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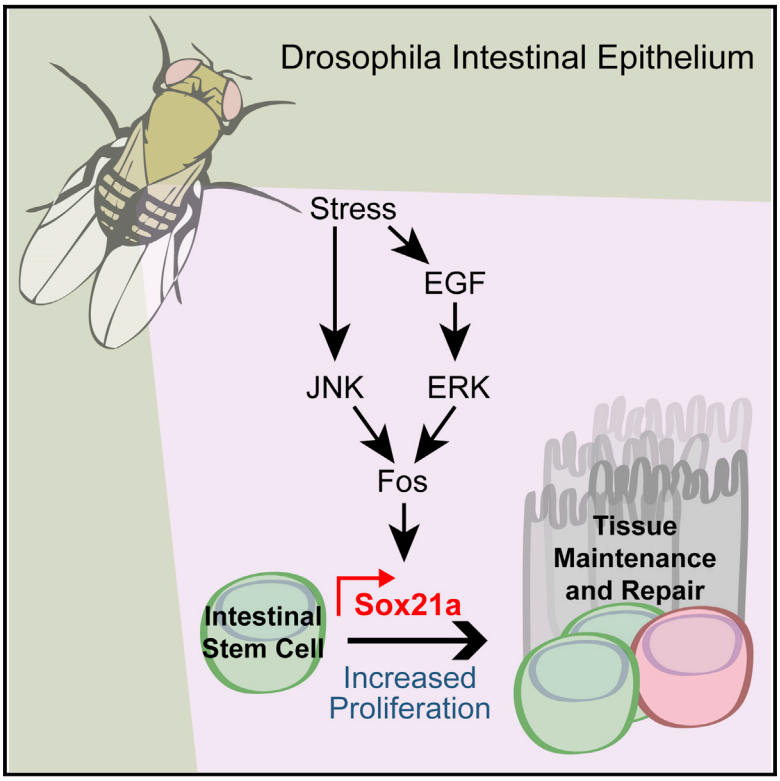
Report

A Sox Transcription Factor Is a Critical Regulator of Adult Stem Cell Proliferation in the *Drosophila* Intestine

Graphical Abstract



Highlights

- Sox21a is specifically required for adult intestinal stem cell proliferation
- Sox21a expression is induced in response to tissue damage
- Sox21a is controlled by the JNK- and ERK-signaling pathways

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In Brief

Meng and Biteau show that a *Drosophila* Sox2 homolog is expressed in adult intestinal stem cells and is essential for proliferation under normal conditions and after tissue damage. They find that this factor is induced by the JNK and ERK pathways to promote epithelial repair.

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A Sox Transcription Factor Is a Critical Regulator of Adult Stem Cell Proliferation in the *Drosophila* Intestine

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SUMMARY

Adult organs and their resident stem cells are constantly facing the challenge of adapting cell proliferation to tissue demand, particularly in response to environmental stresses. Whereas most stress-signaling pathways are conserved between progenitors and differentiated cells, stem cells have the specific ability to respond by increasing their proliferative rate, using largely unknown mechanisms. Here, we show that a member of the Sox family of transcription factors in *Drosophila*, Sox21a, is expressed in intestinal stem cells (ISCs) in the adult gut. Sox21a is essential for the proliferation of these cells during both normal epithelium turnover and repair. Its expression is induced in response to tissue damage, downstream of the Jun N-terminal kinase (JNK) and extracellular signal-regulated kinase (ERK) pathways, to promote ISC proliferation. Although short-lived, *Sox21a* mutant flies show no developmental defects, supporting the notion that this factor is a specific regulator of adult stem cell proliferation.

INTRODUCTION

Resident stem cell populations are essential for the long-term homeostasis of many tissues in organisms ranging from invertebrates to humans. One essential property of these cells is their ability to specifically respond to tissue damage, transiently increasing their proliferation rate to produce new differentiated cells and help restore tissue integrity. Interestingly, the activity of many of the signaling pathways that control this proliferative response in stem cells leads to distinct biological outcomes in non-stem populations. Yet the mechanisms controlling this specificity remain largely unknown in most stem and progenitor populations.

Members of the SRY-box (Sox) transcription factor family are defined by the presence of a specific high-mobility-group box domain first identified in the SRY gene. Sox proteins are expressed in many developing tissues and are critical regulators of cell proliferation, differentiation, or establishment of stem and progenitor populations. More recently, the central role of Sox factors in the control of stem cell identity has been highlighted by the identification of Sox2 as one of the factors originally required to reprogram differentiated cells into induced pluripotent stem cells. Aside from their roles in embryonic stem cells, cell reprogramming, and development, expression of Sox transcription factors has been found in many stem or progenitor cell populations in adult tissues, in which it is essential for the maintenance of tissue-specific stem cells and proper differentiation of progenitors (Sarkar and Hochedlinger, 2013). However, in most cases, the mechanisms regulating the function of Sox transcription factors in adult tissues remain largely unknown.

In recent years, the adult *Drosophila* intestine has emerged as a powerful model to study somatic stem cell regulation in vivo (Micchelli and Perrimon, 2006; Ohlstein and Spradling, 2006). Intestinal stem cells (ISCs) are the only proliferating cells in the fly gut and are essential for the maintenance of the midgut epithelium integrity, metabolic homeostasis, and longevity (Biteau et al., 2011; Jiang and Edgar, 2012). ISC proliferation is tightly controlled by the activity of many signaling pathways during both normal tissue turnover (e.g., insulin and epidermal growth factor/mitogen-activated protein kinase [EGF/MAPK] pathways; Biteau and Jasper, 2011; Biteau et al., 2010; Buchon et al., 2010; Jiang et al., 2011) and tissue repair in response to oxidative stress, tissue damage, or infection (e.g., Jun N-terminal kinase [JNK], JAK/Stat, and Hippo/Yorkie pathways; Beebe et al., 2010; Biteau et al., 2008; Buchon et al., 2009; Jiang et al., 2009; Lin et al., 2010; Liu et al., 2010; Ren et al., 2010; Shaw et al., 2010; Staley and Irvine, 2010; Xu et al., 2011). Yet little is known about the transcriptional network that integrates all these signals into a coordinated proliferative response.

