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No Guts, No Glory - EphB Mediated Signaling in Intestinal Stem and Progenitor Cells

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ABSTRACT

The work presented in this thesis is focused on the intestinal stem cell niche, the crypts, and the Eph tyrosine kinase receptors together with their ligands that are expressed by the crypt epithelial progenitor cells. Two fundamental cellular processes, high turnover coupled to a rapid ordered migration of the newborn cells out from the crypt, characterize the normal intestine. Transformed epithelial cells mirror these processes, gaining an advantage over the untransformed cells by the capacity of going through seemingly endless rounds of division, as well as expanding spatially into surrounding tissues by increased motility. The B class Eph receptors are involved in regulating both proliferation and migration in normal as well as transformed intestinal epithelial cells.

In **Paper I**, we show that EphB receptors, in addition to directing cell migration, regulate proliferation in the intestine. We use both loss and gain of function experiments to correlate the EphB forward signaling to the rate of progenitor cell proliferation. EphB signaling promotes cell cycle reentry of progenitor cells and accounts for up to half the mitogenic activity in the adult intestine. We suggest a model where the restricted Wnt source plays an important role in establishing the EphB-ephrin-B gradients, hence extending the proliferative domain beyond the region directly influenced by the Wnt proteins themselves. In **Paper II** we continue to investigate the dual role of EphB receptors. We show that cell migration and proliferation are controlled independently by the EphB2 receptor. EphB2 regulated cell positioning is kinase-independent and mediated via PI3-kinase, whereas EphB2 tyrosine kinase activity regulates cell proliferation through an Abl-cyclin D1 pathway. Cyclin D1 regulation becomes uncoupled from EphB signaling during the progression from adenoma to colon carcinoma, allowing continued proliferation with invasive growth. The dissociation of EphB2 signaling pathways enables the selective inhibition of the mitogenic signaling pathway without affecting the tumor suppressor function of EphB2. In **Paper III** we investigate the role of the two ligands expressed in the intestinal epithelium, ephrin-B1 and ephrin-B2 and show that, although both ligands can bind EphB2 and EphB3, they have distinct functional outcomes when activating the receptors. Ephrin-B1 selectively affects migration, without influencing proliferation, whereas ephrin-B2 affects proliferation, but not migration.

LIST OF PUBLICATIONS

This thesis is based on the following papers, which will be referred to in the text by their roman numbers:

I. Johan Holmberg*, **Maria Genander***, Michael M Halford, Cecilia Annerén, Marianne Sondell, Michael J Chumley, Robert E Silvany, Mark Henkemeyer, Jonas Frisén
EphB receptors coordinate proliferation and migration in the intestinal stem cell niche
Cell (2006), 125 (6):1151-63

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Dissociation of EphB signaling pathways mediating progenitor cell proliferation and tumor suppression
Cell (2009), 139 (11): 679-692

III. **Maria Genander**, Mark Henkemeyer, Jonas Frisén
Differential engagement of EphB receptors in intestinal progenitor cells
Manuscript (2009)

* These authors contributed equally to this work

LIST OF SELECTED ABBREVIATIONS

Abl	Abelson murine leukemia oncogene
APC	Adenomatosis polyposis coli
APCmin	Multiple intestinal neoplasia
Bmi1	Bmi1 polycomb ring finger oncogene
Bmp	Bone morphogenetic protein
c-Kit	Steel Factor Receptor
Cbc cells	Crypt base columnar cells
CML	Chronic myelogenous leukemia
Eph	erythropoietin-producing hepatocellular (Eph) receptor
FAP	Familial adenomatous polyposis
GFP	Green fluorescent protein
GIST	Gastrointestinal Stromal Tumors
Hes	Hairy and enhancer of split
Lgr5	Leucine rich repeat containing G protein coupled receptor 5
PdgfR	Platelet derived growth factor receptor
PI3-kinase	Phosphatidylinositol 3-kinase
Pten	Phosphatase and tensin homolog
Shh/Ihh	Sonic/Indian Hedgehog
Tcf	T-cell-specific transcription factor
Wnt	Wingless-related MMTV integration site

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INTRODUCTION

The work presented in this thesis is focused on the intestinal stem cell niche, which is defined by the epithelial stem and progenitor cells found in the crypts of the small and large intestine. There are several reasons for this interest in this epithelial tube that normally concerns us only in cases of digestive problems.

Evolution tends to stick to concepts that work. General biological principles or signaling pathways can often be applied to several different cellular contexts, and with minor alterations hold surprisingly true. To understand more about stem cell biology, which tends to be rather complex, it can be beneficial to choose a stem cell system to study which is simple, although still relevant. The knowledge gained from a simpler system can then be used to understand more complex structures. The intestine is composed of repeating units of crypts and villi, built from a single layer of polarized epithelial cells. Each crypt is unique, although the processes taking place in the crypt cells are mirrored by all the other crypts. There are only four different cell types in the intestinal epithelium, in comparison to the numerous types of neurons present in the adult brain. All in all, the intestine offers a somewhat understandable world.

Stem cells are normally elusive and hard to identify, being by definition rare and most times only described by their functional properties. The intestinal stem cell system is an exception to the rule; most likely the identity of crypt stem cells has been revealed. In comparison with the skin or brain stem cells, which are believed to rarely go through the cell cycle, crypt stem cells divide frequently and continuously, at a more or less constant rate producing daughter cells, a character that is sought after when trying to manipulate the stem cells in order to understand more about them.

Mutations in stem cells are believed to be the underlying cause of cancer. In the intestine, this link is clearer than in most other stem cell systems. Genetic alterations in stem cells can lead to the formation of benign tumors, which upon further genetic modifications can progress into malignant tumors and even gain the capability to invade surrounding tissues. The same proteins governing normal intestinal cell functions are also the proteins that trigger the transformation of normal cells into cancer cells when their function is altered by mutations. Again, evolution tends to stick to

successful concepts, and thus we can hope to extrapolate the knowledge gained from normal tissue to understand more about tumor biology.

THE GASTROINTESTINAL TRACT

EMBRYONIC GUT DEVELOPMENT

The vertebrate gastrointestinal tract is a vital and complex organ system derived from a simple tubular structure. The gastrointestinal system starts to form at the end of gastrulation, when invaginations in the anterior and posterior part of the embryo develop and elongate into the embryo, fusing at the midline forming an endodermally derived tube-like structure. In mice, the endoderm undergoes rapid proliferation and forms a single epithelial cell layer at around day E14.5, which starts forming invaginations. The epithelial cells are surrounded by mesoderm, which differentiates and forms smooth muscle cells and connective stromal tissue. Regional specific morphologic development along the anterior-posterior axis forms the foregut (later forming the pharynx, esophagus and stomach), the midgut (small intestine) and hindgut (colon). In the small intestine and colon, villi are formed from mesodermal outgrowth, forming long thin villi in the small intestine and wide and flat villi in the colon. In the first week after birth, the intervillus epithelium is shaped into downward growing, proliferating, pockets of epithelial cells, the crypts. Crypts then multiply by fission between the second and third postnatal week (Calvert and Pothier, 1990).

THE CRYPT-VILLUS UNIT

When morphogenesis is complete, the intestine is composed of crypts and villi. The crypts contain rapidly proliferating cells, dividing every 12-16 hours, generating over 200 cells per crypt per day (Sancho et al., 2004). The newborn cells continuously migrate out from the crypts and feed into the villi, reaching the top of the villi after a few days and eventually ending up being shed into the gut lumen. The turnover time for the entire epithelial cell population in adult mice is around 5 to 7 days, rendering the epithelium a highly dynamic system (Cheng and Bjerknes, 1982, 1983).

Not only is the morphology of the small and large (colon) intestine different, the functions are also somewhat different. The small intestine functions to digest and absorb nutrients, whereas the colon absorbs water and salt, a functional difference which is reflected in the distribution of the cell types present in the epithelium. Most of

the differentiated epithelial cells are columnar enterocytes, with apical microvilli, which together with the folded epithelium greatly increases the absorptive area of the intestine. Enterocytes are the main absorptive cell type of the small intestine, whereas the mucus producing Goblet cells, representing only about 5 percent of the cells present in intestine, secrete a protective lubricating mucous to help trap bacteria so they can be transported out from the intestine. The enteroendocrine cells, which produce hormones that regulate the motility of the gastrointestinal tract, represent an even smaller fraction of the cells in the epithelium. In contrast to the rapid turn over of these three cell populations, the Paneth cells are long lived. After being produced by the mitotic cells in the small intestinal crypts, they resist the upward flow of cells, and instead end up at the crypt bottom, where they reside for up to 20 days, producing antimicrobial agents for the defense of the intestine.

