to play important roles in the regulation of ISC proliferation in *Drosophila* adult midguts during normal tissue homeostasis as well as in response to tissue damage; however, the cell-intrinsic factors that mediate the mitogenic effect of these signaling pathways have remained largely unexplored. Here we present evidence that dMyc is an integrator of these signaling pathways and mediates their effects on ISC proliferation (Figure 5D). Interestingly, dMyc expression is upregulated in precursor cells in response to tissue damage and is required for elevated ISC proliferation and midgut regeneration.

Our previous studies showed that DSS and bleomycin cause distinct tissue damages with DSS affecting the structure of BM and bleomycin affecting ECs [6], and that DSS and bleomycin stimulate ISC proliferation through distinct mechanisms with DSS mainly through the Hpo-Yki pathway and bleomycin through the production of JAK-STAT and EGFR pathway ligands [9]. Consistent with these findings, we found that DSS upregulates dMyc expression through Yki, whereas bleomycin activates dMyc through JAK-STAT and EGFR pathways (Figures 2 and 3). Although inactivation of dMyc blocked ISC proliferation induced by excessive Yki or overactivation of JAK-STAT or EGFR pathways, overexpression of dMyc in precursor cells is not sufficient to drive ISC overproliferation (Supplementary information, Figure S5). These observations suggest that Hpo-Yki, JAK-STAT and EGFR signaling pathways may activate additional factors that act in conjunction with dMyc to fuel ISC proliferation.

We found that tissue damage and multiple signaling pathways upregulate dMyc expression at the transcriptional level, and that transcriptional upregulation of dMyc is required for optimal ISC proliferation in response to tissue damage. We also provided evidence

that tissue damage may regulate dMyc expression at a post-transcriptional level. Two recent studies suggest that Yki-Sd may directly regulate dMyc transcription in wing imaginal discs [33, 34]. We identified a 1 kb *dMyc* enhancer (*dMyc1.0*) that contains multiple consensus Sd- and Stat-binding sites and is capable of mediating the transcriptional regulation of a reporter gene (*dMyc1.0-luc*) by Yki-Sd and Stat in S2 cells. Furthermore, we found that Sd and Stat can be recruited to this enhancer in adult midgut precursor cells. These observations suggest that dMyc is likely to be a transcriptional target of Hpo and JAK-STAT pathways in midgut precursor cells. *dMyc1.0-luc* also contains many putative Pnt-binding sites, but *dMyc1.0-luc* cannot be activated by an active form of Pnt2 (Pnt2-V16) in S2 cells although expression of Pnt2-V16 can activate *dMyc-lacZ* in midgut precursor cells. Further experiments are required to establish whether dMyc is a direct transcriptional target of the EGFR signaling pathway. A recent study suggested that dMyc acts downstream of Wg to mediate its activation of the EGFR ligand Spitz and JAK-STAT pathway ligand Upd3 [24]. Hence, dMyc may act both upstream and downstream of EGFR and JAK-STAT pathways to promote ISC proliferation.

We also demonstrated that the basal dMyc activity is crucial for normal ISC proliferation, maintenance and differentiation in homeostatic midguts. This is in contrast to Yki whose basal activity is not required for normal midgut homeostasis but whose elevated activity in precursor cells can fuel ISC overproliferation [9, 14, 16]. We found that *dMyc* mutant clones (dm^4) were much smaller compared with control WT clones, and only contained 1-2 cells 20 days after clone induction. In addition, *dMyc* mutant cells failed to differentiate into either EEs or ECs. Furthermore, prolonged inactivation of dMyc led to stem cell loss. In wing discs, *dMyc* mutant cells are eliminated by neighboring WT cells in a process called cell competition that depends on apoptosis [35, 36]. However, expression of an apoptosis inhibitor P35 in midgut precursor cells failed to rescue stem cell loss caused by dMyc inactivation (data not shown). The exact mechanisms by which dMyc regulates ISC proliferation, maintenance and lineage differentiation await further investigation.

