EGF signaling regulates the proliferation of intestinal stem cells in *Drosophila*

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**SUMMARY**

Precise control of somatic stem cell proliferation is crucial to ensure maintenance of tissue homeostasis in high-turnover tissues. In *Drosophila*, intestinal stem cells (ISCs) are essential for homeostatic turnover of the intestinal epithelium and ensure epithelial regeneration after tissue damage. To accommodate these functions, ISC proliferation is regulated dynamically by various growth factors and stress signaling pathways. How these signals are integrated is poorly understood. Here, we show that EGF receptor signaling is required to maintain the proliferative capacity of ISCs. The EGF ligand Vein is expressed in the muscle surrounding the intestinal epithelium, providing a permissive signal for ISC proliferation. We find that the AP-1 transcription factor FOS serves as a convergence point for this signal and for the Jun N-terminal kinase (JNK) pathway, which promotes ISC proliferation in response to stress. Our results support the notion that the visceral muscle serves as a functional ‘niche’ for ISCs, and identify FOS as a central integrator of a niche-derived permissive signal with stress-induced instructive signals, adjusting ISC proliferation to environmental conditions.

**KEY WORDS:** *Drosophila*, EGF signaling, Intestinal stem cells

**INTRODUCTION**

In high-turnover tissues, the production of new differentiated cells from stem cells is crucial to maintain homeostasis and prevent attrition. In long-lived organisms, stem cell proliferation has to be precisely regulated to maintain regenerative capacity while preventing overproliferation and cancer (Radtke and Clevers, 2005; Rando, 2006; Rossi et al., 2008; Sharpless and DePinho, 2007). The properties of stem cells are regulated by signals from the environment, the organism and, in many cases, a specialized stem cell niche that provides essential growth factors and thus generates a microenvironment that maintains stem cell function (Barker et al., 2008; Bryder et al., 2006; Crosnier et al., 2006; Gopinath and Rando, 2008; Morrison and Spradling, 2008). All of these inputs need to be integrated within the stem cell population to respond to changing environmental conditions with the production of the appropriate number of differentiated cells. The signaling networks that control stem cell maintenance and proliferation govern the balance between tissue regeneration and tumor prevention in aging animals and are therefore crucial to understand.

In *Drosophila melanogaster*, the integrity of the midgut epithelium is maintained by multipotent intestinal stem cells (ISCs) (Micchelli and Perrimon, 2006; Ohlstein and Spradling, 2006). The ISC lineage consists of a non-dividing ISC daughter cell [enteroblast (EB)] and two differentiated cell types [enterocytes (EC), the main cell type in the intestine; and enteroendocrine cells (EE)] (Micchelli and Perrimon, 2006; Ohlstein and Spradling, 2006). EB differentiation into ECs and EEs is controlled by Notch and JAK (HOP – FlyBase)/STAT (STAT92E – FlyBase) signaling, the decision between these two cell types being regulated by differential Notch and JAK/STAT signaling activities (Beebe et al., 2010; Jiang et al., 2009; Lin et al., 2009; Micchelli and Perrimon, 2006; Ohlstein and Spradling, 2006; Ohlstein and Spradling, 2007). ISC-mediated tissue regeneration is required to maintain tissue homeostasis in the intestinal epithelium after tissue damage due to infection, DNA damage and oxidative stress (Amcheslavsky et al., 2009; Biteau et al., 2008; Buchon et al., 2009a; Jiang et al., 2009; Pitsouli et al., 2009). If control of this regenerative process breaks down, for example in old organisms or under conditions of excessive stress, aberrant stem cell proliferation can occur, leading to dysplasia of the intestinal epithelium. This dysplasia phenotype is characterized by an accumulation of misdifferentiated cells at the basal membrane and by disruption of the apicobasal organization of the epithelium. This effect sensitizes flies to infection (Apidianakis et al., 2009) and shortens lifespan (Biteau et al., 2008; Biteau et al., 2010; Buchon et al., 2009a). Precise regulation of ISC proliferation is thus crucial to limit overproliferation while maintaining regenerative capacity, ensuring long-term functional maintenance of this tissue.

ISC proliferation and self-renewal are regulated by the growth factors insulin and WNT/WG, the JAK/STAT signaling pathway, as well as by the MAP Kinase p38 (Amcheslavsky et al., 2009; Beebe et al., 2010; Buchon et al., 2009a; Jiang et al., 2009; Lee et al., 2009; Lin et al., 2008; Lin et al., 2009; Park et al., 2009). In response to tissue damage, for example after exposure to genotoxic or reactive oxygen species (ROS)-inducing compounds or infection, ISC proliferation is further strongly promoted by multiple stress-responsive signaling systems (JNK, JAK/STAT and PVR/p38) (Amcheslavsky et al., 2009; Apidianakis et al., 2009; Biteau et al., 2008; Buchon et al., 2009a; Buchon et al., 2009b; Chatterjee and Ip, 2009; Choi et al., 2008; Cronin et al., 2009; Jiang et al., 2009). The proper interplay of growth factor and stress signals in ISCs is expected to be important for tissue function and integrity. How these different types of biological information are integrated to regulate proliferation rates of ISCs is unclear.

