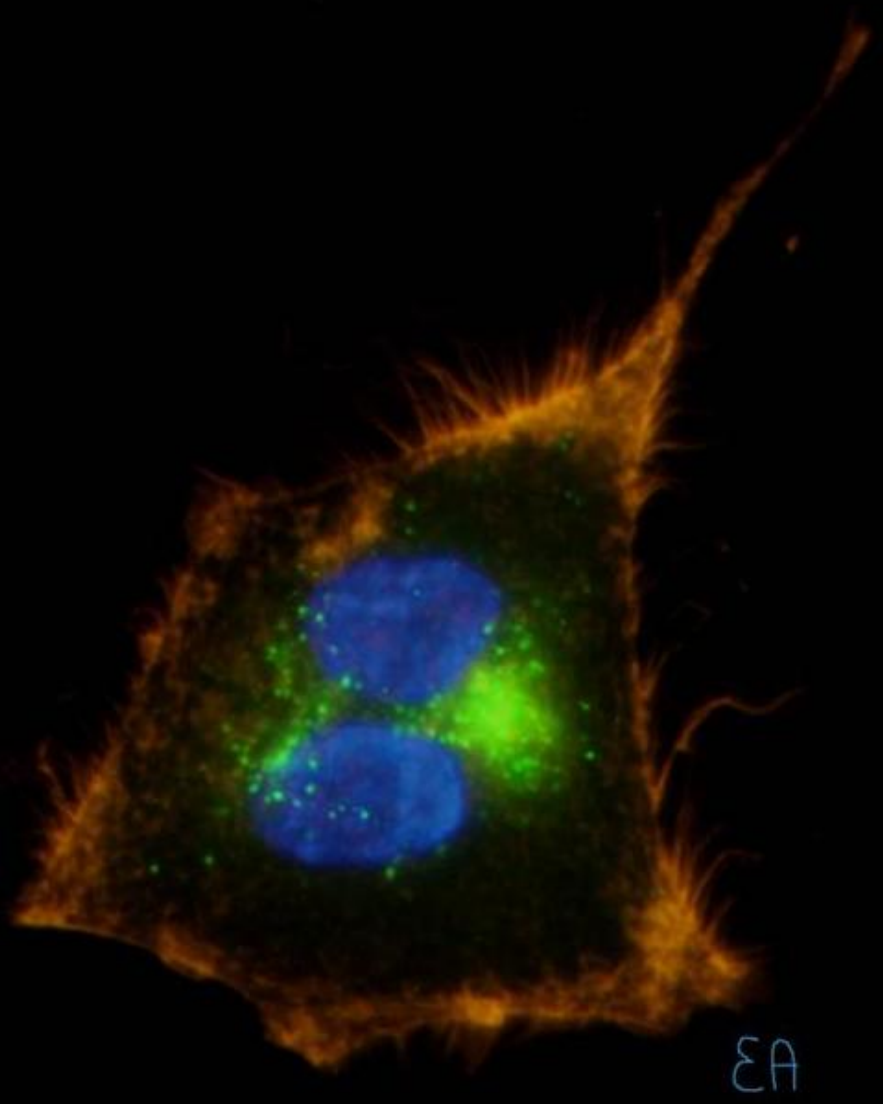
Role of EPHA3 in colorectal cancer



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Role of EPHA3 in colorectal cancer

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To Umberto and Pia

"Every refutation should be regarded as a great success; not merely a success of the scientist who refuted the theory, but also of the scientist who created the refuted theory and who thus in the first instance suggested, if only indirectly, the refuting experiment". Karl Popper, 1902-1994.

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Introduction

1. ANATOMY AND HYSTOLOGY OF HUMAN INTESTINE

The human digestive system is a complex series of organs and glands that processes food. It contains the small and large intestines that form together a continuous tube lined internally by a single layer of columnar epithelium and stretches from the pylorus, the out let of the stomach, to the anus (**Fig.1**).

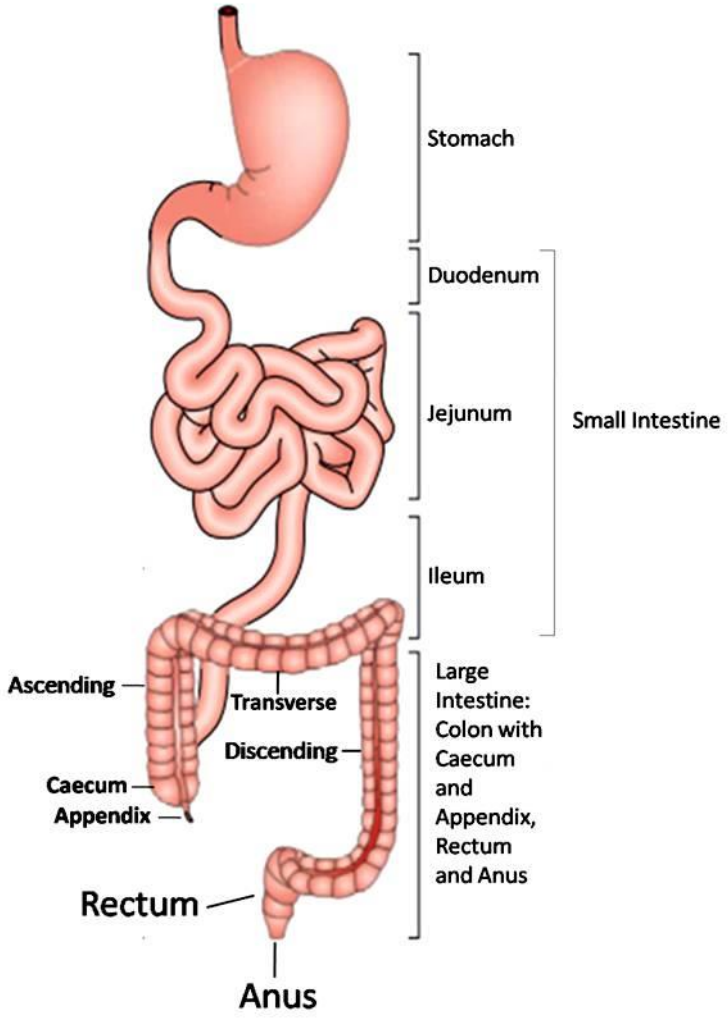

Fig.1. Anatomy of human gastrointestinal system.

1.1. Anatomy

Small Intestine - The small intestine begins at the pylorus and ends at the ileocaecal valve, which is the entry point into the large intestine. It extends by multiple coils about 6–7 m in length and it is divided into three main segments, with the **duodenum** being closest to the stomach, followed by the **jejunum** and then the **ileum**.

The **Duodenum** has received its name from being about equal in length to the breadth of twelve fingers (25 cm). It is the shortest, the widest, and the most fixed part of the small intestine, and has no mesentery, being only partially covered by peritoneum. Ducts from the liver, gallbladder, and pancreas enter the duodenum to provide secretions that neutralize acids coming from the stomach and help digest proteins, carbohydrates, and fats. Absorption of food begins here with the absorption of vitamins, minerals and other nutrients. In particular, before the food passes into the next part of the small intestine iron, calcium and magnesium are absorbed here. The rest of the food is passed into the jejunum.

The **Jejunum** is the second region of the small intestine, with about 2.5 meters of length. The color of the jejunum is deep red because of its extensive blood supply; it contains circular and longitudinal smooth muscles which help the movement of the food along by a process known as peristalsis. Most of the food absorption is done in this part of the digestive tract.

The **Ileum** constitutes approximately 60% of the small intestine in an adult human and is located in the lower part of the abdomen. Its walls are narrow and thinner than the ones of the jejunum. The blood supply is also more limited and the peristaltic movements are slower than in the jejunum. There is no specific demarcation between Jejunum and Ileum and both are suspended within the peritoneal cavity by a thin, broad-based mesentery that is attached to the posterior abdominal wall and allows free movement of the small intestine within the abdominal cavity. Apart from the absorption of bile salts and vitamin B12, the ileum contributes much less to nutrition and it has markedly shorter villi and lower levels of brush border enzymes.

Projected into the lumen of the bowel are the circular folds (also known as "*plicae circulares* of Kerkringi" or "*valvulae conniventes*"), large valvular flaps composed of reduplications of the mucous membrane that retard the passage of the food along the intestines to allow the absorption of the nutrients, and also they afford an increased surface for absorption. Moreover, millions of tiny fingerlike projections called "villi" cover the inner wall of the small intestine. The villi are covered with even thinner projections called "microvilli". The combination of circular folds, villi and microvilli

increases the surface area of the small intestine greatly, significantly improving the absorption of nutrients.

Large Intestine - It begins at the caecum, followed by the ascending (proximal) colon, the transverse colon, the descending (distal) colon and the rectum, terminating at the anus. The colon is wider in diameter and much shorter (~1.5 m) then the small intestine (**Fig.1**). It describes, in its course, an arch which surrounds the convulsions of the small intestine. The first portion of the large intestine, the **caecum**, lies in the right iliac fossa and projects downward as a blind pouch below the entrance of the ileum. The caecum, is a pouch like structure 6 to 8 cm in length, this area allows food to pass from the small intestine to the large intestine. The **ascending colon** extends from the caecum for 12 to 20 cm along the right side of the peritoneal cavity to the hepatic flexure. It is covered with the peritoneum anteriorly and on both sides, thus it constitutes a retroperitoneal organ. At the hepatic flexure, the colon turns medially and anteriorly to emerge into the peritoneal cavity as the **transverse colon**. This longest portion of the colon (40 to 50 cm) is the most mobile segment and drapes itself across the anterior abdomen between the hepatic and splenic flexures. The **descending colon**, about 30 cm in length, travels posteriorly and then inferiorly in the retroperitoneal compartment to the pelvic brim. There, it emerges into the peritoneal cavity as the sigmoid colon. The large intestine produces no digestive enzymes and chemical digestion is completed in the small intestine before the chime (the remaining part of the food) reaches the large intestine. However, the colon performs important functions for the final processing of the chime. Through rhythmic peristaltic contractions of the haustra (the circular folds of the large intestine), the food residues are mixed and forced to move from one haustrum to the next. Vitamins B and K, some electrolytes (Na^+ and Cl^-), and most of the remaining water are absorbed in the colon, in a process that usually takes 24 to 30 hours. Moreover, trillions of commensal bacteria that colonize the large intestine digest waste products and break down indigested foodstuffs by fermentation. The last part of the large intestine, the **rectum**, continues from the sigmoid colon to the anal canal and has a thicker muscular layer. This part is where feces (waste material) are stored before leaving the body through the anus.

1.2. Hystology

The intestinal wall is composed of four distinct functional layers: mucosa, submucosa, *muscularis propria* (muscle layer) and *serosa*.

Small Intestine - The **mucosa** of the small intestine is composed of three layers: the ***epithelium***, the underlying ***lamina propria***, and a thin smooth muscle layer called the ***muscularis mucosae***. Except in the initial length of the duodenum, large crescentic folds

of mucosa project into the lumen of the small intestine, lying either transversely or slightly obliquely to its long axis. Moreover, the mucosa epithelial monolayer of the small intestine is organized in two functionally distinct compartments: the **villi** and the **crypts of Lieberkhün** (**Fig.2**).

The intestinal **villi** are highly vascular projections of the mucosal surface into the lumen of the small intestine. The most abundant cell types covering the epithelium of the villi (more than 90% of the cells) are the **enterocytes** (**Fig. 2**). These are tall columnar cells with apical microvilli that are seen as a brush border in light micrographs. Enterocyte microvilli increases apical membrane surface area, thereby enhancing the nutrient processing and absorptive capacity of the intestinal epithelium as well they represent the major barrier to the resident intestinal microbiota and to pathogens introduced into the gastrointestinal tract (1). Indeed, the functional significance of normal brush border structure is underscored by the fact that disruption of brush border membrane organization is associated with several pathological conditions, including microvillus inclusion disease (2) and gluten-sensitive enteropathies such as Celiac Disease (3). Scattered among the enterocytes there are cup shaped, mucin producing cells known as **goblet cells** (10% of the epithelial cells). The mucus is not only important for the lubrication of the intestinal contents, but has emerged as an important defense mechanism against physical and chemical injury caused by ingested food, microbes and the microbial products (4,5) but also as a substrate and niche for the commensal flora that colonizes the intestine (6). The mucus is a highly charged gel composed of mucin glycoproteins that are directly toxic to many bacteria (7). The extracellular mucus barrier consists of two layers: a thinner inner mucus layer, which is physically difficult to dislodge and is sterile, and a thicker outer mucus layer, which is more easily dispersed and not sterile. The inner layer, much thinner in the small intestine than in the large, is a trait that may be necessary to accommodate the need for nutrient absorption and where antibodies and antimicrobial peptides can adhere (7,8). The largest endocrine system, in terms of total number of cells, is the enteroendocrine system of the digestive tract. In contrast to other endocrine systems, **enteroendocrine cells** (EEC) are scattered as single cells throughout the intestinal tract, located within the intestinal crypts of Lieberkhün and villi, and comprising ~1% of the epithelial cell population. There are at least 15 subtypes of EEC, secreting multiple peptide hormones which elaborately control physiological and homeostatic functions in the digestive tract, particularly postprandial secretion and motility. Their key purpose is to act as sensors of luminal contents, either in a classical endocrine fashion, or by a paracrine effect on proximate cells. Enteroendocrine cells also play a pivotal role in the control of food intake, and emerging data add roles in mucosal immunity and repair (9,10).

The base of each villus is surrounded by multiple epithelial invaginations, termed **crypts of Lieberkhün** after their discoverer Jonathan Nathanael Lieberkühn (1711–1756). It has long been known that crypts are home to multipotent stem cells which give rise to different types of mature epithelial cells (11). Besides the absorptive enterocytes, mucus producing goblet cells and enteroendocrine cells, the intestinal glands also contain **Paneth cells** residing at the base of the crypts. Paneth cells are highly specialized epithelial cells of the small intestine, where they coordinate many physiological functions. These cells synthesize and secrete substantial quantities of bactericidal products such as lysozyme, defensins and mucus. More intriguing, Paneth cells secrete factors that help sustain the epithelial stem/progenitor cells in the crypts of the small intestine (12,13).

The ***lamina propria*** consists of loosely packed connective tissue that forms the scaffolding for the villus, as well as containing the blood supply, lymph drainage and nervous supply for the mucosa. It also contains many cells of the innate and adaptive immune systems. The accumulation of lymphoid follicle can often be seen macroscopically as large white patches commonly known as Peyer's Patches. By their ability to transport luminal antigens and bacteria, the Peyer's Paches can be considered as the immune sensors of the intestine (14). The lamina propria and epithelium form very distinct immunological compartments. Their composition and functions also vary considerably throughout the intestine.

The ***muscularis mucosae*** is a thin smooth muscle layer located underneath the lamina propria all along the intestine and separates it from the *submucosa*. The contraction of the *muscularis mucosae* keeps the mucosal surface and underlying glands in a constant state of gentle agitation to expel contents of glandular crypts and enhances contact between epithelium and the contents of the lumen.

Below the *muscularis mucosae* lies the area of connective tissue known as the ***submucosa***, which is important for its plexus of parasympathetic nerves. Moderately dense irregular connective tissue, blood vessels, lymphatic vessels, nerve network and ganglion neurons are found here. Sometimes the lymphatic tissue of the lamina propria extends through the *muscularis mucosae* into the *submucosa*.

The **muscularis externa** of the small intestine consists of two layers of smooth muscle. The outer, thinner layer contains longitudinal fibers; the inner, thicker layer contains circular fibers. It is responsible for gut movement such as peristalsis, a radially symmetrical contraction and relaxation of muscles which propagates in a wave down the intestine tract in order to push the digested food from the stomach towards the anus.

Finally, the ***serosa*** provides the thick fibrous covering that separates the intestine from the surrounding peritoneal cavity.

Large Intestine - The histological pattern of the large intestine differs from the small intestine mainly in the mucosa. The mucosa of the large intestine shows a columnar epithelium shaped into straight tubular crypts. There are no villi unlike in the small intestine. Regarding the cellular composition, the epithelium of the large intestine resembles that of the small intestine, but with a higher proportion of goblet cells (25% of the epithelial cells), interspersed among the absorptive cells. As no Paneth cells are observed in the crypts of the large intestine, the globet cells represent the largest producers of the mucus (**Fig.2**) (7). The colon has a sophisticated mucus protection system that is under control of the host, even though it is located at a relatively large distance from the epithelial cell surface. Stem cells that constitute the source of the other epithelial cell types are located at or near the base of the intestinal glands. Enteroendocrine cells are located mainly at the base of the glands and secrete basally into the *lamina propria.* The *lamina propria*, *muscularis mucosae* and *submucosa* are similar to the ones of the small intestine. The *muscularis propria* of the large intestine is more prominent when compared to the small intestine, and consists of distinct inner circular and outer longitudinal layers.

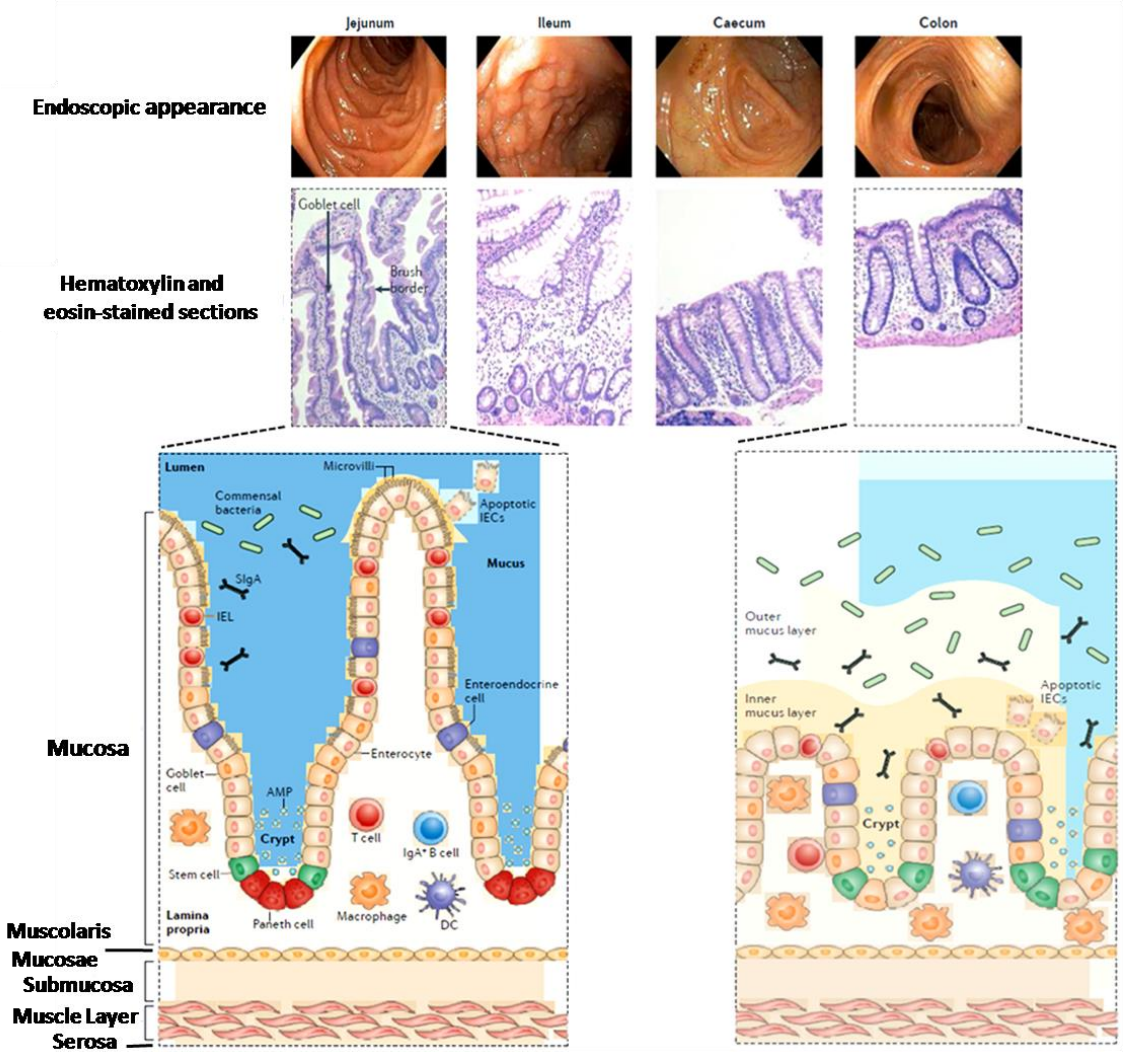

Fig.2. Anatomy and histology of the human intestine. Distinct intestinal segments of the intestine observed from the lumen by endoscopy (top panels). The upper small intestine, as exemplified by the jejunum (middle and lower panels), has long thin villi covered by a surface epithelium that has an extensive brush border (indicated by the arrow) comprising the microvilli that contain digestive enzymes. IECs (Intestinal epithelial cells) are lost from the tip of the villus and are replaced by new cells that migrate upwards from the crypts. As well as the absorptive epithelial cells, stem cells in the crypts give rise to the mucus-secreting goblet cells found on the villus (indicated by the arrow), and to Paneth cells that migrate downwards to the bottom of the crypt. Paneth cells are characterized by dense granules that contain antimicrobial peptides (AMPs). The central part of the villus comprises the lamina propria, where the majority of intestinal immune cells are found, whereas intraepithelial lymphocytes (IELs) are found lying between epithelial cells. The villi become progressively shorter and broader going down the length of the small intestine (the ileum is shown as an example), which is consistent with the lower rates of digestion and absorption that occur in these regions. The caecum is a blind-ended sac comprising the first part of the large intestine and it acts as a large reservoir for the commensal bacteria involved in the fermentative digestion of the complex carbohydrates that cannot be dealt with by small intestinal enzymes. The caecum has no villi and the mucosa consists mainly of crypts with only short regions of flat surface epithelium. Goblet cells are numerous and are found throughout the crypts. No Paneth cells are observed. Villi are absent from all parts of the colon; the main function of the surface epithelium is to reabsorb water from faeces and to act as a barrier to the commensal microbiota. This is assisted by the large number of goblet cells, which produce an extensive and thick layer of protective mucus. IELs are much rarer than in the small intestine. DC, dendritic cell; SIgA, secretory immunoglobulin A. Adapted from (15).

