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EphB Receptors Coordinate Migration and Proliferation in the Intestinal Stem Cell Niche

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SUMMARY

More than 10^{10} cells are generated every day in the human intestine. Wnt proteins are key regulators of proliferation and are known endogenous mitogens for intestinal progenitor cells. The positioning of cells within the stem cell niche in the intestinal epithelium is controlled by B subclass ephrins through their interaction with EphB receptors. We report that EphB receptors, in addition to directing cell migration, regulate proliferation in the intestine. EphB signaling promotes cell-cycle reentry of progenitor cells and accounts for approximately 50% of the mitogenic activity in the adult mouse small intestine and colon. These data establish EphB receptors as key coordinators of migration and proliferation in the intestinal stem cell niche.

INTRODUCTION

The proliferation of stem cells and their generation of progeny need to be tightly regulated, as an overproduction may result in tumor formation and an underproduction in atrophy of the tissue. The proliferation of stem cells is orchestrated, in part, by neighboring cells, forming the stem cell niche (Fuchs et al., 2004; Mikkers and Frisén, 2005; Watt and Hogan, 2000). The study of stem and progenitor cells and their niches in model organisms have provided important insights into their interplay through extracellular signals (Spradling et al., 2001). The often more complex structure of mammalian organs has made it more difficult to elucidate how cells in a niche communicate to maintain homeostasis.

The comparatively simple organization of the intestine offers an attractive model in mammalian stem cell biology. In the intestine, the epithelial lineage is contained within a sheet of cells. Epithelial stem cells reside at or near the bottom of crypts that are formed by the convolution of the epithelial sheet. Members of the BMP (Haramis et al., 2004; He et al., 2004) and hedgehog (Madison et al., 2005; Ramalho-Santos et al., 2000; van den Brink et al., 2004) families direct the positioning of the crypts through an interplay between epithelial and subepithelial cells.

Wnt proteins present at the bottom of the crypts induce nuclear translocation of β-catenin, which is a pivotal feature of intestinal epithelial stem/progenitor cells (van de Wetering et al., 2002). In concert with the Notch pathway, β-catenin maintains intestinal stem cells and controls their differentiation (Fre et al., 2005; Stanger et al., 2005; van de Wetering et al., 2002; van Es et al., 2005). Experimental inhibition of the β-catenin pathway abolishes proliferation in intestinal crypts, and mutations causing overactivation are found in the vast majority of human intestinal cancers, underscoring the central role of this pathway in controlling cell proliferation and differentiation in the intestine (Reya and Clevers, 2005).

As the progeny of the epithelial stem/progenitor cells differentiate, the majority of cells migrate out of the crypt and are shed into the lumen within one week. β-catenin controls the positioning of cells within crypts by regulating the expression of members of the ephrin and Eph families (Batlle et al., 2002). Ephrin ligands and their Eph tyrosine kinase receptors regulate cell migration in many contexts (Holmberg and Frisén, 2002; Palmer and Klein, 2003; Poliakov et al., 2004). Both ephrins and Eph receptors are membrane bound proteins, restricting their interactions to sites of direct cell-cell contacts. The ephrin-Eph receptor interaction allows bidirectional communication, with a signal being conveyed in both the receptor-expressing (forward signaling) as well as in the ligand-expressing (reverse signaling) cell (Cowan and Henkemeyer, 2002; Holmberg and Frisén, 2002; Palmer and Klein, 2003; Pasquale, 2005).

Ephrins and Eph receptors negatively regulate the number of neurons generated from stem/progenitor cells in the brain (Depaepe et al., 2005; Holmberg et al., 2005). The transition from adenoma to colon carcinoma is associated with loss of EphB receptor expression, and this is

an important step in the progression to invasive cancer (Batlle et al., 2005; Guo et al., 2005; Jubb et al., 2005; Lugli et al., 2005). This prompted us to analyze whether ephrins and Eph receptors, in addition to their role in directing cell migration, may participate in the control of proliferative homeostasis in the intestinal stem cell niche.

We find by gain- and loss-of-function experiments that B subclass ephrins and Eph receptors, independently of their influence on cell positioning, promote proliferation in the crypts of the small intestine and colon and account for about 50% of the mitogenic activity. EphB2 and EphB3 kinase-dependent signaling promote cell-cycle reentry of intestinal progenitor cells. These data establish that ephrins and Eph receptors are key coordinators of migration and proliferation in the intestinal stem cell niche.

RESULTS

Reduced Proliferation in Colon Crypts in Mice Lacking EphB2 and EphB3

Progenitor cells in the colon express EphB2 and EphB3, whereas differentiating cells express ephrin-B1 and ephrin-B2 (Figures 1A and S1) (Batlle et al., 2002). As stem cells cannot confidently be identified in the intestine we refer to stem and progenitor cells collectively as progenitor cells. We analyzed mice with null mutations in *EphB2* and *EphB3* (EphB2$^{-/-}$; EphB3$^{-/-}$) as well as mice that lack *EphB3* and have a kinase-inactive (ki) form of EphB2 in which the intracellular domain is replaced by β-galactosidase (EphB2$^{ki/ki}$; EphB3$^{-/-}$) (Henkemeyer et al., 1996; Orioli et al., 1996). Whereas both forward and reverse signaling is abolished in EphB2$^{-/-}$; EphB3$^{-/-}$ mice, reverse signaling through ephrin-Bs is maintained in EphB2$^{ki/ki}$; EphB3$^{-/-}$ mice (Henkemeyer et al., 1996), enabling the dissociation of the relative importance of reverse and kinase-dependent signaling.

Progenitor cells in the colon reside at the bottom of epithelial invaginations known as the crypts of Lieberkühn (Marshman et al., 2002; Sancho et al., 2004; Stappenbeck et al., 1998). They give rise to absorptive enterocytes, the main cell type of the epithelium, mucus-producing goblet cells, and enteroendocrine cells. In both double mutant combinations, we observed an approximately 50% decrease in the number of cells that were immunoreactive for PCNA or Ki-67, markers of proliferating cells, or that incorporated BrdU in colon crypts (Figures 1B and 1D–1F). There was no increase in the number of dying cells that could explain the reduction in proliferating cells (Figure 1C). These data establish EphB receptors as positive regulators of proliferation in colon crypts.

Redistribution of Proliferative Cells in the Crypts of the Small Intestine in Mice Lacking EphB2 and EphB3 Masks Altered Proliferation

In addition to the cell types produced in the colon, another differentiated cell type, the Paneth cell, is generated in the small intestine. Paneth cells are terminally differentiated cells that have antimicrobial functions and are localized

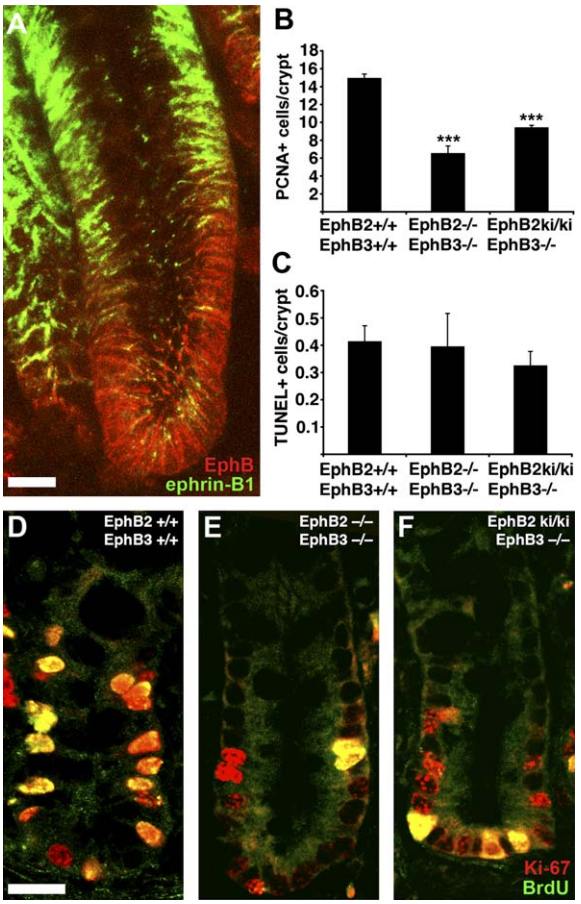

Figure 1. EphB2 and EphB3 Regulate Cell Proliferation in Adult Mouse Colon

(A) EphB receptors, visualized by binding of ephrin-B2-Fc, are expressed in progenitor cells at the bottom of crypts in the colon, with decreasing levels higher in the crypt. Ephrin-B1 is expressed in a countergradient with the lowest levels at the crypt bottom.