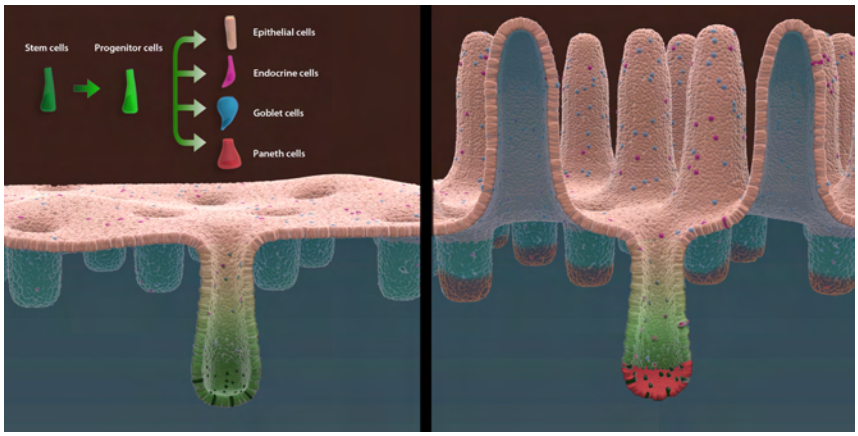

FIGURE 1 SCHEMATIC REPRESENTATION OF COLON AND SMALL INTESTINE

Colon and small intestine are both formed from a single layer of epithelial cells and display many similarities. Stem and progenitor cells localised at the bottom of the crypts continuously produce daughter cells, which migrate up the crypt axis, a process coupled to differentiation. As cells leave the crypt, they exit the cell cycle and become fully differentiated epithelial cells. The intestine produces several differentiated cell types; goblet and endocrine cells are found throughout the gastrointestinal tract, whereas Paneth cells are found exclusively at the bottom of small intestinal crypts.

PAST AND PRESENT - THE IDENTITY OF THE INTESTINAL STEM CELL

Intestinal crypts contain cells which have the capacity to divide and give rise to the four differentiated cell types found in the intestinal epithelium for endless rounds of

divisions. Non-specialized cells, such as the dividing cells in the crypts, with the potential to give rise to progeny that form more specialized or differentiated cell types, like Goblet or Paneth cells, are called stem cells. According to the stem cell definition, intestinal stem cells should not only be able to give rise to all different cell types found in the epithelial lineage, but also self renew and be able to regenerate the tissue after injury (Loeffler and Roeder, 2002; Potten and Loeffler, 1990).

Throughout the years, the small intestinal crypt has been seen as a homogenous column of dividing cell, surrounded by the post-mitotic Paneth cells at the crypt base and the enterocytes in the villi. In the 1950s and 19060s, ideas were put forward about the possible location of the intestinal stem cell, and it was suggested as well as dismissed, based on labeling experiments using 3H-thymidine and careful cell cycle analysis of the dividing crypt cells, that the potential stem cells were positioned at the base of the proliferative column, just above the Paneth cells (Cairnie et al., 1965; Quastler and Sherman, 1959). More recent strategies undertaken in order to pinpoint intestinal stem cells also used DNA labeling techniques. Potten et al injected young mice with 3H-thymidine for several consecutive days in order to target stem cells as they were formed (Potten et al., 2002). After a wash out period, they identified cells in small intestinal crypts which still retained the DNA labeling. Most of the label retaining cells was positioned just above the Paneth cells, at position 4-8 from the crypt bottom. Similar results were obtained after irradiation, where crypts were regenerated from single label retaining cells, fulfilling at least one of the stem cell criteria; the identification of a cell which has the capacity to regenerate the tissue after injury.

As the knowledge about stem cell biology in general evolved, markers began to be identified that were expressed by stem cells, sometimes unique for a specific stem cell system, sometimes more broadly expressed by stem cells in general. The identification of these markers allowed for a more detailed analysis of the stem cells, not only could e.g. cell cycle kinetics be studied, but the purification of cell populations based on a single, or a combination of markers allowed for functional studies. Could a single potential hematopoietic stem cell reconstitute the entire bone marrow in a lethally irradiated mouse? The theoretical criteria set up to define a stem cell could be experimentally explored in more detail.

The first exploratory experiments to identify intestinal stem cells were all focused on the small intestine. The possible location of colon stem cells was rarely investigated or discussed. Perhaps the presence of Paneth cells in small intestine made it tempting to conclude that the +4 position was the perfect stem cell "niche", and the lack of Paneth cells in colon, combined with a different proliferation pattern, made it difficult to draw parallels between the small and large intestine. In 2003, the first report concerning the identity of a colon stem cell was published. Nishimura et al. (Nishimura et al., 2003) reported that the RNA-binding protein Musashi-1, previously identified as a marker for neural precursor cells in the developing central nervous system, was expressed in a few scattered cells in the lower part of colon crypts, and tentatively suggested the presence of a colon stem cell. However, they did not provide any functional data.

As of today, there are only two reports combining the use of potential stem cell markers with the powerful tool of mouse genetics. Promoter-specific expression of Cre, a recombinase of bacterial origin, allows for targeting of subpopulations of cells when combined with a reporter mouse expressing GFP or beta-galactosidase after recombination. Cre recombinase activity can be controlled by fusing Cre to the ligand-binding domain of the estrogen receptor (CreEr), thus allowing recombination to be induced by the administration of an estrogen homolog. In this way, recombination, and the expression of a reporter gene can be controlled and the cells recombined can be detected at precise time points after induction, but importantly, all the progeny derived from a single recombined cell will continue to express the reporter gene and will provide important information about the potential of the primary recombined cell. This type of genetic modification allows for the study of any cell type in its original environment, without an injury paradigm, and is so far the best possible way to investigate whether a cell is a stem cell or not.

Lgr5 (leucine-rich-repeat-containing G-protein-coupled receptor 5) was selected as a potential stem cell marker based on the restricted crypt expression pattern (Barker et al., 2007) in crypt base columnar (CBC) cells in small intestine. CBC cells were described already in 1974, as Cheng et al noticed the presence of slender cycling cell between the post mitotic Paneth cells (Cheng and Leblond, 1974). The CBC cells are distinct from the label retaining cells identified by others, and have occasionally been proposed to harbor stem cell activity (Bjerknes and Cheng, 2005), although the experimental data to support this idea has mainly been lacking. In colon, Lgr5 expressing cells are scattered

in the lower part of the crypts. Recombination of Lgr5 positive cells allowed for the lineage tracing of the progeny, which gave rise to all the cells in the crypts, as well as all the types of differentiated epithelial cells in the villi in small intestine. Lineage tracing of Lgr5 positive colon cells revealed the same result. In the small intestine, only 10% of the targeted cells were located at the +4 position, whereas the majority was scattered between Paneth cells, and thus represent a distinct cell population from what has previously been studied. Lgr5 seems to be a marker for intestinal stem cells, is expressed throughout the gastrointestinal tract, and labels around 6 cells per crypt, leading to the conclusion that each crypt is supported by multiple stem cells. Single Lgr5 positive cells can be kept in culture where they are capable of regenerating an ordered crypt-villus structure (Sato et al., 2009).

In adult mice, scattered Lgr5 positive cell were found in multiple non-gastrointestinal stem cell systems such as eye, mammary gland, reproductive organs and in the hair follicle. So far, characterization and fate mapping of these cells has only been thoroughly conducted in the hair follicles (Jaks et al., 2008), where Lgr5 cycling cells also represent a stem cell population.