The Sox protein family is highly conserved from invertebrates to humans. Eight genes encoding putative Sox transcription factors have been identified in the *Drosophila* genome (Phochanukul and Russell, 2010). The function of SoxNeuro and Dichaete in embryonic development and the developing nervous system is best characterized, whereas Sox100B was identified as a critical regulator of male germline specification (Buescher et al., 2002; Nanda et al., 2009; Soriano and Russell, 1998). However, the potential function of Sox factors in adult somatic stem cell populations has not yet been investigated. Here, we show that Sox21a, one of the *Drosophila* Sox2 homologs, is a critical regulator of ISC function in the adult fly. We found that its expression is required for epithelial turnover and is regulated by the JNK and extracellular signal-regulated kinase (ERK) pathways to control

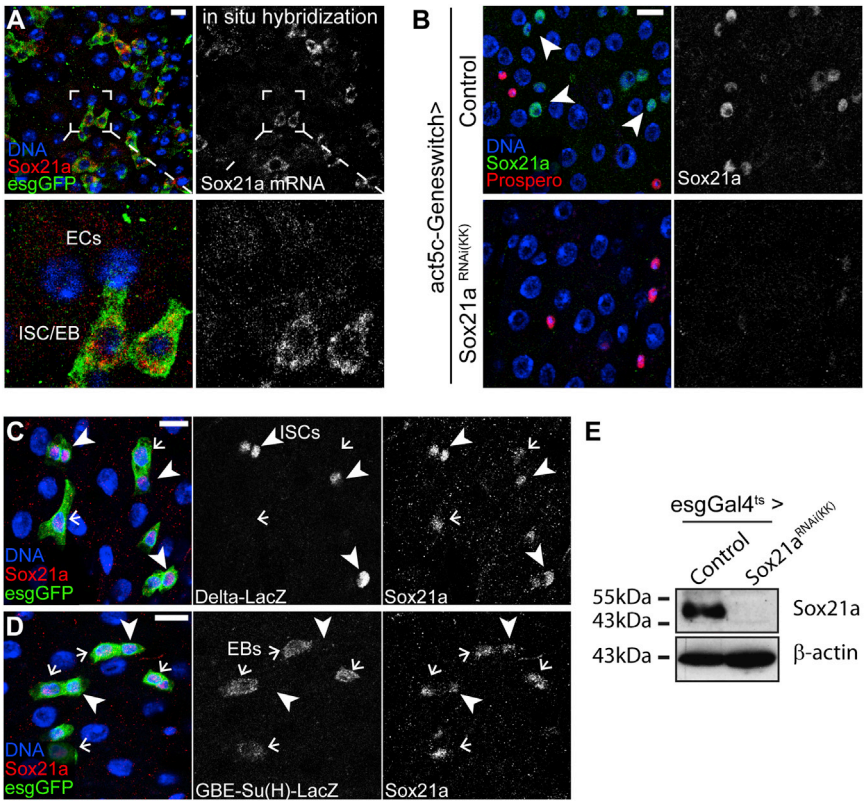

Figure 1. Sox21a Is Specifically Expressed in ISCs and EBs in the *Drosophila* Intestine

(A) In situ hybridization detects *Sox21a* mRNA expression in *escargot* (esg)-positive ISCs and EBs in the posterior midgut (esgGal4 > UAS-GFP).

(B) Sox21a immunocytochemistry of posterior midguts shows that the Sox21a protein is detected in small diploid nuclei (indicated by the arrowhead), but not in differentiated polyploid ECs and Prospero-positive EEs (red). Sox21a protein is not detected when a dsRNA construct directed against *Sox21a* is expressed ubiquitously (act5c-Geneswitch > UAS-Sox21a$^{RNAi(KK)}$).

(C and D) Confocal images of posterior midguts show that the Sox21a protein is expressed in both ISCs and EBs (nuclear; red). GFP expression labels both ISCs and EBs (esgGal4 > UAS-GFP; green). β-galactosidase expression from the reporter lines Delta-LacZ (C) and GBE-Su(H)-LacZ (D) specifically identifies ISCs (arrowheads) and EBs (arrows), respectively.

(E) Western blot analysis shows that Sox21a protein is detected in total protein extracts from intestines but absent when Sox21a$^{RNAi(KK)}$ is expressed in esg-positive ISCs and EBs. β-actin is used as loading control.

In all panels, DNA is detected by Hoechst staining (blue). EB, enteroblast; EC, enterocyte; EE, enteroendocrine cell; ISC, intestinal stem cell. The scale bars represent 10 μm.

ISC proliferation in response to tissue damage. Importantly, Sox21a is dispensable during development, demonstrating that its function represents a novel mechanism regulating cell proliferation specifically in adult stem cells.

RESULTS

Sox21a Is Specifically Expressed in ISCs and EBs in the Intestine

In order to investigate the potential role of Sox transcription factors in the regulation of ISC function, we first asked whether members of this gene family are expressed in the fly intestinal epithelium. Using in situ hybridization, we found that the *Sox21a* mRNA is exclusively detected in esg-positive ISCs and enteroblasts (EBs) in the adult fly intestine (Figure 1A). To confirm that the Sox21a protein is expressed in these cells, we developed a polyclonal antibody against this factor. This antibody specifically recognizes Sox21a protein in the nuclei of diploid cells, distinct from the prospero-positive endocrine cells (EEs) and polyploid enterocytes (ECs) (Figure 1B). We confirmed that these cells are ISCs and EBs using specific markers. Both ISCs and EBs express the escargot marker, whereas ISCs express the Notch ligand Delta (Delta-LacZ; Figure 1C) and EBs show high activity for the Notch reporter GBE-Su(H)-LacZ (Figure 1D; Ohlstein and Spradling, 2007). As suggested by the result of our in situ hybridization analysis of Sox21a expression, the Sox21a protein is detected in both ISCs and EBs throughout the entire midgut epithelium (Figures 1C and 1D). To further confirm the specificity of the observed signal, we expressed a dsRNA directed against *Sox21a* using the temperature-sensitive esgGal4^{ts} driver. This knockdown is sufficient to abolish Sox21a expression in the intestine, as shown by western blot using total protein extracts from dissected guts (Figure 1E), confirming that Sox21a expression is restricted to esg-positive cells in the *Drosophila* intestine.