Here, we show that the EGF Receptor (EGFR) signaling pathway plays a crucial role in the regulation of ISC proliferation. We find that the EGFR ligand Vein is expressed in the circular
muscle surrounding the intestinal epithelium, providing a constitutive signal that activates the ERK (Rolled – FlyBase) protein kinase in ISCs and is essential to maintain an optimal ISC proliferative capacity. We show that the transcription factor FOS (KAY – FlyBase) acts as an integrator of this EGF/ERK signal with stress signals mediated by the Jun N-terminal Kinase (JNK; BSK – FlyBase) pathway in ISCs. FOS is required for EGF-mediated ISC proliferation, but also mediates the JNK-dependent boost in proliferation rates in response to stress. FOS integrates these two specific signals through distinct phosphorylation sites. Our findings thus identify muscle-derived growth factors as crucial regulators of proliferative competence of ISCs, strengthening the notion that the visceral muscle serves as a functional niche for ISCs. The integration of this permissive signal with stress-response signaling pathways by FOS provides a model for the dynamic regulation of ISC proliferation in homeostatic and stress conditions.

MATERIALS AND METHODS

Drosophila stocks and culture

The following strains were obtained from the Bloomington Drosophila Stock Center: w1118, UAS-Ras11V, UAS-Ras11Q, UAS-DERDN, UAS-DER11Q, UAS-Raf95a, rasR12, FRT82B, HowGal4 and tub-Gal80. UAS-EgfrRNAi (#34326 and #107130), UAS-rasRNAi (#106642), UAS-rolledRNAi (#34123) and UAS-bskRNAi (#34138) were obtained from the Vienna Drosophila RNAi Center. The line esgGal4 (#5353) was kindly provided by S. Hayashi (RIKEN Center for Developmental Biology, Kobe, Japan), UAS-Bsk1A by M. Mlodzik (Mount Sinai Medical Center, New York, NY, USA) and NP1Gal4 by D. Ferrand (IBMC, Strasbourg, France).

The original UAS-rolledRNAi carries multiple insertions and is named RolledRNAi2x. A single insertion on the second chromosome is isolated and is referred as rolledRNAi.

UAS-fosRNAi and UAS-fos point mutant constructs were previously described (Hyun et al., 2006). UAS-fosRNAi strong and UAS-fosRNAi weak refer respectively to the F139/15 and F149 lines (Hyun et al., 2006).

The kay allele was described previously (Zeitlinger et al., 1997). The kay1 allele is a transposon insertion in the kayak locus, lethal in trans with the kay1 or kay2 alleles and causing a strong loss-of-function phenotype (not shown).

All flies were raised on standard yeast and molasses-based food, at 25°C and 65% humidity, on a 12-hour light/dark cycle, unless otherwise indicated.

Conditional expression of UAS-linked transgenes

The TARGET system was used to conditionally express UAS-linked transgenes in ISCs and EBs (McGuire et al., 2003). The esg-Gal4, HowGal4 and NP1-Gal4 drivers were combined with a ubiquitously expressed transgenes in ISCs and EBs (McGuire et al., 2003). The esg-Gal4, How-Gal4, derDN, UAS-BskDN by M. Mlodzik (Mount Sinai Medical Center, New York, NY, USA) and BskDN by M. Mlodzik (Mount Sinai Medical Center, New York, NY, USA).

For all experiments, the data is represented as mean ± s.e.m. All P-values were calculated using unpaired two-tailed Student’s t-test.

RESULTS

EGF activity is essential for ISC proliferation

EGF signaling is crucial for the development of the adult midgut in Drosophila (Jiang and Edgar, 2009). The expression of multiple EGF-like ligands in a temporally and spatially defined manner ensures the proper proliferation of progenitors during larval and pupal development. Recent studies indicated that expression of these ligands is maintained in the adult intestine, suggesting that epithelial regeneration might be influenced by EGF signaling (Buchon et al., 2009b; Jiang and Edgar, 2009). To test this hypothesis, we generated Egrf homozygous mutant ISC clones using the temperature-sensitive allele Egrf<sup>ts</sup> (Kumar et al., 1998), or a null allele [Egrf<sup>ASP</sup> (Clifford and Schupbach, 1989)], as well as clones overexpressing a dominant negative form of the EGF receptor (EGF<sup>DN</sup>), in the posterior midgut using somatic recombination through the mosaic analysis with a repressible cell marker (MARCM) method (Lee and Luo, 1999). In wild-type flies, GFP-marked ISC cell clones grew to ~10-12 cells within 7 days (Michelli and Perrimon, 2006; Ohlstein and Spradling, 2006) (Fig. 1A,B). However, Egrf mutant clones and clones expressing DER<sup>DN</sup> showed very limited growth, mostly

**Immunostaining and microscopy**

Intact guts were fixed at room temperature for 45 minutes in 100 mM glutamic acid, 25 mM KCl, 20 mM MgSO<sub>4</sub>, 4 mM sodium phosphate, 1 mM MgCl<sub>2</sub>, 4% formaldehyde. All subsequent incubations were done in PBS, 0.5% BSA, 0.1% TritonX-100 at 4°C.