2. INTESTINAL CRYPTS: DINAMICS IN HOMEOSTASIS, REGENERETION AND CELLULAR LINEAGE

The astounding renewal capacity of the intestinal epithelium has made the intestine one of the favourite tissues in which to study stem-cell regulation. The fact that almost all epithelial cells in the intestinal lining are replaced on a weekly basis puts great demands on the cellular organization of this tissue, and also puts it at serious risk of malignant conversion.

2.1 Self-renewal in the intestinal epithelium

The epithelium of small intestine and colon displays a remarkable self-renewal rate, likely necessitated by the constant barrage from physical, chemical, and biological insult. Indeed, the small intestinal epithelium of the mouse completely renews every 3–5 days. The intense proliferation that fuels this self-renewal process is confined to the crypts. Individual crypts comprise around 250 cells and generate a similar number of new cells each day. Resident stem cells located close to the crypt base produce vigorously proliferating progenitors called transit-amplifying (TA) cells. This cells divide every 12–16 hours migrating upwards as coherent columns toward the crypt/villus border where gradually commit to the absorptive or secretory cell lineages and finally exit the crypts into villi (16). Their migration continues toward the villus tip, where the differentiated cells die and are shed into the lumen (Anoikis). Up to 10 crypts supply new cells to a single villus. The crypt-resident Paneth cells escape this upwardly mobile epithelial conveyer belt. Instead, they migrate downward to occupy the crypt base, where they live for 6–8 weeks (**Fig.3 A, B**). Therefore, due to the stereotypical architecture of the crypt-villus unit and this intensive self-renewal process the intestinal tract represents the best model for studying adult mammalian stem cell biology

2.2 Intestinal stem cell niche

As mentioned above, the cells of the intestinal epithelium are replaced every few days, and this renewal process is maintained by multi-potent intestinal stem cells (ISCs). An intestinal crypt contains about 14 equal ISCs that all divide each day. Their dynamics are consistent with a model in which the resident ISCs double their numbers each day and stochastically adopt either stem or transit-amplifying (TA) fates. Thus, ISCs divide symmetrically while competing for a niche of limited size. As a consequence, their turnover follows a pattern of neutral drift dynamics (17) and crypts tend toward clonality within a period of 1–6 months (**Fig.3 C**).

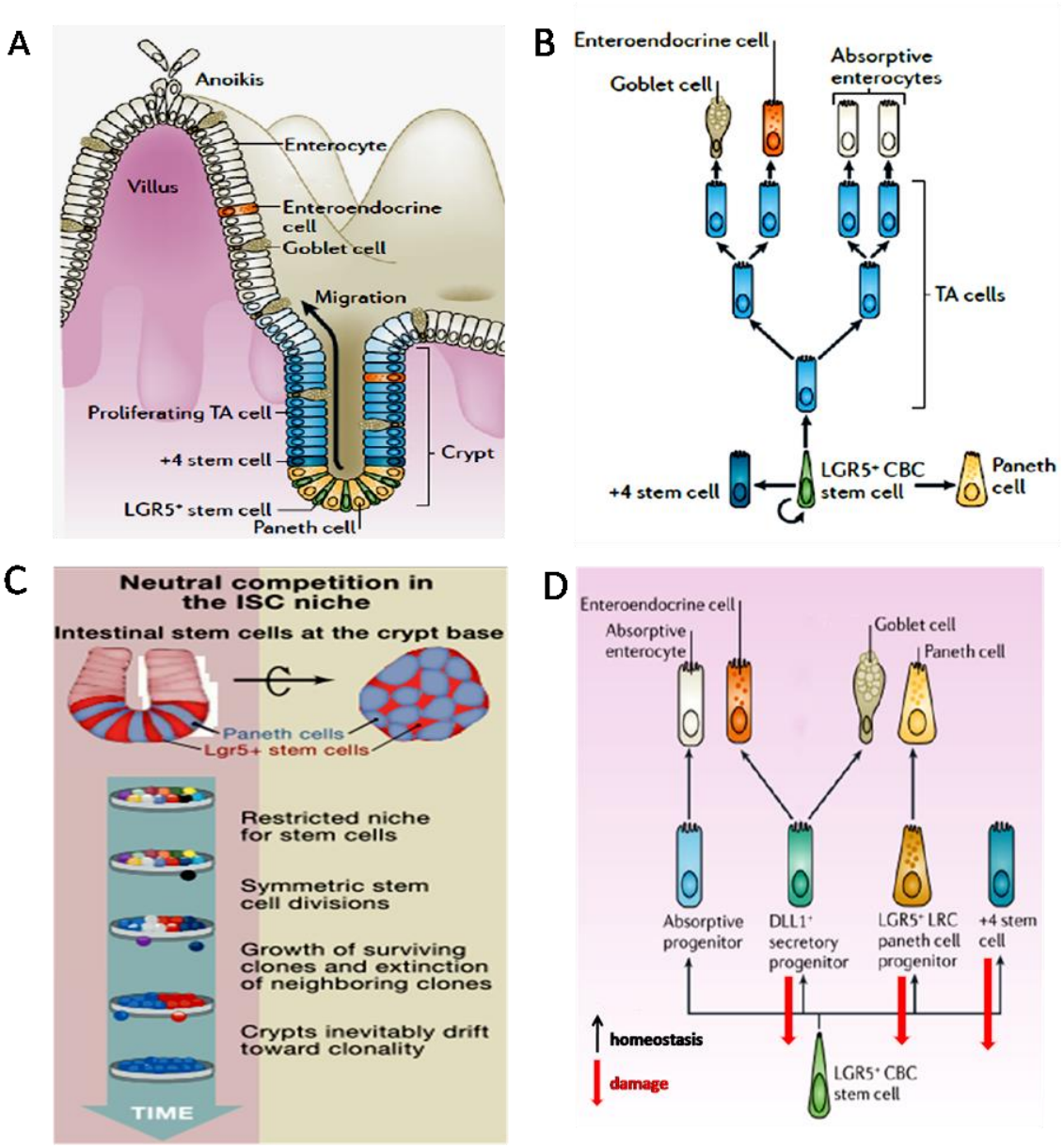

Fig.3. Epithelial self-renewal in the intestine. A) LGR5+ (Leu-rich repeat-containing G protein-coupled receptor 5-expressing) crypt base columnar (CBC) stem cells are intercalated with Paneth cells at the crypt base. During homeostasis, these cells continuously generate rapidly proliferating transit-amplifying (TA) cells, which occupy the remainder of the crypt. **B)** TA cells differentiate into the various functional cells on the villi (enterocytes, goblet cells and enteroendocrine cells) to replace the epithelial cells being lost via anoikis at the villus tip. The +4 'reserve' stem cells (which occupy the fourth position from the crypt base) can restore the LGR5+ CBC stem cell compartment following injury. New Paneth cells are supplied from the TA cells every 3–6 weeks. **C)** Neutral Competition model: intestinal stem cells generate a monoclonal crypt by assuming that at every division ISCs stochastically generate zero, one or two daughter ISCs. When zero daughter ISCs are formed, this specific clone is lost and quickly replaced by a neighboring ISC, hence explaining how several intestinal stem cells can generate monoclonal crypts. **D)** Cell hierarchy and lineage specification (homeostasis versus regeneration): acute injury results in the loss of the proliferating LGR5+ stem cells but leaves the damage-resistant Paneth cell precursors, +4 stem cells and the niche intact. Surviving DLL1+ and LGR5+ LRC secretory progenitors fall back into the surviving niche at the crypt base and are consequently converted into LGR5+stem cells to restore epithelial renewal. Adapted from (18,19).

Therefore, ISCs persist for life as a population, yet only the lineage of one particular ISC is present in each crypt at any given time (20).The location and identity of ISCs has been a subject of much research and debate. Decades ago, two non overlapping crypt stem cell populations were described. On the one hand, Leblond (21) identified a stem cell niche at the crypt base where crypt base columnar (CBC) cells were interspersed between Paneth cells. Much later, the LGR5 protein was discovered (22) to be an exquisite marker for these cells, allowing formal, experimental proof of their "stemness" *in vivo*. Indeed, single LGR5-expressing (LGR5+) cells were shown to form ever-expanding, transplantable mini gut *in vivo* (23). Moreover, additional markers were identified for CBC cells including ASCL2 (24), OLFM4 (25), SMOC2 (26), and SOX9 (27), thus confirming the "stem cell zone" model proposed by Leblond. On the other hand, the presence of DNA-label-retaining cells (LRCs) was noted by Potten (28) directly above the Paneth cells at position +4 (counting Paneth cell nuclei from the bottom of the crypts). The model proposed by Potten, described the +4 cells as proliferative and unusually radiation-sensitive, although not functional data was obtained to validate the hypothesis. Recent efforts to identify +4 stem cells have focused on the identification of cells that are quiescent and radiation-resistant. A number of putative markers for +4 LRCs cells including BMI1 (29), HOPX (30), LRG1 (31,32) and TERT (33,34) providing a distinct population of slowly cycling stem cells that can also generate all intestinal lineage. Instead of constituting irrevocably separated lineages, it seems that LRG5+ and +4 stem cells can interconvert. The highly proliferative LRG5+ base columnar cells appear to be the "workhorse" of daily intestinal renewal (35). Yet, slowly cycling "reserve" +4 stem cells can be recalled to LGR5+ status following tissue injury (36) and viceversa (30). Adding further complexity, the two stem cell lineages may be partially overlapping: LGR5+ cells can coexpress +4 markers (such as BMI1) (26,37). Indeed, whereas the majority of LGR5+ cells are proliferative stem cells, a subset of LGR5+ cells are non-dividing precursors that coexpress +4 markers and are destined to differentiate into Paneth cells (37). These precursors, can be promoted to multipotent stem cell status upon tissue damage to effect intestinal repair (37). Similar observations have been made for DLL1+ cells that are secretory lineage precursors (38). Interestingly, mice lacking the Lgr5+ stem cells under irradiation can convert Dll1+ cells into intestinal stem cells to generate all cell types of the intestinal epithelium (38) (**Fig.3 D**). So, rather than relying on a single, hard-wired stem cell compartment to maintain epithelial homeostasis and effect tissue regeneration following injury, the intestine seems capable of drawing on several pools of highly plastic, reserve stem cell populations in the lower regions of the crypt. Such plasticity is likely to be endowed by the specialized niche environment at the crypt base, which provides the requisite signals to efficiently convert committed progenitor populations into multipotent adult stem cells.

2.3 How are crypts and villi made? Signaling and morphogenetic pathways

Villi begin to form at embryonic day 15; crypts form substantially later, around postnatal day 7. What, then, is the mechanism that organizes the formation of villi and crypts? It has long been known that the modeling of the intestinal epithelium depends on epithelial mesenchymal interactions (39): the **hedgehog**, **platelet-derived growth factor (PDGF)**, and **BMP** signaling pathways represent key mediators of these two-way communications. Indeed, mutations in these pathways derange the construction of crypts and villi. Moreover, within the epithelium, cells signal to one another through the **WNT**, **NOTCH** and **EPH/Ephrin** pathways: mutations that affect these pathways cause marked changes in the distribution of cell types along the crypt–villus axis. The challenge is to understand not just the action of each type of signal individually, but how the whole set of signals operates as a system to organize the crypt–villus architecture and to control the patterning and renewal of the gut epithelium (**Fig.4 A, B**).

Hedgehog demarcates villus from crypt - In the small intestine of the developing mouse, expression of the two ligands Sonic hedgehog (SHH) and Indian hedgehog (IHH) is restricted to the epithelium and becomes progressively concentrated in the intervillus regions of the epithelium as villus morphogenesis proceeds. Meanwhile, expression of the receptors patched-1 (PTCH1) and patched-2 (PTCH2), and the effectors of hedgehog signaling, GLI1, GLI2 and GLI3, is restricted to the underlying mesenchyme (40). Hedgehog signaling from the gut epithelium to the mesenchyme is crucial for development of the connective-tissue coat around the gut tube, but it is no less important for the behavior of the epithelial cells themselves, which are powerfully affected by feedback from the mesenchymal cells. Blocking of the hedgehog signal by strong overexpression of an inhibitor, hedgehog-interacting protein (HHIP), leads to a complete absence of villi and the persistence of a highly proliferative intestinal epithelium with increased activation of the Wnt pathway and a deficit of properly differentiated cells (40). Furthermore, conditional deletion of patched-1, a negative regulator of the pathway, leads to premature enterocyte differentiation, myofibroblasts accumulation and colonic crypt hypoplasia (41). Then, the hedgehog signaling from epithelium to mesenchyme is required for the formation of villi and the concomitant restriction of proliferation to the intervillus regions within the intestinal epithelium. This process must depend on a feedback loop in which mesenchymal cells respond directly to hedgehog from the epithelium potentially through BMP secretion (41) and deliver a signal back to the epithelium by some other signaling pathways (**Fig.4 C**).

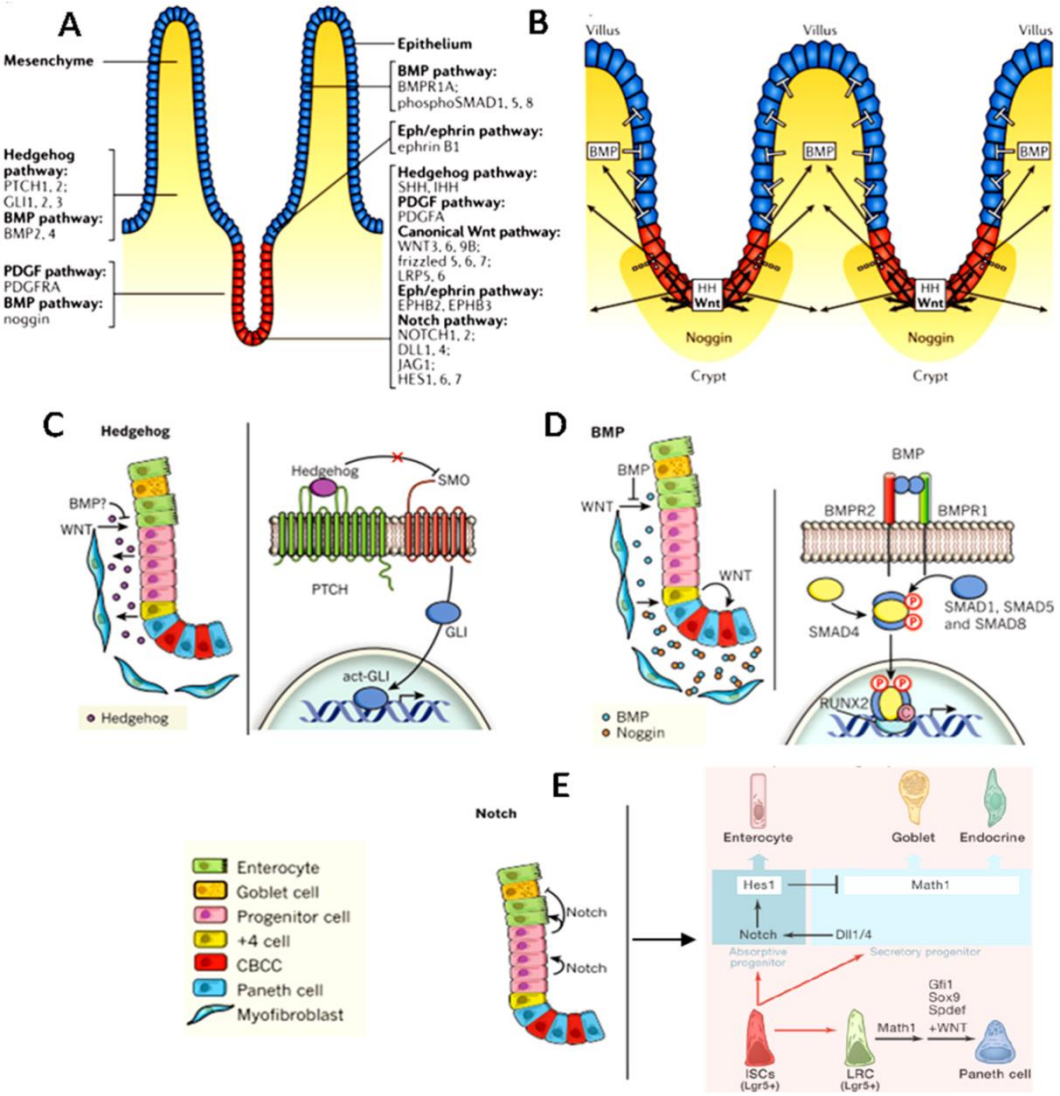

Fig.4. Signaling pathways in the small intestine. A) Components of the hedgehog (HH), platelet-derived growth factor (PDGF), bone morphogenetic protein (BMP), WNT, EPH/Ephrin and NOTCH pathways are expressed in different regions along the crypt–villus axis, some in the epithelium and some in the mesenchyme. The brackets list, for each indicated region, the pathway components that are expressed there. **B)** A model of how the HH, BMP and Wnt signaling pathways combine to organize the pattern of intestinal villi and crypts. Epithelial cells in each crypt form a signaling centre, which functions as a source of long-range inhibition through the HH–BMP relay, and of short-range auto-activation through Wnt signaling. HH signaling activates the expression of BMP in the mesenchyme. BMP feeds back on the intestinal epithelium to repress Wnt signaling. The expression of the BMP inhibitor noggin in the neighborhood of the crypts counteracts the effect of BMP so that Wnt activity is maintained in the crypt epithelium. **C)** Hedgehog signaling relies on the interaction between patched (PTCH) and smoothened (SMO). Patched represses smoothened, but is blocked when bound to Hedgehog. Derepressed smoothened activates GLI transcription factors (act-GLI), which translocate directly to the nucleus and drive the transcription of Hedgehog target genes. **D)** BMP proteins counteract proliferative WNT signals and thereby halt proliferation and drive differentiation, at the crypt bottom BMPs are blocked by noggin. Signaling by BMPs depends on the heterodimerization of the BMPR1 and BMPR2 receptors, leading to phosphorylation of SMADs protein and association with SMAD4. The complex translocates to the nucleus driving the transcription of BMP target genes. **E)** NOTCH signaling is involved in lineage-fate decisions: MATH1 promotes elevated levels of delta-like ligands (Dll1/Dll4) in secretory precursors. DELTA ligands on the surface of one cell activate NOTCH receptors on neighboring cells. In contrast, expression of MATH1 is repressed in enterocytes by the NOTCH downstream effector HES1. Adapted from (19,42,43).

Hedgehog is not the only signal passing from epithelium to mesenchyme. PDGFA, like SHH and IHH, is made by the epithelial cells, and its receptor, PDGFRA, is expressed in the mesenchyme. PDGF signaling helps to control the behaviour of the mesenchyme and the shaping of villi, but does not, apparently, evoke signals that act back on the epithelium to regulate its proliferation or differentiation (44).

Bone morphogenetic protein (BMP) inhibits crypt formation - If hedgehog and PDGF signals are both delivered from the epithelium to the mesenchyme, other molecules convey signals from the mesenchyme back to the epithelium to control its regional differentiation. One pathway, at least, has been identified: BMP2 and BMP4 are both expressed in the mesenchyme, where they are positively regulated by hedgehog signaling (40); their receptor, BMPR1A, is expressed in the epithelium. The BMP antagonist noggin is expressed in the neighborhood of the crypts, whereas activation of the BMP pathway, as indicated by the presence of phosphoSMAD1, 5 and 8, is seen most strongly in the epithelium of the villi. When the receptor is knocked out, or the antagonist noggin is overexpressed, excessive quantities of crypt like structures develop. In the noggin overexpression mutant, these occur on the sides of the villi (45). Similar abnormalities are seen in humans with juvenile polyposis syndrome, which can be traced to mutations in BMPR1A or SMAD4 (a key downstream effector of BMP signaling) (46,47). This evidence strongly suggests that BMP signaling is a key factor, if not the key factor, that mediates the action of hedgehog, blocking the formation of ectopic crypts, and that the expression of noggin in the neighborhood of each crypt base protects the epithelium in this region from the action of BMPs, thereby enabling proliferation to continue (**Fig.4 D**).