(B–F) The number of BrdU-incorporating, Ki-67- and PCNA-immunoreactive cells is reduced in EphB2; EphB3 mutant mice, but cell death is unaltered. Data are represented as mean ± SEM. *** = $p < 0.001$. Scale bars = 20 μm.

at the bottom of the crypt (Ayabe et al., 2004). Progenitor cells in the small intestine reside just above the Paneth cell compartment (Marshman et al., 2002; Sancho et al., 2004; Stappenbeck et al., 1998). Paneth cells express mainly EphB3, which is required for their positioning, and the progenitor cells above them in the crypt express mainly EphB2, although there is an overlap in the expression domains of EphB2 and EphB3 (Figures 2A and 2B) (Batlle et al., 2002). Ephrin-B1 is expressed in a countergradient in wild-type mice (Figures 2A and 2B), whereas ephrin-B1-immunoreactive cells are scattered throughout the crypt in EphB2; EphB3 mutant mice (Figure 2C) (Batlle et al., 2002).

Analysis of the small intestine of adult EphB2; EphB3 mutant mice did not reveal any significant difference in the total number of dividing cells, but a redistribution of

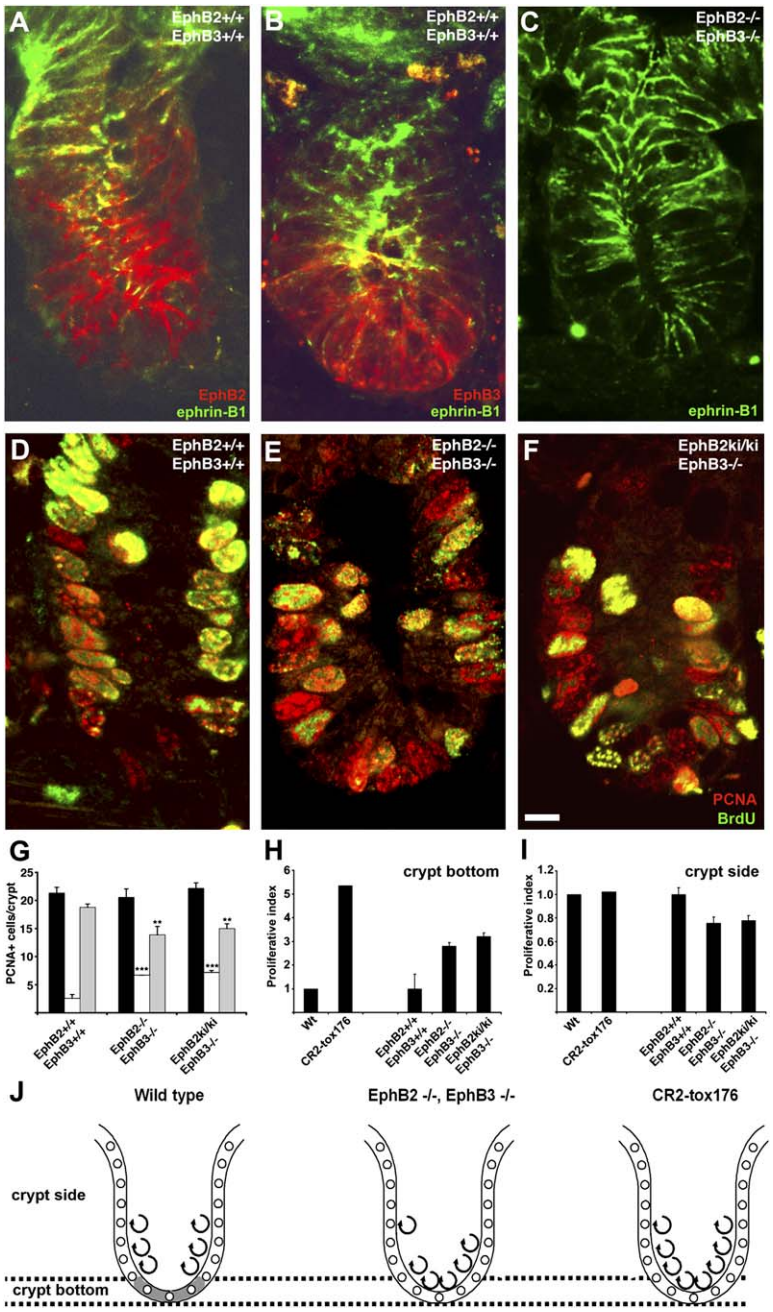

Figure 2. Redistribution of Proliferating Cells in the Small Intestine of Mice Lacking EphB2 and EphB3

(A and B) EphB3 is predominantly expressed by Paneth cells and EphB2 by progenitor cells in the small intestine, but their expression overlaps. Ephrin-B1 immunoreactivity is localized to differentiating cells higher in the crypt (A and B), but these cells are distributed throughout the crypt in the absence of EphB signaling (C). EphB receptors and ephrin-B1 form countergradients and overlap in the middle of the crypt (yellow in A and B). (A) and (B) show the same crypt in adjacent sections.

(D–G) The total number of proliferative cells in crypts of the adult small intestine of EphB2; EphB3 mutant mice is not different from wild-types (black bars in G), but their distribution within the crypts is altered. Paneth cells are mislocalized in the mutant mice, resulting in a larger number of dividing cells at the very bottom of the crypt (white bars in G). Proliferation is reduced in the progenitor niche in the side of the crypts (gray bars in G).

(H and I) The proportion of cells that are dividing (proliferation index) in the different compartments of the crypt in EphB2; EphB3 mutant mice is compared to that in CR2-tox176 mice (which lack Paneth cells; Stappenbeck et al., 2003) to address the relative effect of EphB signaling and cell redistribution on cell proliferation. The result from mice of different genotypes is compared to their respective wild-type controls, which were set to 1. Data on proliferation index in non-Paneth cells together with data on the proportion of Paneth cells at the crypt bottom from Stappenbeck et al. (2003) were used to calculate the total proliferation index at the crypt bottom in CR-2tox176 mice.

(J) shows an illustration of how the loss of Paneth cells (gray) at the bottom of crypts allows proliferative cells (indicated by circular arrows) to occupy this position and mask the reduced proliferation in the normal stem/progenitor cell compartment in the side of the crypt in the absence of EphB signaling. Data are represented as mean ± SEM. ** = $p < 0.01$, *** = $p < 0.001$. Scale bar = 10 μm.

proliferating cells within the crypt was observed (Figures 2D–2G). The postmitotic Paneth cells are displaced from the bottom of the crypts in the absence of EphB signaling (Batlle et al., 2002), and this position becomes occupied by progenitor cells, resulting in an increase in the number of proliferating cells at the bottom of the crypt (white bars in Figure 2G). This is reminiscent of the situation in mice lacking Paneth cells, in which proliferation in the crypt is increased (Garabedian et al., 1997; Stappenbeck et al., 2003). However, analysis of cell proliferation in the side of the crypt, where the majority of proliferating progenitor cells normally are located, revealed a significant reduction in cell proliferation in this compartment in EphB2; EphB3 mutant mice relative to wild-type mice (gray bars in Figure 2G). Thus, the mislocation of postmitotic Paneth cells allows proliferation at the bottom of the crypt, whereas proliferation in the normal progenitor niche is reduced, thus resulting in similar total numbers of proliferating cells in the crypts of EphB2; EphB3 mutant and wild-type mice.