Altogether, these results demonstrate that Sox21a is specifically expressed in ISCs and EBs in the intestinal epithelium.

Sox21a Is Specifically Required in ISCs for Stem Cell Proliferation

The Sox21a expression pattern in the adult gut strongly suggests that this transcription factor specifically functions in ISCs. Therefore, we tested whether Sox21a is required for stem cell proliferation. To this end, we identified a transposable element insertion in the *Sox21a* locus (*Sox21a*^{f04672}) that strongly impairs Sox21a expression in the intestine (Figure 2A), without affecting the proportion of ISCs or EEs in the gut epithelium (Figure S1A). We assessed the effect of this mutation on ISC proliferation after tissue damage. Exposure to dextran sulfate sodium (DSS) induces a robust proliferative response that can be easily quantified by counting the number of cells positive for the mitotic marker phospho-histone H3 (pH3) in the midgut (Amcheslavsky et al., 2009). Consistent with previous reports, DSS induces ISC proliferation in wild-type or heterozygous animals; however, this response is abolished in *Sox21a*^{f04672} homozygous flies (Figure 2B), suggesting that Sox21a is essential for ISC proliferation. We have

previously shown that flies with greatly impaired ISC proliferative capacity are short lived (Biteau et al., 2010). Thus, to test the functional requirement for Sox21a, we backcrossed the *Sox21a*^{f04672} allele in two different genetic backgrounds and analyzed the lifespan of control, heterozygous, and homozygous mutant animals. Consistent with the critical role of Sox21a in intestinal homeostasis, we found that *Sox21a*^{f04672} homozygous females are significantly shorter lived than their siblings (Figures S1B and S1C).

To better characterize this Sox21a loss-of-function proliferation defect, we next analyzed ISC lineages by generating positively marked stem cell clones (MARCM; Lee and Luo, 1999) in the adult posterior midgut. Consistent with previous studies, control clones reach an average size of 8 to 12 cells/clones, 7 days after induction (Figure 2C). However, clones expressing two distinct RNAi constructs directed against *Sox21a* are much smaller than their respective controls (Figure 2C), demonstrating that ISCs in which Sox21a is knocked down are essentially incapable of proliferation. Of note, comparable numbers of clones were observed in all conditions (data not shown) and Sox21a^{RNAi}-expressing single-cell clones retain Delta expression (Figure 2C), confirming that Sox21a knockdown specifically impairs ISCs proliferation but does not affect their survival or self-renewal. To support these results, we used the esgGal4^{ts} driver to specifically express three independent RNAi constructs in all ISCs and EBs and found that these manipulations are sufficient to reduce ISC proliferation under normal conditions and strongly inhibit the DSS-induced proliferative response (Figure 2D).

We show that Sox21a is expressed in both ISCs and EBs (Figures 1C and 1D). Previous studies have reported that defects in EBs are capable of signaling back to the ISCs, preventing further stem cell division (Choi et al., 2011). To exclude such non-cell-autonomous effect, we tested whether Sox21a is required in ISCs themselves to permit cell proliferation. We expressed the Sox21a$^{RNAi(KK)}$ construct using the ISC-specific Delta-Gal4^{ts} driver and the EB-specific GBE-Su(H)-Gal4^{ts} driver (Zeng et al., 2010). Similar to what we observed using the esgGal4^{ts} driver, knockdown of Sox21a in ISCs only is sufficient to significantly impair cell proliferation in response to DSS, whereas Sox21a knockdown in EBs does not affect ISCs proliferation in these conditions (Figure 2E).

Altogether, these results demonstrate that Sox21a is specifically required in ISCs to maintain their proliferative capacity under homeostatic conditions and during tissue repair.

Sox21a Expression Is Induced to Promote ISC Proliferation

The essential role of Sox21a in ISC proliferation prompted us to investigate the possibility that its expression is regulated to control the stem cell stress response. We found that Sox21a protein level in the intestine increases dramatically after DSS treatment (Figures 3A, S2A, and S2B). Importantly, this stress-induced expression is absent in esgGal4 > Sox21a^{RNAi} animals, confirming that, even under stress conditions, Sox21a expression is limited to ISCs and EBs. Previous studies by us and others have shown that the population of esg-positive cells expands after exposure to stress or in aging flies (Amcheslavsky et al., 2009; Biteau et al., 2008; Buchon et al., 2009; Jiang et al., 2009). To confirm that the observed Sox21a induction is caused by an increased expression in ISCs, rather than an increased number of esg-positive Sox21a-expressing cells, we exposed flies to DSS for short periods of time and detected increased Sox21a protein level as early as 24 hr, a time when no supernumerary esg-positive cells are present in the intestinal epithelium, as confirmed by similar GFP expression levels (Figure 3B). Similar induction was observed when flies are exposed to paraquat, a compound that leads to the production of reactive oxygen species in the intestinal epithelium and increases ISC proliferation (Biteau et al., 2008; Choi et al., 2008; Figure 3C). In parallel to our western blot analysis, we used a blind scoring approach to evaluate the intensity of the Sox21a immuno-staining in the intestine of control and DSS- or paraquat-treated flies. We found a greater proportion of intestines showing moderate to high Sox21a protein level in stressed animals compared to untreated animals (Figures 3D and S2C). In addition, we used immunostaining to show that the expression of Sox21a protein is uniquely induced in esg-positive cells after DSS exposure and used the ISC-specific marker Delta to confirm that Sox21a expression specifically increases in ISCs and EBs in response to tissue damage (Figures 3E and S2D). Finally, we show that *Sox21a* mRNA level is induced in response to DSS, suggesting that this factor is regulated at the transcriptional level (Figure 3F).