A similar genetic labeling approach to identify intestinal stem cells was undertaken using the Bmi1 promoter. Bmi1 is known to be involved in self-renewal of both neuronal and hematopoietic cells. Intestinal cells labeled shortly after recombination were predominantly found in the +4 position, and were able to give rise to all differentiated cell lineages in the intestinal epithelium (Sangiorgi and Capecchi, 2008) for extended periods of time. Although these cells display functional stem cell characteristics, Bmi1 positive cells are not homogenously distributed throughout the gastrointestinal tract, but are present in a gradient, with more crypts labelled in the duodenum, getting progressively fewer in the first part of jejunum and finally absent in ileum. The expression pattern in colon has not been reported.

The non-uniform expression of Bmi1 suggests that these cells are not the only stem cell population present in the intestinal crypts. Perhaps the Bmi1 population is a subpopulation of the Lgr5 expressing cells. If so, it is possible that the Bmi1 positive population is the same as the previously identified +4 label retaining population, and would argue for heterogeneity within the larger Lgr5 population, with the rapidly proliferating CBC cells and the quiescent Bmi1 subpopulation.

MULTIPLE SIGNALING PATHWAYS REGULATE THE DEVELOPMENT AND HOMEOSTASIS OF THE INTESTINE

Multiple signaling pathways control the development and homeostasis of the intestinal epithelium. Several of the players involved in regulating these pathways are activated or inactivated through mutations in both hereditary and sporadic colorectal cancers. The phenotype of the cancer can often be referred back to the function of the mutated pathway. This relationship is particularly evident, and has been extensively studied, in the case of Wnt signaling. A short overview of important pathways active in intestinal cells is presented here.

HEDGEHOG SIGNALING

The Hedgehog family of morphogens includes three members in most vertebrates; Sonic (Shh), Indian (Ihh) and Desert hedgehog. The soluble ligands signal through transmembrane receptors, activating the Gli family of transcriptions factors. Both Shh and Ihh are produced by the endodermal epithelium from early developmental stages. Most likely, interplay by the Hedgehog expressing epithelial cells and the surrounding Hedgehog-responsive mesenchyme are critical for the patterning of the gastrointestinal axis. In the E18.5 small intestine, both Shh and Ihh are expressed by the intervillus epithelial cells; mutant mice, however, show both overlapping and unique phenotypes (Ramalho-Santos et al., 2000). Both Sonic and Indian hedgehog mutants analyzed at E18.5 show gut malrotation, whereas the Shh -/- display imperforate anus - colon termination without fusion to the surface ectoderm (also reported in the Gli2-/- and Gli3-/- embryo (Kimmel et al., 2000; Mo et al., 2001)) as well as duodenal obstruction, caused by villi overgrowth. Interestingly, Ihh -/- mice have shorter villi, and a reduction in the proliferation of cells in the intervillus stem cell compartment. Since these gene deletions are not compatible with postnatal life, analyses of these mice in adult are not possible. Inhibition of Hegdehog signaling using cyclopamine in the adult suggests however that they play a role in restricting proliferation to the bottom of the crypt, possibly through down regulation of the Wnt signaling cascade (van den Brink et al., 2004)

BMP SIGNALING

Bone morphogenetic protein (BMP) is a member of the transforming growth factor β family of proteins, and has a key role during intestinal development. BMP proteins bind to their receptors (BMPR1A or BMPR1B) and the signal is conveyed into the nucleus through the SMAD family of transcription factors. BMP signaling has been coupled to the regulation of postnatal localization of the intervillus pockets, forming the crypts, to regions opposed to the muscular layer. Inhibition of BMP signaling by transgenic expression of Noggin resulted in the formation of ectopic crypts perpendicular to the crypt-villus axis (Haramis et al., 2004), a phenotype similar to the human syndrome of juvenile polyposis (JP). In JP patients, mutations in Smad4 or BMPR1A resulting in truncated and functionally deficient proteins have been identified (Houlston et al., 1998; Howe et al., 2001; Howe et al., 1998). Inducible inactivation of the BMPR1A in mice suggests that BMP signaling acts to suppress Wnt signaling and balance the control of self-renewal of intestinal stem cells. Removal of this "brake" would allow for stem cell expansion and crypt fission (He et al., 2004).

NOTCH SIGNALING

Notch signaling is used by many systems to regulate stem cell differentiation and lineage specification. Activation of this pathway, by cleavage of the transmembrane Notch receptor, leads to the translocation of the intracellular Notch fragment to the nucleus, where it affects transcription after binding to CSL. In the intestine, activation of Notch signaling by transgenic overexpression of the intracellular part of Notch-1 leads to a depletion or reduction of all secretory cell types present in the intestine (Fre et al., 2005), in line with the observation that mutant mice lacking the Notch transcriptional target genes Hes1 or Math1 also display a shift in the differentiation of cells within the secretory cell lineage, without affecting the number of normal columnar enterocytes (Jensen et al., 2000; Yang et al., 2001). These findings suggest that the intestinal stem cells give rise to two different progenitor cells, one which is committed to the secretory lineage and negatively regulated by Notch, whereas the other progenitor cell which differentiates into enterocytes upon Notch activation.

WNT SIGNALING

Wnts are a large family of secreted glycoproteins with roughly 20 members identified in mammals. Although Wnts are secreted from cells, they are poorly soluble, due to a cysteine palmitoylation, which is crucial to Wnt function. Three different signaling pathways can be activated upon Wnt signaling activation; the canonical, non-canonical planar cell polarity (PCP) and the Ca^{2+} pathway. The canonical pathway signals through β-catenin, and is not only the best understood but also the major mitogenic signaling pathway in the intestinal stem cell niche. As long as the Wnt receptors Frizzled (Fz) and LRP are not bound by ligand, intracellular β-catenin is bound in a destruction complex containing Axin, APC and GSK3β. This complex phosphorlylates β-catenin, rendering it a target for the E3 ubiquitin ligase βTrCP which results in rapid degradation of β-catenin through the proteasome. The levels of β-catenin are kept low in the cytoplasm. Once Wnt has bound and activated Fz and LRP receptors, an intracellular signaling cascade is set of, which results in the disruption of the β-catenin degradation complex, and the subsequent stabilization of β-catenin. β-catenin is transported into the nucleus where it displaces the transcriptional repressor Groucho and interacts with TCF/Lef enhancer family and activate transcription of β-catenin target genes (Clevers, 2006; Leedham et al., 2005). β-catenin not only functions as a transcriptional activator during Wnt signaling, but also participates in the formation of cell contacts, forming adherens junctions when binding to E-cadherin and α-catenin. The β-catenin bound in cell junctions is highly stable, and it is believed that the two different functions of β-catenin, adhesion and signaling, represent two distinct pools, so that the β-catenin bound in junctions rarely becomes available for signaling.

Several studies have established the importance of Wnt signaling in intestinal development and adult homeostasis. Ambitious attempts to pinpoint the Wnts and its corresponding receptors and transcriptions factors in the intestine (Gregorieff et al., 2005) have revealed a complex pattern where expression is detected in both epithelial and mesenchymal cells, rendering it difficult to draw any conclusions on function or signaling based on expression pattern. However, nuclear β-catenin, a read out for canonical Wnt signaling, is only detected in Paneth cells and epithelial cells situated at the bottom of crypts (Batlle et al., 2002a; van de Wetering et al., 2002), indicating that these cells, perhaps not produce Wnts, but at least are capable to bind and respond to

soluble Wnts, and that the poorly soluble Wnts most likely are produced by cells in close proximity. Although the localization of the Wnt producing cells is not entirely clear, the function is well elucidated; mice with a genetic ablation of Tcf-4, the transcription factor complexing with nuclear β-catenin, die peri-natally with an intestinal epithelium composed only of differentiated enterocytes, and completely lack the proliferative pockets harboring stem and progenitor cells in the small intestine (Korinek et al., 1998). Similarly, overexpression of the Wnt antagonist, Dickkopf-1, in intestinal epithelium results in reduced proliferation and a subsequent absence of crypts (Pinto et al., 2003), whereas injection of human R-spondin, an activator of Wnt signaling results in β-catenin stabilization and increased crypt cell proliferation (Kim et al., 2005).