This finding suggested that the loss of postmitotic Paneth cells from the bottom of the crypt, allowing progenitor cells to occupy this niche, might mask an effect of EphB receptors on proliferation in the progenitor compartment

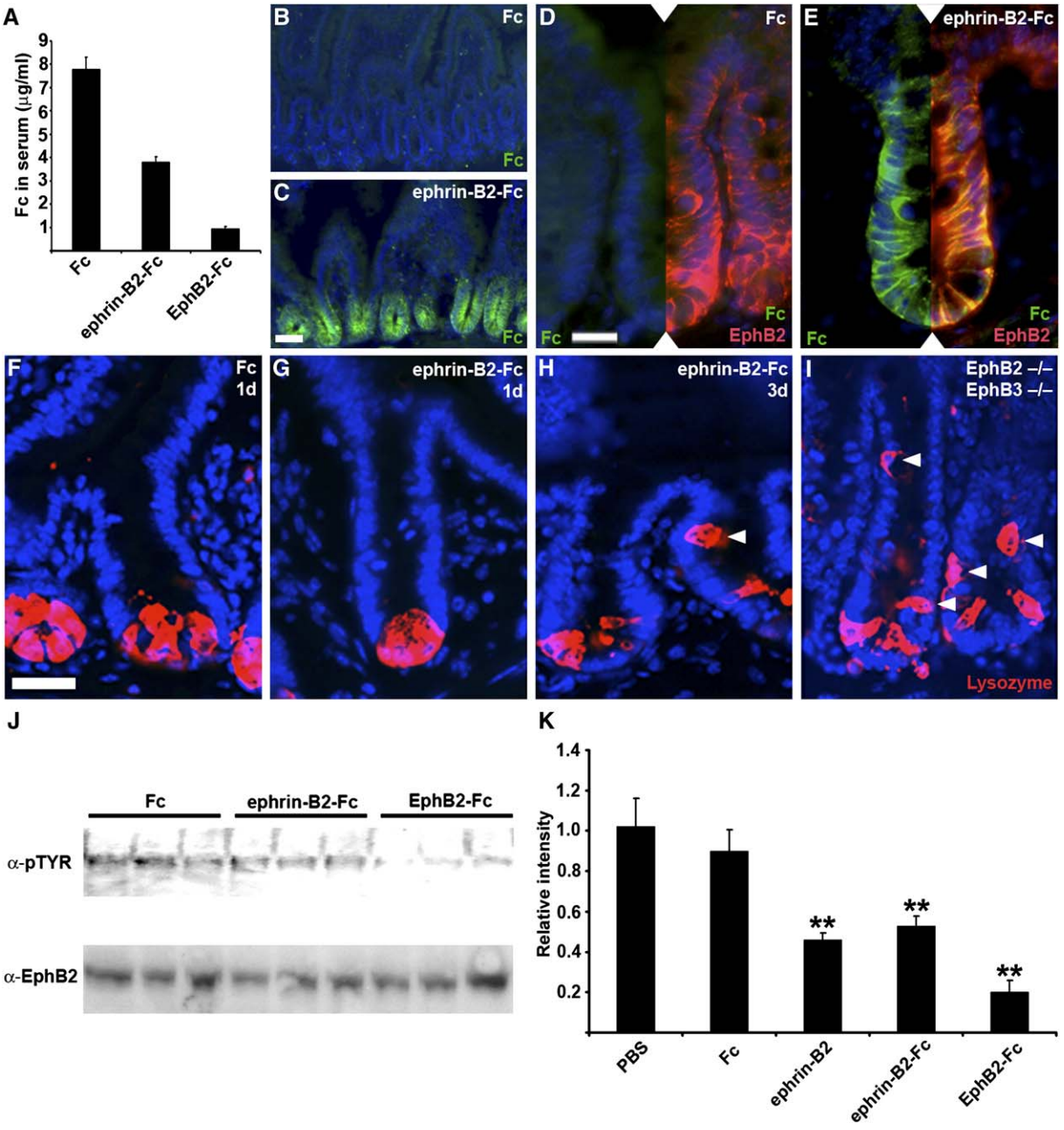

Figure 3. Acute Inhibition of EphB Signaling In Vivo

Monomeric ephrin-B2 ectodomains (ephrin-B2), ephrin-B2-Fc, EphB2-Fc, Fc protein, or vehicle (PBS) was injected intravenously in adult mice.

(A) ELISA analysis of serum from injected animals demonstrates substantial levels of infused Fc proteins (detected with an antibody against human Fc) 3 days after the injection.

(B–E) Visualization of injected proteins with anti-human Fc antibodies in tissue sections of the small intestine fail to detect binding in Fc-injected animals (B and D) but shows strong labeling restricted to crypts in animals receiving ephrin-B2-Fc (green in C and E). The binding of ephrin-B2-Fc closely matches the pattern of EphB2 immunoreactivity (red) in the crypt (anti-Fc and anti-EphB2 are shown in the right half of the photo, and only anti-Fc in the left half in D and E).

(F and G) Paneth cells (lyzozyme-immunoreactive, red) normally reside at the very bottom of the crypts and their position is unaltered in mice receiving Fc as well as 1 day after an ephrin-B2-Fc injection.

(H and I) A few Paneth cells (indicated by arrowheads) are seen outside their normal compartment 3 days after injecting ephrin-B2-Fc (H), although not as many as in EphB2; EphB3 mutant mice (I).

(J and K) The tyrosine phosphorylation of EphB2 in colon is reduced after ephrin-B2, ephrin-B2-Fc, or EphB2-Fc injections. Tyrosine phosphorylation of the immunoprecipitated EphB2 receptor was visualized with phosphotyrosine-specific antibodies (upper panel in J) and normalized to EphB2

in the side of the crypt in small intestine. To address this possibility, we compared proliferation in the different compartments of the crypt in EphB2; EphB3 mutant mice to that in transgenic mice expressing the diphtheria toxin A subunit under the Paneth cell-specific cryptidin-2 promoter (CR2-tox176 mice) (Garabedian et al., 1997; Stappenbeck et al., 2003). CR2-tox176 mice lack Paneth cells but are otherwise normal and thus allow the examination of the effect of cell mislocation within the crypt on proliferation, without perturbing EphB signaling. In both EphB2; EphB3 mutant mice and CR2-tox176 mice, there was an increase in the number of proliferating cells in the crypt bottom compared to wild-type mice due to the loss of postmitotic Paneth cells from this compartment (Figure 2H). The relative increase in the number of proliferating cells was larger in this compartment in CR2-tox176 mice (approximately 5-fold) than in EphB2; EphB3 mutant mice (approximately 3-fold), compared to their respective wild-type controls. Most importantly, whereas there was a significant reduction in proliferation in the normal progenitor domain in the side of the crypt in the EphB2; EphB3 mutant mice, there was no decrease in proliferation in this compartment in CR2-tox176 mice (Figure 2I). The absence of Paneth cells from the crypts in CR2-tox176 mice, but not in EphB2; EphB3 mutant mice, is accompanied by increased total proliferation (Garabedian et al., 1997). Thus, the redistribution of proliferative cells in the crypts of EphB2; EphB3 mutant mice, due to the loss of postmitotic Paneth cells from the bottom of the crypts, masks an effect on proliferation of EphB receptors in the small intestine (illustrated in Figure 2J). This is in contrast to the colon, which lacks Paneth cells, and where there is an approximately 50% reduction in the number of proliferative cells in the crypts in EphB2; EphB3 mutant mice (Figure 1).

Analysis of either EphB2 or EphB3 single mutant mice did not reveal significant alterations in cell proliferation in any crypt compartment, indicating that these receptors have, at least partly, redundant function in the regulation of proliferation. Furthermore, cell death in small intestine crypts was not significantly affected by abolished EphB signaling (1.1 ± 0.2 in wild-type, 0.6 ± 0.1 in EphB2$^{-/-}$; EphB3$^{-/-}$, and 0.7 ± 0.2 TUNEL+ cells/crypt and section in EphB2$^{ki/ki}$; EphB3$^{-/-}$ mice, mean ± SEM, $p > 0.05$). Proliferation was reduced by a similar magnitude in EphB2$^{-/-}$; EphB3$^{-/-}$ and EphB2$^{ki/ki}$; EphB3$^{-/-}$ mice in both the colon and in the side compartment in small intestine crypts (Figures 1B and 2G), establishing that the effect on proliferation is mediated by kinase-dependent signaling.