Notch Signaling: the first decision between absorptive and secretory cell fate - Notch and its ligands of the Delta (DLL1, DLL4) and Serrate/Jagged subfamilies are transmembrane proteins that mediate cellular communication by direct cell to cell contact. When the Notch pathway is partially inactivated in mouse by deletion of *Hes1* (hairy and enhancer of split 1), the most important Notch downstream effector, excessive numbers of goblet and enteroendocrine cells are produced (48). Stronger inhibition of Notch signaling by use of a γ-secretase inhibitor that prevents the release of N^{ICD} (49,50) ('activated Notch', the active intracellular domain of Notch), results in a more extreme effect: the intestinal epithelium of the mouse becomes almost exclusively composed of goblet cells. Increased activity of Notch signaling has the opposite effect on intestinal cell differentiation: mice that express N^{ICD} constitutively in the gut epithelium show a severe reduction of all three secretory cell types (51).

In the normal tissue, the cells that become secretory are those that escape neighbors from differentiating in the same way (**Fig.4 E**). The expression of MATH1 (mouse atonal homologue 1) which encodes bHLH transcription factor commits precursor cells to a secretory phenotype. MATH1 also promotes elevated levels of delta-like ligands (DLL1/DLL4) in secretory precursors. In contrast, the enterocytes repress MATH1 by Notch downstream effector HES1. *Math1* mutant mice lack all three secretory gut cell types but still generate absorptive cells (52). The Notch-controlled choice between an absorptive fate (MATH1 negative, receiving lateral inhibition) and a secretory fate (MATH1 positive, delivering lateral inhibition) might therefore be the first of the decisions made by daughters of stem cells as they become committed to differentiation (**Fig.4 E**).

Canonical Wnt signaling maintains stem cell proliferation and makes cells competent for secretory fate - The discovery of the common origin of the Drosophila segment polarity gene Wingless and the murine proto-oncogene *Int-1* (53) laid the keystone of a signaling pathway now commonly referred to as the canonical Wnt cascade (**Fig.5**). The Wnt signaling pathway was the first (54) to be implicated in the control of the gut stem-cell system, and a large body of evidence shows that activation of the Wnt pathway is the key factor that maintains the crypt cell population in a proliferative state. When the pathway is overactivated, crypts enlarge; when the pathway is blocked, they disappear. For instance, inactivating mutations in adenomatous polyposis coli (APC) or activating mutations in β-catenin (CTBNN1) both key Wnt signaling factors drive intestinal hyperplasia (55). Similarly, overexpression of the Wnt activator R-spondin-1 induces ISC expansion in vivo (56). Conversely, transgenic expression of the Wnt inhibitor DKK1 (57) or deletion of the transcription factor TCF4 (58) results in a block in Wnt signals and subsequent depletion of intestinal proliferative compartments in fetal mice. Although Wnt proteins are expressed in a highly complex fashion by both epithelial and mesenchymal cells, nuclear localization of β-catenin is only observed at the crypt bottom. More data show that Paneth cells residing next to ISCs are one of the main sources of WNT3A, and they consequently spatially constrain ISCs to the crypt bottom (59). Furthermore, the Paneth cells dictate the size of the stem-cell pool: their deletion decrease the number of ISCs in the crypt (60). Not less important is the role of Wnt signals in guiding the cells to a secretory fate. Indeed, the Paneth cells show signs of sustained Wnt pathway activation. They reside at the base of crypts, where WNT protein is plentiful, and Wnt signaling drives their differentiation (61,62).

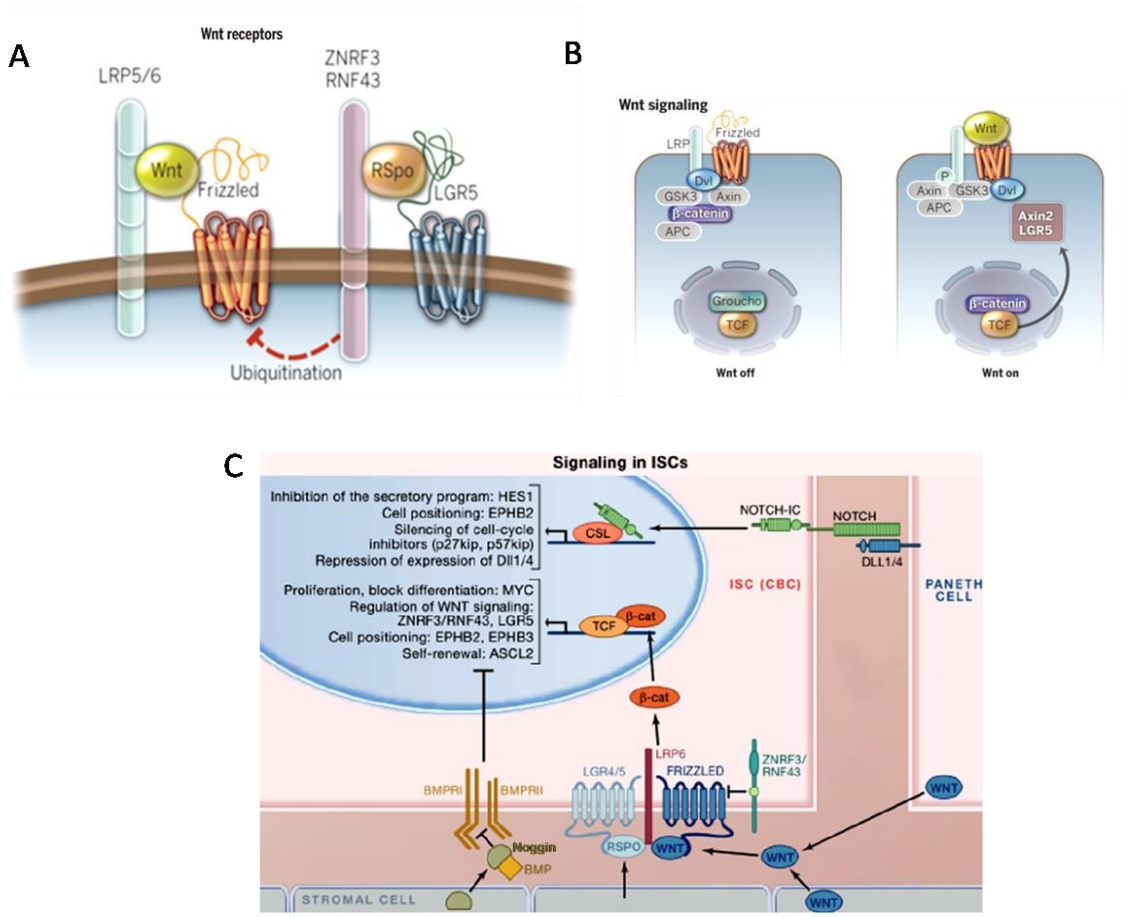

Fig.5. Wnt signaling mechanisms. A) Wnt reception on the cell surface: Wnt ligands bind to the Frizzled and LRP5/6 receptors, activating downstream signaling. The membrane proteins ZNF3 and RNF43 are ubiquitin ligases that continually down-regulate Frizzleds through ubiquitination. Binding of R-spondin to ZNRF3 and RNF43 and the LGR4/5/6 receptor relieves ZNRF3 and RNF43 activity, thus stabilizing Frizzleds. **B)** Wnt signaling in target cells: (Left) in the absence of Wnt, a destruction complex consisting of AXIN, APC, and GSK3 resides in the cytoplasm, where it binds to and phosphorylates β-catenin, which is then degraded. DVL (Disheveled) is required for activating the pathway as well. In the nucleus, Tcell factor (TCF) is in an inactive state as the consequence of binding to the repressor Groucho; (Right) binding of Wnt to its receptors induces the association of AXIN with phosphorylated lipoprotein receptor-related protein (LRP). The destruction complex falls apart, and β-catenin is stabilized, subsequently binding TCF in the nucleus to up-regulate target genes, including AXIN2 and LGR5. **C)** Signaling in ISCs is specified by high levels of Wnt signaling in the crypts. Paneth cells secrete the WNT3 ligand constitutively, but an additional Wnt source also exists in the surrounding stroma. NOTCH mainly acts by inhibiting the secretory fate in ISCs pool. NOTCH ligands (DLL1 and DLL4) are expressed by surrounding secretory cells, including the Paneth cells. BMPs are mainly expressed by stromal cells that surround the epithelium, whereas ISCs are protected from their action by the presence of local inhibitors, including Noggin. Adapted from (19,63).

All the other terminally differentiated intestinal cell types absorptive, goblet and enteroendocrine maintain their differentiated characters in areas where canonical Wnt signaling is not active, but under the influence of Wnt signaling or shortly after the cells seem to be maintained in a secretory fate. When Wnt signaling is blocked by overexpression of DKK1, the absorptive cells still differentiate normally, but all classes of secretory cells seem to be lost (57). Indeed, the same phenotype is observed in *Math1* mutant mice. Therefore, Wnt/β-catenin pathway is essential for the maintenance of MATH1-positive progenitors for the secretory cell lineage. Nonetheless, MATH1 itself did not appear to be a direct target gene of TCF4 (57). In the opposite circumstance, where the Wnt pathway is overactivated by the loss of APC, there is a more general failure to differentiate, leading to reduced expression of markers of absorptive, goblet and enteroendocrine cells, but with an overproduction of Paneth cell precursors (61).

WNT and NOTCH jointly maintain stem cells - As discussed above, Wnt pathway has an important role in maintaining crypt cell population in a proliferative state while Notch signaling controls the choice between differentiating as an absorptive or secretory cell. The truth, however, seems to be more interesting. NOTCH, DELTA and HES proteins are in fact chiefly expressed in the neighborhood of the crypt base, in the stem-cell region (64). When Notch signaling is blocked, secretory cells are overproduced. However, this does not only occur at the expense of differentiated absorptive cells, indeed, it seems that the whole cell population of the adult intestinal crypt is converted to a secretory character and surprisingly stops proliferating (49). Overactivation of the Wnt signaling pathway, at least as seen in adenomas of mice Apc^{Min}, is not sufficient to overcome this proliferation failure. When the mice are treated with a γ-secretase inhibitor that abolishes Notch signaling, proliferation is blocked within the intestinal adenomas (49). The opposite combination of signals, overactivation of the Notch pathway along the villus epithelium, where the canonical Wnt pathway is inactive is equally unable to drive proliferation (65). Forced expression of N^{ICD} (“activated” Notch) in the new born mouse increase the population of proliferating cells, but mainly in the intervillus regions, where Wnt signaling is active. Therefore, it seems that all the proliferating cells, including the stem cells, depend on Notch and Wnt signals in combination to keep them in a proliferating state: neither Wnt pathway activation nor Notch pathway activation is sufficient by itself.

WNT controls EPH/Ephrin signilling - Through selective cell migration, the different categories of cells in the intestinal epithelium are segregated into separate regions: the cluster of WNT-activated cells avoids becoming diluted with differentiated cells that lack WNT activity (Paneth cells being a special case), whereas the population of differentiated cells on the villi avoids contamination with proliferative stem cells. WNT

levels define the tissue boundary between proliferative and differentiated compartement by modulating the expression of EPHB2 and EPHB3 receptors as well as Ephrin-B1 ligand (discussed in detail in chapter 5) (66).

Despite stringent homeostatic maintenance in the intestine, the high number of patients with colorectal cancer (CRC) indicates that these regulatory mechanisms often fail in protecting against malignant transformation. Both environmental and genetic risk factors have been defined, and not surprisingly the deregulation of morphogenetic pathways, mainly Wnt signaling, play a key part in colorectal cancer development.

3. CANCER

Cancer is, in essence, a genetic and epigenetic disease. Alterations in three types of genes are responsible for tumorigenesis: oncogenes, tumor-suppressor genes and stability genes. Mammalian cells have multiple safeguards to protect them against the potentially lethal effects of cancer gene mutations, and only when several genes are defective does an invasive cancer develop. Therefore, most of mutated cancer genes contribute to, rather than cause, the cancer. Oncogenes are mutated in ways that render the gene constitutively active or active under conditions in which the wild-type gene is not. Tumor suppressor genes are targeted in the opposite way by genetic alterations: mutations reduce the activity of the gene product. Such inactivations arise from missense mutations at residues that are essential for its activity, from mutations that result in a truncated protein, from deletions or insertions of various sizes or from epigenetic silencing. A third class of cancer genes, called stability genes or caretakers, promotes tumorigenesis in a completely different way when mutated. This class includes the mismatch repair (MMR), nucleotide-excision repair (NER) and base-excision repair (BER) genes responsible for repairing subtle mistakes made during normal DNA replication or induced by exposure to mutagens. Stability genes keep genetic alterations to a minimum, and thus when they are inactivated, mutations in other genes occur at a higher rate (67). All genes are potentially affected by the resultant increased rate of mutation, but only mutations in oncogenes and tumor suppressor genes affect net cell growth and can thereby confer a selective growth advantage to the mutant cells (68). In keeping with this, an interesting study revealed about 140 genes that, when altered by intragenic mutations, can promote or “drive” tumorigenesis (69). A typical tumor contains two to eight of these “driver gene” mutations, the remaining mutations are passengers and do not confer any growth advantage. Moreover, driver genes can be classified into one or more of 12 pathways that regulate three core cellular processes: cell fate, cell survival, and genome stability (**Fig.6**).

3.1 Signaling pathways in tumors

Cell fate - Numerous studies have demonstrated the opposing relationship between cell division and differentiation, the arbiters of cell fate. Dividing cells that are responsible for populating normal tissues (stem cells) do not differentiate, and viceversa. Many of the genetic alterations in cancer abrogate the precise balance between differentiation and division favoring the latter. This causes a selective growth advantage, because differentiating cells eventually die or become quiescent. Pathways that function through this process include APC, HH and NOTCH all of which are well known to control cell fate in organisms ranging from worms to mammals (70). Genes encoding chromatin-

modifying enzymes can also be included in this category. In the normal development, the heritable switch from division to differentiation is not determined by mutation, as it is in cancer, but rather by epigenetic alteration affecting DNA and chromatin proteins.

Cell survival - Though cancer cells divide abnormally because of cell-autonomous alterations, such as those controlling cell fates, their surrounding stromal cells are perfectly normal at genetic level. The most obvious ramification of this asymmetry is the abnormal vasculature of tumors. As opposed to the well-ordered network of arteries, veins, and lymphatics that control nutrient concentrations in normal tissues, the vascular system in cancers is tortuous and lacks uniformity of structure (71,72). As a result, a cancer cell acquiring a mutation that allows it to proliferate under limiting nutrient concentrations will have a selective growth advantage (73). Mutations of this sort occur, for example, in the *EGFR*, *HER2*, *FGFR2*, *PDGFR*, *TGFβRII*, *RAS*, *RAF*, *PIK3CA*, and *PTEN* genes. Some of these genes encode receptors for the growth factors themselves, whereas others relay the signal from the growth factor to the interior of the cell, stimulating growth when activated (74). For instance, mutations in *KRAS* or *BRAF* confer on cancer cells the ability to grow in glucose concentrations that are lower than those required for the growth of normal cells or the cancer cells that do not have mutations in this gene (75,76). In addition, progression through the cell cycle (and its antithesis apoptosis) can be directly controlled by intracellular metabolites, and driver genes that regulate the cell cycle or apoptosis, such as *MYC* or *BCL2* are often mutated in cancer. Another driver gene is *VHL*, whose mutations enhance cell survival through secretion of vascular endothelial growth factor, thus stimulating angiogenesis.

Genome maintenance - As a result of the exotic microenvironments in which they reside, cancer cells are exposed to a variety of toxic substances, like the reactive oxigens species. Even without microenvironmental poisons, cells make mistakes while replicating their DNA or during cell division (77) and check-points exist to either slow down these cells or make them commit to suicide (apoptosis) under such circumstances (78). Although it is good for the organism to remove damage cells, tumor cells have the capacity to avoid those mechanisms that underlie the programmed cell death. Therefore, it is not surprising that genes whose mutations abrogate these checkpoints, such as *TP53* are often mutated in cancer. Defects in these genes can also indirectly confer a selective growth advantage by allowing cells that have a gross chromosomal change favoring growth, like a translocation or an extra chromosome, to survive and divide. Analogously, genes that control point mutation rate, including *MLH1* or *MSH2*, are mutated in cancer or in germ line of patients predisposed to cancer because they accelerate the acquisition of mutations that function through mechanisms that regulate

cell survival or cell fate. What better way than to promote cancer by increasing the incidence of the mutations that drive the process?

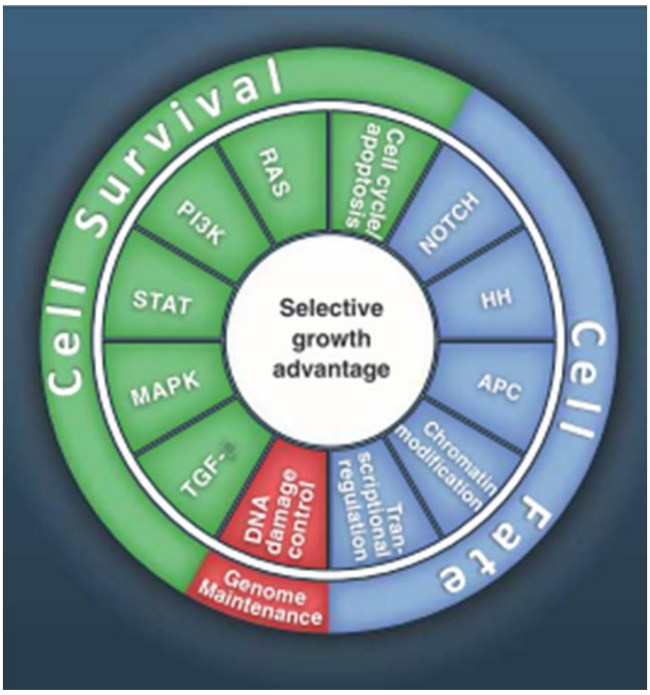

Fig.6. Cancer cell signaling pathways and the cellular processes that they regulate. All drivers genes can be classified in one or more of 12 pathways (middle ring) that confer a selective growth advantage (inner circle). These pathways can themselves be further organized into three cores cellular processes (outer ring) (69).

4. COLORECTAL CANCER

Colorectal cancer (CRC) is a major cause of cancer morbidity and mortality. After lung cancer which is predicted to cause in EU nearly 280 000 deaths in both sexes combined in 2015 corresponding to over 20% total cancer deaths, the cancer with the second largest impact is intestinal cancer (colon and rectum), with predicted rates of 16.6 and 9.4/100,000 in men and women, corresponding to 5.3% and 8.1% cancer related death, respectively. Intestinal cancer deaths represent 13% of total cancer mortality in the EU in 2015, with 172,600 projected deceases (**Fig.7**) (79).

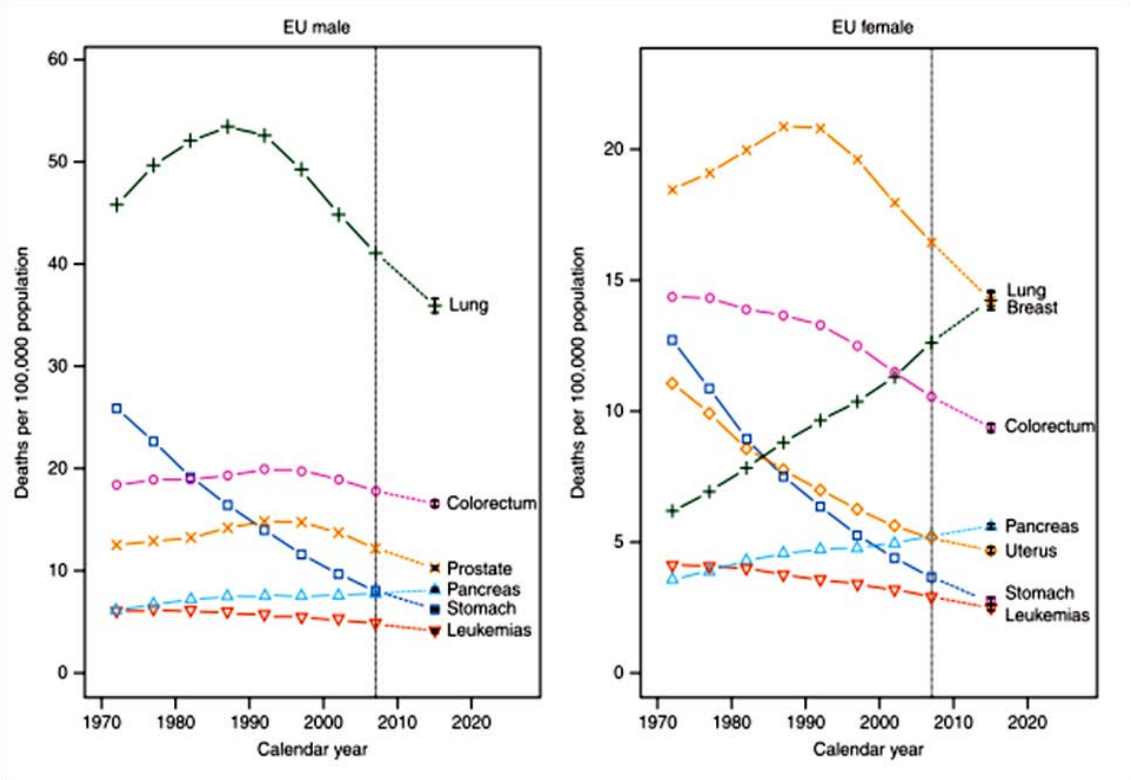

Fig.7. Age-standardised (world population) EU male and female cancer mortality rate trends. Quinquennia from 1970–1974 to 2005–2009 and predicted rates for 2015 with 95% prediction intervals (PIs). Men: stomach (squares), colorectum (circles), pancreas (triangles), lung (crosses), prostate (xs) and leukaemias (inverted triangles). Women: stomach (squares), colorectum (circles), pancreas (triangles), lung (crosses), breast (xs), uterus (diamonds) and leukaemias (inverted triangles). Adapted from (79).