EPH RECEPTORS AND THEIR LIGANDS

In 1987, the first Eph receptor was identified and cloned from a hepatoma cell line (Hirai et al., 1987), a finding which quickly lead to the discovery of the largest family of tyrosine kinase receptors. Eph receptors are activated by their ligands, the ephrins. Ephrins can be subdivided into two groups based on their membrane attachment and binding preference for Eph receptors, ephrin-A ligands (ephrin-A1 to A5) are tethered to the cell membrane through a glycosylphosphatidylinositol (GPI) anchor, and bind EphA (EphA1-A7) receptors, whereas ephrin-B ligands (ephrin-B1 to B3) are transmembrane proteins with a cytoplasmic part that bind EphB (EphB1-B6) receptors. There are however exceptions to the rule; EphA4 have been found to bind ephrin-B2 and ephrin-B3 (Gale et al., 1996a; Gale et al., 1996b), and EphB2 can bind ephrin-A5 (Himanen et al., 2004).

Ephrins bind Eph receptors with high affinity, forming heterodimers which creates complementary interaction surfaces that result in the joining of dimer pairs into tetrameric complexes. The formation of high order complexes is necessary for proper activation and signaling to occur. Engagement of ligand to the receptor also induces a conformational change in the cytoplasmic portion of the Eph receptor (Wybenga-Groot et al., 2001) by phosphorylation of tyrosine residues in the juxtamembrane domain, relieving the structural inhibition imposed by the juxtamembrane domain on the kinase domain, and allowing for activation of the kinase domain. Similarly, ephrin-B ligands

also become phosphorylated at a conserved tyrosine residue in the cytoplamic portion, resulting in a conformational change and activation of signaling (Bruckner et al., 1997; Holland et al., 1996; Kalo et al., 2001). The notion that signals can not only be conveyed into the receptor-expressing cell (forward signaling) but also by the ligand-expressing cell (reverse signaling) adds an extra layer of complexity to the possible biological outcomes upon ligand-receptor binding. Ephrin-A ligands lack the cytoplasmic part present in the B class, and presumably convey intracellular signals through a co-receptor (Lim et al., 2008).

The Eph-ephrin system is traditionally seen as a chemotactic guidance system, steering moving cells or axons to a specific position or maintaining cellular organization by preventing cell intermingling. Eph signaling can result in either repulsion or attraction and adhesion depending on the cellular context or developmental stage, outcomes most of which can be explained by Eph mediated rearrangement of the cellular cytoskeleton. There are however also findings pointing in the direction of Eph regulated cellular functions that are not explained by the regulation of the cytoskeleton, such as apoptosis and survival, synapse formation and proliferation.

EXPRESSION PATTERN OF EPH AND EPHRINS IN THE INTESTINE

A clue to understanding the function of Eph signaling in a system is to look at the expression pattern of receptors and ligands. Receptors and ligands are commonly expressed in gradients, allowing for the guidance of cells or axons, or exclusively in different cell compartments when establishing cell borders. Hence, the regulation of Eph expression must be tightly regulated. In the intestinal epithelium, Ephs and ephrins are transcriptional targets of β-catenin (Batlle et al., 2002a; van de Wetering et al., 2002), as shown by modulation of β-catenin signaling in colorectal cancer cells. EphB receptors are positive targets of β-catenin signaling, whereas the ephrin-B ligands are inversely regulated, and hence down regulated in cells with active β-catenin signaling.

In all crypts, both in colon and small intestine, EphB2 and EphB3 are expressed in gradients. EphB3 is expressed mainly by the cells at the bottom of the crypt, in the region defined by the Paneth cells in the small intestine, whereas cells situated above the Paneth cell compartment loose their EphB3 expression in a graded manner. Similarly, EphB2 is also expressed in a gradient, although not as restricted to the

Paneth cell compartment, but at high levels in the lower part of the crypts and with decreasing expression as cells move up the crypt axis. Inversely, the ephrin-B ligands, ephrin-B1 and ephrin-B2, are expressed at relatively low levels by the dividing progenitor cells in the crypts, but with increasing level as cells start differentiating and move outside the crypt to the villi (Batlle et al., 2002a). Since Wnts are poorly soluble ligands, the expression pattern of EphBs and ephrinBs in intestinal crypts suggests that Wnt ligands are produces either by the epithelial cells at the bottom of the crypt or by mesenchymal cells closely associated with the lower part of the crypt, in order to establish this intricate Eph/ephrin expression pattern. The counter gradient of ligand and receptor allows for the guidance of progenitor cells as they migrate up the crypt-villus axis, and genetic removal of either EphB receptors or ephrin-B ligands results in distorted cell migration (Batlle et al., 2002a; Cortina et al., 2007).

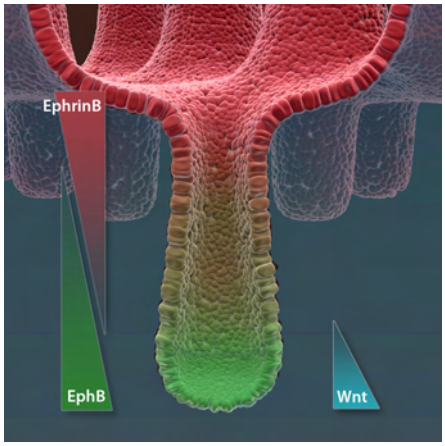

FIGURE 2 EXPRESSION PATTERNS OF EPHB RECEPTORS AND THEIR EPHRINB LIGANDS
Wnts are produced and secreted by cells somewhere in or just outside the crypt bottom. The poor Wnt solubility restricts the target range of these crypt mitogens, but allows for the establishment of an EphB-ephrinB counter gradient. EphB receptors are expressed highly in the bottom of the crypt, being positive targets of Wnt signaling, whereas ephrin-Bs are inversely regulated, and thus expressed at high levels by the more differentiated cells.

EPHB RECEPTORS COORDINATE MIGRATION AND PROLIFERATION IN THE INTESTINAL STEM CELL NICHE

In the adult neural stem cell niche, Eph-ephrin signaling have been shown to negatively regulate the number of neurons generated from stem and progenitor cells (Holmberg et al., 2005), a finding which suggested that the same could hold true in other stem cell niches. In Paper I, analysis of the EphB2; EphB3 double mutant mice together with the EphB2 lacZ/lacZ; EphB3-/- mutant (where the intracellular EphB2 domain is replaced by β-galactosidase), revealed that EphB receptor forward signaling is necessary for

regulating proliferation in intestinal crypt cells, since these mutant mice display up to a 50 percent reduction in the number of dividing cells when compared to wild type mice. Genetic ablation of EphB2 and EphB3 not only leads to reduced stem and progenitor proliferation, but also to the loss of the positional cues normally guiding cells up the crypt and villi in an ordered manner. As a consequence, cell migration is disturbed (Batlle et al., 2002a). Acute inhibition of EphB signaling by the use of soluble ephrin-B2-Fc proteins (Davis et al., 1994) resulted in a reduction in proliferation without affecting cell sorting, establishing the dual, independent, roles of EphB receptors.

In the EphB2$^{F620D/F620D}$ mutant mice, harboring a point mutation in the *EphB2* gene rendering the kinase domain constitutively active, proliferation of crypt cells is increased, further underlining the importance of kinase dependent receptor signaling and establishing the EphB receptors as positive regulators of intestinal progenitor cell proliferation.

Wnt proteins are the master regulators of proliferation in the intestinal system. It may seem counterintuitive that EphB receptors, being Wnt target genes, also are able to regulate proliferation in the intestinal stem cell system. The explanation might lie in the poor diffusion capacity of Wnt ligands, and hence their limited range of action. Although nuclear β-catenin is only detected in the lowest part of the crypt, the EphB gradient, being a direct β-catenin target extends higher up in the crypt, suggesting that the expression of EphB receptors is maintained for longer time than the actual β-catenin transcriptional activation. Thus by regulating the expression of more stable membrane bound receptors, the short ranged Wnt gradient can be expanded to a larger domain beyond the region of high nuclear β-catenin.

IDENTIFICATION OF A PROLIFERATION SIGNALING PATHWAY

The finding that EphB receptors are responsible for conveying a large part of the proliferative signals in the intestinal stem cell niche raised the question of how these mitogenic signals are transmitted into the cell nucleus in order to promote cell division. Since EphB receptor expression is dependent of Wnt signaling, perhaps the EphB mediated proliferation is conveyed through the modulation of the Wnt pathway? By analysis of the TOPGAL mice, a β-catenin signaling reporter mouse which express β-

galactosidase in cells with active β-catenin signaling (DasGupta and Fuchs, 1999), receiving either an inhibitory ephrin-B2-Fc injection or control protein, we concluded that EphB signaling is independent of Wnt signaling (Paper II).