Acute Inhibition of EphB Signaling in the Intestine

The analyses of mutant mice demonstrate a role for EphB2 and EphB3 in regulating proliferation in the intestine, but it remains difficult to dissociate the effect on migration from an effect on proliferation, especially in the small intestine. Moreover, it is not possible from the analyses of mutant mice to exclude the possibility that EphB receptors may regulate an earlier developmental step and that the intestinal progenitor cells at later time points are intrinsically different and less proliferative. We therefore sought to acutely modulate EphB signaling in wild-type mice to analyze the role of EphB signaling on progenitor proliferation independent of cell positioning.

Ephrins have to be clustered in a cell membrane or artificially with, for example, antibodies to efficiently activate Eph receptors (Davis et al., 1994; Stein et al., 1998). Unclustered soluble monomeric or dimeric ephrins bind Eph receptors but are often weak agonists or antagonists (Vearing and Lackmann, 2005). We administered unclustered monomeric ephrin-B2 ectodomains (ephrin-B2), ephrin-B2-Fc, antibody-clustered ephrin-B2-Fc, EphB2-Fc, control Fc protein, or vehicle (PBS) systemically by intravenous injection to modulate EphB signaling acutely in vivo in wild-type mice. The serum concentrations of injected proteins were sufficiently high to maintain significant systemic levels for several days (Figure 3A). Visualization of injected proteins in sections of the intestine revealed prominent ephrin-B2-Fc binding specifically in the crypts of the intestine but did not reveal any bound protein in animals receiving the control Fc protein (Figures 3B–3E). Visualization of injected ephrin-B2-Fc and endogenous EphB2 showed a close overlap indicating that ephrin-B2-Fc specifically binds to EphB receptors in the intestine (Figure 3E). We were unable to detect antibody-clustered ephrin-B2-Fc complexes in the intestine (data not shown), suggesting that they may be too large to exit the vasculature.

One day after the injection of recombinant proteins, the distribution of EphB2-expressing progenitor cells was unaffected and no Paneth cells were seen outside their normal domain at the bottom of the crypt (Figures 3F and 3G). However, in animals analyzed 3 days after the injection, a few displaced Paneth cells started to appear, phenocopying the EphB2; EphB3 mutant mice, indicating that EphB signaling was inhibited (Figures 3H and 3I).

We next asked whether EphB receptor phosphorylation was affected by the injection of ephrin-B2, ephrin-B2-Fc, or EphB2-Fc. We found significantly reduced tyrosine phosphorylation of EphB2 in the colon of ephrin-B2, ephrin-B2-Fc, and EphB-Fc injected mice compared to control Fc or vehicle-injected mice (Figures 3J and 3K), establishing these reagents as antagonists of EphB signaling in this context.

Acute Inhibition of EphB Signaling Reduces Proliferation in the Small Intestine and Colon

Analysis of cell proliferation revealed that reduction in EphB2 phosphorylation (Figure 3) was accompanied by

protein levels (lower panel in J). The tyrosine phosphorylation of EphB2 relative to PBS-injected animals is shown in (K). Cell nuclei are visualized with DAPI, which appears blue. Data are represented as mean ± SEM. ** = $p < 0.01$ relative to PBS, Student's t test.
Scale bar in (C) is 50 μm, 25 μm in (D), and 40 μm in (F).

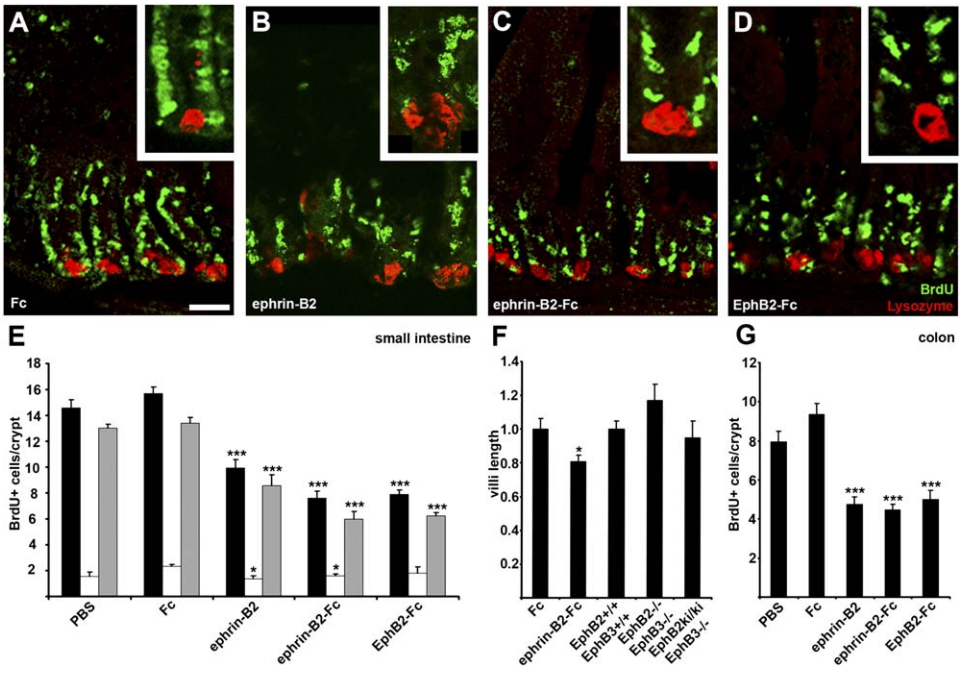

Figure 4. Inhibition of EphB Signaling Reduces Progenitor Proliferation

(A–E) Reduced proliferation in the small intestine 24 hr after injection of monomeric ephrin-B2 ectodomains (ephrin-B2), ephrin-B2-Fc, or EphB2-Fc.

(E) Quantification of BrdU-positive cells in the small intestine 24 hr after injection. Black bars represent the entire crypt, white bars represent the Paneth cell compartment at the crypt bottom, and gray bars represent the side compartment of the crypts.

(F) The length of villi in the small intestine, relative to the respective control (which was set to 1), is reduced 3 days after an injection of ephrin-B2-Fc, but not in EphB2; EphB3 mutant mice.

(G) The injection of ephrin-B2, ephrin-B2-Fc, or EphB2-Fc significantly reduces the number of proliferating cells in colon crypts 1 day later. Data are represented as mean ± SEM. * = $p < 0.05$, ** = $p < 0.005$, *** = $p < 0.001$ relative to Fc, Student's t test. The scale bar is 50 μm.

a significant decrease in the total number of proliferating cells in crypts of the small intestine as soon as 1 day after the injection of ephrin-B2, ephrin-B2-Fc, or EphB2-Fc compared to the control Fc protein (Figures 4A–4E). Quantification of cell proliferation in the different compartments of the crypt revealed that proliferation was unaffected or mildly reduced in the Paneth cell compartment at the bottom of the crypt but reduced to 45%–64% of that seen in control Fc-injected animals in the progenitor domain in the side of the crypt 1 day after the injection of ephrin-B2, ephrin-B2-Fc, or EphB2-Fc (all $p < 0.0005$, Figure 4E). The reduced proliferation upon acute inhibition of EphB signaling was accompanied by a reduction in the length of the villi in the small intestine after 3 days (Figure 4F). This was in contrast to what was observed in EphB2; EphB3 mutant mice, where total proliferation (Figure 2) and villi length were similar to that seen in wild-type mice (Figure 4F). A reduction in the number of BrdU-incorporating cells to 48%–54% of that in Fc-injected animals was also seen in the colon after injection of ephrin-B2, ephrin-B2-Fc, or EphB2-Fc (all $p < 0.001$, Figure 4G), mimicking the effect of EphB2 and EphB3 deletion (Figure 1). Apoptotic cell death in crypts was not significantly affected by the inhibition of EphB signaling (0.53 ± 0.03 and 0.42 ± 0.08 TUNEL+ cells/crypt and section in ephrin-B2-Fc and Fc-injected animals, respectively, mean ± SEM, $p > 0.05$). Since cell positioning was unaffected 1 day after the ephrin-B2-Fc or EphB2-Fc injections, and only mildly affected after 3 days, this experiment dissociates the role of EphB signaling on cell migration and proliferation. The strong reduction in proliferation establishes a major role for EphB receptors in regulating intestinal progenitor proliferation, independent of their function in cell positioning.