This regulation strongly suggested that elevated Sox21a protein level promotes ISC proliferation during tissue repair. To test this notion, we first overexpressed the endogenous *Sox21a* gene using an EP line (*Sox21a*^{d03399}; a P-element carrying UAS sites inserted in the *Sox21a* promoter region). When combined with the esgGal4^{ts} driver, this insertion is sufficient to significantly induce *Sox21a* mRNA level (Figure 3G) and leads to a robust increase in cell proliferation in the intestine (Figure 3H). In addition, we generated a UAS-driven Flag-tagged Sox21a transgene. Expression of this fusion protein was confirmed by western blot and immunochemistry (Figure 3I) and is sufficient to promote ISCs proliferation when driven by the esgGal4 (ISC/EBs) or the DeltaGal4 (ISCs only) driver (Figure 3J). Finally, although Sox21a-overexpressing MARCM clones grow larger than control (Figure S3A), we found no evidence that Sox21a overexpression affects the ability of the ISC lineage to differentiate into EEs and ECs (Figures S3B–S3D).

Collectively, these results demonstrate that Sox21a expression is induced in ISCs to promote cell proliferation in response to tissue damage.

Sox21a Is a Critical Mediator of the JNK and Ras/ERK Pathways in the Control of ISC Proliferation

Previous studies have established that ISC proliferation requires the activity of multiple signaling pathways (Biteau et al., 2011; Jiang and Edgar, 2012; Pasco et al., 2015). The JNK and EGF/Ras/ERK pathways are two critical components of the regulatory network involved in the maintenance of ISC proliferative capacity during normal tissue turnover and essential for the increased proliferation under stress conditions (Biteau et al., 2008; Biteau and Jasper, 2011; Buchon et al., 2010; Jiang et al., 2011; Xu et al., 2011). The expression of Sox21a and its function in the control of ISC proliferation lead us to investigate the potential

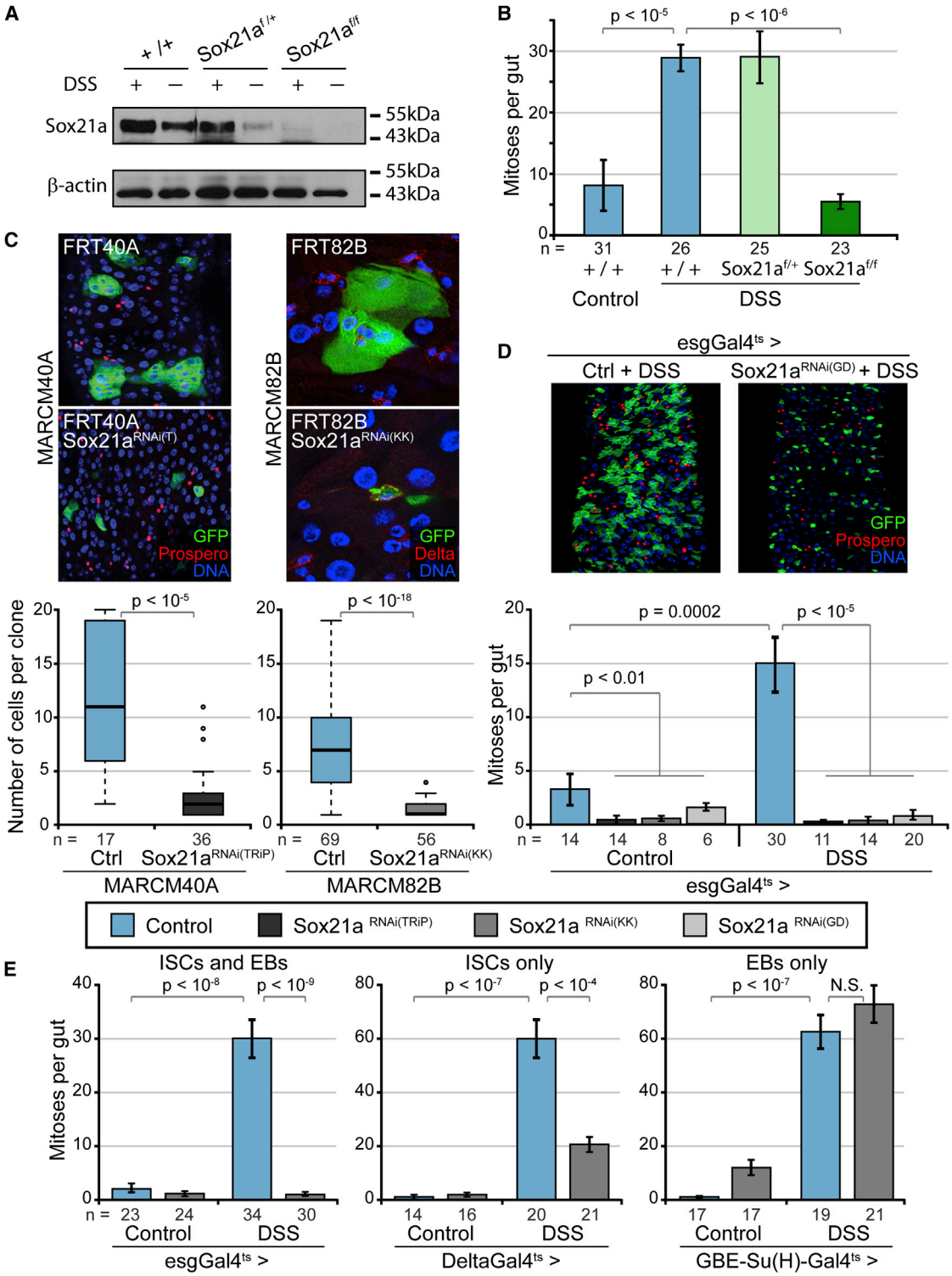

Figure 2. Sox21a Is Required in ISCs for Stem Cell Proliferation under Both Homeostasis and Stress Conditions

(A) Western blot analysis of total intestinal proteins shows that *Sox21a*^{f04672} homozygous flies have significantly reduced Sox21a expression compared to control and heterozygous animals. β-actin is used as loading control.