Although deletion of c-Myc rescued the neoplastic phenotype caused by loss of APC [37], deletion of c-Myc in otherwise WT small intestine only led to reduced cell sizes, but did not halt cell proliferation or differentiation of crypt cells, and the normal homeostasis of adult intestinal epithelium could be still maintained in the absence of c-Myc [38]. This is in contrast to our finding here that dMyc is required for ISC proliferation, maintenance and lineage differentiation during *Drosophila* adult midgut homeostasis. Mammalian *myc* gene family contains three members: c-*myc*, N-*myc* and L-*myc*, which might have overlapping functions in mouse intestine. Inactivation of c-Myc alone may not be sufficient to cause a profound homeostatic phenotype, which could explain the difference observed between our study in *Drosophila* intestine and previous reports in mouse intestine. It is also possible that basal Myc activity plays a minor role in normal homeostasis of mammalian intestines, but its elevated activity is essential for abnormal proliferation due to oncogenic mutations or loss of APC. In this regard, Myc may resemble Yap, which appears to be dispensable for normal homeostasis of the intestine but is required for overproliferation of intestine cells induced by injury or oncogenic mutations [39, 40]. It would be interesting to determine whether Myc is also regulated by Hpo signaling and plays a role in adult tissue regeneration in mammals. Finally, activation of Stat3 has been implicated in inflammation-associated colon cancers [41-43]. Our finding that the JAK/STAT pathway can activate dMyc raises an interesting possibility that Stat3 may also regulate Myc expression in colon cancer cells.

Materials and Methods

Drosophila genetics and trangenes

The following fly strains were used for this study: *esg-Gal4/tub-Gal80^{ts}* [3]; *Dl-Gal4/tub-Gal80^{ts}* [44]; *UAS-dMyc-RNAi* (VDRC #2947; VDRC #2948; BL #25783; BL #25784); *esg^{ts}F/O* (*esg-Gal4 tubGal80^{ts} UAS-GFP; UAS-flp Act>CD2>Gal4*), *10XSTAT-dGFP*, *UAS-Dome-RNAi* and *UAS-Upd* [5]; *UAS-Wts-RNAi* (VDRC #106174); *UAS-Yki-RNAi* and *UAS-Yki* [27]; *UAS-EGFR-RNAi* (VDRC #43267); *UAS-EGFR^{A887T}* (BL #9534); *dMyc-lacZ* (BL #11981 and #12247) [45]; *UAS-3HA-STAT92EΔNΔC* (from Erika Bach); *UAS-pntp2-vp16* [26]. dm^4 is a null allele of *dMyc* [46]. Mutant clones for dm^4 were generated using the MARCM system [32]. Fly stocks were crossed and cultured at 18 °C. Five-day-old F1 female adults with the appropriate genotypes were subjected to heat shock at 37 °C for 1 h. After clone induction, flies were raised at 18 °C for the indicated period of time before dissection. For experiments involving *esg-Gal4/tub-Gal80^{ts}*, crosses were set up and cultured at 18 °C to restrict Gal4 activity. Two- to three-day-old F1 adult flies were then shifted to 29 °C to inactivate Gal80^{ts}, allowing *esg-Gal4* to activate *UAS* transgenes. PCR-based site-directed mutagenesis was employed to introduce base pair substitutions in the Stat-binding sites in *dMyc1.0* to generate *dMyc1.0m*. To construct *dMyc1.0-luc*, *dMyc1.0m-luc* and *dMyc1.0-GFP*, DNA fragments were amplified by PCR and inserted between *Kpn*I and *Nhe*I digestion sites into the pGL3 and pH-Stinger vectors, respectively.

Feeding experiments

In general, 5- to 10-day-old female adult flies were used for feeding experiments. For dMyc RNAi, 2- to 3-day-old adult females were shifted to 29 °C for 7 days before feeding. Flies were cultured in an empty vial containing a piece of 2.5 × 3.75-cm chromatography paper (Fisher) wet with 5% sucrose solution as feeding medium. Flies were fed with 5% of DSS (MP Biomedicals) or

25 μg/ml bleomycin (Sigma) dissolved in 5% sucrose for 2 days at 29 °C. Infection with *Pe* was carried out as previously described [5].