Enhanced EphB Signaling Increases Progenitor Proliferation

To further examine the potential role of EphB signaling on intestinal progenitor cell proliferation, we devised two gain-of-function strategies to examine the effect of increased EphB signaling. In the first approach, we overexpressed the ligand ephrin-B2 in the intestinal epithelium by electroporation in explant culture. The ephrin-B2 expression construct contained an IRES followed by GFP, allowing us to visualize ephrin-B2-overexpressing cells and to quantify the proliferation of adjacent cells (Figures 5A–5D). Overexpression of ephrin-B2 resulted in a statistically significant 44% increase in proliferation compared to the GFP control construct (Figure 5E). Since these experiments are performed in explant culture where the blood

circulation is disrupted, they argue against the effects of EphB signaling on proliferation in the intestinal epithelium as secondary to altered EphB function in the vasculature.

In a second gain-of-function approach, we generated a point mutation in the mouse *EphB2* gene to create a constitutive, overactive kinase that does not require interactions with ephrin ligands to transduce forward signals. Based on the crystal structure of the autoinhibitory juxtamembrane region of EphB2 and in vitro biochemical data (Wybenga-Groot et al., 2001), we replaced the highly conserved phenylalanine codon for residue 620 in EphB2 with an aspartic acid codon (F620D) in the mouse germline (Figure 5F). F620 is notable because it contributes to the helical hydrophobic pocket of the unphosphorylated autoinhibitory segment that binds to and distorts the small N-terminal lobe of the kinase domain, preventing it from attaining an active conformation. The F620D substitution is predicted to electrostatically disrupt the hydrophobic contacts made by F620 with the surrounding negatively charged groups in a manner mimicking phosphorylation of Y610 (Wybenga-Groot et al., 2001). Thus, the catalytic domain of the F620D mutant protein does become constitutively active, as shown in vitro (Wybenga-Groot et al., 2001). Since the F620D substitution does not interfere with the two conserved juxtamembrane tyrosine residues known to become phosphorylated and bind multiple SH2 domain-containing proteins, it is anticipated that the full complement of forward signaling molecules should be activated by this substitution.

Analysis of EphB2 tyrosine phosphorylation in the intestine of mice heterozygous for the F620D mutation revealed a statistically significant 67% increase compared to wild-type littermates (Figures 5G and 5H). Analysis of proliferation in the small intestine revealed significantly more BrdU-incorporating proliferative cells in mice heterozygous (12%) or homozygous (19%) for the F620D mutation compared to wild-type littermates (Figure 5I). The number of BrdU-incorporating cells in the colon was 17% larger in $EphB2^{+/F620D}$ ($p > 0.05$) and 30% larger in $EphB2^{F620D/F620D}$ ($p < 0.05$) mice compared to wild-type littermates (Figure 5J). Importantly, we could not detect any signs of migration defects within the intestinal epithelium of adult mice hetero- or homozygous for the F620D mutation (Figure S2), dissociating the effect of EphB2 signaling on proliferation and migration. Thus, EphB2 kinase activity promotes proliferation in intestinal crypts in a dose-dependent manner.

EphB Kinase-Dependent Signaling Conveys the Mitogenic Signal

A similar reduction in intestinal progenitor cell proliferation in mice lacking EphB2 and EphB3 signaling irrespective of whether reverse signaling is abolished ($EphB2^{-/-}$; $EphB3^{-/-}$) or maintained ($EphB2^{ki/ki}$; $EphB3^{-/-}$) establishes that kinase-dependent EphB signaling mediates the effect on proliferation. We next asked whether the reduction in proliferation is in the EphB receptor-expressing cells or whether it takes place in another cell type in the epithelium secondary to signaling in the receptor-expressing cells. We quantified the number of proliferating cells with respect to their expression of ephrin-B1 or EphB2 after acute inhibition of the ephrinB-EphB interaction by an ephrin-B2-Fc injection. Analysis of BrdU incorporation in ephrin-B2-Fc and Fc control protein-injected animals did not reveal a significant difference in the proliferation of ligand-expressing cells (Figure 6A), whereas there was a large decrease in the proliferation of receptor-expressing cells accounting for all the reduction in proliferation in ephrin-B2-Fc-injected animals (Figure 6B). Thus, the proliferative effect of the ephrin-B/EphB pathway in the intestine is mediated by kinase-dependent signaling in EphB-expressing cells. This is in contrast to the neural lineage, where EphA receptor signaling in stem cells is inhibited by the expression of an endogenous dominant-negative splice form of the receptor (Holmberg et al., 2000), and ephrin-A reverse signaling negatively regulates progenitor proliferation (Holmberg et al., 2005).

EphB Signaling Regulates Cell-Cycle Reentry

To gain insights into how EphB signaling regulates proliferation in the intestine, we determined the cell-cycle length and the cell-cycle reentry frequency in intestinal progenitors. The length of the cell cycle is largely determined by the duration of the G1 phase, whereas the S phase is subject to little variation. Analysis of the proportion of cells in cycle (PCNA positive) that are in S phase (BrdU incorporating within 1 hr) therefore gives a relative indication of the cell-cycle length (Schmal, 1983). There was no difference in the BrdU/PCNA labeling index between Fc- and ephrin-B2-Fc-injected animals (Figure 6C), arguing against EphB signaling affecting cell-cycle length.

The frequency of cell-cycle reentry can be quantified by first labeling cells in S phase by a BrdU pulse and then analyzing what proportion of these cells are in cycle 24 hr later (Chenn and Walsh, 2002). The lag time between BrdU administration and analysis allows cells either to exit (PCNA negative) or reenter the cell cycle (PCNA positive). There were 32% fewer cells reentering the cell cycle in the crypts of ephrin-B2-Fc-injected mice compared to Fc-injected mice in the small intestine (Figure 6D) and 35% fewer in the colon. Thus, EphB kinase signaling controls proliferation of intestinal progenitor cells by promoting cell-cycle reentry.

EphB Signaling Promotes Proliferation in Adenomas

The expression of EphB receptors is downregulated during the progression of colorectal cancer and the loss of EphB receptor expression correlates with a poor prognosis (Batlle et al., 2005; Guo et al., 2005; Jubb et al., 2005; Lugli et al., 2005). A direct role for EphB receptors acting as tumor suppressors was demonstrated by the development of invasive adenocarcinoma in a mouse model of adenomatous polyposis ($APC^{Min/+}$) when EphB signaling was inhibited (Batlle et al., 2005). We tested whether EphB signaling affects proliferation in adenomas by injecting ephrin-B2-Fc in $APC^{Min/+}$ mice. Blocking