EPHB SIGNALING REGULATES CYCLIN D1-MEDIATED PROLIFERATION

Since EphB signaling regulates cell cycle reentry (Paper I), and cyclin D1 is necessary for cells to complete the G1-S transition, we analyzed the role of cyclin D1 in EphB signaling. Cyclin D1 is an important regulator of proliferation in the normal intestinal epithelium as well as in adenoma and colon cancer cells (Arber et al., 1996; Hulit et al., 2004; Maeda et al., 1998; McKay et al., 2000). However, its regulation has been controversial. Cyclin D1 was suggested to be a target of β-catenin mediated transcription in the intestine, based on *in vitro* studies of colon carcinoma cell lines (Shtutman et al., 1999; Tetsu and McCormick, 1999). Cyclin D1 is also over-expressed in human colon carcinomas as well as in mouse models of tumorigenesis (Arber et al., 1996; Hulit et al., 2004; Maeda et al., 1998; McKay et al., 2000). However, overactivating the β-catenin signaling pathway specifically in the crypts of the intestine by conditionally deleting APC did not result in a direct dysregulation of cyclin D1 in APC deficient cells, leading to the conclusion that cyclin D1 is not a direct transcriptional target for β-catenin (Sansom et al., 2005). However, cyclin D1 levels were increased at later time points, but only in the first APC deficient cells that migrated up along the villi and bordered wild type cells (Sansom et al., 2005). These results could be explained by EphB-mediated regulation of cyclin D1 expression. Since EphB expression is positively regulated by β-catenin, ablation of APC in crypt cells increases EphB receptor levels. In contrast ephrin-B expression is negatively regulated by β-catenin, resulting in low ligand expression in the APC-negative cells (Batlle et al., 2002b). As the mutant cells migrate out from the crypt, they will border wild type cells expressing progressively increasing levels of ephrins, resulting in strong EphB forward signaling and the up-regulation of cyclin D1 in APC-negative cells in the villi.

Analysis of the level of cyclin D1 protein in either wild type mice receiving ephrin-B2-Fc or control protein, or the constitutively active EphB2 $^{F620D/F620D}$ mice compared to wild type litter mates, revealed a correlation between the EphB receptor activation and

the amount of cyclin D1 protein present in the intestinal progenitor cells. In addition, injection of ephrin-B2-Fc into wild type mice significantly reduced the number of proliferating cells in both colon and small intestine as compared to mice receiving control protein, whereas quantification revealed no difference in the number of diving cells in cyclin D1 mutant mice receiving either ephrin-B2-Fc or control Fc. These data suggest that in the intestinal epithelium, cyclin D1 is not a β-catenin target, but rather is under the control of EphB receptor signaling.

EPHB REGULATES CYCLIN D1 LEVELS AND PROLIFERATION VIA ABL

Abl binds to EphB4 in an activity-dependent manner and regulates proliferation and tumorigenicity in breast cancer cell lines (Noren et al., 2006; Yu et al., 2001). Abl acts by regulating phosphorylation of Crk at tyrosine 221, creating a binding site for the Crk SH2 domain (Feller et al., 1994), inhibiting Crk from forming complexes with downstream adaptors. Abl has been shown to be able to bind EphB2 (Yu et al., 2001). Abl mutant mice show reduced proliferation in the intestinal epithelium of both colon and small intestine to a similar degree as the EphB2; EphB3 double mutant mice (Paper II). We used Gleevec (Imatinib mesylate), an Abl tyrosine kinase inhibitor, alone or in combination with ephrin-B2-Fc, to demonstrate that Abl is downstream of EphB receptors and regulates proliferation through cyclin D1 in intestinal progenitor cells. Thus, we establish a signaling pathway, EphB-Abl-cyclin D1, mediating mitogenic signals in the intestinal epithelium.

INTESTINAL TUMORIGENICITY– MOUSE VRS HUMAN

At least 50 percent of the western population develops a colorectal tumor by the age of 70, most of which does not progress to malignancy. Epidemiological studies have suggested that around 15 percent of colorectal cancer occurs in dominantly inherited patterns (Cannon-Albright et al., 1988). Molecular studies demonstrated tight linkage of the disease to markers on chromosome 5q21, and the adenomatous polyposis coli (*APC*) gene was later identified and proven to cause the hereditary syndrome of familial adenomatous polyposis (FAP) (Kinzler and Vogelstein, 1996).

FAP patients harbor a germline mutation in the *APC* allele but in order for tumorigenesis to be initiated in the intestine, a second mutation in a somatic *APC* allele is necessary (Lamlum et al., 2000). Mutations are most commonly found around codon 1300, a region called the "mutation cluster region", and are associated with allelic loss, whereas mutations outside this region tend to result in a truncated protein (Rowan et al., 2000). *APC* loss of function mutations results in loss of β-catenin degradation and hence β-catenin is stabilized and translocates into the nucleus where transcription of β-catenin target genes is enhanced. Cells homozygous for *APC* mutations are highly proliferating, rapidly producing daughter cells which are insensitive to the normal differentiation cues present in the intestine. Microadenomas develop mainly in the colorectum, but also to a smaller extent in the small intestine.

A mouse model resembling the human FAP syndrome was identified using a random mutagenesis approach (Moser et al., 1990), and the mutation was later found to be a truncating mutation in codon 850 of APC. The mouse was named APCmin because of the phenotype observed (multiple intestinal neoplasia), and develops around 30 small intestinal polyps and a smaller number of colorectal lesions. Other mouse models harboring different APC mutations also develop adenomas to different extents (McCart et al., 2008; Oshima et al., 1995) establishing the role of β-catenin in intestinal tumorigenesis.

ARE HUMANS AND MICE THE SAME?

Although the same molecular mechanisms that underlie the formation of tumors in human sporadic or FAP patients are the same as in the APCmin model, there are obvious differences between the two which are not easily explained. Firstly, humans develop adenomas, which later turn into carcinomas, mainly in the colon and rectum, hence the commonly used name of colorectal tumors. In mice, on the other hand, adenomas are mainly found in the small intestine, with increased tumor load in the second half of the small intestine. Colorectal adenomas are found in the APCmin mouse, but in much lower numbers than what is found in the small intestine. Secondly, whereas human adenomas commonly progress into invasive carcinomas, the adenomas found in mice grow in size, but rarely gain invasiveness. Perhaps the shorter life span in mice contribute to the lack of carcinomas, which most likely require more time to

develop. It has been argued that because of the discrepancy between the human disease and the mouse model, the APCmin mouse is not a good enough model to study intestinal tumorigenesis. However with the increased longevity seen in FAP patients due to prophylactic colectomy, small intestinal carcinoma is now a common cause of death in this group (Arvanitis et al., 1990; Nugent et al., 1993). Hence, both man and mice develop tumors throughout the gastrointestinal system following APC inactivation, although the primary location seems to differ.

IDENTITY OF THE TUMOR INITIATING CELL IN MOUSE ADENOMAS

Although the genetic alterations required for tumor formation has been know for quite some time, the cellular origin of microadenomas has remained elusive. Based on histological analyses, different models of tumor origin developed, suggesting that neoplastic cells either first appear in the upper part of colon crypt and then spread down to out compete the untransformed cells localized further down in the crypt bottom (Shih et al., 2001), or origin in the lower stem cell compartment after which the adenomatous cells fill up the entire crypt and spread either by crypt fission or by growth down into adjacent crypts (Preston et al., 2003). With the identification of stem cell marker such as Lgr5 and Bmi1, targeting of these cells allowed for induction of tumorigenesis in a specific cell population at a defined localization in the crypt. Barker et al (Barker et al., 2009) elegantly showed that genetic removal of *APC* in Lgr5-expressing stem cells results in massive adenoma formation throughout the gastrointestinal system, and that a single transformed stem cell at the bottom of the crypt is enough for the formation of a neoplastic clone of cells which eventually fills the entire crypt in both colon and small intestine. Conversely, stochastic removal of APC in cells through out the crypt-villi axis results in a low number of adenomas, arguing that stem cells are the main tumor initiating cell in intestinal cancer although not ruling out the possibility that transit amplifying cells and differentiated cells can initiate tumors although at a much lower frequency. A similar approach (Sangiorgi and Capecchi, 2008) using an activated form of β-catenin in Bmi1 expressing stem cells also resulted in the formation of adenomas, further supporting the role of stem cells as the main tumor initiating cell in intestinal cancer.