The etiological factors and pathogenetic mechanisms underlying CRC development appear to be complex and heterogeneous. Contributory agents and mechanisms in CRC include dietary and lifestyle factors and inherited and somatic mutations. Among the most significant dietary and lifestyle risk factors for CRC appear to be a diet rich in unsaturated fats and red meat, total energy intake, excessive alcohol consumption, and reduced physical activity. In contrast to the modest progress achieved in defining lifestyle and environmental risk factors, there has been significant progress in identifying the specific gene defects that underlie inherited predisposition to CRC, as well as the constellation of somatic (i.e., arising in non germ cells during the patient's lifetime) alterations that are present in sporadic CRCs.

4.1 Characteristics and clinical classification

Benign gastrointestinal tumors are a varied group, but localized lesions that project above the surrounding mucosa are commonly termed polyps. In humans, most colorectal polyps, particularly small polyps less than 5 mm in size, are hyperplastic (80). Most data indicate that hyperplastic polyps are not a major precursor to CRC; rather, the adenomatous polyp, or adenoma, is probably the important precursor lesion (80). Adenomas arise from glandular epithelium and are characterized by dysplastic morphology and altered differentiation of the epithelial cells in the lesion. The epithelium of adenomas can form glands (tubular adenoma), finger-like projection (villus adenoma), or a combination of both (tubulovillus adenoma). More rarely, adenomas can present a flat or depressed morphology, these are called serrated adenomas and include traditional serrated adenoma (TSA), mixed polyp, and sessile serrated adenoma (SSA) (81). There is a high risk of CRC in individuals whose adenomas are not removed, and polypectomy decreases the risk of CRC (82). Foci of adenocarcinomas can often be detected in adenomatous polyps and residual regions of adenomatous epithelium are often observed in CRC specimens. Importantly, only a fraction of adenomas progress to cancer, and progression probably occurs over years to decades. For instance, adenomas roughly 1 cm in size may have an approximately 10% to 15% chance of progressing to carcinoma (83). Colorectal cancer is the end result of a multistep process of colon neoplasia. Once cancer forms in the large intestine, it can grow through the lining and into the wall of the colon or rectum. Cancers that have invaded the wall can also penetrate blood or lymph vessels. Cancer cells typically spread first into nearby lymph nodes then can also be carried in blood vessel to the liver, lung or other organs. The process through which cancer cells travel to distant parts of the body through blood or lymphatic vessels is called metastasis (**Fig.8**).

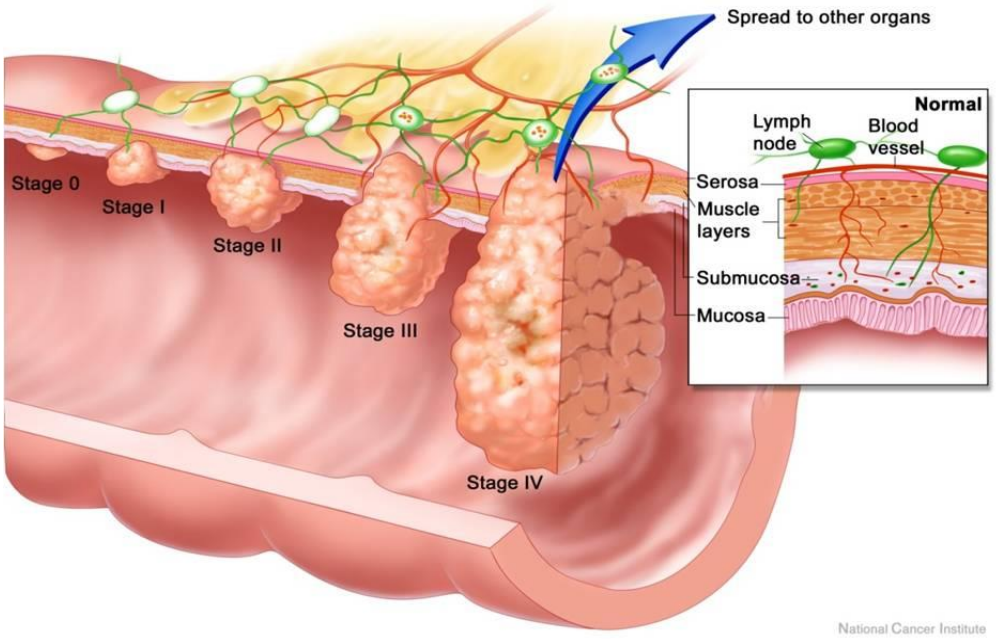

Fig.8. The growth from polyp to metastatic tumor. In stage 0, abnormal cells are found in the mucosa of the colon wall. These abnormal cells may become cancer and spread. Stage 0 is also called carcinoma *in situ*. In stage I, cancer has formed in the mucosa of the colon wall and has spread to the submucosa. Cancer may have spread to the muscle layer of the colon wall. Stage II tumors have spread through the muscle layer of the colon wall to the serosa. In stage III, cancer has spread through the mucosa to the submucosa and to nearby lymph nodes. In stage IV the cancer has spread through the blood and lymph nodes to other parts of the body, such as the lung, liver, abdominal wall, or ovary. Image from the Terese Winslow, US Govt.

Currently, the gold standard to predict cancer prognosis remains clinicopathological staging. The recommended staging system is the TNM classification, although systems based on that proposed by Dukes (Dukes staging system) in 1932 are also used (84). The TNM staging system is based on the size and extension of the primary tumor, its lymphatic involvement, and the presence of metastases to classify the progression of cancer. This system provides 3 key pieces of information: “T” describes how far the main (primary) tumor has grown into the wall of the intestine and whether it has grown into nearby areas; “N” describes the extent of spread to regional lymph nodes; “M” indicates whether the cancer has spread (metastasized) to other organs of the body. The numbers appearing after this letter (from 0 to 4) serve to indicate increasing severity. The letter X means "cannot be assessed because the information is not available". Once the TNM categories of a person have been determined, usually after surgery, this information is combined in a process called stage grouping. The stage is expressed in Roman numerals from stage I (the least advanced) to stage IV (the most advanced), whereas, the letters from A to D reminds to Dukes classification. “Dukes A” indicates that the cancer is only affecting the innermost lining of the colon or rectum or slightly growing into the muscle layer; in “Dukes B” the cancer has grown through the muscle layer of the colon or rectum; “Dukes C” is characterized by the spreading of the cancer to at least one lymph node in the area; in “Dukes D” the cancer has spread to distant organs in the body, commonly the liver or the lungs (see **Table 1**).

Another factor that can affect the outlook for survival is the grade of the cancer. Grade is a description of how closely the cancer resembles normal colorectal tissue when looked at under a microscope. The scale used for grading colorectal cancers goes from G1 (where the cancer looks much like normal colon tissue) to G4 (where the cancer looks very abnormal). The grades G2 and G3 fall somewhere in between. The grade is often simplified as either "low-grade" (G1 or G2) or "high-grade" (G3 or G4). Low-grade cancers tend to grow and spread more slowly than high-grade cancers.

T—primary tumour				
TX	Primary tumour cannot be assessed			
T0	No evidence of primary tumour			
Tis	Carcinoma in situ: intraepithelial or invasion of lamina propria			
T1	Tumour invades submucosa			
T2	Tumour invades muscularis propria			
T3	Tumour invades through the muscularis propria into subserosa or into non-peritonealised pericolic or perirectal tissues			
T4	Tumour directly invades other organs or structures and/or perforates visceral peritoneum			
N—regional lymph nodes				
NX	Regional lymph nodes cannot be assessed			
N0	No regional lymph node metastasis			
N1	Metastasis in 1 to 3 regional lymph nodes			
N2	Metastasis in 4 or more regional lymph nodes			
M—distant metastasis				
MX	Distant metastasis cannot be assessed			
M0	No distant metastasis			
M1	Distant metastasis			
Stage (Dukes)	**T**	**N**	**M**	**5-year overall survival**
Stage I	T1, T2	N0	M0	80–95%
Stage IIA	T3	N0	M0	72–75%
Stage IIB	T4	N0	M0	65–66%
Stage IIIA	T1, T2	N1	M0	55–60%
Stage IIIB	T3, T4	N1	M0	35–42%
Stage IIIC	Any T	N2	M0	25–27%
Stage IV	Any T	Any N	M1	0–7%

Table 1. TNM staging system for colorectal cancer and published survival rates for different stages. Source (85).

4.2 Multistep genetic model of colorectal cancer : "Vogelgram"

In 1990, Fearon and Vogelstein proposed the first progressive development model of colon cancer and presented some of the key genetic changes associated with the stages of progression (86). They showed a schematic presentation nicknamed "Vogelgram" which correlates the genetic/genomic changes with the stages of colon cancer progression. The inactivation of the tumor suppressor adenomatous polyposis coli (*APC*) is observed at an early stage of colon tumor development, activation of the *KRAS* oncogene is associated with transition from early adenoma to intermediate adenoma. Genomic level changes such as loss of chromosome 18q, along with loss of deleted in colon cancer (*DCC*) loci, are observed in transition from intermediate adenoma to late adenoma and loss of tumor suppressor p53 (*TP53*) is associated with late adenoma-carcinoma transition (**Fig.9**). Although a preferred order for the genetic alterations exists, this model suggests that the progressive accumulation of these changes rather their order is the most important feature of the clinical and histopathogical progression of colorectal tumors. Despite the "Vogelgram" is still accepted, newer information from genomics, cytogenetics, and tumor mass sequencing is being added to advance our understanding. At present, three different pathways seem to be implicated in the development of colorectal cancer: 1) chromosomal instability (CIN); 2) microsatellite instability (MSI); 3) CpG island methylator phenotype (CIMP). Of note, these three phenotypes are not mutually exclusive and often can coexist in the same tumor (87).

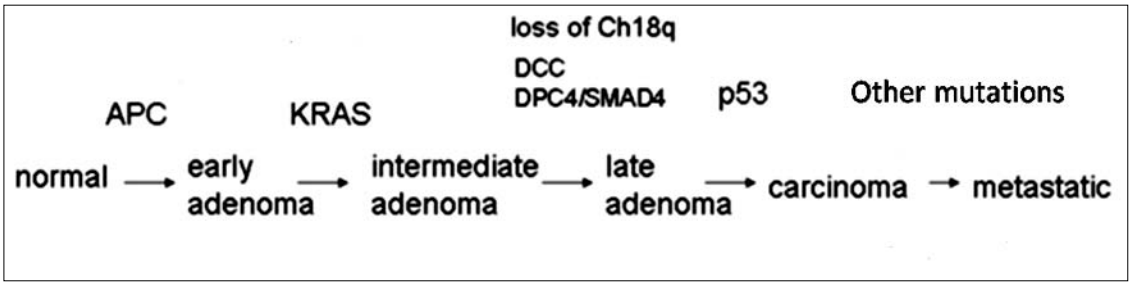

Fig.9. "Vogelgram". Loss of adenomatous polyposis coli (*APC*) which causes the adenoma formation is the initial step of intestinal tumorigenesis. Larger adenomas acquire mutations in *KRAS* gene, followed by loss of chromosome 18q with *DPC4/SMAD4*. Mutations in *TP53* are responsible of late adenoma-carcinoma transition. Tumors continue to progress once carcinomas have formed and accumulation of genetic alterations correlates with the ability of the carcinoma to metastasize and cause death (88).

4.3 The distinct paths of colorectal cancer

DNA mismatch repair gene defects in colorectal cancer: Microsatellite Instability (MSI) - Microsatellite sequences are repeating stretches of DNA located throughout the entire genome, including intronic parts of genes, gene promoters, untranslated terminal regions and exonic parts of genes. From one to six base pairs long and scattered through the human genome many thousand times (89), the microsatellite sequence constitute one of the most abundant classes of repetitive DNA families and show a great susceptibility to insertions and deletions at the time of replication. The DNA replication requires high level of fidelity because replication errors might induce mutations in every daughter cell. Because of this, the human cells need proofreading specialized mechanisms and one of the most important is called mismatch repair (MMR). An intact MMR lowers the rate of mutation from one hundred to six hundred times (90). The hereditary form of colorectal cancer with microsatellite instability is known as Lynch Syndrome or hereditary nonpolyposis colorectal cancer (HNPCC), and it represents about 1-3% of all CRC incidence. These tumors arise from a germline mutation in one of mismatch repair genes (commonly *MLH1* and *MSH2* (91)), followed by a second hit of mutation on the wild type copy (inherited from the unaffected parent) that often occur via loss of heterozygosity (LOH) or point mutations (92). However, most cases of CRC associated with MSI are not inherited (familial), but arise through sporadic methylation-induced silencing of *MLH1*. When the *MLH1* promoter is methylated, MMR activity fails and MSI ensues. Thus, the mutational profile of sporadic colorectal tumors with microsatellite instability frequently include CIMP signature. These sporadic forms of CRC develop approximately in 15% of all CRC cases (93) and often arise from sessile serrated adenomas (SSA) with frequent mutation in *BRAF* (usually V600E) (94,95). Among the most frequently mutated genes in MSI tumors approximately 90% of the DNA mismatch repair deficient colorectal carcinomas display inactivating mutations in the *TGFβII* receptor gene (96). Frequent somatic deletions or insertions in coding mononucleotide repeats have also been detected in other tumor suppressor genes like *IGFR-II* (insulin-like growth factor 2) and the pro-apoptotic factor *BAX* (97,98). Moreover, mutational analysis of CRC tumors with MSI including hereditary nonpolyposis show gene alterations in WNT signaling regulators such as *APC*, *CTNNB*, *AXIN-1*, and *TCF4* (99).

Chromosome intsabilty (CIN) or microsatellite stabilty (MSS) in colorectal tumors - Karyotyping analyses of primary CRCs revealed that many cancers had numerical chromosomal alterations, including frequent loss of chromosomes 18 and 17p as well as gains of chromosomes 13 and 20 (100,101). Comprehensive array-based approaches have defined the chromosome regions most commonly affected by gains and losses. Notably, the gains affecting chromosomes 8q, 13, and 20q and the losses affecting

chromosomes 8p, 17p, and 18q in colorectal cancer lead to strong influences on average gene expression for a number of genes that reside on the affected chromosomes (102). One of the critical steps for the identification of tumor suppressor genes is loss of heterozygosity analysis (LOH). Loss of one allele at a specific locus is caused by a deletion mutation or loss of a chromosome from a chromosome pair. When this occurs at a tumor suppressor gene locus where one of the alleles is already abnormal, it can result in neoplastic transformation. Allelotyping studies of colorectal cancer found that approximately 15% of the cancers had no apparent loss of heterozygosity (LOH) (103). It is known that colorectal cancer with very few or no allelic losses display MSI phenotype. Further studies have been undertaken to better understand the differences between the 15% of cancers with the microsatellite instability and the 85% of cancers that display frequent allelic losses. Key factors that underlie chromosome instability (CIN) in CRC are poorly defined, but some clues to their identity have emerged. Presumably, defects in genes that regulate formation of the mitotic spindle and the proper alignment and segregation of chromosomes at mitosis may contribute to the CIN phenotype (104). To date, only a few specific gene defects seem to be responsible of chromosome instability, these include alterations in mitotic checkpoint genes such as *MAD2*, *BUBR1*, *BUB3* (105). Moreover, *APC* inactivation has been suggested to play a role in CIN (106). However, because many tumors that are MMR defective, but not characterized by chromosome instability, carry *APC* mutation, *APC* is unlike to be the primary determinant of CIN phenotype. Therefore, the chromosome instability in CRC seems to be much more complex and heterogeneous than the straightforward relationship between MMR gene defects and the MSI signature. Colorectal tumors showing chromosome instability can be familial or sporadic. Germline mutations in *APC* gene underlie FAP (familial adenomatous polyposis) syndrome: although a fraction of germline mutations in FAP patients cause APC gene–expression silencing (107), more than 95% of known mutations are frame-shift or nonsense that lead to a premature truncation of protein synthesis. FAP is an autosomal dominant syndrome that affects approximately 1 in 12,000 individuals and accounts about 1% of all CRCs. Hundreds to thousands of adenomas can arise in the colon and rectum of affected individuals by the third or fourth decade of life, but only few adenomas progress to CRC (101). In addition to have a key role in FAP syndrome, *APC* has also prominent role in sporadic colorectal tumors with CIN phenotype. Biallelic inactivation of *APC* gene is observed in adenomas and carcinoma of both FAP and sporadic colorectal tumor patients, suggesting that loss of *APC* is the earliest genetic event required for developing adenomas. The adenoma-carcinoma transition is induced by coupling chromosomal defects with additional mutations, most frequently in tumor suppressor genes like *TP53*, E3 ubiquitin ligase complex member *FBXW7,* TGFβ signal transducer *SMAD4*, transcription factor *TCF7L2*,

or in oncogenes including *KRAS* and the kinase *PIK3CA.* Although the inactivation of *APC* reprents the first genetic event most frequently observed in sporadic colorectal tumors with chromosome instability, some exceptions exist. Indeed, mutations in *BRAF* or *KRAS* have been observed in two different subgroups of MSS patients with serrated adenomas lesions (108) and CpG island methylator phenotype (CIMP). Thus, confirming the heterogeneity and complexity within the fraction of sporadic MSS tumors (109).

CpG island methylator phenotype (CIMP) - The promoters of approximately 50% of all genes contain CpG islands. Hypermethylation of these CpG islands seems to be associated with silencing of downstream transcriptional units, which may reflect an epigenetic mechanism that reinforces long-term gene silencing following more transient chromatin modifications (110). In CRC cells there is a generalized decrease in the total level of DNA methylation (i.e., hypomethylation) compared with adjacent normal tissues, and DNA hypomethylation is also observed in adenoma-carcinoma transition (111). Although the global trend in CRC cells is hypomethylation, CpG islands at various promoters show increased methylation that is often linked with the transcriptional silencing (112). In fact, a group of CRCs shows concomitant hypermethylation of numerous genes, suggesting that the normal regulation of DNA methylation may be globally disrupted in at least some CRCs. The group of CRCs with hypermethyation changes at many different CpG-rich elements fits the CIMP model (113). Adenomatous precursor lesions of CIMP CRCs often present a sessile serrated adenoma (SSAs) morphology. Depending on the gene methylation levels, it is common to separate tumors with CIMP-high (H-CIMP) from those with CIMP-low (L-CIMP) status. A subset of CIMP tumors shows hypermethylation of the *MLH1* MMR gene, and these tumors represent a major fraction of sporadic MSI tumors. These cases often harbor gain-of-function mutations in *BRAF*, suggesting a link between CIMP-high and microsatellity instability. In contrast, tumors with no methylation are frequently associated with chromosome imbalance. However, recent studies in a microsatellity-stable (MSS) group identified two subgroups of patients with high-CIMP and low-CIMP phenotypes that significantly correlated with mutations in *BRAF* and *KRAS* respectively and worse outcome than those with nonmethylated MSS tumors (87,114).