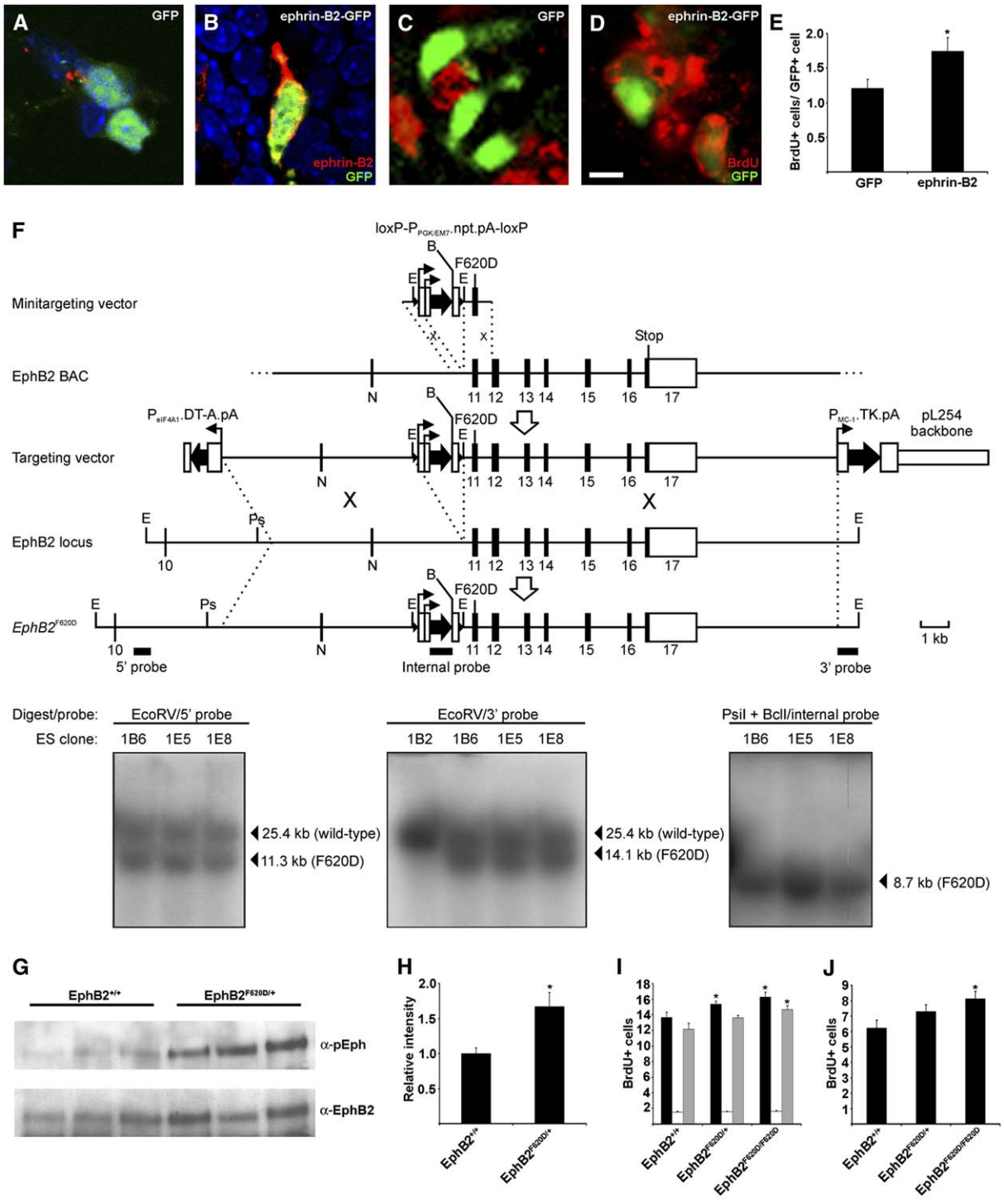

Figure 5. Enhanced EphB Signaling Increases Proliferation

Misexpression of GFP (A) or ephrin-B2:IRES:GFP (B) by plasmid electroporation of intestinal explants. (B) ephrin-B2 immunoreactivity in an electroporated cell. A higher proportion of cells in contact with ephrin-B2-overexpressing cells incorporate BrdU compared to those adjacent to cells expressing GFP only (C–E). (F) Targeting of the F620D (codon 620: TTT→GAT) mutation to the mouse EphB2 gene. Coding sequences are represented by filled boxes (exons) or filled arrows (positive or negative selectable markers, or DT-A). Filled arrowheads represent loxP sites. The origin of probes used in Southern blotting analysis and relevant EcoRV (E), PsiI (Ps), and BclI (B) sites are indicated. Southern blotting analysis of three targeted ES cell clones (1B6, 1E5, 1E8) subsequently used to create chimeric mice and establish germline transmission is shown below the targeting strategy. A non-targeted clone (1B2) analyzed with the 3′ probe is included to illustrate the increased mobility of the targeted allele. (G and H) Phosphorylation of

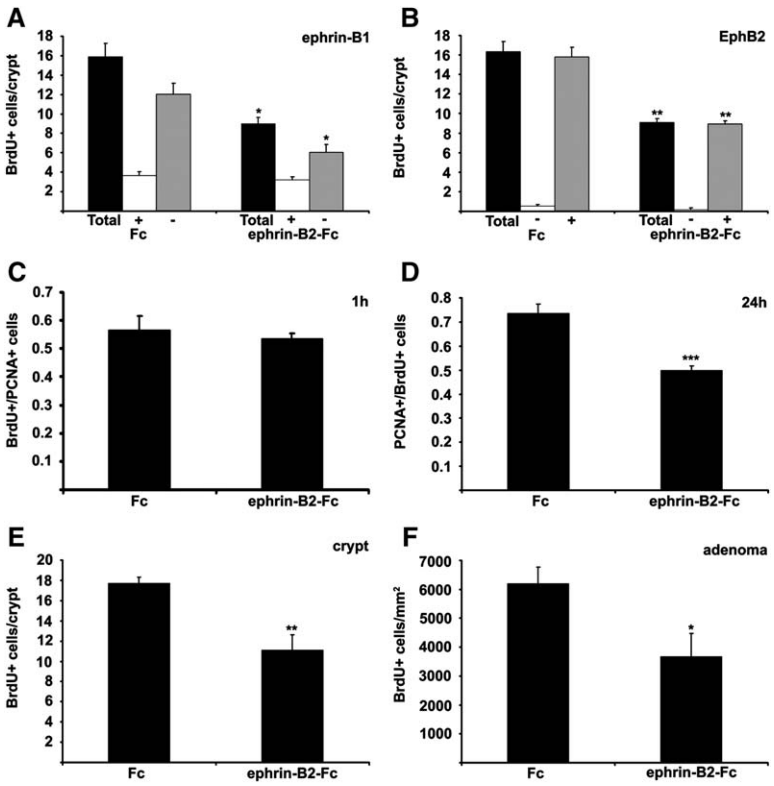

Figure 6. EphB Signaling Promotes Progenitor Cell-Cycle Reentry

(A and B) Analysis of BrdU incorporation in cells immunoreactive (+) or not (–) for ephrin-B1 (A) or EphB2 (B) 1 day after an injection of ephrin-B2-Fc.

(C) The cell-cycle length is unaffected in ephrin-B2-Fc-injected animals, but the frequency of cells reentering the cell cycle is significantly shortened (D). Injection of ephrin-B2-Fc in $APC^{min/+}$ mice results in decreased proliferation 1 day later in small intestine crypts (E) and in adenomas (F). Data are represented as mean ± SEM. * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, Student's t test.

EphB signaling reduced proliferation in small intestine crypts in $APC^{Min/+}$ mice to a similar degree as in wild-type mice (Figure 6E) and reduced the number of BrdU-incorporating cells in adenomas by 41% 3 days after the injection of ephrin-B2-Fc (Figure 6F). This suggests that the tumor cells in the absence of EphB signaling may gain invasiveness (Clevers and Batlle, 2006) at the expense of proliferation.

EphB Signaling Controls the Size of the Proliferative Domain but Not the Number of Stem or Progenitor Cells

We next asked whether the altered proliferation in crypts of EphB2; EphB3 mutant mice was due to a reduction in the number of stem cells. Intestinal crypts each contain an average of 1–2 stem cells, and the rest of the proliferative cells are progenitor cells (Marshman et al., 2002; Sancho et al., 2004; Stappenbeck et al., 1998). Stem cells in the intestine can be identified retrospectively by their label-retaining capacity (Potten et al., 2002). If EphBs regulate stem cell number, one would expect fewer label-retaining cells in the absence of EphB signaling, but we did not find any difference between wild-type animals and EphB2; EphB3 mutants (Figure 7A). This also demonstrates that EphB2 and EphB3 are not required to keep stem cells from migrating out of the crypt, and that other molecules mediate their anchoring in the niche, at least over the time course and conditions of these experiments.

Intestinal progenitor cells express musashi-1 (Kayahara et al., 2003; Potten et al., 2003). We measured the length of the domain of musashi-1-immunoreactive cells in the crypt in ephrin-B2-Fc-, EphB2-Fc-, and Fc-injected animals to assess whether EphB signaling regulates the progenitor cell pool size. The size of the progenitor cell domains was independent of the EphB signaling status (Figure 7B).

We next analyzed the length of the domain of proliferative cells in the crypt. Interestingly, the BrdU-incorporating cells did not extend as far from the bottom of the crypt in ephrin-B2-Fc or EphB2-Fc as in Fc-injected animals (Figures 4A–4C and 7C). Thus, EphB signaling regulates the size of the proliferative compartment without an apparent effect on the number of stem or progenitor cells.