EPHB RECEPTORS IN ADENOMAS

Adenomas develop from a cell that has lost the capacity to down regulate β-catenin signaling and as a consequence, all transformed cells express high levels of β-catenin target genes, such as the EphB receptors, and low levels of ephrinBs which are negative β-catenin targets. As the mutated EphB positive adenoma cells expand, they bud out from the crypt, forming outpocketing pouches into the surrounding stroma tissue (Oshima et al., 1997), eventually resulting in a large continuous, but folded, layer of transformed cells kept under the untransformed villi cells. The transformed layer of EphB positive cells is repelled by the surrounding ephrinB expressing villi cells, resulting in an *in situ* adenoma growth (Batlle, Henderson et al. 2002; Cortina, Palomo-Ponce et al. 2007).

Since EphB mediated proliferation in intestinal progenitor cells in independent of Wnt signaling, we argued that perhaps EphB receptor signaling still mediate mitogenic signals in transformed adenoma cells. Infusion of ephrin-B2-Fc or injection of Gleevec into APCmin mice resulted in significantly reduced levels of proliferation in small intestinal adenomas when compared to control mice. Animals treated with ephrin-B or Gleevec also displayed reduced levels of cyclin D1 protein, suggesting that the same signaling pathways active in normal epithelium is also regulating proliferation in adenomas (Paper II). It is not evident to understand how the folded layer of EphB expressing adenoma cells is able to activate its receptors, since ligand expression is restricted to the outer villi layer surrounding the entire adenoma structure. If only the adenoma cells close to the villi border would have active EphB signaling, the effect seen on proliferation after ephrin-B2-Fc or Gleevec injection would be localized to these regions, a phenomenon which is not seen. One possible explanation is that the three dimensional folding of the adenoma layer allows for most EphB-expressing cells to establish contact with a ligand expressing cell. Recently, Noren et al found that overexpression of EphB4 in cancer cells resulted in increased phosphorylation of the receptor, although there is virtually no expression of ligand in these cells (Noren et al., 2009), raising the possibility that Eph receptors could have ligand-independent functions in transformed cells.

ADENOMA TO CARCINOMA TRANSITION

As tumor cells divide, they acquire new mutations, turning into more aggressive carcinomas. In colorectal tumors K-Ras and p53 are commonly mutated, resulting in overactivation and inactivation, respectively. Carcinomas are classified depending on their capacity to invade the surrounding tissue, eventually gaining the ability to establish metastasis in lymph nodes and distant organs. As the tumor gains the ability to form secondary tumors, it also loses the EphB expression (Batlle et al., 2005). Loss of EphB receptors not only correlates to carcinoma progression, but also to survival (Davalos et al., 2006; Guo et al., 2006), where low EphB expression significantly correlates to poor survival prognosis. The mechanism of EphB receptor downregulation is not clear, but it has been suggested that promoter hypermethylation or microsatellite instability could explain the sudden protein downregulation observed (Alazzouzi et al., 2005; Song et al., 2007; Wu et al., 2007). However, the percentage of patients found harboring these types of genetic alterations are quite low, and most likely other mechanisms are also involved in modulating the EphB expression in carcinomas.

We analyzed human colorectal tumor samples ranging from adenomas to late stage carcinomas (Paper II) and found that although EphB2 expression is downregulated as the tumor progresses from adenoma to carcinoma, the level of proliferation and cyclin D1 is sustained, suggesting that while cyclin D1 is dependent on EphB receptor signaling in normal epithelium and adenomas, its expression is EphB independent in carcinomas. Work in colorectal carcinoma cell lines show that Abl and cyclin D1 still mediate proliferative signals, and that in the absence of EphB receptors, Abl might be regulated by EGF and/or IGF receptors (Paper II). EGF and IGF receptor pathways are overactivated in human colon carcinoma, and monoclonal blocking antibodies to the EGF receptor are used to treat colon cancer in humans (Ciardiello and Tortora, 2008; Davalos et al., 2006; Donovan and Kummar, 2008). Both EGF and IGF receptors activate Abl in other contexts (Davalos et al., 2006; Srinivasan and Plattner, 2006; Srinivasan et al., 2008).

TUMOR METASTASIS AND EPHB RECEPTORS

Due to the general lack of invasive tumors in APCmin mice, several studies have focused on genes believed to be important in the adenoma to carcinoma transition in attempts to recapitulate invasive disease in the mouse. As a result, a handful of genes have been identified as modulators of adenoma to carcinoma transition, for example estrogen receptors α and β, PTEN, K-Ras, members of the Smad family of transcription factors and EphB receptors (Alberici et al., 2006; Batlle et al., 2005; Cho et al., 2007; Hamamoto et al., 2002; Sansom et al., 2006; Shao et al., 2007). Most of these genetic modulations result not only in the development of invasive carcinomas, but in a general increase in the tumor load and tumor size of APCmin mice, raising the question of whether increased tumor load and size *per se* is enough to allow for carcinomas to develop, simply because there are more adenomas which have the possibility to do so?

However, the dual role of EphB receptors as mediators of both proliferation and migration suggests that there is a direct mechanism explaining the observed increase in tumor invasiveness upon EphB loss. As tumor cells downregulate EphB receptors, they

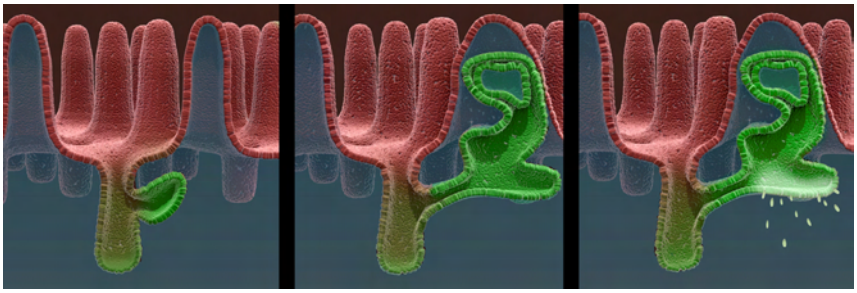

FIGURE 3 EPHB RECEPTORS DUAL ROLES IN TUMOR FORMATION
Activation in the Wnt signaling cascade leads to the formation of adenomas, as well as the upregulation of EphB receptor expression in the transformed cells (green). The high EphB expression drive proliferation of the transformed cells which form outpocketings from the crypt into the surrounding stroma, eventually forming large proliferating adenomas *in situ*. Due to the repulsive effect mediated by the surrounding ephrinB layer of villi cells (red), the adenoma is unable to invade the surrounding tissue. At later stages, tumor cells loose EphB receptor expression and are no longer repelled by ephrinB ligands, hence gaining the ability to metastasise.

no longer have the ability to respond to and be repelled by the surrounding ephrinB expressing cells, and hence single cells can migrate away and leave the large mass of adenomatous cells, potentially reaching lymph vessels and finally settle in new organs. Interestingly, the spatial restrictions enforced by the EphB expression on tumor cells are mediated by the adhesion molecule E-cadherin (Cortina et al., 2007). E-cadherin is the main adhesion molecule in epithelia, and is frequently lost in human epithelial cancers (Takeichi, 1993), and suggested to regulate adenoma to carcinoma transition in pancreatic β-cell cancer (Perl et al., 1998). Analysis of E-cadherin expression in human colon tumor samples revealed high E-cadherin expression in adenomas followed by low expression as tumors progressed into carcinomas (data not shown). In adenomas of APCmin mice, E-cadherin is highly expressed by the ephrinB positive cells compartmentalizing the adenoma cells (data not shown), suggesting that E-cadherins have a role in regulating invasiveness in colon tumors.