The following primary antibodies were used: mouse anti-dpErk (Sigma; 1:100); mouse anti-BrdU (Becton Dickson; 1:200); mouse anti-Prospero, anti-Armadillo and anti-β-galactosidase (Developmental Studies Hybridoma Bank; 1:250, 1:100 and 1:500, respectively); rabbit anti-pH3 (Upstate; 1:1000). Fluorescent secondary antibodies were obtained from Jackson Immunoresearch.

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

**Western blot**

Intact guts were dissected and proteins extracted in Laemmli buffer, separated on 10% acrylamide gel and transferred according to standard procedures. Antibodies directed against dpERK (MB159; 1:1000 dilution) and ERK (M5670; 1:5000) are from Sigma. Total proteins from four guts and one gut were used for dpERK and total ERK, respectively.

**Analysis of gene expression in the gut**

Young mated females were transferred for 7 days at 29°C to allow the expression of RNAi constructs. Total RNA from six dissected guts 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 q5 detection system using the following primers (5’ to 3’): EGFR forward TGGCCGATCGTTAAGTCATCCCTGT; EGFR reverse TGACTGATCGGAGCAAACATTGTGC; venus forward TCTCGC-GCTATAGTGGCGTC; venus reverse ATAGACCTCTCGTGATT-GCGGAG; actin5c forward CTCGCCCTTGCGTGTAGAC; actin5c reverse TGTATTGGCTGGTGTGTC. Relative expression of Egfr and venus were normalized to Actin5c levels.

**Paracquat and bleomycin treatments**

For all stress experiments, young mated females were cultured on standard food at 29°C for 2 days, in order to induce transgene expression. Flies were then starved for 6 hours in empty vials at room temperature and re-fed with a sucrose solution (5%:mock) with or without 5 mM paracetamol or 10 μg/ml bleomycin sulfate. Flies were maintained at 29°C and dissected 24-48 hours later.

**Statistical analysis**

For all experiments, the data is represented as mean ± s.e.m. All P-values were calculated using unpaired two-tailed Student’s t-test.
consisting of single cells that maintain the expression of Delta, a specific marker for ISCs in the posterior midgut, indicating that ISC proliferation was significantly reduced, whereas ISC survival was not affected (Fig. 1A-C). Accordingly, these single cell clones were maintained in the intestinal epithelium for at least 15 days (see Fig. S1A,B in the supplementary material). EGFR is thus essential for ISC proliferation, but is not required for ISC survival.

Analysis of EGFR-deficient single cell-clones further suggests that EGFR signaling is not required for EE or EC differentiation: as somatic recombination occurs in the G2 stage of the cell cycle of asymmetrically dividing ISCs, only 50% of the marked cells generated by the MARCM method became clone-generating ISCs, whereas the other 50% became EBs, which will undergo differentiation into a single GFP-positive EE or EC. When Egfr-null or EGFRDN-expressing clones were generated, single GFP-positive EEs and ECs were observed in frequencies comparable with wild-type conditions (see Fig. S1C in the supplementary material).

To confirm further that EGFR is required for ISC proliferation, we used an inducible system to express two independent dsRNA constructs directed against the Egfr mRNA (EgfrRNAi) in ISCs and EBs [using esgGal4 together with ubiquitously expressed temperature-sensitive Gal80, tubGal80ts; combined with UAS-GFP, this system is termed esgGFPts here (Micchelli and Perrimon, 2006)]. Expression of either one of the EgfrRNAi constructs efficiently repressed Egfr expression in the intestine (Fig. 1D), confirming that Egfr is expressed in ISCs and EBs using the temperature-sensitive driver esgGFPts (esgGal4; tubGal80ts) is sufficient to prevent age-related induction of proliferation and intestinal dysplasia. Proliferation was quantified by counting the number of pH3+ cells per gut after immunostaining, 15 days after transgenes induction at 29°C. Representative confocal images are shown to illustrate the reduced number of pH3+ cells (indicated by arrowheads) and limited intestinal dysplasia observed in esgGFPts>EgfrRNAi flies. Boxed areas are magnified in the lower panels. (F) Reduction of Egfr activity prevents NotchRNAi-induced tumor formation. EgfrRNAi transgenes were expressed together with NRNAi; 10 days after induction, tumors composed of esg+ and PROS+ cells accumulate in the posterior midgut of control flies (N RNAi alone), whereas the intestinal epithelium architecture is preserved when EGFR activity is inhibited. In A, C, E and F, GFP expression is shown in green, cell boundaries are stained using β-Catenin/Armadillo (red, membrane), EEs are marked by the expression of Prospero (red nuclei) and DNA is labeled using Hoechst (blue). EB, entoblast; EE, enteroendocrine cells; ISC, intestinal stem cell; MARCM, mosaic analysis with a repressible cell marker.
proliferation was strongly increased owing to activation of stress signaling pathways. Loss of Notch prevents EB differentiation into ECs, and causes unchecked expansion of ISCs and EEs into tumor-like structures (Biteau et al., 2008; Choi et al., 2008; Micchelli and Perrimon, 2006; Ohlstein and Spradling, 2006). When EGFR expression was knocked down, the prevalence of pH3+ cells and the accumulation of esgGFP+ cells at 15 days of age was significantly reduced, whereas paraquat-induced proliferation of ISCs was inhibited by expression of EGFRDN (Fig. 1E; see Fig. S1D in the supplementary material). Similarly, the formation of Notch mutant ISC and EE tumors was significantly impaired, indicating that loss of EGFR is sufficient to prevent ISC proliferation independently of Notch signaling (Fig. 1F). Altogether, our results demonstrate that the activity of the EGF receptor is thus essential for ISC proliferation under normal conditions, as well as in response to stress or mitogenic signals, without affecting ISC survival or differentiation in the ISC lineage.