(B) *Sox21a*^{f04672} homozygous flies show reduced ISCs proliferation after a 48-hr dextran sulfate sodium (DSS) treatment.

(legend continued on next page)

role of Sox21a downstream of JNK and Ras signaling. To this end, we first genetically induced ISCs proliferation by activating the JNK and Ras/ERK pathways through overexpression of JNKK/Hep or expression of activated Ras (Ras^{V12}) under the control of the $esgGal4^{ts}$ driver. In both conditions, the expression of the Sox21a protein in the gut is dramatically increased (Figures 4A and S4A), recapitulating the induction observed in response to stress. Next, because JNK and Ras are critical for stress-mediated proliferation (Figure 4B), we asked whether these pathways are required for Sox21a expression in response to DSS. We found that expressing a dominant-negative form of JNK/Bsk or an RNAi directed against *Ras* using the $esgGal4^{ts}$ driver is sufficient to abolish Sox21a expression in the intestine of DSS-treated animals (Figure 4C). Last, we tested whether Sox21a is essential for Hep- and Ras-induced proliferation and found that knocking down Sox21a prevents the hyperproliferation induced by overexpression of Hep/JNKK or Ras^{V12} (Figures 4D and S4B), confirming that Sox21a is a critical mediator of JNK and Ras signaling in the control of ISC proliferation.

We have previously established that the AP-1 transcription factor Fos (*kayak* in *Drosophila*) integrates the activity of both JNK and Ras/ERK signaling in ISCs and is essential for proliferation downstream of these pathways (Biteau and Jasper, 2011; Figure S4B). Thus, we hypothesized that Sox21a is an essential target of Fos for the control of ISC proliferative rate. To test this notion, we first exposed flies that expressed RNAi constructs directed against *Fos* in esg-positive cells to DSS. In the intestine of these animals, we found that the DSS-mediated induction of Sox21a protein is strongly inhibited and that this inhibition correlates with the efficacy of the Fos knockdown (Figure 4E). Consistent with this result, we also found that Fos is essential for Hep- and Ras-mediated Sox21a expression (Figure 4F). Finally, we reasoned that, if Sox21a is a major target of the Ras/ERK pathway and Fos in the regulation of ISC proliferation, ectopic expression of Sox21a might be sufficient to bypass the requirement for these signaling components. To test this hypothesis, we simultaneously expressed the $Sox21a^{Flag}$ construct with either Ras^{RNAi} or Fos^{RNAi} in esg-positive cells. Whereas Ras^{RNAi} fully inhibits DSS-mediated proliferation (Figure 4B), we found that Sox21a expression partially but significantly rescues this proliferation defect (Figure 4G). Similarly, although Fos^{RNAi} completely blocks Hep- and Ras- mediated proliferation (Figure S4B), Fos knockdown has little to no effect on Sox21a-mediated proliferation (Figure 4H). Together, these results support a model in which Sox21a expression is controlled by Ras, JNK, and Fos to promote ISC proliferation (Figure 4I).

DISCUSSION

In this work, we demonstrate that Sox21a, a member of the Sox2 sub-family of transcription factors, is essential for cell proliferation in the adult *Drosophila* intestine under homeostatic conditions and in response to stress. Strikingly, although we found that *Sox21a* mutant adult flies have dramatic ISCs proliferation defects and are short lived (Figure S1), they do not display any visible developmental phenotype, recapitulating a reported analysis of null *Sox21a* mutants (Phochanukul and Russell, 2010). Thus, this demonstrates that ISCs use stem-cell-specific mechanisms to control cell proliferation. Further studies will be required to understand how Sox21a interacts with other transcription factors that have been shown to regulate ISC proliferation, such as Myc, Nrf2, Stat92E, and Yorkie (Amcheslavsky et al., 2011; Beebe et al., 2010; Buchon et al., 2009; Hochmuth et al., 2011; Jiang et al., 2009; Lin et al., 2010; Liu et al., 2010; Ren et al., 2010; Shaw et al., 2010; Staley and Irvine, 2010). In addition, we anticipate that the identification of Sox21a transcriptional targets in ISCs will be required to fully decipher the mechanism by which this factor controls ISC cell cycle and/or quiescent state. This also constitutes a unique opportunity to study the adult-specific functions of a Sox factor, apart from their requirement during development.

Here, we show that JNK and Ras/ERK signaling, as well as the AP-1 transcription factor Fos, are required for Sox21a induction in response to tissue damage. Although our data support a model in which the activity of Fos is regulated by JNK and ERK to control Sox21a expression (Figure 4I), further studies will be necessary to investigate the potential mechanisms of such regulation and test whether Fos directly binds to the *Sox21a* locus and controls its transcription. Whereas the transcriptional response to various stresses or the activation of these pathways has been investigated in developing tissues and other adult organs, Sox21a has not been identified as a target of these pathways (Asha et al., 2003; Girardot et al., 2004; Wang et al., 2003). Thus, our findings suggest that unidentified stem-cell-specific factor(s) cooperate with Fos to control Sox21a expression in ISCs and EBs. Additional work will be necessary to carefully describe the regulation of Sox21a and the possible role of ISC-specific factors, such as esg (Korzelius et al., 2014; Micchelli and Perrimon, 2006). It will also be interesting to test whether other signaling pathways, such as JAK/STAT and Hippo/Yorkie, are involved in the regulation of Sox21a expression. The identification of potential common transcriptional targets will help to

(C) MARCM clones expressing dsRNA constructs directed against *Sox21a* fail to grow. Clones are labeled by GFP expression (green), EEs are detected by prospero staining (nuclear; red), and DNA is stained with Hoechst (blue). Box plots representing the size distribution of control and $Sox21a^{RNAi}$-expressing clones, 7 days after induction, are in the lower panels. Results for two independent dsRNA constructs $Sox21a^{RNAi(TRiP)}$ and $Sox21a^{RNAi(KK)}$ are shown.