4.4 Signaling pathwys and gene mutations in colorectal cancer

Constitutive activation of β-catenin/TCF signaling - The tumor suppressor gene *APC* encodes a roughly 300 kDa protein that may regulate proliferation, differentiation, cell-cell adhesion, cell migration and chromosomal segregation in the colonic crypt (115,116). Restoration of APC protein in colorectal cancer cells that lack endogenous APC expression promotes rapid cellular differentiation and reestablishes crypt homeostasis in colorectal cancer (117). The primary amino acid sequence of the APC

protein has some recognizable sequence motifs and binding sites for varied cellular proteins. Although the APC protein more than likely has multiple critical cellular functions, the best-established role for APC in the cancer process is as a major binding partner and regulator of β-catenin protein in the so-called canonical or β-catenin-dependent Wnt signaling pathway (115,116). As discussed in chapter 2, in the absence of Wnt ligand, APC binds and collaborates with the scaffold protein Axin and GSK3β to promote phosphorylation of several conserved serine/threonine residues in the N-terminal region of β-catenin, thereby targeting β-catenin for ubiquitination and subsequent proteosome degradation. In a physiological setting, the Wnt ligands, following binding to their cognate receptor complex of Frizzled proteins and LRP5/6 proteins, exert inhibitory effects on GSK3β and Axin, the net consequence is stabilization of the free pool of β-catenin in the cytoplasm and nucleus. In the approximately 80% of CRCs wherein both APC alleles are inactivated, the coordinated phosphorylation and destruction of β-catenin are abolished. This process essentially mimics the constitutive activation of Wnt signaling. As a result, β-catenin accumulates in the cytoplasm and translocates to the nucleus (115) where it binds members of the TCF family and converts these WNT effectors from transcriptional repressors into activators (118,119). The collection of genes regulated by β-catenin/TCF include proto-oncogenes, such as *CMYC* and *cyclin-D1* as well as genes encoding membrane factors, such as *matrix metalloproteinase-7 (MMP-7)/Matrilysin* and *CD44* and Wnt pathway feedback regulators including AXIN-2 and Dickkopf-1. Microarray studies performed by van de Wetering et al. (120) were the first to highlight that the transcriptional program induced by the constitutive activation of β-catenin/TCF resembles the transcriptional program in presumptive tissue stem cells at the base of the intestinal/colon crypt (**Fig.10**). Further work (66) discovered that β-catenin play not only a role in the establishment of the crypt progenitor phenotype but also in the spatial organization and migratory pattern of the cells in the continuous renewal of crypts by modulating EPH/Ephrin signaling. Thus, it is not surprising to observe during early stages of intestinal tumorigenesis a massive upregulation of the β-catenin/TCF4 downstream targets, including those with tumor suppressor activity such as EPHB receptors (described in chapter 5).

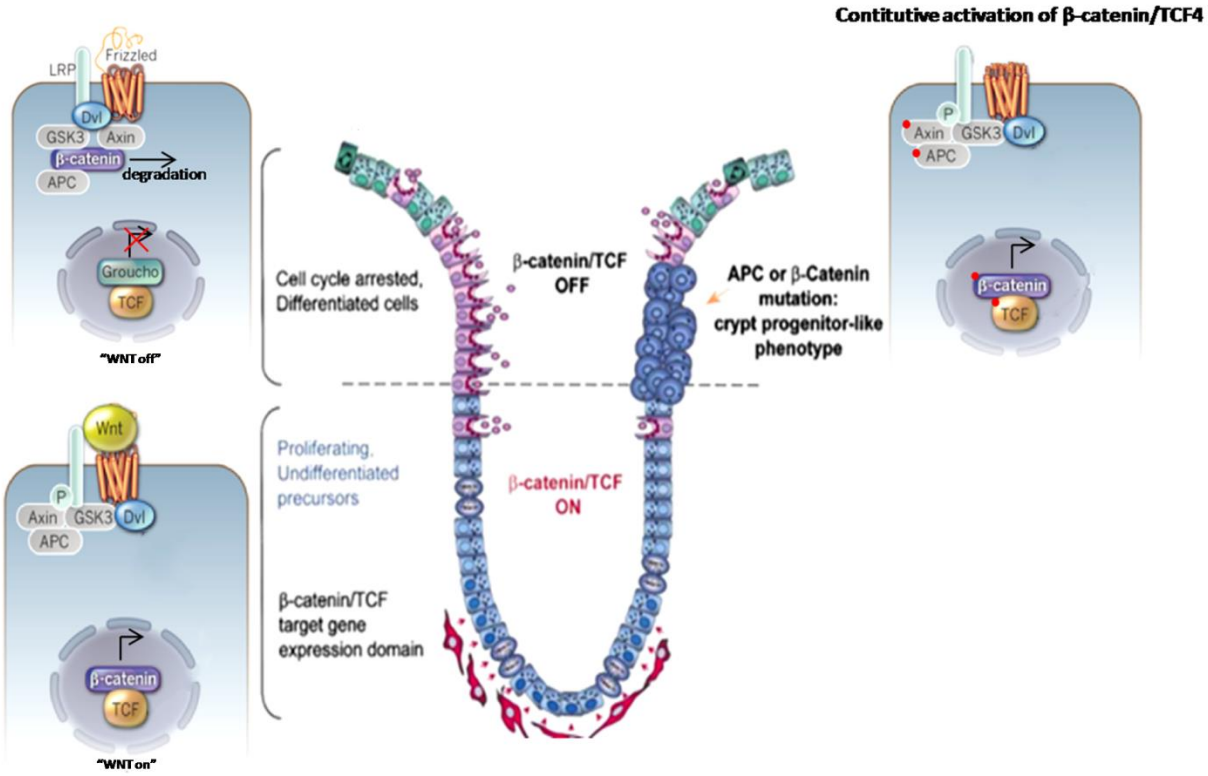

Fig.10. Role of β-Catenin/TCF in the early stages of intestinal tumorigenesis. (In the middle) Colon crypt and polyp formation. At the bottom of the crypt, the progenitor proliferating cells accumulate nuclear β-catenin. Consequently, they express β-catenin/TCF target genes; "Wnt on" (left down). An important source of WNT ligand comes from the Paneth cells as well as the mesenchymal cells surrounding the bottom of the crypt. As the cells reach the mid crypt region, β-catenin/TCF activity is downregulated resulting in cell cycle arrest and differentiation; "Wnt off" (left up). Cells undergoing mutation in *APC* or *CTNNB1* (β-catenin) become independent of the physiological signals controlling β-catenin/TCF activity. As a consequence, they continue to behave as crypt progenitor cells in the surface epithelium, giving rise to aberrant crypt foci (ACFs) (120); (right up) constitutive activation of Wnt signaling; (red circle) the most frequently mutated members of Wnt signaling in colorectal cancer.

Consistent with the notion that *APC* plays a key physiological role in regulating β-catenin levels and localization and that *APC* inactivation is selected in CRCs primarily due to the resultant dysrelugation of β-catenin-dependent Wnt signaling, a fraction of CRCs that lack *APC* mutation have defects in other canonical Wnt pathway (121). Somatic mutations in the *CTNNB1* gene that affect key amino acids involved in β-catenin phosphorylation and ubiquitination, although more common in other cancer types, have been observed also in a small subset of CRCs: these changes mainly increase the β-catenin stability (122). Moreover, inactivating mutations in *AXIN-1* gene, a negative regulator of the Wnt pathway, have been also identified in several CRCs. In some tissues, AXIN-2 may substitute AXIN-1 in the β-catenin degradation complex. Mutations in *AXIN-2* gene have been reported in MSI tumors but germline mutations in this gene have been suggested as predisposing to colorectal cancer as well (123). However, the functional relevance of these genetic alterations is still debated. Finally, mutations in the 3' region of *TCF4* gene that convert the transcription factor in a more active form, have been observed in microsatellite-unstable colorectal cancers (124).

Somatic mutations affecting TGF-β pathway - LOH of chromosome 18q is observed in about 70% of CRC, around 50% of large, late-stage adenonoma and fewer than 10% of small, early stage adenomas (86). *SMAD2* and *SMAD4* genes, located on chromosome 18q, are frequentely mutated in colorectal cancer. Both genes encode proteins that function downstream of the TGF-β receptor complex, and the role of SMAD2 and SMAD3 protein is regulated by TGF-β-mediated receptor phosphorylation (125). Phosphorylation of SMAD2/3 allows the proteins to traffic to the nucleus and complex with the SMAD4, this complex can then bind to specific sequence elements and regulate gene transcription. Some of the downstream targets of TGF-β signaling are important cell-cycle checkpoint genes. Mutations that inactivate *SMAD4* are found in ∼10–15% of CRCs, and *SMAD2* mutations are found in ∼5% of CRCs (126). Moreover, inactivating mutation of *SMAD3,* wich maps on chromosome 15, are aslo found in ∼5% of CRCs (126). TGF-β signaling is initiated by the binding of TGF-β ligands to type II TGF-β receptors (TGFβRII). Once bound to TGF-β, TGFβRII recruits and phosphorylates the type I TFG-β receptor (TGFβRI), which stimulates TGFβRI protein kinase activity (**Fig.11**). Inactivating mutations in *TGFβRII* are found in approximately 25% of CRCs. The coding region of the *TGFβRII* gene contains a long mononucleotide adenine tract, and more than 90% of MSI CRCs harbor somatic insertion or deletion in the sequence tract of both alleles, which lead to inactivation of TGFβRII function (127). Further support for *TGFβRII* role as a tumor suppressor gene in colorectal cancer come from the demonstration of *TGFβRII* mutations in colorectal cancer cell lines that are microsatellite stable (128).

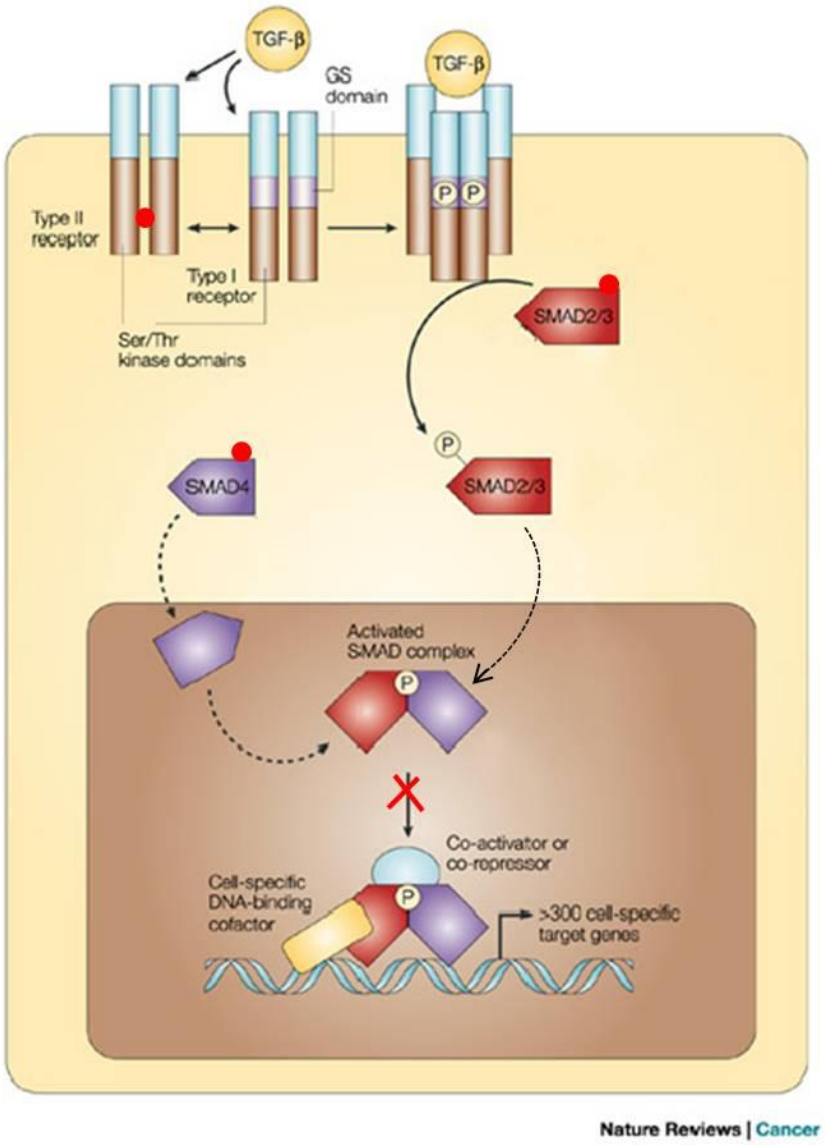

Fig.11. Diagram of the TGF-β signaling pathway. Trasforming growth factor-β binds type II receptor and recruits type I to this complex. The type II receptor phosphorylates the type I TGFβR which then phosphorylates receptor-associated SMADs (SMAD2/3). SMAD2/3 complex forms dimers or trimers with SDMD4 end traslocates to the nucleus where it interacts with DNA-binding cofactors and co-activators or co-repressors to modulate trascription of TGF-β target genes (129). The most frequently mutated members of TGF-β pathway (red circle) in colorectal cancer.

KRAS/BRAF and PI3K/AKT pathways in colorectal cancer - The RAS family of small-G proteins function as molecular switches downstream of multiple receptors with tyrosine kinase activity, such as the epidermal growth factor receptor (EGFR) (130). EGFR activation results in signaling through the MAP kinase and PI3K/AKT pathways, causing cellular growth, angiogenesis and invasion. At present, two different monoclonal antibodies (Cetuximab and Panitumumab) can selectively bind the extracellular domain of EGFR leading to the inactivation of downstream signaling and consequently blocking cell growth and proliferation. However, treatment with monoclonal antibodies anti-EGFR is effective in only a small percentage of patients and frequently resistance to this therapy is caused by activating mutations in *KRAS* gene. KRAS, HRAS and NRAS, the three members of RAS GTPase proteins, are common targets for somatic mutations in many human cancers (131). Somatic mutations in *KRAS* have been found in approximately 40% of CRCs. The vast majority of *KRAS* mutations affect exon 2 (codon 12 and 13) and exon 3 (codon 61) and compromise the intrinsic GTPase activity of the KRAS protein, leading to reductions in GTP hydrolysis capacity and maintaining the protein in its active state (Ras-bound GTP form) (86,132). Constitutive activation of KRAS contributes to colorectal adenoma development but is certainly not required for adenoma initiation. Despite the observation that *KRAS* mutations can be present in some colorectal lesions with minor malignant potential (e.g., aberrant crypt foci and hyperplastic polyps), mutant *KRAS* alleles, when present, play a critical role in driving the behavior of advanced CRC cells. The RAS proteins exert effects on several downstream signaling cascades, including the mitogen-activated protein kinase (MAPK) and PI3K pathways. As such, it is unsurprising that selected components of these pathways are mutated in a fraction of CRCs. More than half of *KRAS* wild type patients do not respond to the anti-EGFR therapy. The gene *BRAF*, which encodes for a downstream effector of KRAS (**Fig.12**), is mutated in approximately 5–10% of CRCs (133). Mutations in *KRAS* and *BRAF* are thought to be mutually exclusive. Thus, as for *KRAS*, patient with *BRAF* mutations are predicted to lack the response to anti-EGFR therapy. The other second key messenger with effects on cell growth, proliferation, and survival is phosphatidylinositol-3,4,5-triphosphate (PIP3). At the cell membrane, formation of PIP3 from phosphatidylinositol-4,5-biphosphate (PIP2) depends on the activity of PI3K, which are physiologically activated by upstream EGFR (Fig.12) (134). Somatic mutations in the *PIK3CA* gene are found in approximately 15–25% of CRCs (132). Further studies showed that these mutations activate PIK3CA kinase activity, thereby increasing production of PIP3 in affected cells (135). Notably, although KRAS can activate PI3K/AKT pathway, like EGFR, *KRAS* mutations seem to cosegregate to some degree with mutations in *PIK3CA* and this is because mutant forms of KRAS are not very efficient in activating PI3K signaling (136).

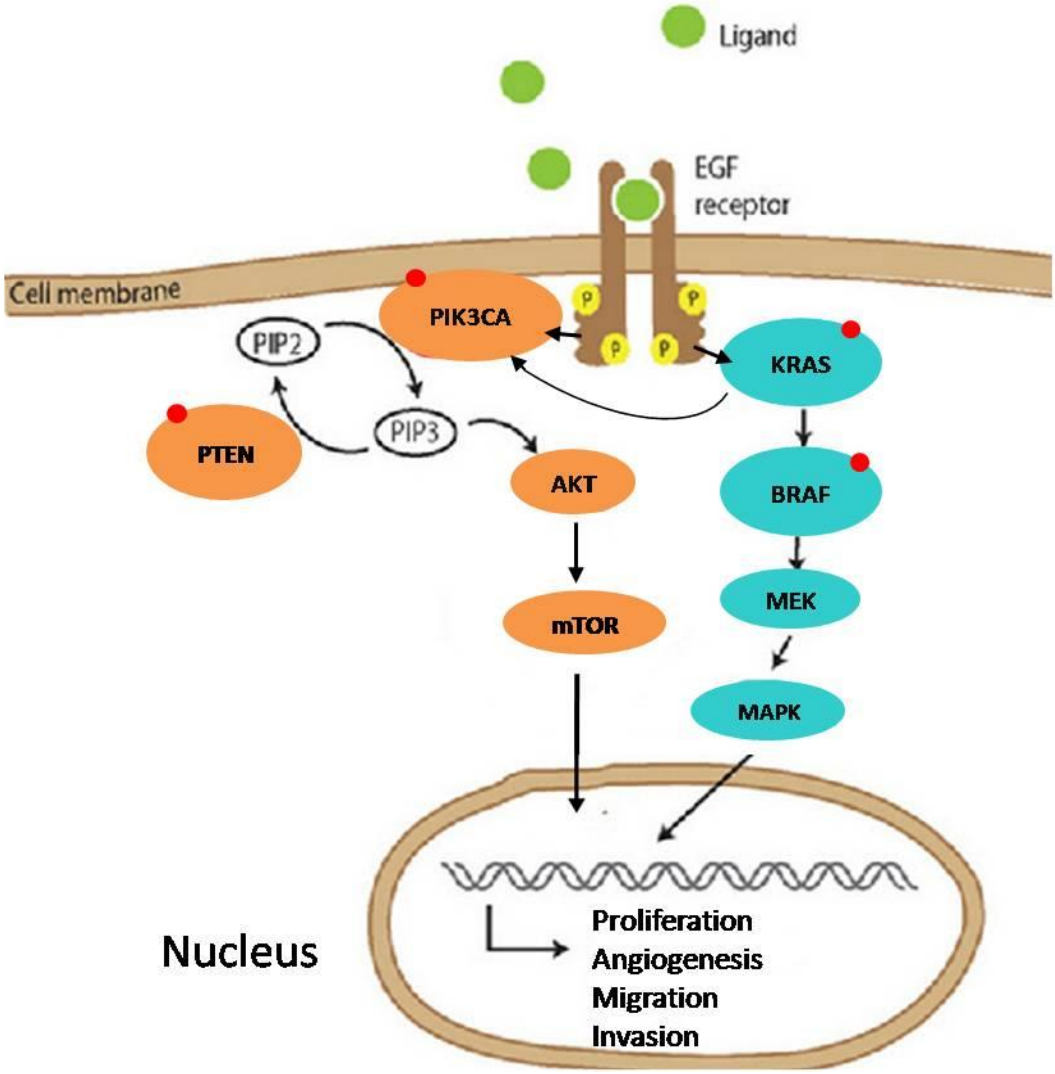

Fig.12. KRAS/BRAF and PI3K/AKT pathways. Ligand binds to the extracellular domain and results in receptor dimerization and phosphorylation of the intracellular domains. Activated EGFR leads to activation of the oncogene *KRAS* which in turn activates the oncogene *BRAF*, the mitogen-activated protein kinase Kinase (MEK), and the mitogen-activated protein Kinase (MAPK) leading to expression of growth promoting genes. In addition to promote KRAS, EGFR also activates the oncogene *PI3KCA,* which phosphorylates phosphatidylinositol-2-phosphate (PIP2) to phosphatidylinositol-3-phosphate (PIP3) which in turn activates AKT and several downstream effectors such as mTOR, leading to protein synthesis, cell growth, survival, migration, invasion and angiogenesis. Of note, KRAS alone can activate PI3K/AKT pathway. Genes most frequently mutated in colorectal cancer (red circle). Source (137).

The PTEN protein is a phospholipid phosphatase that mediates dephosphorylation of PIP3 to PIP2, acting as negative regulator of PI3K/AKT signaling. Inactivating somatic mutations in *PTEN* gene were found in about 10% of CRCs (132). Loss of PTEN activity or activating mutations in PI3K results in hyperphosphorylation of AKT and increased cell growth. As shown for *BRAF* mutants*, KRAS* wild type patients carrying mutation in *PTEN* or *PI3K* fail to respond to anti-EGFR treatment (138,139).

p53 in CRC - As shown for the chromosome 18q, 70% of patients with CRC present 17p LOH (86). The *TP53* gene is thought to be the main target of 17p LOH because, in most CRCs with 17p LOH, the remaining *TP53* allele carries a somatic mutation (140). Approximately 85% of the *TP53* mutations in CRC are missense defects. Importantly, a small minority of CRCs lacking 17p LOH have *TP53* mutations, and most adenomas lack 17p LOH as well as mutations in *TP53* (140). Therefore, both mechanisms seem to be closely associated with the adenoma-carcinoma transition. Under normal conditions, p53 protein is negatively regulated by MDM2, an E3-ubiquitin ligase, and the related protein MDM4 (also known as MDMX), which bind to the transactivation domain of p53 and target it for degradation by ubiquitination. In cells with a high level of stress, the interactions between MDM2, MDM4, and p53 are disrupted, allowing activated p53 to exert its transcriptional activity. Defined as the "guardian of the genome", p53 is a master regulator that controls the transcription of hundreds of genes involved in DNA metabolism, apoptosis, cell cycle regulation, senescence, angiogenesis and numerous other processes. Some of the best-studied gene targets of p53 are the cell-cycle inhibitor such as $P21^{WAF/CIP1}$, or apoptosis regulators including *PUMA* and *BAX* (141). The wild-type p53 protein plays also transcription-independent roles outside the nucleus; one of these is the inhibition of autophagy that seems to be associated with an oncogenic activity. Some interesting studies suggested that this particular function of wild-type p53 is retained by mutant forms associated with cancer and, indeed, p53 mutated still inhibits autophagy in the cytoplasm but fails to activated pro-apoptotic genes into the nucleus. Thus, the predicted net effect is less autophagy in mutant cells than those wild-type or *TP53* null mutant alleles (141,142). Therefore, the loss of heterozygosis together with selective mutations of *TP53* that retain its oncogenic activity, may explain the key role of this protein in adenoma-carcinoma transition.