The MAPK signaling pathway is required for ISC proliferation

EGFR signaling is transduced by the MAPK signaling pathway in Drosophila, including the small GTPase RAS (encoded by Drosophila Ras85D) and the MAP kinase ERK [encoded by Drosophila gene rolled (rl)] (Shilo, 2005). We assessed whether these downstream components are also required for stem cell proliferation using the MARCM and flip-out lineage tracing techniques (Lee and Luo, 1999; Pignoni and Zipursky, 1997). Similar to Egfr loss-of-function conditions, ISCs homozygous for Ras loss-of-function alleles or expressing a dominant negative form of RAS or rolledRNAi failed to generate multi-cell clones (Fig. 2A-D). Inhibition of RAS and ERK is also sufficient to inhibit NRNAi and paraquat-induced proliferation (Fig. 2E; see Fig. S1D in the supplementary material), confirming that the MAPK/ERK pathway is required to maintain proliferative competence of ISCs.

Expression of the EGFR ligand Vein in the muscle is partially required for ISC proliferation

The requirement of EGFR/ERK signaling activity for ISC proliferation suggests that an EGF-like ligand is secreted from a neighboring source, generating a niche-like microenvironment for ISCs that maintains proliferative competence. During the development of the adult intestine, the muscle surrounding the intestinal epithelium expresses the neuregulin homolog vein, one of the Drosophila EGFR ligands, and it has been suggested that this expression is preserved in adult intestines (Jiang and Edgar, 2009).

Using a reporter construct expressing nuclear β-galactosidase, we confirmed that vein promoter activity is maintained in the adult visceral muscle, as indicated by the colocalization of β-galactosidase positive nuclei with GFP expressed under the control of the muscle-specific HowGal4 driver and with phallolidin staining, as well as with a basement membrane component, vkgGFP (also known as collagen IV) (Fig. 3A-C). Importantly, expression of two independent veinRNAi constructs in the muscle using the HowGal4 driver was sufficient to strongly reduce the expression of vein in the whole intestine, as determined by qRT-PCR, whereas expressing these dsRNA constructs in ISCs/EBs or ECs had no effect (Fig. 3D). The visceral muscle is thus the primary source of Vein in the adult midgut and is a candidate for providing the signal required for ISC proliferation. We first tested this idea by assessing whether vein expression in the muscle is required for ISC proliferation under normal conditions. Indeed, inhibition of vein expression in the muscle for 5 days did not affect the number of ISCs (identified by the expression of Delta; Fig. 3E), but significantly reduced the number of EBs in the intestinal epithelium (identified by the expression of GBE-Su(H)-lacZ reporter; Fig. 3F), suggesting that ISC division is reduced in these conditions. We assessed further the requirement for vein expression in stress-induced ISC proliferation. Compared with wild-type controls, the number of pH3+ cells detected after exposure to paraquat or to the genotoxic compound bleomycin (Amcheslavsky et al., 2009) was significantly lower in animals expressing veinRNAi in the muscle (Fig. 3G,H). vein expression in the muscle is thus required for optimal ISC proliferation.