(D) Representative images showing that Sox21a knockdown in ISCs and EBs, using the $esgGal4^{ts}$ driver, abolishes DSS-mediated expansion of esg-positive cells. Quantification of proliferation, as measured by the number of phospho-Histone H3 (pH3)-positive cells per gut, in control treatment and after 48 hr DSS exposure, is shown in the lower panel for three independent dsRNA constructs. EEs are labeled with nuclear prospero straining (red). DNA is detected with Hoechst staining (blue).

(E) Cell-type-specific Sox21a knockdown using the ISC-specific $DeltaGal4^{ts}$ driver and the EB-specific GBE-Su(H)-$Gal4^{ts}$ driver shows that Sox21a is specifically required in ISCs, but not in EBs for ISCs proliferation in response to DSS.

N represents total number of guts included in each analysis. The data are represented as average ± SEM. All p values are calculated using unpaired two-tailed Student's t test. See also Figure S1.

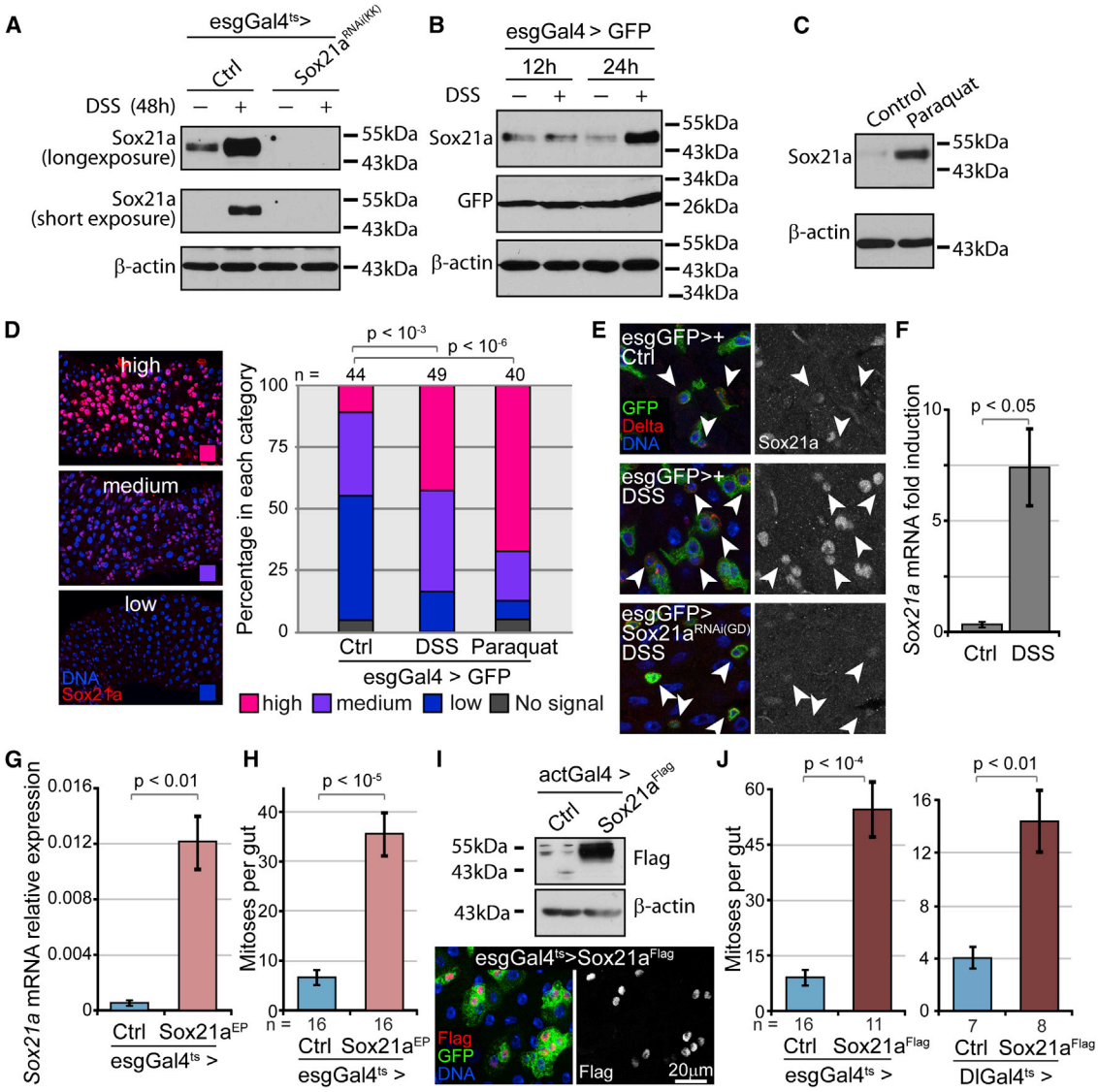

Figure 3. Sox21a Expression Is Induced upon Stress Exposure and Sufficient to Promote ISC Proliferation

(A–C) Western blot analysis shows that Sox21a expression is strongly induced in response DSS treatment (A and B), as early as 24 hr after treatment, and in response to paraquat (C). No Sox21a protein is detected when Sox21a is knocked down in ISCs and EBs using the esgGal4^{ts} driver (A). GFP expression is used to approximate the number of ISCs and EBs in (B). β-actin serves as loading control.

(D) Representative confocal images of posterior midguts illustrating the categories used to score Sox21a expression. Scoring analysis of Sox21a expression in sucrose-treated (Ctrl), 24 hr DSS-treated (DSS), or paraquat-treated (Paraquat) flies is shown on the right panel.

(E) Confocal images demonstrating that the Sox21a protein is specifically induced in ISCs (esg- and Delta-positive cells) and EBS (esg-positive Delta-negative cells). Additional images are provided in Figure S2D.

(F) qRT-PCR shows that *Sox21a* mRNA expression is induced in the intestine after 48 hr DSS treatment.

(G and H) qRT-PCR shows that the *Sox21a*^{d03399} (Sox21a^{EP}) insertion is sufficient to induce intestinal *Sox21a* mRNA expression when combined with the esgGal4^{ts} driver and increase ISCs proliferation compared to control animals.