Mutations in *CMYC*, *Cyclin E*, and *FBXW7* - The role of the human *CMYC* gene in cancer development was highlighted approximately three decades ago by the identification of chromosomal translocations and gene amplifications that affect the *CMYC* gene in lymphomas and lung cancer, respectively (143). The protein is a trascription factor that regulates genes with roles in cell-cycle progression, survival, and various aspects of cellular metabolism in normal and neoplastic cell (143). High copy amplification of the

CMYC gene in CRC is observed in approximately 5–10% of CRCs (126), although moderate copy amplification may be found in more than 30% of CRCs (144). As mentioned above, *CMYC* is a dowstream target of the β-catenin/TCF trascriptional complex, and deregulated gene expression in many CRCs may be attributable in part to *APC* inactivation (120).

The cyclin-E protein functions together with the CDK2 to regulate cell-cycle progression. High copy amplification of the *cyclin E* gene (*CCNE1*) is observed in only 5% or fewer of CRCs, although modest to moderate increases in *CCNE1* copy number are found in approximately 15–20% of CRCs (145). More commonly, elevated expression of cyclin-E is due to inactivating mutations in the *FBXW7* gene, which encodes an F-box protein that acts as a substrate recognition component for the SCF ubiquitin ligase complex (146). Indeed, approximately 20% of CRCs have somatic mutations that inactivate FBXW7 function (132). Interestingly, in addition to regulating the levels of cyclin-E, FBXW7 may also regulate the levels of other potential oncogenic factors (146). Thus, loss of FBXW7 function may be commonly selected for in CRC due to the resultant dysregulation of multiple different proteins and pathways with oncogenic activity.

4.5 Colorectal cancer treatment

Surgery - Surgery is the main treatment for CRC cure. Surgical resection is highly effective for early stage colon cancers, providing cure rates of over 90% in stage I and 75% in stage II disease, and up to 73% of cases of stage III disease are curable by surgery combined with adjuvant chemotherapy (after surgery). In metastatic CRC treatment, chemotherapy can be used as a complement to metastases potentially curative by surgery as neoadjuvant treatment (before the surgery) to achieve resectability of initially unresectable disease or as palliative treatment. Stage IV disease is usually incurable but treatment improvements in the last years have been shown to prolong the overall survival of these patients. The regimens most commonly used in **chemotherapy** consist in different combination of the following drugs: 1) the inhibitor of thymidylate synthase such as, fluoropyrimidine 5-fluorouracil (**5-FU**) often used in synergistic combination with folinic acid (**leucovorin**), or **capecitabine**, an anaolgue of 5-FU amministrated orally with a lower percentage of adverse effects; 2) the topoisomerase I inhibitor **Irinotecan**; 3) the platinum-based antineoplastic agent (DNA alkylating) **Oxaliplatin**. At present, the drug combinations most commonly used in colorectal cancer are, **FOLFOX**: 5-fluorouracil (5-FU), leucovorin, and oxaliplatin; **FOLFIRI**: 5-FU, folinic acid, irinotecan; **FOLFOXIRI**: 5-FU, folinic acid, oxaliplatin and irinotecan; **CapeOx**: Capecitabine together with oxaliplatin. In the last years, monoclonal antobodies against the vascular endothelial growth factor (VEGF) (**Bevacizumab**, **Avastin**) and the

epidermal growth factor receptor (EGFR) (**Cetuximab**, **Erbitux** and **Panitumumab**, Vectibix) have also been approved by regulatory agencies. **Radiotherapy** aims to reduce local recurrence and improve survival for patients with rectal cancer. Traditionally used in combination with chemotherapy, adjuvant radiotherapy (long course) is considered standard care for patients with stage II and III rectal cancer. Neoadjuvant radiochemotherapy (short course of higher dose) is reserved for advanced rectal cancers. Recently, neoadjuvant methods have been advocated for stage II and III patients too on the basis of better local tumor control and lower morbidity (85,147).

However, although most of the increased survival of colorectal cancer patients comes from better treatments, prevention as well as early detection still remain the most effective strategies to limit morbidity and mortality of this disease.

The **Fig.13** shows a schematic representation of the most recurrent molecular pathways, prognosis and response treatment found in colorectal cancer.

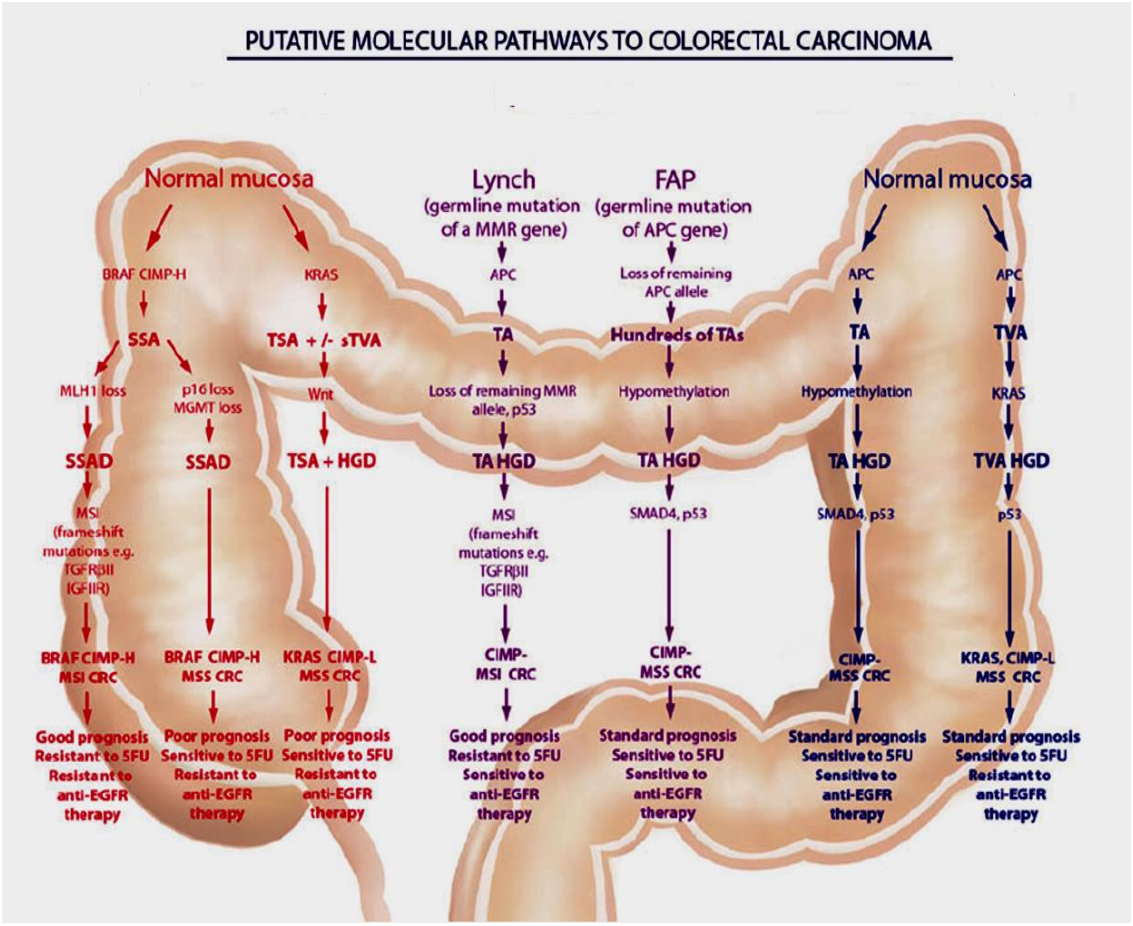

Fig.13. Schematic representation of the most frequent molecular pathways in colorectal cancer, prognosis and treatment response. (Red) Serrated pathways; (Purple) Familial pathways; (Blue) Conventional pathways. SSDA, sessile serrated adenoma; TSA traditional serrated adenoma; TA, tubular adenoma; TVA, tubulovillous adenoma, (HGD) High-grade dysplasia (109).

5. EPH, AN INTRUIGUING RECEPTOR SUBFAMILY: FROM EMBRIOGENESIS TO TUMOROGENESIS

5.1 The largest Receptor Tyrosine Kinase subfamily

It was in 1987, in the lead-up to the human genome project when was discovered in a hepatoma cell line the first receptor tyrosine kinase (RTK) (148). It became evident very quickly that EPH was the funding member of the populous RTK family. An exponentially growing interest over the past two decades (**Fig.14**) leaves us today with an intriguingly complex picture and help us to understand better these proteins. The genes that encode EPHs and their Ephrins ligand are present throughout the animal kingdom and have an origin that possibly predates the parazoan-eumetazoan bifurcation (149). Conservation of both the structure and function of Eph and Ephrin gene products throughout evolution contrasts with the dramatic increase in the number of members of each family in vertebrates. Considering signaling by RTKs as one of the universal concepts of cell-cell communication, it is tempting to speculate that the expansion of the EPHs to the largest of all RTK subfamilies reflect the evolution of the complex vertebrate body plan (149,150). In this context, it is interesting to consider that the function of EPH receptors started with a single, primordial *Caenorhabditis elegans* EPH receptor VAB-1 (151) which interacts with four Ephrins (EFN-1 to EFN-4) in different cell types and during different stages of embryogenesis (152). In the vertebrates the EPH receptors are classified into two subgroups, namely EPHAs or EPHBs based on sequence homology and binding affinity to their ligands. The human EPHA subgroup includes 9 receptors (EPHA1–8 and EPHA10), whereas the EPHB subgroup includes five members (EPHB1–4 and EPHB6) (**Fig.15**). Their ligands, the Ephrins, are diveded in two subclass, the A subclass (ephrinA1–ephrinA5) and the B subclass (Ephrin-B1–Ephrin-B3). EPHA receptors typically bind to most or all A-type ligands, and EPHB receptors bind to most or all B-type ligands (Fig.15). Exceptions to this rule is EPHA4, which can bind most Ephrin-A but also Ephrin-B2 and Ephrin-B3 ligands (153). Moreover, EphrinA5 can effectively activate type A EPHs as well as EPHB2 receptors (154).

Several features distinguish the EPH–Ephrin system from other RTKs. First, whereas all other RTKs bind to soluble ligands, which can diffuse considerable distances, Ephrins require membrane attachment, limiting the action of this system to cell-to-cell communication. Second, whereas the classical activated RTK consists of a receptor dimer, functional EPH–Ephrin signaling requires higher-order cluster (155).

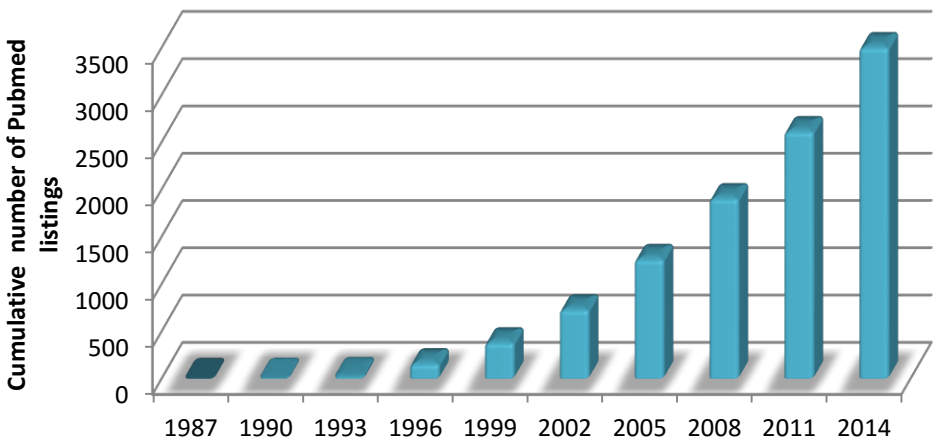

Fig.14. The scientific interest in EPH-ephrin biology is measured by the number of publications. The graph illustrates the cumulative number of publications that appear in a PubMed search with "ephrin or EPH receptor" as a search term, starting from the first citation in 1987 (148).

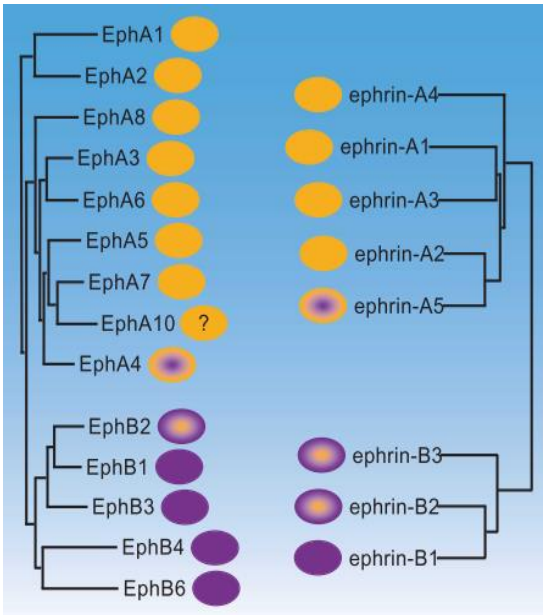

Fig.15. Binding interactions and sequence relationships of EPH receptors and Ephrins. Yellow and purple indicate high binding affinity to partners of A and B subclass, respectively; yellow with a purple center indicates high binding affinity to partners of A subclass and lower binding affinity to partners of the B subclass; and purple with a yellow center indicates high binding affinity to partners of the B subclass and lower binding affinity to partners of the A subclass. The question mark indicates that that the binding preference of the EPHA10 has not yet been determined (156). EPH receptors and Ephrins are arranged according to the phylogenetic trees determined using the Clustal program. The dendogram for the EPH receptors was constructed using the sequences of the ephrin-binding domains (157) of the human EPH receptors, and the dendogram for the Ephrins was constructed using the sequences of the Eph receptor-binding domains (157) of the human Ephrins. The lengths of the horizontal branches are proportional to sequence divergence between proteins and the arrangement of the branches indicates putative phylogenetic relationships. EPHA9, EPHB5 and ephrin-A6 are not shown because these proteins were identified in chicken and do not appear to be present in the human and mouse genome. Adapted from (153).

Soluble forms of Ephrins bind to EPH receptors, but do not trigger activation unless artificially clustered (158). Third, despite high-affinity multimeric binding sites between EPHs and ephrins, the cellular response to EPH–Ephrin engagement is often repulsion between the two cells. This raises the question about the molecular events that turn adhesion into repulsion. Fourth, Ephrins have their own signaling potential. Following Ephrin binding to its receptor, in addition to stimulating signaling cascades within the Eph-bearing cell (referred to as 'forward signaling'), Ephrins can elicit signals within the Ephrin-bearing cell ('reverse signaling'). Therefore, EPH–Ephrin interaction or '*trans*' interaction has the potential to mediate bidirectional signaling between adjacent cells, with each component acting as both 'receptor' and 'ligand'. Both signaling events can happen simultaneously, and the relative contributions can vary depending on cellular context. Fifth, '*cis*' interactions between EPHs and Ephrins when co-expressed in the same cell are also possible.

In mammals EPHs and Ephrins are predominantly expressed and active during development but their roles in adult tissue maintenance and homeostasis as well as their aberrant expression in a wide range of cancers is increasingly recognised, wherein both tumorigenic and tumor suppressive functions have been largely described (159,160).

5.2 Domain organization of EPH and Ephrin protein

The domain organization of EPH receptors (**Fig.16**) is conserved across different animal phyla. The extracellular domain consists of an N-terminal globular domain responsible for Ephrin binding (ligand binding domain, LBD), a cysteine-rich region followed by a Sushi and an epidermal growth factor (EGF)-like domain. The globular, together with the cysteine-rich domain, is additionally involved in Ephrin-independent receptor dimerization and clustering (161–163). Analogous to other RTKs, EPH receptors contain a single transmembrane spanning domain. The intracellular domain is composed of a juxtamembrane region containing two conserved regulatory tyrosine residues that control kinase activity, a single tyrosine kinase domain, a SAM (sterile α motif) protein-protein interaction domain and a C-terminal postsynaptic density protein/disc large/zona occludens (PDZ)-binding motif which can bind PDZ domain-containing proteins serving as scaffolds for the assembly of multiprotein signaling complexes (164). Amongst RTKs the presence of a SAM domain is unique to the EPH receptor family and

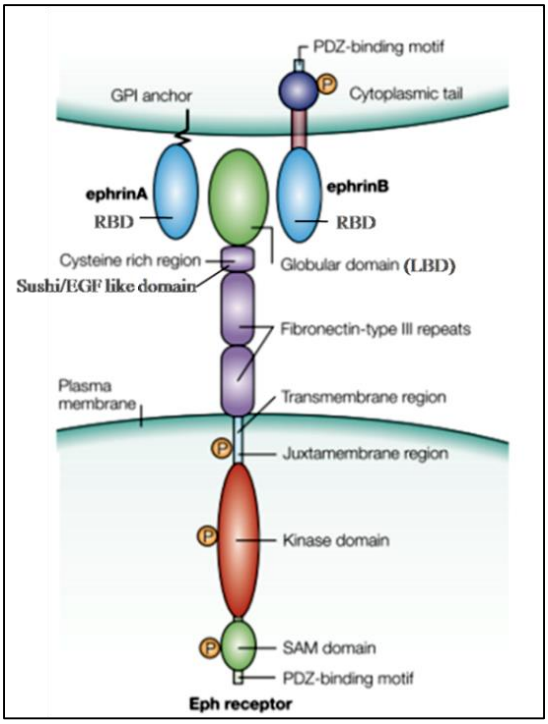

Fig.16. General features of Eph receptors and ephrins. Interaction between Ephrin-expressing cell (top) and EPH-expressing cell (bottom). GPI, glycosylphosphatidylinositol; SAM, sterile α-motif; P, thyrosine phpsphorylation sites. Adapted from (165).

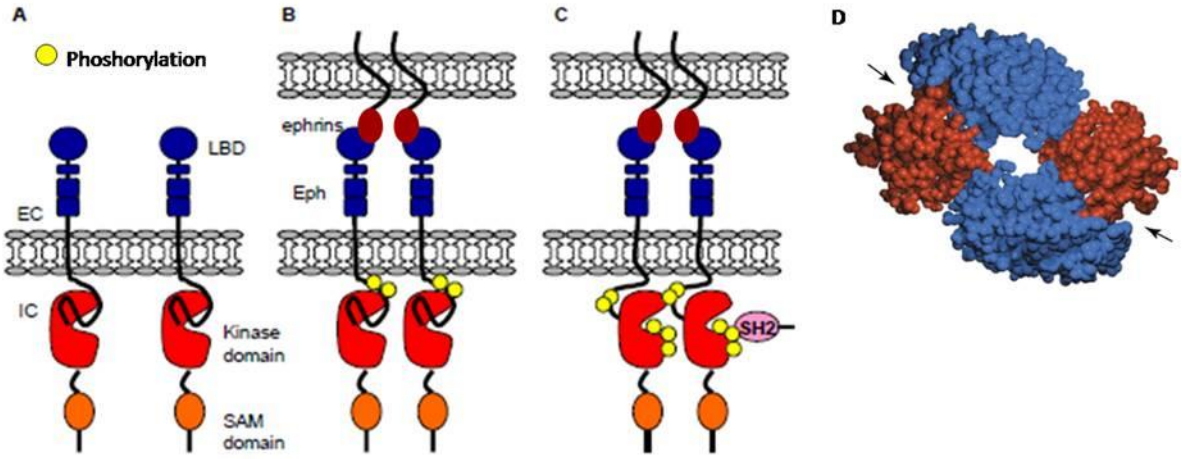

Fig.17. EPH receptor activation. A) In the absence of Ephrin binding, the kinase domain of the EPH receptor is maintained in an inhibited state through interaction of the juxtamembrane region (and the carboxy tail of the kinase regions). **B)** Following ligand-mediated dimerization of the EPH receptors, cytoplasmic domains become juxtaposed potentially with higher order EPH aggregates containing active kinase domains. This allows trans-phosphorylation of tyrosine residues in the juxtamembrane region to occur. **C)** Following phosphorylation, conformational changes in inhibitory segment from the juxtamembrane region removes the autoinhibition, and allows potential recruitment of SH2 domain proteins that recognize specifically phosphorylated tyrosine residues. **D)** The EPH-Ephrin tetramer; the EPH receptors are blues and Ephrins are red, the high-affinity dimerization interfaces are indicated by arrows, adapted from (164).

may, along with the ligand-binding and cysteine-rich domains within the extracellular region, play a role in receptor-receptor interactions aiding homo- or heterotypic oligomerization (166,167) as well as in regulating receptor endocytosis. In addition, the SAM domain also acts as binding site for low molecular weight protein tyrosine phosphatase (LMW-PTP) (168). With respect to the Ephrin ligands (**Fig.16**), the N-terminal receptor binding domain (RBD) are highly conserved, even among the A and B class ligands (164). Following the receptor binding domain, EphrinA proteins are tethered to the cell membrane by a glycosylphosphatidylinositol (GPI) anchor that can interact with other transmembrane co-receptors in *cis* (169,170). By contrast, Ephrin B proteins have a transmembrane helix, an intracellular part with several conserved tyrosine residues and a C-terminal PDZ-binding motif. Tyrosine residues of the EphrinB cytoplasmic part are also phosphorylated upon engagement with EPH receptors (165).