MIGRATION OF PROGENITOR CELLS

In order to be able to dissociate from the primary tumor and spread through the lymphatic system, metastasizing cells have to be highly motile. Untransformed epithelial cells in the intestine are in constant movement, migrating rapidly, but in an ordered manner, up the crypt-villus axis. Cancer cells normally employ similar signaling pathways as untransformed cells, suggesting that identification of signaling pathways regulating migration in normal intestinal progenitor cells could also have implications for the understanding of tumorigenesis.

Because of the highly organized migration pattern seen in crypts and villi, the intestine offers a good model to identify signaling pathways involved in regulating this cell placement. Migration could be analyzed by (at least) two parameters; the speed of which epithelial cells move up the villi, or the distribution of Paneth cells, which normally are situated at the bottom of the small intestinal crypts. Both strategies have been employed. Loss of either APC (Sansom et al., 2004) or Foxl1 (Takano-Maruyama et al., 2006) results in the mislocalisation of Paneth cells, which in both cases could be explained by dysregulation of β-catenin signaling and hence aberrant EphB expression. In comparison, overexpression of E-cadherin resulted in slowed cell movement up the villi (Hermiston et al., 1996), suggesting that both EphB receptors and cadherins are implicated in regulating migration of intestinal progenitor cells.

Genetic ablation of EphB3, but not EphB2, also results in mislocalised Paneth cells (Batlle et al., 2002a), however, removal of both receptors results in a dose dependent increase in cell mislocalisation (Paper II), suggesting that both receptors are able to mediate migratory signals. Analysis of mice with different EphB2 mutations show that the EphB mediated effect on cell positioning is kinase independent (Paper II), in line with previous *in vitro* results showing EphB3 (Miao et al., 2005) or EphA8 (Gu and Park, 2003) mediating kinase independent migration, both using cell lines. Gu et al further concluded that the p110ϒ subunit of PI3-kinase is required for the effects. PI3-kinase has also been shown to regulate migration through Eph receptors in other contexts (Brantley-Sieders et al., 2004; Maekawa et al., 2003), as well as to be able to bind to the EphB2 juxtamembrane region (Holland et al., 1997). We assessed the influence of PI3-kinase signaling on intestinal epithelial cells by using a PI3-kinse inhibitor (LY294002), and found an increased number of displaced Paneth cells 3 and 7 days after PI3-kinase inhibition (Paper II), similar in magnitude to what is seen after ephrin-B2-Fc injection. Colon carcinoma cells expressing EphB receptors were no longer repelled by ephrin-B2 after treatment with the same inhibitor (Paper II), establishing PI3-kinase as a candidate for mediating the migratory kinase independent EphB signaling pathway.

LIGAND DEPENDENT ACTIVATION OF EPHB RECEPTORS REGULATES MIGRATION

Not much is known about the function of the individual ephrin-Bs expressed in the epithelium. Based on the rather unspecific binding preferences and high sequence homology, it is generally thought that most ephrin-Bs can bind all EphB receptors in a redundant manner. EphB2 and EphB3 receptors have redundant functions and are involved in mediating both proliferative and migratory signals (Paper I and II). However, the specific functions for the two most prominent ephrin-B ligands expressed in the intestinal epithelium are not known. Analysis of mutant mice as well as ephrin-B1-Fc or ephrin-B2-Fc injections revealed distinct functions for the two, where ephrin-B1 mainly is involved in regulating migration and ephrin-B2 promotes proliferation (Paper III). How can EphB receptors discriminate between the two ligands?

Immunohistochemical detection of infused ephrin-B1-Fc or ephrin-B2-Fc suggests that there is no binding preference for either ligand to any EphB receptor (data not shown), where neither ephrin-B1-Fc nor ephrin-B2-Fc binding is restricted to the EphB3 expressing domain of the crypts. Thus, both ligands are capable of binding both receptors, yet the outcome from ephrin-B1 versus ephrin-B2 binding is distinct.

Ephrin-B1 shares significant sequence homology (43% amino acid identity) with ephrin-B2 (Nikolov et al., 2005), and the overall secondary structure of ephrin-B1 and ephrin-B2 is very similar. The most distinct difference between the ligands can be found in the receptor binding loop, where the ephrin-B2 loop not only is involved in recognition of the receptor, but also in ephrin homodimerization, whereas the ephrin-B1 loop is unable to participate in ephrin-ephrin interactions (Nikolov et al., 2005) suggesting that both receptor and ephrin-B1 ligand must undergo conformational changes in order to form the high order complexes necessary for activation of signaling. Thus, ephrin-B1 is more likely to exist as a monomer, not having formed pre-dimerized units on the cell surface. Furthermore, ephrin-B1 contains a more disordered N-terminal end compared to ephrin-B2, which in ephrin-B2 is used to mediate the formation of ligand-receptor heterotetramers.

Together Nikolov´s findings lead to the proposal of at least two different hypothesis of the differential EphB activation of ephrin-B1 and ephrin-B2. The structural differences found in ephrin-B1 suggest that this ligand is less prone to form both ligand-ligand and ligand-receptor binding, hence less probable to form the heterotetramers that are necessary for the complete activation of receptor-ligand signaling. Or, if heterotetramers are formed, the process is slower and requires higher density of both receptors and ligands to be efficient. The outcome could be that ephrin-B1 mostly complexes with EphB receptors as a monomer, whereas ephrin-B2 binding more readily forms high order signaling complexes. The consequences of the monomer hypothesis could be that ephrin-B1 binding to EphB receptors is less likely to result in subsequent phosphorylation of the receptor kinase domain. Previous work suggests that EphB mediated signaling regulating migration is kinase independent (Paper II). Inhibition of EphB signaling by injection of soluble ephrin-B2-Fc results in reduced levels of EphB phosphorylation in colon tissue (Paper I), and investigation of the phosphorylation status of EphB receptors after binding of soluble ephrin-B1-Fc would

give a clue as to whether ephrin-B1 mimics ephrin-B2, or is unable to affect the phosphorylation of the receptor kinase domain.

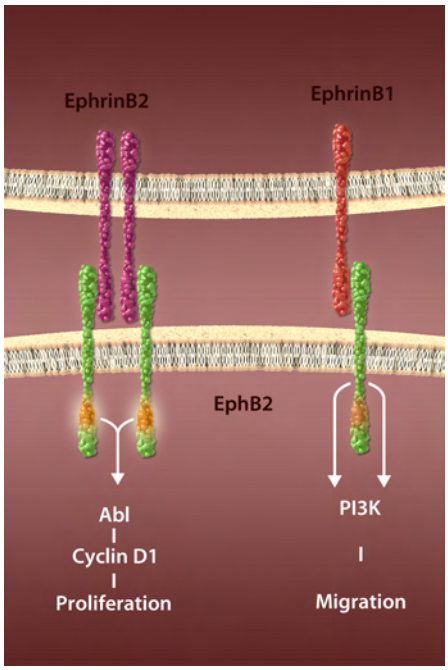

FIGURE 4 SUGGESTED MODEL FOR THE DISTINCT EPHB ENGAGEMENT OF EPHRIN-B1 AND EPHRIN-B2

Based on the differences found in ephrin-B1 and ephrin-B2 protein structure, we suggest a model where ephrin-B2 binding to EphB2 results in dimerisation and subsequent clustering of ligand-receptor heterodimers. Ephrin-B2 binding can hence activate the receptor kinase activity, mediating the Abl-cyclin D1 proliferative signaling cascade. Ephrin-B1 is more likely to exist as a monomer and binding to the EphB2 receptor would not result in the formation of heterodimers, but rather involve receptor conformational changes without kinase activation. EphB2 forward signaling could regulate kinase independent migration though PI3-kinase binding to the juxtamembrane region of EphB2.

Alternatively, Eph kinase activity is already activated at low level of receptor dimerization, and high order clustering would not lead to a further increase in Eph receptor activity, but rather to the recruitment of a new set of cytoplasmic effectors, a scenario suggested in the analysis of a constitutively active EphA4 mouse (Egea et al., 2005).

TO BROADEN THE VIEW….