(I) Western blot and immunostaining show that the Sox21a^{Flag} transgene allows overexpression of Flag-tagged Sox21a protein, using the ubiquitous actGal4 driver or the esgGal4^{ts} driver.

(J) Overexpression of the Sox21a^{Flag} in ISCs and EBs (esgGal4^{ts}) or only in ISCs (DeltaGal4^{ts}) is sufficient to drive ISC proliferation.

In (H) and (J), pH3-positive cells per gut were used to monitor ISCs proliferation. In (F)–(H) and (J), values are presented as average ± SEM. All p values are calculated using unpaired two-tailed Student's t test. See also Figures S2 and S3.

understand how these signaling pathways crosstalk in ISCs and how different signals are integrated into a coordinated proliferative response.

Interestingly, like the activation of stress-signaling pathways, expression of Sox factors is essential for tumor formation in many tissues (Gracz and Magness, 2011; Liu et al., 2013).

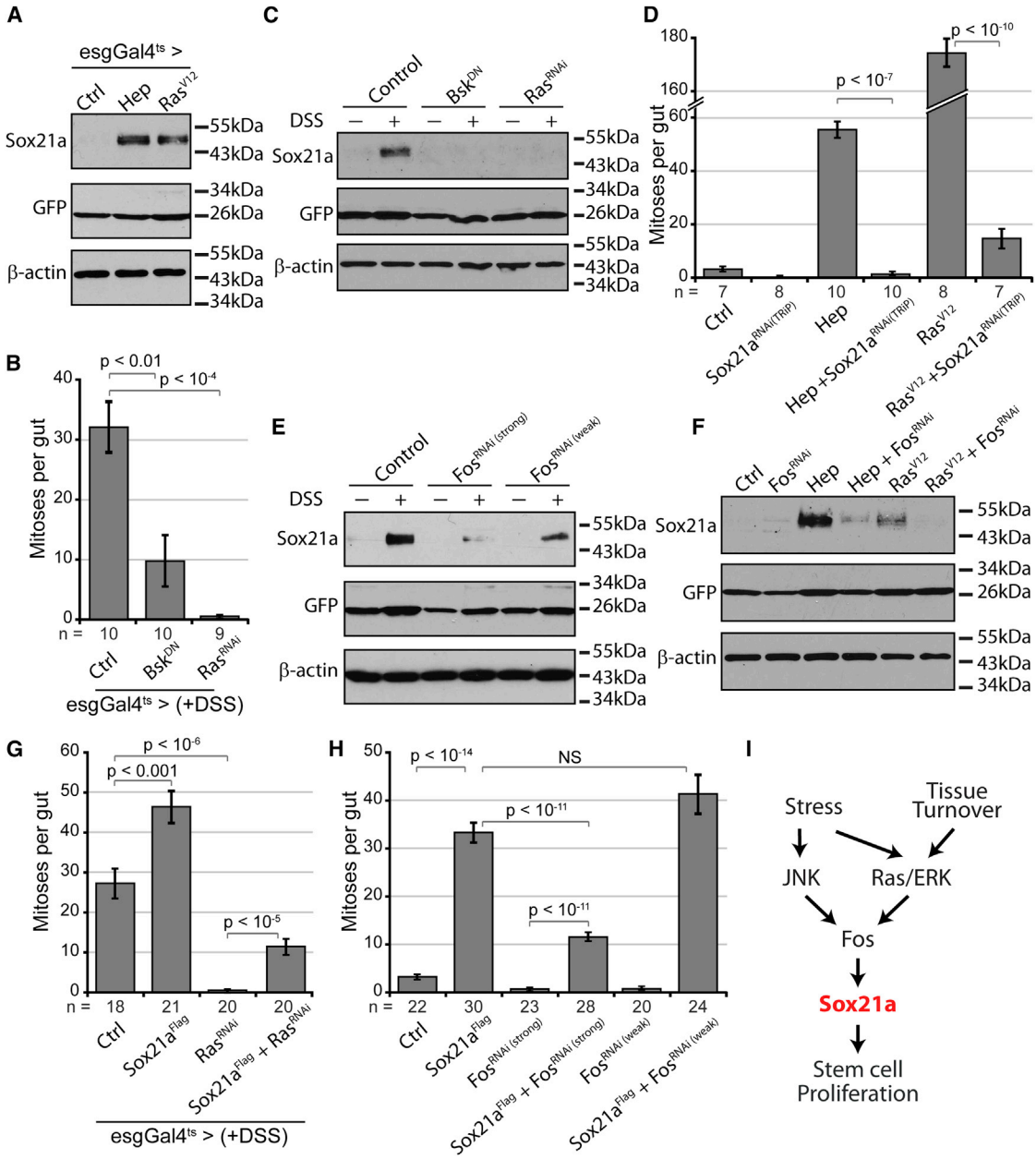

Figure 4. Sox21a Is a Critical Mediator of the JNK and Ras/ERK Pathways in the Control of ISC Proliferation

(A) Activation of JNK (esgGal4^{ts} > UAS-Hep) and Ras (esgGal4^{ts} > UAS-Ras^{V12}) is sufficient to induce Sox21a expression. Intestinal proteins were extracted 18 hr after transgenes induction at 29°C.

(B) Expression of a dominant-negative form of JNK/Bsk (UAS-Bsk^{DN}) or a dsRNA directed against *Ras* (UAS-Ras^{RNAi}) in ISCs and EBs is sufficient to significantly affect proliferation response to DSS exposure.

(C) Sox21a induction (24 hr DSS treatment) is impaired when Bsk^{DN} or Ras^{RNAi} are expressed in ISCs and EBs.

(D) ISCs proliferation induced by JNKK/Hep and Ras^{V12} expression is significantly inhibited when Sox21a$^{RNAi(TRiP)}$ is expressed simultaneously.

(E) Western blot analysis showing that Fos is required for DSS-mediated Sox21a induction (24 hr treatment).