ERK activation is a permissive signal required to promote cell proliferation

To monitor the activity of the EGFR signaling pathway in the intestinal epithelium, we detected the active, double-phosphorylated (dp) form of ERK using immunohistochemistry (Gabay et al.,
In the posterior midgut of young flies, dpERK could be detected in ISCs (Fig. 4A) and enteroendocrine cells (not shown), but not in ECs. Interestingly, the vast majority of these cells were dpERK positive (>80% of esg-positive cells), even though under unstressed conditions most ISCs were not or only slowly dividing (Biteau et al., 2008; Choi et al., 2008; Michelli and Perrimon, 2006; Ohlstein and Spradling, 2006) (Fig. 4E; in young flies, only 10% of esg	extsuperscript{+} cells incorporate BrdU within 48 hours, not shown). This suggests that the level of ERK activity in ISCs under such basal, non-stressed conditions is not sufficient to promote high rates of cell division. The lack of mitotic activity in the majority of cells does not seem to be due to fluctuations of ERK activity, as the intensity of dpERK staining in pH3	extsuperscript{+} and pH3 ISCs was indistinguishable (Fig. 4B). Although we cannot formally exclude the possibility that ERK is activated very transiently during early phases of the cell cycle, we deduce that ERK activation in these cells provides a permissive, but not inductive, signal for proliferation. Interestingly, however, expression of an oncogenic form of RAS is sufficient to promote widespread ISC division (Apidianakis et al., 2009), indicating that strong EGFR gain-of-function conditions can overcome a threshold and provide a sufficient signal for ISC division. To confirm this interpretation, we expressed activated forms of EGFR [DER-ellipse (Baker and Rubin, 1989)], RAS [rasV12 (Karim and Rubin, 1998)], RAF [raf	extsuperscript{S65} (Brand and Perrimon, 1994)] and ERK [rolle	extsuperscript{SEM} (Brunner et al., 1994)] using the esgGFP	extsuperscript{+} driver. Expression of any of these constructs for 2 days resulted in strongly increased levels of activated ERK in the intestine (Fig. 4C) and correspondingly very high numbers of pH3	extsuperscript{+} cells in the intestinal epithelium accompanied by a dramatic expansion of esg	extsuperscript{+} cells (Fig. 4D,E). Strong activation of ERK is thus sufficient to promote ISC proliferation. Importantly, however, expression of the JNK Kinase Hemipterous [HEP (Biteau et al., 2008)] strongly increased ISC proliferation without significantly increasing ERK phosphorylation in the intestine (Fig. 4E,F), suggesting that, as in other biological contexts, RAS and HEP signal through independent signaling pathways to induce ISC proliferation (Ciapponi et al., 2001; Kockel et al., 2001; Luo et al., 2007; Suzanne et al., 2001; Weston and Davis, 2002). These findings further support the notion that elevated ERK phosphorylation is not required to induce ISC proliferation under stress conditions, and that the basal activity of ERK that is maintained by muscle-derived Vein constitutes a permissive signal for ISC division. In this model, EGFR signaling is required to maintain proliferative competence of ISCs, whereas activation of JNK signaling or other stress-responsive signaling pathways is required to stimulate proliferation of ISCs in response to oxidative stress, tissue damage or infection (Amcheslavsky et al., 2009; Apidianakis et al., 2009; Biteau et al., 2008; Buchon et al., 2009a; Buchon et al., 2009b; Chatterjee and Ip, 2009; Choi et al., 2008; Cronin et al., 2009; Jiang et al., 2009). Accordingly, JNK [encoded by the Drosophila gene basket (bsk)] is
required for oxidative stress and DNA damage-induced proliferation of ISCs, but not for proliferation under homeostatic conditions: inhibition of JNK, by expressing either dominant-negative bsk [Bsk$^{DN}$ (Weber et al., 2000) or a dsRNA against bsk [BskRNAi (Hull-Thompson et al., 2009)] in ISCs and EBs significantly decreased paraquat- and bleomycin-induced proliferation (see Fig. S2B,C in the supplementary material). Levels of total ERK protein serve as loading control. 

**The transcription factor FOS is required for JNK- and ERK-induced ISC proliferation**

The proliferative response of ISCs both to activation of JNK and of EGFR/ERK signaling pathways raises the possibility that a common downstream effector might mediate these responses and thus integrate permissive and inductive signals. The AP-1 transcription factor FOS is a well-described target of JNK signaling in *Drosophila* and has been shown to also respond to EGFR signaling in various biological contexts, such as developing imaginal discs (Ciapponi et al., 2001). FOS might thus serve as a convergence point for JNK and ERK responses in ISCs. To test this idea, we first assessed the requirement for FOS in proliferating ISCs. We generated homozygous mutant clones for the fos loss-of-function alleles kay$^2$ and kay$^3$. The resulting GFP-positive clones