5.3 EPH receptor activation

The current model of EPH-ephrin interaction involves the formation of a tetrameric EPH-Ephrin complex where each ephrin ligand forms contacts with two EPH receptors and each receptor with two ephrin ligands (164). EPH is the only family of RTK in which tetramer formation is required for biological activity (158,168). Consistent with this, x-ray crystallographic showed two distinct Ephrin-binding sites located on opposite sides of the EPH ectodomain. One site mediates the initial, high affinity (nanomolar) association between the receptor and the ligand, while the second site mediates further assembly of two EPH-Ephrin heterodimers into an activated circular tetramer (164). Prior to cell-cell contact, EPH receptors are loosely preclustered on lipid rafts (155). Following initial contact, both EPH and Ephrin undergo conformational changes to expose the receptor-binding interfaces. EPH-ephrin heterodimerization then creates complementary interaction surfaces that result in the joining of dimer pairs into tetrameric complexes. Together, these actions are thought to form the active tetramer whose orientation is able to induce *trans* phosphorylation of the receptors. The first phosphorylation events occur at the tyrosine residues of the juxtamembrane region (**Fig.17**). Phosphorylation of these residues causes distortion of a helix which disrupts the inhibitory interactions of the juxtamembrane segment with the kinase domain. In brief, the phosphorylation cause steric and electrostatic forces that push the juxtamembrane segment away from the kinase relieving the structural constraints that distort the active site. In addition to enhancing kinase activity of the receptor, the exposed juxtamembrane region becomes available for interactions with downstream signaling proteins.

5.4 EPH receptors and cellular mechanisms: repulsion versus adhesion, what defines the choice?

The repulsion or inhibition of growing axons plays a critical role in controlling their motility and guidance (171,172). Repulsion involves collapse of the leading edge and lamellipodia, followed by axon or cell retraction. The ability of cells to respond to repulsive cues is fundamental to numerous developmental processes. For example, repulsive cues are critical for guiding axons to their targets in many neural development such as retinotectal mapping and midline crossing (173,174). Additionally, repulsive action is important for regulating cell migration throughout events including cell sorting and tissue boundary formation during somitogenesis, hindbrain development, vascular patterning and intestine homeostasis (175). Ephrin–EPH interaction and both forward as well as reverse signaling tightly regulate the balance between cell repulsion and adhesion. Extensive KO and transgenic animal studies provide compelling evidence that EPHs and Ephrins are key regulators of both cellular mechanisms and the "interaction mode", strongly dependent on the cellular context, determines the biological outcomes. For cell-cell repulsion to proceed after EPH-Ephrin interactions, the resulting multivalent molecular tethers between opposing cells must be broken: a key event that not only provides a switch between cell-cell repulsion and adhesion but also determines the fate of the signaling cluster and consequently the type of resulting signaling cascade. It is now evident from several studies that, whereas clustering is clearly essential for phosphotyrosine-mediated EPH and Ephrin signaling, it also triggers tyrosine independent functions, in particular, cellular adhesion and migration (176,177). Considerable experimental evidence confirms that the composition and dynamic regulation of EPH-Ephrin signaling influence the nature and the strength of the responses (175,178).

First, EPH function is regulated by phosphorylation of the juxtamembrane tyrosines, which modulate the conformation, accessibility and activity of the kinase domain but also provide SRC homology 2 domain (SH2 domain)-docking site for downstream molecules (179). Clearly, the ability to activate downstream pathways necessarily depends on EPH tyrosine kinase signaling capacity, and modulating the ratio of kinase-active to kinase-inactive receptors will switch responses from repulsion to adhesion. Protein tyrosine phosphatases (PTPs) play an important role in modulating EPH function. One potential regulator of EPH kinase activity is low molecular weight (LMW)–PTP, which is believed to modulate EPHB2-induced cell adhesion and capillary assembly (168).

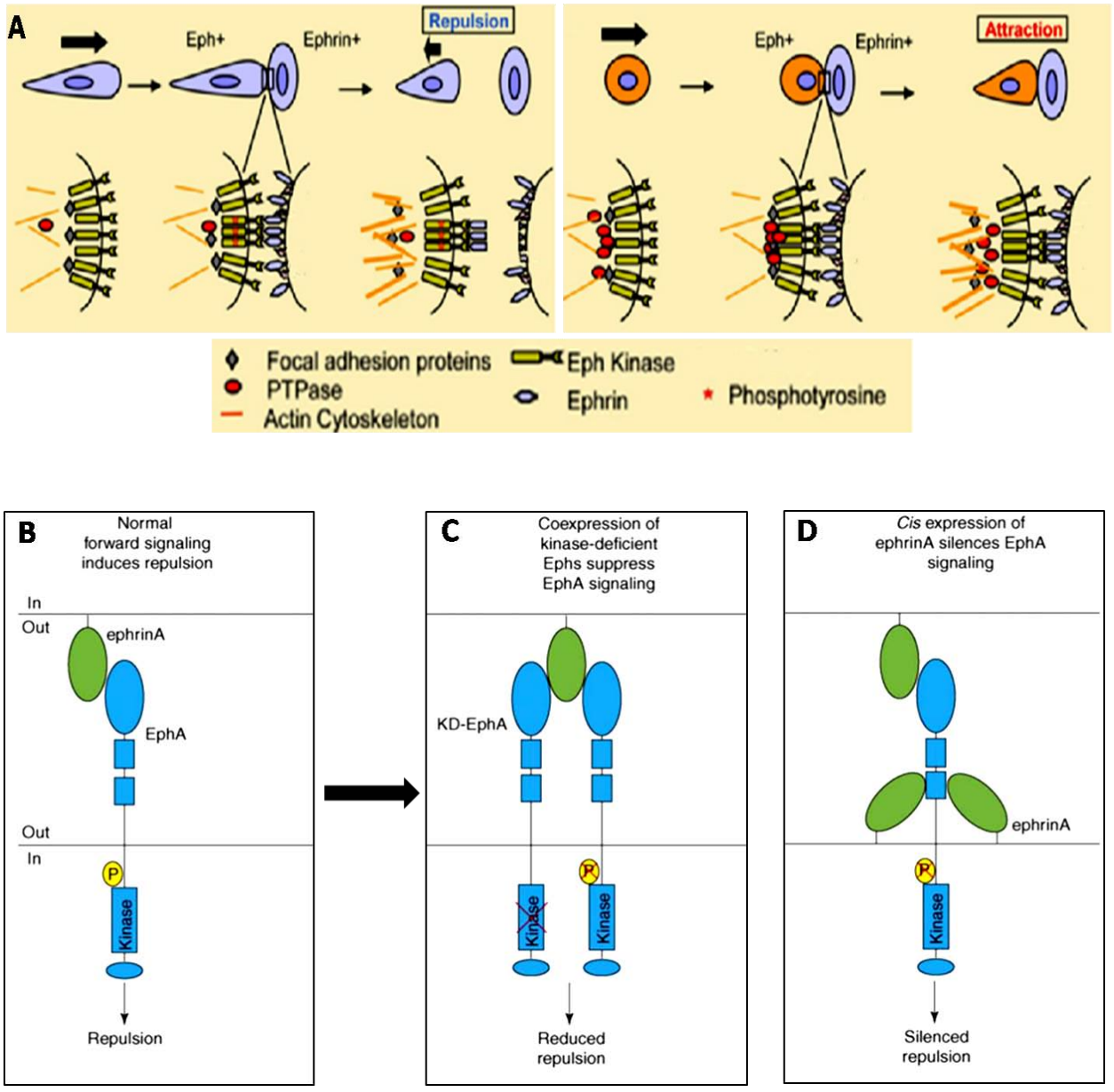

Fig.18. Regulation of EPH-ephrin signaling to determine repulsion or adhesion. **A)** Repulsive versus attractive outcome upon cell-contact induced EPHA/ephrin-A interactions. In a typical response, EPHA3 activation by autophosphorylation (denoted by an asterisk) is followed by cleavage of ephrin-A5, enabling cell separation. High levels of endogenous PTPases (red circle) prevent EPHA3 activation by ephrin-A5. This together with the lack of ephrin-A5 cleavage supports cell adhesion (180). **B)** Normal forward signaling induces repulsion of the EphA-expressing cell after interaction with ephrinA in *trans*. **C)** Coexpression of kinase-deficient EPHs with kinase-active EPHs suppresses phosphorylation of the kinase active EPH to reduce forward signaling and repulsion of the EPH expressing cell. **D)** *Cis* expression of ephrinA silences forward signaling by interacting with EPHA's first fibronectin III domain, which prevents the EPHA cytoplasmic phosphorylation necessary for signaling, adapted from (181).

Moreover, in LK63 leukemia cells high levels of endogenous protein tyrosine phosphatases (PTPases) appear to play a major role in maintaining EPHA3 receptor in an unphosphorylated and presumably inactive state even after ligand stimulation. This suggests that the presence of PTPase activities in LK63 cells suppresses EPHA3 phosphorylation and shifts the response from repulsion to adhesion (**Fig.18 A**) (180). In addition to PTPases, 2 other mechanisms have been reported previously that shed light on how initial EPH/Ephrin interaction may turn into stable cell-cell adhesion or repulsion. Whereas full-length EPHA7 mediates repulsive responses, 2 splice variants of EPHA7 cause truncation of the kinase domain, and the truncated variants inhibit Ephrin-A5–induced phosphorylation of full-length EPHA7 in a dominant-negative manner, turning repulsion into adhesion (**Fig.18 B, C**) (182). Indeed, mutant mice deficient in *Ephrin-A5*, which is required for the proper guidance and mapping of retinal axons in the mammalian midbrain (183,184), in addition to having defects in the axons projections also show severe craniofacial malformation that reflect improper closure of neural tube. Moreover, *EphA7* null mutant mice develop neural tube defects at a similar frequency to the *Ephrin-A5* null mice (182). This demonstrates that depending on the cellular context, different splice forms of an EPH receptor can determine whether a cell responds to its ligand with repulsion or adhesion. Another reported mechanism that can shift the balance from repulsion to adhesion is the *cis* inactivation of EPH receptors by Ephrins expressed on same cell. It is reported that EPHAs and EphrinAs are expressed in multiple areas of the developing brain in overlapping countergradients, notably in the retina and tectum. The *cis* interaction site on EPHA3 which is independent of the ligand-binding domain, abolishes the induction of tyrosine phosphorylation of EPHA3 and results in a loss of sensitivity of retinal axons to Ephrin-A5 in *trans* (185) (**Fig.18 B, D**).

Second, the abundance of EPH and Ephrin in gradients directly influences the signaling outcome and the underlying principles involved have been extensively explored *in vitro* (168,186) and *in vivo* (187,188). Interestingly, low levels of EPH forward signaling can mediate attractive effects that change to repulsion when signaling level increases (189). Moreover, the coexpression of both EPH and Ephrin proteins that segregates laterally into distinct membrane domains can signal opposing effects on the growth cones: EPHAs direct growth cone collapse/repulsion and Ephrin-As signal motor axon growth/attraction. This subcellular arrangement of EPH-Ephrin proteins enables axons to discriminate between *cis* versus *trans* configurations of ligand/receptor proteins, thereby allowing the utilization of both EPHs and Ephrins as functional guidance receptors within the same neuronal growth cone (190) (**Fig.19 A**).

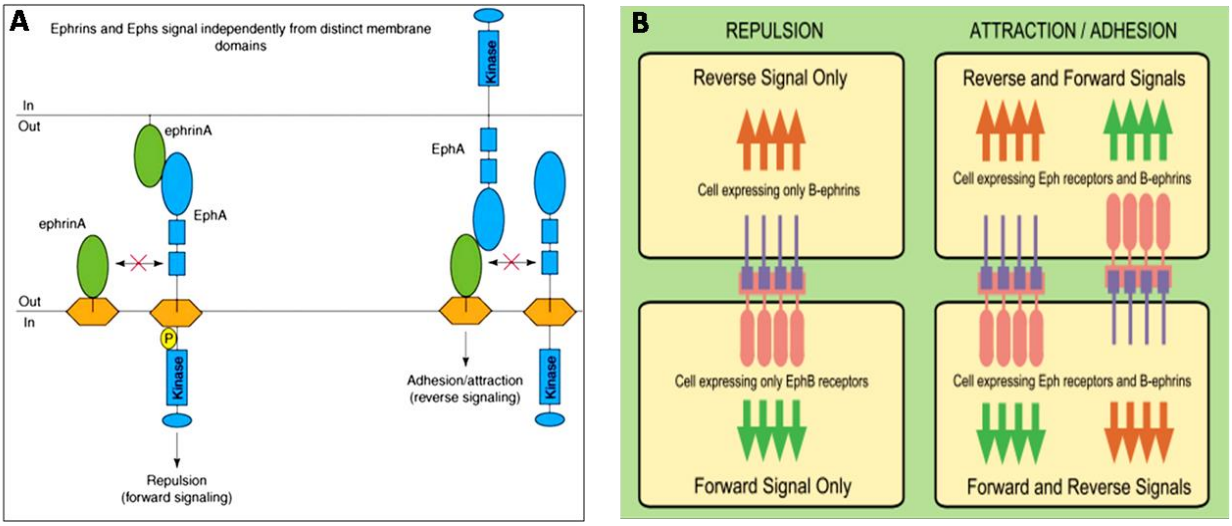

Fig.19. Effects of Ephrin-A and EPHA expression within the cell membrane. A) Expression of ephrin-A and EPHA from distinct domains within the same cell membrane prevents their cis interaction, allowing either forward (repulsion) or reverse (adhesion/attraction) signaling. **B)** Proposed mechanism to explain Eph/ephrin-mediated repulsion versus adhesion. (Left) Upon juxtaposition of a cell expressing only Ephrins with a cell expressing only EPH receptors, the interaction leads to unidirectional transduction of the forward signal into the bottom cell and the reverse signal into the top cell. This interaction is proposed to lead to repulsion. (Right) Upon juxtaposition of a cell coexpressing both Ephrins and EPH receptors with another cell also coexpressing both Ephrins and EPH receptors, the interaction leads to transduction of both the forward and reverse signals into both cells. This interaction is proposed to lead to attraction or adhesion. Adapted from (191).

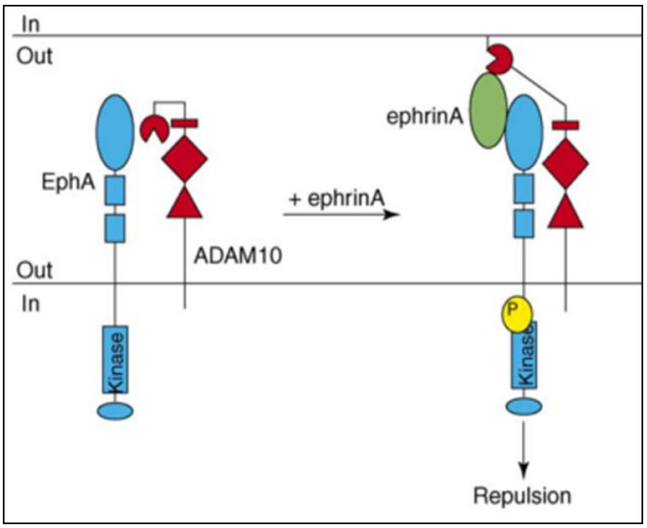

Fig.20. Example of EPH-Ephrin complex disruption and cell detachment. ADAM10 cleaves EPHA bound ephrin-A in *trans* to initiate repulsion. ADAM10 and unbound EPHA interact in *cis*. Upon EPHA binding to ephrinA, ADAM10 activates its metalloproteinase to cleave the *trans* ephrin-A and allow the EPHA/ADAM10 expressing cell to retract, adapted from (181).

Finally, genetic evidence suggests that forward and reverse signals that occur in the same cell might mediate attractive responses (**Fig.19 B**). EphB2 and Ephrin-B2 are required for the epithelial fusion that separates the urethra endoderm into the urinary and alimentary tracts (191). The incomplete septation of the urethra observed in *EphB2* or *Ephrin-B2* mutant mice leads to a hypospadia phenotype similar to a common human birth defect (191). Either receptor or ligand are coexpressed in cells that meet at the fusion site, suggesting that both regulate adhesive responses. Importantly, the analysis of gene knockins in which the cytoplasmic domain of EphB2 or Ephrin-B2 is removed reveals that signaling through both components is required for the septation (191).

Third, it is apparent that regulated disruption of the molecular EPH-Ephrin tether between cells fulfills a gatekeeper function in the progression to either cell-cell repulsion or adhesion. Two mechanisms have been identified that achieve controlled termination of EPH-Ephrin–mediated cell-cell contacts. A form of endocytosis removes the adhesive EPH–Ephrin complexes from the cell surfaces, allowing the cells to disengage. The internalized vesicles contain intact EPH–Ephrin complexes and both their surrounding plasma membranes (192,193). It is not know whether EPH–Ephrin internalization also induces internalization of other colocalized proteins, promotes the exchange of proteins between cells, or allows EPH–Ephrin signaling from the cell interior. Another strategy to eliminate EPH–Ephrin complexes and allow cell detachment involves proteolytic cleavage by ADAM10 (a Disintegrin and Metalloprotease 10) also known as Kuzbanian, which cleaves the extracellular domain of the Ephrin (194). More recently, it was shown that ADAM10 is associated in *cis* with EPHA3, and only cleaves Ephrin-A5 when bound to EPHA3 (195) (**Fig.20**). Thus, transendocytosis and proteolytic cleavage are two means by which EPH-Ephrin repulsion is initiated, and represent two potential targets for precise regulation of the adhesion–repulsion balance.

5.5 EPH receptors signaling

Considering the interest that EPH-Ephrin biology has attracted, and the large number of molecules that are known to participate in downstream signaling cascades, the understanding of the pathways that execute the various responses attributed to EPH-ephrin signaling is surprisingly limited. To some extent, this may reflect the difficulty of dissecting pathways that rely on kinase activation and the generation of SH2 domain–docking sites, as well as on the assembly of multimeric receptor clusters (even in the absence of kinase activity).

EPH signaling through RHO family GTPases: cytoskeletal remodeling - Given the effects of EPH receptors and ephrins on cells shape, adhesion, and migration, it is not surprising

that multiple, critical connections are emerged between EPH receptors and small GTPases of the Rho family. These GTPases are molecular switches that cycle between an inactive GDP-bound state and an active GTP-bound state. Activation is facilitated by guanine nucleotide exchange factors (GEFs), which promote the exchange of GDP for GTP, and is inhibited by GTPase activating proteins (GAPs), which promote the hydrolysis of GTP to GDP. EPH activation and phosphotyrosine dependent signaling commonly leads to rapid depolymerization and reorganization of actin fibres (196) and the loss of focal adhesions and cell-cell contacts, resulting in loss of substratum contact and cell segregation (197,198). The opposite response, cell-cell adhesion is likely to reflect lower effective EPH-Ephrin signaling cluster density and thus reduced signal strength. This concept also applies to oncogenic patterning in which tumor cells expressing active EPHs are prevented from spreading until mutations reduce or ablate EPH kinase function and thus allow tumor cell invasion and spreading (199,200). The three best characterized members of Rho GTPase family are RHOA, RAC-1, and CDC42. RHOA regulates stress fiber and focal adhesion formation and cell contractility, whereas RAC-1 and CDC42 activation results in the formation of protrusive structures such as lamellipodia and filopodia, respectively. The activation and/or direct binding of GEFs (guanine exchange factors) is one of the major mechanisms by which EPH receptors and ephrins regulate cytoskeletal remodeling in neuronal cells (201). EPHA receptors can activate RHO GTPases through the exchange factor Ephexin (202). Interestingly, Ephrin-A-1 treatment of cultured neurons potentiates Ephexin-mediated exchange on RHOA. The activation of RHOA and its downstream effectors, propagate Ephrin-A-induced signals to initiate growth cone collapse (203). EPHB2 signaling in hippocampal neurons regulates dendritic spines morphogenesis and involves the GEFs “intersectin”(204) and “kallirin”(205), which activate CDC42 and RAC-1, respectively. In addition, the binding of EPH receptors to adaptor proteins, including NCK, RAS-GAP and CRK (206), leads to modulation of RHO family GTPase activity. In HEK-293T and melanoma cells, Ephrin-A-induced Rho activation causes the retraction of cell processes, cell rounding/detachment, and membrane blebbing, and this appears to depend on the adaptor protein CRKII (198). Ephrin-A5-mediated stimulation of EPHA3-positive Jurkat cells promotes CRKII recruitment and inhibits cell adhesion to fibronectin (207). CRK family adapter proteins are known regulators of integrin-dependent cell adhesion and motility, that recruit via SH2 and SH3 domain downstream molecules including p130CAS, DOK180 and C3G (208) all of which are known to be involved in EPH signaling and cytoskeletal organization (**Fig.21**).