Naturally, the Eph and ephrin expression is not restricted to the gastrointestinal system, but is found in a multitude of tissues throughout development into adulthood, where they have been found to regulate a large amount of cellular responses and behaviors. Interestingly, the Eph receptors are commonly expressed in stem cell niches (Arthur et al., 2009; Tumbar et al., 2004; Vaught et al., 2008), although the great number of members of the tyrosine kinase family does not allow for the pinpointing of single

receptors or ligands being more common then others. In the adult brain, the subventricular zone and the dentate gyrus of the hippocampus, the two adult neurogenic areas, display complementary expression patterns of receptors and ligands (Chumley et al., 2007; Holmberg et al., 2005). Progenitor cells in the subventricular zone express ephrin-A2, and ligand reverse signaling negatively regulates progenitor proliferation, whereas EphB receptor signaling in the subgranular layer of the dentate gyrus positively regulates hippocampal neurogenesis. Thus, the effect of receptor ligand interaction on proliferation can differ depending on the cellular setting. However, some assumptions can be made. The dentate gyrus stem/progenitor cell niche is similar to the intestinal crypts in that progenitor cells in both tissues express high levels of receptors, whereas the ligand is expressed by a more differentiated cell type. Hence, receptor signaling positively regulates the number of progenitor cells produced. In the subventricular zone, on the other hand, the inverse is true. Receptor expression is limited to the quiescent layer of ependymal cells, whereas progenitor cells express the ligand. Ligand signaling into these cells functions as a brake to reduce the number of progenitors produced. In the skin stem cell niche, inhibition of Eph signaling by soluble ephrin-Fc proteins reduce proliferation of hair follicle keratinocytes (unpublished data), most likely as a result of disrupted ligand signaling. Thus, the Eph-ephrin effect on progenitor cells seems to correlate to the expression pattern, and hence to receptor or ligand signaling.

In line with the silencing of EphB expression in colorectal adenocarcinomas, other tumors also display differential receptor expression when compared to their untransformed tissue of origin. EphA7 is downregulated in human prostate tumors (Guan et al., 2009), and EphB2 mutations was identified in a cell line derived from metastasizing prostate tumor (Huusko et al., 2004), consistent with a causal role for Eph receptors functioning as tumor suppressors. However, high levels of Eph receptors have been reported in diverse types of cancer, including mammary adenocarcinomas and glioblastoma multiforme (Brantley-Sieders et al., 2008; Noren et al., 2006; Wang et al., 2008a; Wang et al., 2008b), corroborating the role of Eph receptors in tumor promotion. Thus, different types of cancer display distinct patterns of Eph expression during tumor progression, perhaps as a result of the differences found in the untransformed tissue where the function of Eph receptors is dependent on the cellular context.

There are indications that Eph receptors can have ligand-independent functions in tumors (Miao et al., 2009; Noren et al., 2006; Noren et al., 2009), as exposure of cancer cells to ephrin ligands tends to inhibit tumorigenesis. Downregulation of ligands instead of receptors may be an alternative way of transformed cells to avoid the tumor suppressor functions of Eph receptors.

FUTURE PERSPECTIVES

GLEEVEC – A POTENTIAL TREATMENT OF INTESTINAL EPITHELIAL TUMORS?

The development of Gleevec (Imatinib), started in the 1960´s with the realization that a majority of cells in chronic myelogenous leukaemia (CML) were characterized by an abnormally small chromosome 22. Investigations revealed an exchange of chromosomal material between two chromosomes, 22 and 9, resulting in the formation of a large tyrosine kinase, Bcr-Abl (Weinberg, 2007). Depending on the breaking point in chromosome 22, three different versions of Bcr-Abl is found, resulting in CML, but also ALL (acute lymphoblastic leukaemia) and CNL (chronic neutrophilic leukaemia). Bcr-Abl is a large constitutively active tyrosine kinase, with the ability to interact with a multitude of signaling pathways employed by the cell, hence its transforming capacity. Decades later, Gleevec was developed; a small molecule able to bind the ATP-binding pocket of Abl, thereby leading to the stabilization of catalytically inactive Abl. Structural resemblance of the catalytic domain of tyrosine kinases renders Gleevec able to bind and inhibit the function of PDGFR α and β and the c-Kit receptor. Activating mutations in the c-Kit receptors is found in GIST (gastrointestinal stromal tumors) (Miettinen and Lasota, 2003), which together with CML are the major clinical diagnoses treated with Gleevec. Gleevec is administered daily over long time to CML and GIST patients, and the drug is fairly well tolerable.

In Paper II, administration of Gleevec to adult APCmin mice results in a significant reduction in the number of proliferating cells, coupled to a down regulation of cyclin D1 expression in small intestinal tumors, suggesting that Abl inhibitors could be used as a therapeutic strategy to suppress adenoma growth. However, reduced cell proliferation does not necessarily equal reduced malignancy. To assess the potential of Abl inhibitors, administration of Gleevec for extended periods of time followed by analysis of tumor size and load and importantly survival will hopefully answer this question. The normal dose of Gleevec administered to patients ranges from 400 to 800 mg per day, which is about a ten fold lower then the dose used in Paper II, raising the

possibility of unexpected side effects when using Gleevec as a long time treatment in mice.

CML patients taken off treatment often relapse into disease. Gleevec predominantly targets the cancer progenitor cells, whereas the leukaemia initiating stem cells remain largely unaffected, hence allowing for the reoccurrence of disease. In the intestine, it is not known which cells are the primary target of Gleevec, or whether Gleevec also could potentially affect the rare tumor initiating cells found in human colon carcinomas (O'Brien et al., 2007; Ricci-Vitiani et al., 2007), a prerequisite for any curative treatment.

MIGRATION SIGNALING DOWNSTREAM OF PI3-KINASE?

Overexpression of E-cadherin in intestinal epithelial cells leads to a reduction in the migration of these cells up the villi (Hermiston, Wong et al. 1996). E-cadherin has also been shown to compartmentalize small intestinal tumor cells by meditating the repulsive effects of EphB-ephrin signaling (Cortina et al., 2007). Inhibition of PI3-kinase signaling also affects the migration pattern of cells in small intestine, to a similar extent as inhibition of EphB signaling. Both cadherins and PI3-kinase are hence linked to EphB receptors, suggesting that they are both part of the same signaling network. We have analyzed the expression of E-cadherin in both gain and loss of function models of EphB signaling, and find that both the expression level, and the localization of cadherins correlate to the kinase activity of EphB2 (data not shown). Thus, inhibition of EphB signaling leads to accumulation of β-catenin bound E-cadherin in the cell membrane of crypt cells, whereas the activation of EphB receptors lead to reduced levels of cadherins and a shift towards increased intracellular localization. The regulation of adherens junctions thus correlates to receptor activation, but does it correlate to migration, or is it simply a result of an altered proliferative rate? Cells that undergo rapid cell division are perhaps less prone to, and have less time, to establish functional cell contacts with its neighbouring cells? Although in our models where we directly affect EphB receptors, adherens junction formation correlates to changes in proliferation, Hermiston et al (Hermiston, Wong et al. 1996) were able to dissociate the effects seen on cell division from migration, suggesting that alterations in E-cadherin have a direct effect on cell migration in the intestine.

Several reports link Eph receptors to adherens junctions. Overexpression of EphA2 in mammary epithelial cells weakens E-cadherin based cell-cell contact (Fang et al., 2008), whereas increased EphB3 expression was found to redistribute E-cadherin to the cell membrane in colon carcinoma cells (Chiu et al., 2009). Likewise, *in vitro* work suggests that PI3-kinase is recruited to, and required for, the proper formation of adherens junctions (Chartier et al., 2006; Kovacs et al., 2002; Laprise et al., 2002). Applying these findings to the intestinal system suggest that treatment with a PI3-kinase inhibitor would lead to a reduction in cell-cell contacts and increased cell motility. Administration of PI3-kinase inhibitor for several consecutive days do result in displaced cells (Paper II), similarly to mice injected with ephrin-B2-Fc. It is not clear how the PI3-kinase inhibitor affects cadherin junctions *in vivo*, however, ephrin-B2-Fc injection leads to an increase in the level of E-cadherin based junctions. To further elucidate the possible relationship between PI3-kinase and the formation of adherens junctions *in vivo*, there is a need for a functional link between cadherins and cell localization.

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