(F) Western blot shows that Sox21a induction by JNKK/Hep and Ras^{V12} overexpression is impaired when Fos is knocked down. The dsRNA directed against *Fos* with higher knockdown efficiency was used.

(G) Sox21a^{Flag} expression is sufficient to significantly rescue the proliferation defects of Ras knockdown ISCs, 24 hr after DSS exposure.

(H) Sox21a overexpression rescues ISCs proliferation defects caused by Fos loss of function.

(I) A model presenting the role and regulation of Sox21a in the control of ISC proliferation.

In all panels, the esgGal4^{ts} driver was used to allow transgenic constructs expression in ISCs and EBs. In (A), (C), (E), and (F), GFP expression is used to approximate the number of ISCs and EBs and β-actin serves as loading control. In (B), (D), (G), and (H), pH3-positive cells per gut were used to monitor ISCs proliferation, and values are presented as average ± SEM. All p values are calculated using unpaired two-tailed Student's t test. See also Figure S4.

Therefore, it will be interesting to test whether, similarly to the regulation we describe here in ISCs, stress pathways, such as JNK and Ras/ERK, directly control the expression of Sox factor(s) in mammals. In this context, our results may provide new insights in the mechanisms that control tissue repair and tumorigenesis in higher organisms, including in humans.

EXPERIMENTAL PROCEDURES

Additional experimental procedures are described in Supplemental Information.

Conditional Expression of UAS-Linked Transgenes

The esgGal4, DeltaGal4, and Su(H)GBEGal4 drivers were combined with a ubiquitously expressed temperature-sensitive Gal80 inhibitor (tubGal80^{ts}). Crosses and flies were kept at 18°C (permissive temperature) and 3- to 5-day-old females were then shifted to 29°C for 2 or 3 days to allow expression of the transgenes before analysis or additional treatment.

In order to induce UAS-driven gene expression with the Act5CGeneswitch driver, food vials were supplemented with 100 μl of a 5 mg/ml solution of mefiprestone, resulting in a final concentration of 0.2 mg/ml.

Mosaic Analysis with a Repressible Cell Marker Clone

Positively marked clones were generated by somatic recombination using the following MARCM stocks: hsFlp;FRT40A,tubGal80;tubGal4,UAS-GFP (MARCM40A; gift from B. Ohlstein) and hsFlp,UAS-GFP;;tubGal4,FRT82B, tubGal80 (MARCM82B). Three- to five-day-old mated female flies were heat shocked for 45 min at 37°C to induce somatic recombination. Flies were transferred to 25°C, and clones were observed 7 days after induction. Only isolated ISC clones in the posterior midgut were included in our analysis.

DSS and Paraquat Treatments

For all stress experiments, young mated females were cultured on standard food. Flies were starved for 6 hr in empty vials and re-fed with a 5% sucrose (AMRESCO) solution with or without 5 mM paraquat (Sigma-Aldrich) or 4% DSS (Sigma-Aldrich; 9 KDa∼20 KDa). Flies were then dissected at the indicated time points for western analysis and immunocytochemistry.

Western Blot Analysis of Intestinal Proteins

Intact guts were dissected in PBS and proteins extracted in Laemmli buffer, separated on 10% acrylamide gel and transferred according to standard procedures. Antibodies directed against β-actin (Cell Signaling Technology; 1:5,000 dilution), Flag tag (Sigma; 1:5,000 dilution), and GFP (Invitrogen; 1:5,000 dilution) were used and Sox21a (this study; 1:50,000 dilution). Total proteins were extracted from 12 guts, and the equivalent of 1.2, 4.8, and 4.8 guts was used for β-actin, Sox21a, and GFP detection, respectively.

Analysis of Gene Expression in the Gut

Total RNA from eight dissected guts from young mated females or three whole flies was extracted using Trizol (Invitrogen), according to manufacturer instructions. cDNA was synthesized using an oligo-dT primer. Real-time PCR was performed on a Bio-Rad iQ5 detection system using the following primers: *Sox21a* forward 5′-GCCGAGTGGAAATTACTCACCGAA-3′; *Sox21a* reverse 5′-TGCGACGTGGTCGATACTTGTAGT-3′; *actin5c* forward 5′-CTCGCCACTT GCGTTTACAGT-3′; and *actin5c* reverse 5′-TCCATATCGTCCCAGTTGGTC-3′. Relative expression was calculated using the ΔΔCt method and normalized to *actin5c* levels.

Fluorescent In Situ Hybridization

In situ hybridization protocol was adapted from previously described procedure (Lécuyer et al., 2008). An ∼550-bp sequence of the *Sox21a* cDNA was amplified, using the 5′-GCCGAGTGGAAATTACTCACCGAA-3′ and 5′-AGGGTGGAGTTTCCGGACTTATCA-3′ primers, and cloned in the pCRII-TOPO vector to generate the antisense RNA probe.

Immunocytochemistry and Microscopy

Intact fly intestines were dissected in PBS and fixed at room temperature for 45 min in 100 mM glutamic acid, 25 mM KCl, 20 mM $MgSO_4$, 4 mM sodium phosphate, 1 mM $MgCl_2$, and 4% formaldehyde. Tissues were blocked in PBS, 0.5% BSA, and 0.1% Triton X-100 and incubated in the same buffer at 4°C. For Delta and Sox21a staining, dissected intestines were fixed in PBS+4% formaldehyde, dehydrated with 100% methanol, and progressively rehydrated in the staining buffer.

The anti-Delta (1:100 dilution) and anti-Prospero (1:250 dilution) were obtained from the Developmental Studies Hybridoma Bank and the anti-phosphoHistoneH3 (1:2,000 dilution) from Millipore. Fluorescent secondary antibodies were obtained from Jackson Immunoresearch. Hoechst was used to stain DNA.

Confocal images were collected using a Leica SP5 confocal system and processed using the Leica software and Adobe Photoshop CS5.

For all experiments, the data are represented as average ± SEM. All p values are calculated using unpaired two-tailed Student's t test unless stated otherwise.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and four figures and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2015.09.061.

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