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**Fig. 4. The MAPK signaling pathway is active in ISCs and its activation is sufficient to promote ISC proliferation.** (A) The active, double-phosphorylated (dp) form of ERK can be detected by immunostaining in the ISCs under normal conditions (red, left-hand panel; monochrome, right-hand panel). (B) Similar dpERK staining is observed in dividing (pH3-positive; open arrowhead) and non-dividing ISCs (solid arrowhead). (C) Expression of activated forms of the EGFR (DER$^{act}$) or RAS (ras$^{V12}$), using the esgGFP$^+$ driver, dramatically increases the level of activated ERK in the intestine. dpERK is detected by Western blot from dissected midguts 2 days after transgene induction at 29°C. Levels of total ERK protein serve as loading control. (D,E) Activation of the EGFR/MAPK and JNK signaling pathways is sufficient to induce ISC proliferation, resulting in intestinal dysplasia. Activated forms of DER (DER$^{act}$), RAS (ras$^{V12}$), RAF (raf$^{GOF}$) and ERK (rolled$^{SEM}$; rl$^{SEM}$) or HEP (Hep) were expressed for 2 days in ISCs using the temperature-sensitive driver (esg-Gal4, UAS-GFP; tub-Gal80$^{ts}$). Proliferation rates in the midgut were quantified (E) by counting the number of pH3$^+$ cells per gut in the same genetic conditions. (F) Activation of JNK in ISCs and EBs does not significantly elevate dpERK levels. dpERK is detected by Western blot from dissected midguts 2 days after transgene induction at 29°C. Levels of total ERK protein serve as loading control. (G) Overexpression of HEP in ISCs and EBs results in dysplastic phenotype in the intestinal epithelium (esgGFP$^+$>Hep). Co-expression of dominant forms of EGFR (DER$^{DN}$) and ras (ras$^{N17}$), or a dsRNA construct directed against ERK (rolledRNAi), is sufficient to prevent HEP-induced dysplasia. (H) RAS-induced dysplasia specifically requires ERK activity. Inhibiting ERK (rolledRNAi) prevents the expansion of esg$^+$ cells observed when Ras$^{V12}$ is expressed under the control of the esgGFP$^+$ driver. Co-expression of a JNK dominant form (Bsk$^{DN}$) does not affect this phenotype. Boxed areas are enlarged in lower panels. EB, enteroblast; ISC, intestinal stem cell.
failed to grow, and often remained restricted to a single stem cell (Fig. 5A, B). This result suggests that FOS is required for ISC proliferation. kay" [a hypomorphic mutant allele that does not result in cell lethality (Zetlinler et al., 1997)] clones were induced at frequencies comparable to wild-type clones, but clones homozygous for the strong loss-of-function allele kay" (a P-element insertion into the kay locus) were recovered at much lower frequency, suggesting that strongly reducing FOS function in ISCs affects stem cell survival. To confirm these effects of fos loss-of-function on ISC proliferation and survival, we used two distinct transgenic constructs allowing mild or strong expression of dsRNA directed against FOS [fosRNAi (Hyun et al., 2006)]. Flip-out clones expressing fosRNAi weak show much reduced growth compared with wild-type clones (see Fig. S4A, B in the supplementary material). In addition, expression of fosRNAi strong for 10 days in ISCs and EBS using esgGal4 results in the complete loss of esg+ cells in the intestinal epithelium. This cell loss could be rescued by overexpressing the anti-apoptotic protein p35, demonstrating that strong knockdown of FOS in ISCs results in their death by apoptosis (Fig. 5C). Similarly, strong inhibition of FOS in a Notch loss-of-function background (esgGFPts>NRNAi) prevented ISC overproliferation and resulted in the apoptotic death of esg-positive cells. However, preventing apoptosis of these Notch mutant ISCs did not restore proliferation (Fig. 5D), supporting the conclusion that FOS activity is essential for both ISC proliferation and survival, and demonstrating that these functions of FOS are separable.

Next, we wanted to investigate whether FOS relays EGFR/ERK and JNK signaling in the regulation of stem cell proliferation. To test this idea directly, we assessed the effect of impairing FOS function on JNK- or RAS-induced ISC proliferation. Intestines 5 days after induction of HEP or rasV12 together with fosRNAi, using the temperature-sensitive esgGal4 driver. The HEP- and rasV12-induced expansion of esg+ cells is entirely blocked by fosRNAi, demonstrating that FOS is required downstream of JNK and RAS. Note that knocking down FOS does not block RAS-induced cell growth. (F) Paraquat-induced ISC proliferation requires FOS. Expression of two different fosRNAi constructs, using esgGFPts (for 2 days at 29°C prior to treatment), significantly reduces Paraquat-induced proliferation in the intestinal epithelium, as shown by the limited number of pH3+ cells per gut 48 hours after paraquat exposure. In A, C and D GFP is shown in green, armadillo (Arm) outlines cell boundaries (red), prospero (Pros) identifies EEs (nuclear red), DNA is shown in blue. AHS, after heat shock; EE, enteroendocrine cells; ISC, intestinal stem cell; MARCM, mosaic analysis with a repressible cell marker.

Distinct phosphorylation sites in FOS mediate JNK- and RAS/ERK-induced ISC proliferation

These results strongly suggest that FOS is essential for both JNK and EGFR-mediated proliferation in ISCs. It remains possible, however, that FOS acts primarily as an ERK target and is thus...
generally required for ISC proliferation, but does not directly respond to the JNK-mediated inductive signal. Interestingly, a function of FOS directly downstream of JNK and ERK in *Drosophila* has been suggested, as the two kinases can phosphorylate FOS on overlapping and distinct sites (Ciapponi et al., 2001). This suggests a potential mechanism by which FOS might integrate permissive and instructive signals to regulate ISC proliferation. In vitro, JNK phosphorylates two residues located in the N-terminal part of the FOS protein, whereas both ERK and JNK can phosphorylate residues in the C-terminal domain (Ciapponi et al., 2001) (Fig. 6A). The importance of these distinct phosphorylation sites for FOS function in vivo has been tested in developmental contexts. Expression of a mutant form of FOS carrying alanine substitutions of the N-terminal phosphorylation sites (FOSN-Ala) dominantly interferes with JNK-dependent thorax closure, but has no effect on ERK-dependent wing vein formation. Conversely, expression of a mutant form in which the C-terminal phosphorylation sites are replaced (FOSC-Ala), recapitulates ERK mutant phenotypes in the developing wing, without affecting JNK function during thorax closure (Ciapponi et al., 2001). These mutants thus provide unique tools to selectively perturb JNK-or ERK-specific signaling to FOS.