EphB2 is increased in anti-EphB2 immunoprecipitates from colon lysates of adult mice expressing the F620D substitution. This is accompanied by increased BrdU incorporation in the small intestine (I) and colon (J). In (I), black bars represent the total crypt, white bars the crypt bottom, and gray bars the crypt side. Abbreviations used in (F): P, promoter; PGK, phosphoglycerate kinase; Npt, neomycin phosphotransferase; pA, polyadenylation signal; eIF4A1, eukaryotic initiation factor 4A1; DT-A, diphtheria toxin-A chain; TK, HSV thymidine kinase; N, novel exon. Data are represented as mean ± SEM. * = $p < 0.05$, Student's t test. The scale bar is 10 μm.

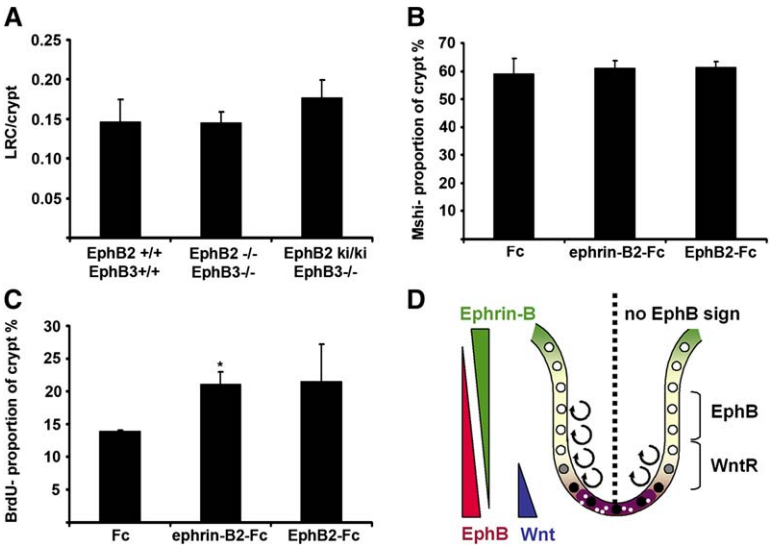

Figure 7. Disruption of EphB Signaling Does Not Alter the Progenitor or Stem Cell Number but Reduces the Size of the Proliferative Compartment

(A) Quantification of label-retaining cells did not reveal a difference in stem cell numbers between EphB2; EphB3 mutant and wild-type mice.

(B) The size of progenitor cell pool, as indicated by musashi-1 (mshi) immunoreactive cells, is unaltered in injected animals.

(C) shows the proportion of crypt in small intestine that lacks BrdU-positive cells after injection of ephrin-B2-Fc or EphB2-Fc.

(D) Wnt proteins are present at the bottom of crypts and interact with receptors (WntR) in epithelial cells, resulting in nuclear β-catenin (indicated by filled nuclei) driving proliferation (indicated by circular arrows). β-catenin drives the expression of EphB receptors, which interact with ephrin ligands higher in the crypt, extending the domain of proliferation. Postmitotic Paneth cells (purple with granulae) occupy the bottom of crypts in the small intestine. Data are represented as mean ± SEM. * = $p < 0.05$, Student's t test.

DISCUSSION

We report the identification of EphB receptors as major regulators of proliferation in the intestinal stem cell niche. Analysis of mutant mice and acute inhibition of EphB signaling indicate that this pathway accounts for approximately 50% of the mitogenic activity in this lineage. EphB kinase-dependent signaling in intestinal progenitor cells promotes cell-cycle reentry and extends the proliferative domain in the crypts. The expression of ephrins and Ephs in multiple adult stem cell systems (Figure S3) suggests that they may control similar features in several organs and may be attractive drug targets in regenerative medicine.

Coupling of Migration and Proliferation in the Intestinal Stem Cell Niche

The distorted migration in the small intestine caused by the absence of EphB2 and EphB3 results in redistribution of proliferative cells toward the source of Wnt proteins at the bottom of the crypt, masking EphB receptors' mitogenic effect. The terminally differentiated Paneth cells are lost from the crypt bottom and proliferative cells now occupy this domain, whereas proliferation is reduced in the side of the crypt, where dividing progenitor cells are positioned.

The acute inhibition of EphB2 signaling by systemic delivery of ephrin-B2, ephrin-B2-Fc, or EphB2-Fc as well as the generation of mice with constitutively active EphB2 receptors allowed us to discriminate between effects on cell migration and proliferation. One day after the injection of the inhibitory proteins, the distribution of EphB2-immunoreactive progenitor cells and Paneth cells is unaltered. In this situation, there is, as in control mice, little proliferation in the Paneth cell compartment at the bottom of the crypts. However, proliferation in the progenitor domain at the side of the crypt is then reduced to levels similar to EphB2; EphB3 mutants. By avoiding the displacement of Paneth cells and the mispositioning of cells along the crypt-villus axis seen in EphB2; EphB3 mutant mice, we were able to establish the role of EphB2 and EphB3 in proliferation independently of their roles in controlling cell migration. Moreover, in mice expressing the EphB2 F620D substitution, rendering the receptor constitutively active, there is a dose-dependent increase in proliferation in the progenitor domain in the absence of any migration defect. The same challenge in dissociating migration and proliferation is not as apparent in the colon, where there are no Paneth cells, and the reduction in proliferation was similar in EphB2; EphB3 mutant mice and after acute inhibition of EphB signaling.

Eph receptor signaling has not previously been reported to promote cell proliferation in vivo. It is possible, however, that Ephs may couple migration and proliferation in several developmental contexts and that their mitogenic effects are masked by perturbed cell positioning in loss-of-function models, as in the small intestine of the mutant mice described in this study.

Ephrins and Eph Receptors in Intestinal Homeostasis and Cancer

The expression of EphB receptors is frequently lost during the progression of colorectal cancer, and this correlates with a poor prognosis (Batlle et al., 2005; Guo et al., 2005; Jubb et al., 2005; Lugli et al., 2005). The development of invasive cancer in a mouse model of adenomatous

polyposis when EphB signaling was inhibited established EphB signaling as inhibitory for cancer progression (Batlle et al., 2005).

How can EphB receptors promote progenitor proliferation in the normal intestine and still act as tumor suppressors in intestinal neoplasia? The mechanism by which EphB signaling inhibits the development of invasive cancer is unclear, but Clevers and Batlle (2006) have postulated that EphB signaling inhibits tumor cells from invading surrounding repulsive ephrin-B-expressing areas. This is supported by the fact that EphB2 expression is more commonly lost in metastases than in primary cancers (Guo et al., 2005; Jubb et al., 2005). We found that inhibition of EphB signaling reduced cell proliferation in adenomas in $APC^{min/+}$ mice. This supports the hypothesis that the tumor suppressor role of EphB receptors may be related to restricting invasive growth, rather than inhibiting proliferation. The loss of EphB expression allows invasive growth but may come at the expense of a reduction in proliferation. It is possible, however, that later stage cancer cells have acquired mutations maximizing proliferation independently of EphB signaling. The larger size of tumors in the absence of EphB signaling (Batlle et al., 2005) is likely secondary to the invasive growth, with loss of spatial restriction and possibly increased access to mitogens in the tissue.

EphB Receptors Extend the Range of Wnt-Mediated Proliferation

Wnt proteins are master regulators of cell proliferation in the intestine. Their proliferative effects are, in part, mediated by positive regulation of c-Myc expression and suppression of p21 expression (van de Wetering et al., 2002). EphB receptor expression in the intestine is under the control of Wnt proteins, and inhibition of this pathway results in abolished EphB expression (Batlle et al., 2002; van de Wetering et al., 2002). The present data demonstrate that EphB receptor kinase signaling promotes intestinal progenitor cell-cycle reentry and accounts for approximately 50% of the mitogenic activity in the intestine. Thus, an important mechanism by which Wnt proteins regulate proliferation in the intestine is by promoting EphB expression.