Immunostaining

Female flies were used for gut immunostaining in all experiments. The entire gastrointestinal tract was taken and fixed in 1× PBS plus 8% EM grade formaldehyde (Polysciences) for 2 h. Samples were washed and incubated with primary and secondary antibodies in a solution containing 1× PBS, 0.5% BSA and 0.1% Triton X-100. The following primary antibodies were used: mouse anti-Delta (DSHB), 1:100; mouse anti-Prospero (DSHB), 1:50; mouse anti-dMyc (gift from Dr Paola Bellosta, City college of the City University of New York), 1:2; rabbit anti-dMyc (Santa Cruz), 1:200, rabbit anti-lacZ (Cell signaling), 1:1 000; rabbit anti-PH3 (Upstate Biotechnology), 1:2 000; rabbit anti-GFP (Santa Cruz), 1:500; rabbit anti-Pdm1 (gift from Xiaohang Yang, Institute of Molecular and Cell Biology, Singapore), 1:2 000; rabbit anti-dpERK (Cell Signaling Technology), 1:500; DRAQ5 (Cell Signaling Technology).

Cell Culture, transfection and luciferase reporter assay

S2 cells were cultured in *Drosophila* Schneider's Medium (Invitrogen) with 10% fetal bovine serum, 100 U/ml of penicillin and 100 μg/ml of Streptomycin. Transfection was carried out using Calcium Phosphate Transfection Kit (Specialty Media) according to manufacturer's instructions. A ubiquitin-Gal4 construct was co-transfected with *pUAST* expression vectors for all the transfection experiments. For Luciferase reporter assays, S2 cells were transfected with *dMyc1.0-luc* or *dMyc1.0m-luc* and copia-renilla luciferase reporter constructs in 12-well plates together with constructs expressing different genes. Cells were incubated for 48 h after transfection and the luciferase reporter assay was performed using the Dual-Luciferase reporter assay system (Promega). Dual-Luciferase measurements were performed in triplicate using FLUOstar OPTIMA (BMG LABTECH).

ChIP experiment

Formaldehyde-crosslinked chromatin was prepared from midguts that express *UAS-Notch-RNAi* alone or together with *UAS-HA-Sd + Myc-Yki* or *UAS-HA-StatΔNΔC* with *esg-Gal4/tubGal80^{ts}* were dissected in serum-free SS3 medium (Sigma) and stored on ice before formaldehyde fixation. Groups of 150 guts were fixed 10 min at a time, and immunoprecipitation were performed using the ChIP assay kit (Upstate Biotechnology) according to the manufacture's recommended protocol. One tenth of the DNA from each immunoprecipitation was used in each PCR reaction. Pairs of PCR primers were used for amplification of the following segments of a *dm*-enhance element: dMyc enhancer ChIP F Fragment 1: 5′-AACGCAACGACTTCAAAAATC-3′; dMyc enhancer ChIP R Fragment 1: 5′-GATTCTTAACATTCC-TTCTGAGTTCC-3′; dMyc enhancer ChIP F Fragment 2: 5′-GTTGATATTCCAATTTTACTATGATTC-3′; dMyc enhancer ChIP R Fragment 2: 5′-TAAACGGTCCTTTTACCGATAAA-3′; dMyc 2 kb upstream ChIP F: 5′-GAAACAAAAAAAAAGTGCAGA-3′; dMyc 2 kb upstream ChIP R: 5′-TACAACTGCGTCACA-GA-CAAAA-3′. PCR scheme: 94 °C for 5 min, once; 94 °C for 30 s, 52 °C for 30 s, 72 °C for 1 min, 30 times; 72 °C for 5 min, once. The amplified DNA was separated on 1.5% agarose gel and visualized with ethidium bromide.

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(**Supplementary information** is linked to the online version of the paper on the *Cell Research* website.)