To assess the effect of ERK or JNK-specific FOS phosphorylation on ISC proliferation, we generated MARCM clones overexpressing fosC-Ala and fosN-Ala 7 days after induction. Expression of FOS carrying substitution ERK phosphorylation sites (fosC-Ala) prevents the formation of large clones. Inset shows enlarged cells. Clone size (number of cells per clone) is quantified in C. (D) Expression of FOS mutant forms in ISCs and EBs does not affect the architecture of the posterior midgut. (E) ERK phosphorylation sites are required for N loss-of-function tumor formation. Intestines 10 days after induction of NRNAi and fos mutants carrying substitution of JNK and/or ERK phosphorylation sites. Expression of the mutant forms lacking ERK phosphorylation prevents NRNAi-induced ISC overproliferation. (F) ERK and JNK phosphorylation sites are required for HEP-induced proliferation. Representative confocal images of intestines 5 days after induction of JNK and HEP together with the wild-type or mutant forms of FOS in ISCs/EBs. The HEP-induced expansion of esg+ cells is blocked by all the FOS mutant forms. (G) Overexpression of FOS mutant forms partially prevents paraquat-induced stem cell proliferation, as shown by reduced number of pH3+ cells, 48 hours after paraquat exposure. In B, D and E, GFP is shown in green, armadillo (Arm) outlines cell boundaries (red), prospero (Pros) identifies EEs (nuclear red), DNA is shown in blue. (H) Model representing the role of the EGFR signaling pathway and circular muscle acting as a niche for ISC and the integration of EGFR and JNK signaling by FOS in ISCs to regulate proliferation. EB, enteroblast; EE, enteroendocrine cells; ISC, intestinal stem cell; MARCM, mosaic analysis with a repressible cell marker.
expressing FOS mutants in which the ERK phosphorylation sites were replaced, strongly reduced veinRNAi-induced proliferation and tumor formation, whereas mutating JNK phosphorylation sites had no effect (Fig. 6D-F). This strongly suggests that ERK-dependent phosphorylation of FOS is a permissive signal required for stem cell proliferation, and confirms that JNK-dependent regulation of FOS is not required under normal conditions.

To further confirm this model, we assessed the consequences of expressing these FOS variants in conditions in which ISC proliferation is induced by JNK. The expression of any of the three mutant forms of FOS (JNK-specific, FOSN-Ala; ERK-specific, FOSC-Ala; and combined, FOSN/Ala) was sufficient to prevent HEP-induced expansion of esg<sup>-</sup> cells (Fig. 6F), and significantly reduced paraquat-induced proliferation (Fig. 6G), further supporting the notion that JNK-mediated phosphorylation of FOS is required in addition to ERK phosphorylation to mediate the instructive signal promoting ISC proliferation in response to stress.

**DISCUSSION**

Our findings establish a crucial role for EGF signaling in the regulation of ISC proliferation, and thus support the notion that the visceral muscle surrounding the intestinal epithelium has the characteristics of a functional niche. *vein* expression in the muscle maintains the competence of ISCs to enter rapid proliferation in responses to stress and JNK signaling, and is thus expected to regulate epithelial homeostasis. Interestingly, we find that both the EGFR-mediated permissive signal and the JNK-derived inductive signal are relayed by FOS, establishing an integrated molecular mechanism for the control of ISC proliferation (Fig. 6H).

**The visceral muscle: a niche for ISCs?**

Many stem cell populations are regulated by their microenvironments, and larval ISC progenitors are regulated by a transient niche (Mathur et al., 2010). However, ISCs in adult flies apparently lack such a closely associated cell population within the intestinal epithelium. By contrast, control of ISC maintenance by muscle-derived Wingless suggested this tissue as a potential functional niche for adult ISCs (Lin and Xi, 2008; Lin et al., 2008). Our results support and extend this idea by identifying a second growth factor derived from the visceral muscle that controls ISC proliferation. In its regulation of stem cell function through Wingless and Vein, and in the close association of ISCs and muscle cells, the muscle thus shares characteristics of stem cell niches in other systems, yet it also differs from these in important ways. In mammals, as well as in the *Drosophila* and *C. elegans* gonads, the niche of most stem cell populations maintains stem cell quiescence and prevents differentiation (Jones and Wagers, 2008; Morrison and Spradling, 2008; Voog and Jones, 2010). The EGF signal originating from the muscle, however, maintains the capacity of ISCs to divide, allowing these cells to respond to stimulating signals while not affecting ISC differentiation. Interestingly, EGFR signaling has not been described so far as crucial for interactions between the niche and stem cell populations in other systems, and our findings raise the possibility that this signaling pathway might also regulate the function of other stem cell populations in both invertebrates and vertebrates.