Wnt proteins are poorly soluble palmitoylated proteins (Willert et al., 2003), and their physical range of effect may be rather limited. Only cells close to the bottom of the crypts display nuclear β-catenin (Batlle et al., 2002). EphB expression extends substantially higher up in the crypt than the domain of cells with nuclear β-catenin (Batlle et al., 2002). As cells move out of reach of the Wnt proteins, they thus still maintain EphB receptor expression for some time, despite the fact that EphB expression is under the control of the Wnt pathway. This may be related to translation of EphB mRNAs continuing for some time after the transcription of *EphB* genes or that the EphB proteins are stable in the cell membrane until the cells encounter ligand higher in the crypt. Importantly, the expression of ephrin-Bs is negatively regulated by β-catenin (Batlle et al., 2002), which limits cells expressing EphB to encounter ligand until they move out of the domain of high Wnt protein concentration.

By controlling the expression of a class of stable membrane bound signaling proteins, and negatively regulating the expression of its cognate ligand, it appears that the effect of a localized Wnt protein morphogen gradient can be extended to a larger domain beyond the region of high Wnt protein concentration (Figure 7D). This is supported by the shortening of the domain of proliferative cells toward the bottom of the crypts after inhibition of EphB signaling. The coupling of cell positioning and proliferation by the ephrin-Eph pathway may thus serve to amplify and extend the mitogenic effect of a localized source of Wnt proteins in intestinal crypts.

EXPERIMENTAL PROCEDURES

Animals

Tissues from EphB2 and EphB3 mutant mice on CD1 genetic background (Henkemeyer et al., 1996; Orioli et al., 1996) were dissected and coded by genotype in one laboratory (M.H.) and all analyses were done blind to genotype in the other laboratory (J.F.). Adult male C57/Bl6 mice were used for injection experiments.

Generation of Mice with Constitutively Active EphB2

A contig was assembled in pBeloBAC11 from previously described genomic mouse EphB2 λ phage DNA clones 5.2 and 7.2 (Henkemeyer et al., 1996) using homologous recombination in *E. coli*. EphB2 exon 11 was then targeted, again using homologous recombination in *E. coli*, with a pL452-based minitargeting vector containing EphB2 homology arms (a wild-type 400 bp 5′-arm and a codon 620-mutated (TTT→GAT) 3′-arm) to yield pBeloBAC11.EphB2.F620D. An EphB2 targeting vector was produced by homologous recombination-mediated retrieval of an F620D-containing fragment from pBeloBAC11. EphB2.F620D into pL254 (a modified form of pL2451 [Liu et al., 2003] with a DT-A expression cassette and unique AscI site for linearization). All sequences and details of DNA engineering are available upon request. R1 ES cells were electroporated with AscI-linearized pL254.EphB2.F620D, then subjected to selection and screened by Southern blotting with the 5′-probe. The floxed PPGK/EM7.npt.pA cassette was deleted in the mouse germline by crossing to Krox20-Cre recombinase-expressing males. Genotyping was performed using a two-allele three-primer touchdown PCR using the oligonucleotides neoF2 (5′- TTGGCTACCCGTGATATTGCTGAA-3′), F620DgF1 (5′-GGGGGACTCTTCACCGACTAA-3′), and F620DgR1 (5′-CAAGGGGAACAGAGATCAGAAAAG-3′). Products were diagnostic of wild-type (257 bp), F620D (635 bp), and F620Δ neo (389 bp) alleles.

Analysis of Proliferation and Cell Death

BrdU (100 mg/kg in 0.9% NaCl) was injected intraperitonealy 2 or 24 hr prior to sacrifice. For visualization of label-retaining cells, BrdU was injected once every 12 hr for 3 days, and the mice sacrificed 3 weeks later (Potten et al., 2002). Data on proliferation index in non-Paneth cells together with data on the proportion of Paneth cells at the crypt bottom from Stappenbeck et al. (2003) was used to calculate the proliferation index in germ-free CR-2tox176 mice. Apoptotic cells were detected with the Apoptag Peroxidase In Situ Apoptosis Detection Kit (Chemicon International).

Immunohistochemistry

Mice were perfused transcardially with 4% formaldehyde in PBS, the isolated tissues cryoprotected in 20% sucrose in PBS overnight, and sections were cut on a cryostat. Goat anti-EphB2 and anti-EphB3

were used at 1:500 (R&D Systems), rat anti-ephrin-B1 at 1:2.5 (RPMI 1640, gift from H. Okano), rat anti-BrdU at 1:200 (BD Immunocytometry Systems), anti-PCNA at 1:400 (Oncogene), rabbit anti-lyzozyme at 1:250 (DakoCytomation), and rat anti-musashi-1 at 1:10,000 (Kaneko et al., 2000). Sections used to visualize BrdU- and/or PCNA-immunoreactive nuclei were pretreated with 2M HCl and 0.5% Triton X-100 (Sigma) for 30 min at 37°C. To detect infused Fc-chimeras, sections were incubated with either AlexaFlour 488 conjugated goat anti-human or Cy3 donkey anti-human at 1:500. For all immunohistochemistry, control studies included exclusion of the primary antibody, which resulted in the absence of immunoreactivity. In addition, the specificity of antisera against EphB2 and EphB3 was confirmed by abolished labeling in sections from the respective null mice (Figure S1).

Delineation of Crypt Compartments

Crypts in the small intestine were divided into a Paneth compartment and a side compartment for quantification. This was done based on area rather than localization of Paneth cells since they are mispositioned in the mutant mice. The area of the Paneth compartment was determined in tissue sections in the microscope in five wild-type crypts in which Paneth cells were visualized with antibodies against lyzozyme. The average area was calculated from this measurement, and the corresponding area at the bottom of all crypts in the small intestine was defined as the Paneth compartment. The part of the crypt not harboring Paneth cells was defined as the side compartment. Together the Paneth compartment and the side compartment compose the total crypt.

Protein Injections

Recombinant mouse ephrin-B2, ephrin-B2-Fc, EphB2-Fc, or human Fc (100 μg/mouse, R&D systems) in PBS were injected intravenously via the tail vein, and serum concentrations were measured by ELISA. Monomeric ephrin-B2 ectodomains were generated by cleaving ephrin-B2-Fc with Factor Xa protease at the IEGR sequence between Fc and the ephrin-B2 ectodomain. Fc fragments and uncleaved protein were removed with Protein A-sepharose, and the purity of the correct fragment was verified by SDS-PAGE.

Immunoprecipitation and Immunoblotting

Mice were sacrificed 24 hr after injection with Fc proteins, the ascending colon was dissected out, and the tissue solubilized in 2 ml of lysis buffer (50 mM Tris-HCl pH 8.0, 300 mM NaCl, 1% Triton X-100, 20 mM Hepes, 10% glycerol, 0.1% BSA, 20 mM sodium fluoride, 2 mM PMSF, 100 μM sodium orthovanadate, 100 U/ml Trasylol [aprotinin], and 0.05 mM Leupeptin) and centrifuged at 13,000 g for 10 min. For immunoprecipitation of EphB2, lysates were incubated with 2 μg/ml of goat anti-mouse EphB2 antibody (R&D Systems) and 40 μl protein A Sepharose for 5 hr. The beads were washed three times in 1% Triton X-100 in PBS and the protein samples were separated by 4%–12% NuPAGE followed by Western blot analysis with an anti-phosphotyrosine antibody (4G10, Upstate), an anti-phospho-Eph antibody (Dalva et al., 2000), or anti-EphB2 antibody (R&D Systems).

Electroporation and Explant Culture

Small intestine from E16 embryos was dissected out and expression plasmids for ephrin-B2 and GFP or GFP only (10 mg/ml) were injected into the lumen of 0.5 cm long segments. The tissue was submerged in PBS and subjected to five pulses (80 V and 50 ms) utilizing an ECM830 electroporator from BTX. After electroporation, the tissue was put in catenary culture as described (Hearn et al., 1999) and grown in DMEM:F12 with 10% FBS and 1% penicillin/streptomycin for 15 hr. BrdU (40 μg/ml) was added to the medium 1 hour before fixation in 4% formaldehyde.

Supplemental Data

Supplemental Data includes three figures and references and can be found with this article online at http://www.cell.com/cgi/content/full/125/6/1151/DC1/.

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