**Potential function for additional EGF-like ligands**

Whereas knocking down the expression of *vein* in the muscle partially affects the ability of ISCs to proliferate under normal conditions and in response to stress, the inhibition of EGFR completely abolishes stem cell division. This might reflect the inefficiency of the veinRNAi constructs used in our study, but might also suggest a contribution of other EGFR ligands to the regulation of ISC function. Accordingly, a genome-wide analysis of the transcriptional response of the adult intestine to bacterial infection suggests that expression of *vein*, as well as of two other genes encoding EGFR ligands, *Keren* and *spitz*, is increased after immune challenge (Buchon et al., 2009b). However, the potential role for these additional EGF-like ligands in regulating ISC function remains to be investigated and the cells expressing *spitz* and *Keren* in the adult intestine have yet to be identified.

**Integration of mitogenic and stress signals by FOS**

ISC function is regulated by systemic [insulin-like peptides expressed by neurosecretory cells in the brain (Amcheslavsky et al., 2009)], muscle-derived [*vein* and *wingless* (this study)] (Lin et al., 2008), local [*unpaired* cytokines expressed by ECs (Buchon et al., 2009a; Buchon et al., 2009b; Chatterjee and Ip, 2009; Cronin et al., 2009; Jiang et al., 2009; Lin et al., 2009]) and cell-intrinsic [JNK and PVR/p38 signaling (Biteau et al., 2008; Buchon et al., 2009a; Choi et al., 2008; Park et al., 2009)] signals. These multiple signals are integrated in ISCs to adapt their proliferation rate and differentiation program to environmental and physiological challenges. To fully understand stem cell regulation in this high-turnover tissue, the molecular structure of this signaling network has to be unraveled. Our findings introduce the transcription factor FOS as a crucial regulator of ISC proliferation that integrates mitogenic and stress signals, and indicate that JNK and ERK regulate FOS activity directly by phosphorylation on distinct residues, controlling ISC proliferation in a combinatorial fashion. This signal-specific mode of FOS regulation by ERK and JNK in *Drosophila* had previously been described in the context of morphogenetic movements (in which FOS is regulated by JNK) and of eye and wing growth during development (in which it is regulated by ERK and JNK) (Ciapponi et al., 2001).

How FOS promotes ISC proliferation remains unclear. In developing imaginal discs, inhibition of FOS causes an accumulation of cells in the G2/M phase of the cell cycle, probably owing to a loss of Cyclin B expression, an essential regulator of the G2/M transition (Hyun et al., 2006). Interestingly, in ISCs, expression of FosRNAi not only inhibits stress-induced accumulation of pH3<sup>+</sup> cells, but also represses BrdU incorporation (see Fig. S4D in the supplementary material), indicating that FOS regulates entry into S phase. In these cells, FOS might thus regulate the transcription of essential S phase components. Further studies will be required to identify such ISC-specific FOS target genes.

**AP-1 and intestinal homeostasis**

The maintenance of stem cells in a primed state, ready to respond to inductive mitogenic stress signals, is likely to be crucial for high-turnover tissues like the intestinal epithelium, which require rapid activation of stem cell division for an efficient regenerative response to tissue damage. At the same time, this enhanced mitogenic potential of ISCs might contribute to the loss of tissue homeostasis in the aging gut (Biteau et al., 2008), and contribute to cancer formation in mammalian intestinal epithelia (Barker et al., 2009; van der Flier and Clevers, 2009). Interestingly, a conserved role of AP-1 transcription factors and JNK signaling in the regulation of intestinal stem cell proliferation and intestinal cancer is emerging in mice. JNK activation is sufficient to induce cell proliferation in the intestinal crypt and increases tumor incidence and tumor growth in an inflammation-induced colon cancer model (Sancho et al., 2009). These effects of JNK signaling
are mediated by the FOS binding partner JUN, as shown by the requirement for JNK-mediated phosphorylation of JUN for APCmin-induced tumorigenesis (Nateri et al., 2005). Strikingly, ISC-specific activation of WNT signaling, by mutating APC or expressing an active form of β-catenin or wingless itself, is sufficient to induce the formation of tumor-like stem cell clusters in the fly intestine (Lee et al., 2009, Lin et al., 2008). A potential interaction of WNT signaling with JNK and JUN or FOS in ISCs remains to be tested in Drosophila. Interestingly, increased FOS activity has also recently been shown to be sufficient to promote hematopoietic stem cell self-renewal in mice, further illustrating the conserved function of FOS in the regulation of stem cell function (Deneault et al., 2009). AP-1 transcription factors are thus emerging as conserved essential regulators of stem cell function and our findings provide an important starting point for further studies characterizing stem cell-specific signaling networks that integrate mitogenic, survival and stress signals to control stem cell maintenance, quiescence and proliferation, and thus influence the balance between regeneration and tumor suppression in high turnover tissues.

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Competing interests statement
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