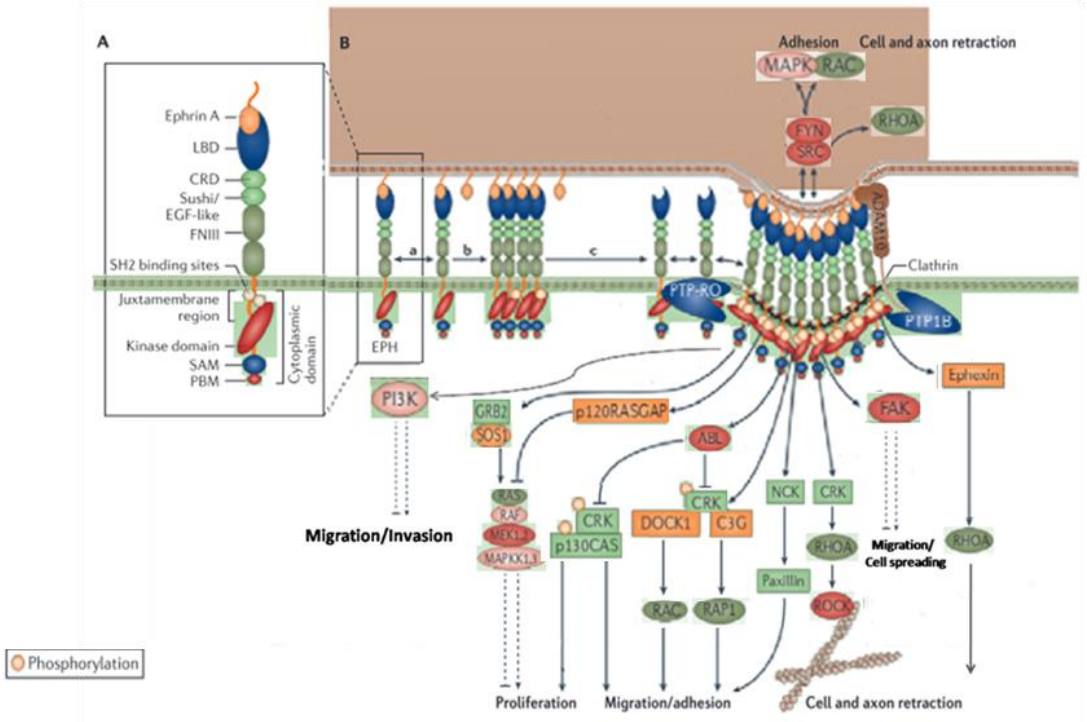

Fig.21. EPH receptors and signaling concepts. **(a, b, c)** Generally, only membrane-bound or artificially clustered soluble Ephrins can activate EPHs, and tetrameric EPH clusters are effective in initiating signaling. Signal activation involves oligomeric EPH–ephrin signaling clusters between interacting cells. Depending on EPH and ephrin cell surface densities, additional EPH–EPH interactions within the ligand-binding domain (LBD) and cysteine-rich domain (CRD) expand this oligomer into multimeric signaling clusters. **A)** Forward signaling: EPH phosphorylation is controlled by protein tyrosine phosphatases (PTPs), including PTP-RO and PTP1B. For simplicity, signaling mediators of A- and B-type EPHs are illustrated together, including tyrosine kinases (red), serine/threonine kinases (pink), SH2 adaptors (light green), RHO GTPases (green), GTPase-activating proteins (GAPs) and guanine nucleotide exchange factors (GEFs) (orange). Principle EPH signaling pathways : **1)** RHOA, RAC-1 and repressor/activator protein 1 (RAP1) modulate cell cytoskeleton changes involving a range of signaling modulator, CT10 regulator of kinase (CRK), CRKII, non-catalytic region of tyrosine kinase adaptor protein (NCK1) and GEFs (including Ephexin), **2)** Focal adhesion kinases (FAK) interconnect EPH receptors with integrin signaling modulating both migration and cell spreading in a cell-type specific manner, **3)** Opposite effects of EPH activation on RAS–MAPK kinase signaling: GRB2-SOS1 complex is a positive regulator of MAPK kinase pathway, whereas the most common negative regulator is p120RASGAP, the cellular outcome is highly context dependent, **4)** EPH activation via ABL phosphorylation of CRK disrupts CRK–p130CAS and CRK–DOCK1 complexes, thereby inhibiting cell migration, adhesion and proliferation, **5)** Crosstalk between EPH receptors and phosphatidylinositol 3-kinase (PI3K) can either promotes or inhibits the cell migration. **B)** Ephrin-A signaling (reverse signaling) remains poorly defined (209): clustering causes recruitment and activation of SRC kinases and facilitates cytoskeletal changes by RAC and RHOA GTPases. Adapted from (210).

Interestingly, EPHB receptors control actin cytoskeleton in colorectal cancer cell lines (CRC). In LS174T cells actin is mainly organized into polymerized bundles that extend to focal contacts shaping membrane protrusions. Ephrin-B1 treatment recruited polymerized actin to the cell cortex. The rounded morphology observed in LS174T after Ephrin-B1 stimulation correlates with a decreased in RAC-1 activity (66). Furthermore, EPHB3-Ephrin-B1 interaction in HEK293T cells induces cell rounding and inhibits integrin mediated cell adhesion in a kinase dependent manner, whereas inhibition of cell migration seems to be kinase independent as demonstrated by using a kinase deficient form of EPHB3; activation of both mechanisms show a dramatic reduction in CDC42 and RAC-1 activities (211).

Focal adhesion crosstalk - Cell migration is a highly coordinated process involving precise regulation of cell adhesion and deadhesion to extracellular matrix (ECM) proteins. Therefore, molecules involved in cell adhesion signaling represent potential targets for activated EPH kinases. Integrins are the primary receptors for ECM molecules. A critical element in integrin signaling, that connects EPH receptors with integrins, is the focal adhesion kinase (FAK) (212) (**Fig.21**). However, the biological outcome may be cell-type specific. Indeed, EPHA2 activation in NIH3T3 mouse embryonic fibroblast increases FAK phosphorylation and enhances cell spreading in a FAK-dependent fashion (213). On the other hand, EPHA2 activation in PC-3 prostate carcinoma cells causes dephosphorylation of FAK. This correlates with inhibition of integrin-mediated adhesion, cell spreading and cell migration (212). Similarly, activation of EPHB2 and EPHB3 receptors with Ephrin-B1 in LS174 colon cancer cells induces lost of lamellipodia and cell extensions with a negative effect on the migration/adhesion through the inhibition of FAK activity (66).

RAS/MAP kinases signaling - The RAS GTPase proteins mediate their effects by activation of RAS/MAPK signaling (214). The mitogen-activated protein kinase (MAPK) pathway is commonly activated by receptor tyrosine kinases and indeed is viewed as a hallmark of RTK signaling (215). This pathway plays a central role in regulation of key developmental processes, such as proliferation, differentiation, and cell survival but it also important for cell migration, neurite outgrowth and axon guidance (216). Unlike many other RTKs, EPH receptors can engage both positive and negative regulators of the MAPK pathway (**Fig.21**). In addition, signal transduction via Ephrin-A proteins leads to MAPK activation (217). A positive effect of EPH receptors on MAPK activity has been found in many cell types. Indeed, depending on the cellular context, EPH-mediated MAPK activation results in decreased of cell matrix adhesion in breast cancer cells (218), activation of chemotaxis in endothelial cells (219), stimulation of cell proliferation in T cells (220,221) or differentiation of neuronal precursors (222). Intriguingly, a recent

study demonstrated that EPHA3, able to induce cell fate of neuronal precursors in a kinase dependent manner by activation of MAPK pathway (222), plays an important role in maintaining tumor cells of glioblastoma multiforme (GBM) in an undifferentiated, self-renewing state through a mechanism which is kinase-independent and that limits MAPK signaling (223). EPH receptors can also negatively regulate RAS/MAPK activity, resulting in suppression of cell proliferation (224), inhibition of cell matrix adhesion (225) and neurite retraction (226). The opposite effects of EPH activation on MAPK activity may be partially explained by the specificity of binding to inhibitors and activators of the MAPK pathway. In most cases, the activating effect of EPH receptors is mediated by direct or indirect recruitment of the GRB2-SOS1 complex, which acts as a RAS-specific GEF (219). While activated EPH receptors downregulate the RAS/MAPK pathway by direct recruitment of p120RASGAP (120kDa RAS GTPase-activating protein), a negative regulator of RAS (**Fig.21**). EPHB2 can also inhibit MAPK activity through direct interaction and phosphorylation of R-RAS, which can then no longer bind and activate the MAPK activator RAF-1 (225). These distinct pathways, activated by different EPH receptor family members, could underlie cell type-specific responses.

Crosstalk with other signaling pathway downstream of EPH receptors - The Abelson **(ABL)** and Abelson related gene **(ARG)** are non-receptor tyrosine kinases with SH2 domains which regulate the actin cytoskeleton in the developing nervous system, as well as in other tissues. A new signaling connection that links regions of ABL and the ABL-related gene (ARG) to the EPHB2 receptor was found in a yeast two-hybrid screen (227). Indeed, activated EPHB2 causes tyrosine phosphorylation of ABL/ARG and viceversa. ABL and EPHB2 are co-expressed in the developing brain and retina; suggesting that EPH receptors, via ABL/ARG signaling, influence axon guidance through changes in cell adhesion and actin cytoskeleton organization. Moreover, EPHB4 inhibits breast cancer cell motility and invasion both *in vitro* and *in vivo* when its tyrosine kinase activity is stimulated by Ephrin-B2 ligand. The signaling pathways initiated by EPHB4 and Ephrin-B2 lead to ABL activation and inactivation of CRK adaptor function through phosphorylation by ABL. A likely mediator of the effects of CRK in breast cancer cells is the RAC-1 GTPase. CRK can promote RAC-1 activation by forming signaling complexes with the scaffolding protein p130Cas and the RAC-1 exchange factor DOCK180, and Ephrin-B2 treatment does indeed disrupt this signaling complexes in breast cancer cell lines (228) (**Fig.21**). In the normal intestine EPHB2 interaction with Ephrin-B ligands increases cyclin D1 protein levels through ABL thereby promoting cell proliferation, a mechanism that require EPHB2 kinase activity. In intestinal adenomas, EPHB2 is overexpressed and also promotes proliferation (229,230).

Class I phosphatidylinositol 3-kinases (**PI3Ks**) are heterodimers composed of a p85 regulatory subunit and p110 kinase subunit. Stimulation of the PI3K pathway sets off a complex chain of events, which influences cell survival, gene regulation, cell metabolism and cytoskeletal rearrangements. The role of EPH receptors and their membrane ligands have been extensively explored in capillary remodeling during development and in adult neovascularization (231) including tumor angiogenesis (232); experimental evidences suggest that the crosstalk between PI3K and EPH receptors is a critical event for endothelial cell migration and assembly into new blood vessels. In a yeast two-hybrid screen was discovered that the p85 subunit of PI3K binds EPHA2 (233). The p85 subunit contains two SH2 domains and one SH3 domain and activation of EPHA2 with Ephrin-A1 in vascular smooth muscle cells increases PI3K activity (233), but the significance of this interaction is not clear. Moreover, EPHB4-Ephrin-B2 interaction and both signaling, forward in human microvascular endothelial cells (234) and reverse in retinal endothelial cells (235), are mediated by the PI3K pathway and regulate cell proliferation and migration. EPHA2 regulates endothelial cell assembly and migration through PI3K pathway (236). Furthermore, Ephrin-B2-Fc induces migration of human umbilical vein endothelial cells (HUVEC) and promotes corneal neovascularization in adult mice via PI3K pathway (237). Additionally, a crosstalk between PI3K and EPH receptors has been explored also outside the vascular tissue. In the normal intestine EPHB2 interaction with Ephrin-B ligand upregulates transcripts encoding the p110α isoform of PI3 kinase and inhibits cell migration, the PI3 kinase inhibitor LY294002 blocks this response *in vivo* (229). Furthermore, this mechanism seems to be kinase-independent, as demonstrated by using a kinase mutant form of EPHB2 (229). Similarly, EPHA8 can increase and recruit p110Ɣ subunits of phosphatidylinositol 3-kinase through a kinase-independent mechanism but the cellular response is promoting integrin mediated adhesion and cell migration (238,239). Therefore, the interaction between EPH receptors and PI3K mainly modulates cell migration but, again, the cellular outcomes in either promoting or inhibiting the migration are strongly dependent on cell type (**Fig.21**).

5.6 Cell positioning, cell sorting and boundary formation as central mechanisms underlying EPH function

EPH receptors primarily function during embryogenesis to position motile cells and cell layers within surrounding tissues. They control the direction of cell movement and the choice of interacting cells or cell layers by initiating assembly or disassembly of cell-cell contacts. During embryonic development EPH and ephrins are expressed in temporally and spatially restricted patterns in developing tissues and organs, where they participate in germ layer formation, gastrulation, organogenesis and tissue patterning. EPH/Ephrin-mediated cell positioning relies on cell-cell contact since both receptors and

ligands are membrane bound. Thus, migration of an EPH-expressing cell into an area of graded Ephrin expression is directed by contact-dependent cell-cell segregation, instructing a change of direction to avoid the Ephrin-rich area and a final destination. In many developing tissues, areas where EPH receptors are expressed confine areas where Ephrins ligand are expressed (240). Consistent with this expression pattern, the EPH receptor-ligand system restricts intermingling between adjacent populations of cells (197). Initial evidence for roles of Eph-ephrin signaling in boundary formation came from studies of the segmentation of the vertebrate hindbrain to form rhombomeres. Cell intermingling is restricted across rhombomere boundaries owing to distinct cellular properties of odd versus even numbered segments. This correlates with the expression of EPHA4 and specific EPHB receptors in rhombomeres r3 and r5, whereas corresponding Ephrin-B ligands are expressed in r2, r4, and r6 (241,242) (**Fig.22 A, B**). Moreover, reciprocal expression of EPH receptors and Ephrins in mice embryo has been implicated in boundary formation during somitogenesis (243), cell segregation in the limb (244,245) and during cell segregation that defines the demarcation between arterial/venous domains in vascular development (231) (**Fig.23**). Another interesting example is the role of counter gradients of EPHB and Ephrin-B expression in maintaining the segregation of progenitor and differentiating cells in intestinal crypts (66), which are also relevant for tumor metastasis (199). These studies raise the question of the mechanisms by which EPH receptor and Ephrin expressing cells segregate.

5.7 EPHB/Ephrin-B signaling in the intestinal epithelium

The genetic program driven by β-catenin/Tcf complex seems to dictate three different sets of instructions that collectively regulate the biology of the crypt cells. As mentioned in the beginning, the core module enforces the undifferentiated-proliferative phenotype of progenitor crypt cells. Mice genetically manipulated to lack β-catenin/Tcf activity in the intestine lack proliferative progenitors (246). This core set of instructions also determines the proliferative undifferentiated phenotype of colorectal cancer cells. Blockage of β-catenin/Tcf–mediated transcription in colorectal cancer cell lines results in cell cycle arrest and differentiation even in the presence of multiple alterations in other tumor suppressors and oncogenes (59). The second module of the β-catenin/Tcf program is necessary for Paneth cell maturation (62). The third module of the β-catenin/Tcf program controls the compartmentalization of epithelial cells along the crypt axis and regulates their ordered migration (247). The main effectors of this function are the β-catenin/Tcf targets EPHB2 and EPHB3 receptors.

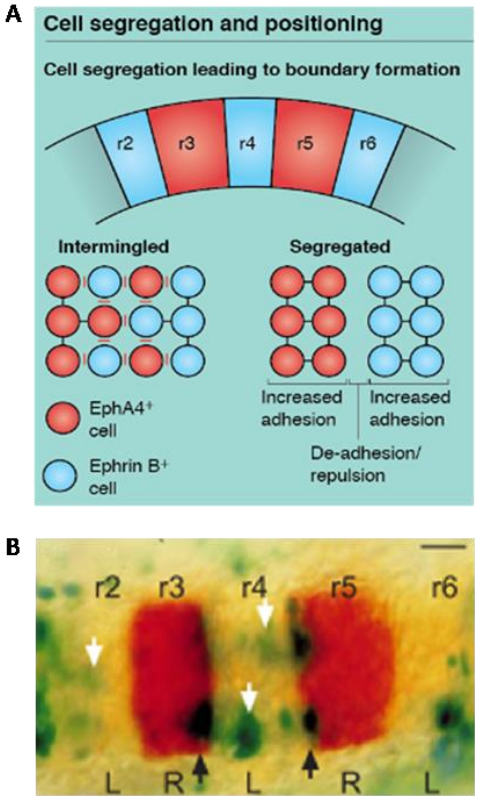

Fig.22. EPH receptors and tissue patterning. **A)** Top, schematic expression pattern of EPHA4 (red) and transmembrane Ephrin-Bs (blu) in Zebrafish hindbrain. Bottom, a partly intermingled population of EPHA4 and Ephrin-B positive cells segregate and form a sharp interface at hindbrain rhombomere. This process involves repulsive interaction (red bars) between EPHA4^{+} and Ephrin-B^{+} and adhesive interaction (black bars) between cells of the same cohort. **B)** Cell sorting after mosaic expression of Ephrin-B2 in Zebrafish embryo. Activation of EPHA4 in odd rhombomeres (R, orange) and Ephrin-B in even segments (L, yellow). Co-injection of lacZ and Ephrin-B2 RNA: expressing cells have a scattered distribution in r2/r4/r6 (white arrows indicate examples), but sort to the boundaries of r3/r5 (black arrows). Adapted from (241,248).

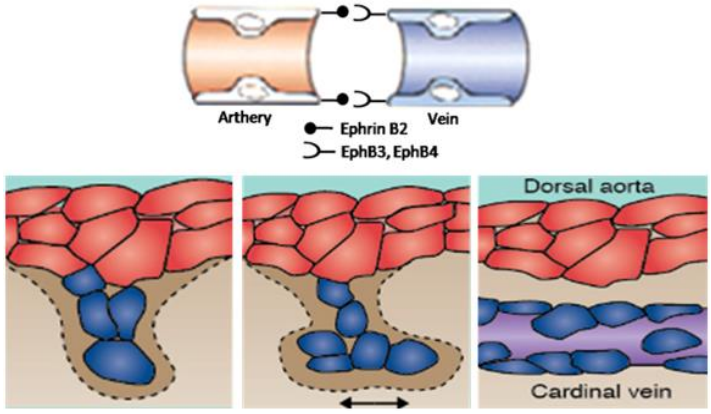

Fig.23. Mechanisms and sites of action of Ephrins and EPH receptors during remodeling of the vasculature in mice. Top) interaction of Ephrin-B2 ligand expressed on arteries and EPHB3 and EPHB4 receptors expressed on veins demarcates the boundary between arterial and venous domains. Bottom) new blood vessel formation by arterial venous segregation, Ephrin-EphB signaling drives the ventral migration of venous angioblasts (blue) from the dorsal aorta (red) to contribute to the cardinal vein primordium. Adapted from (231,248).

EphB2 and EphB3 establish a boundary between proliferative and differentiated cells in the intestinal epithelium of newborn mice - In the small intestine of newborn mice, EphB2 is expressed in the intervillus pockets of the epithelium. Its expression domain coincides with that of Ki67, a proliferation marker. This cell population also expresses EphB3 receptor in a largely overlapping pattern. However, Ephrin-B1 is highly expressed by all epithelial cells excluding those localized at the bottom of intervillus pockets (**Fig.24 A-C**). In *EphB2/EphB3* double-mutant mice, the boundary between the proliferative and the differentiated cells is largely absent, as demonstrated using cellular markers that stain specifically the proliferative or the differentiated compartments (**Fig.24 D, E**). Therefore, the concerted expression of EphB2 and EphB3 positions cell population within neonatal intestinal epithelium and restricts cell intermingling (**Fig.24 F**).

EphB/EphrinB expression in the adult mouse intestine - A more complex organization occurs in the adult intestine, in which proliferating cells are located above the base of the crypts, and whereas most differentiating cells move toward the villus, Paneth cells move in the opposite direction into the base. There is an overlapping complementary gradient of Ephrin-B1 (highest in differentiated cells in the villi) and EphB2 (highest in the most basal proliferating cells), while EphB3 expression occurs at high levels specifically in both Paneth and proliferative cells. In *EphB3* and double *EphB2/EphB3* null mutants, Paneth cells are evident throughout the crypt (**Fig.24 G, H**). Moreover, disruption of EphB gradient alters positioning of Ephrin-B positive cells along the villus. These findings suggest that the levels of EphB and ephrinB1 expression regulate the positioning and migration of intestinal cells. As differentiating cells progressively downregulate EphB2 and upregulate Ephrin-B1, they move down the gradient of EphB2 and up the gradient of EphrinB1 expression, such that there is a unidirectional flow of migration. In contrast, the high-level expression of EphB3 by Paneth cells underlies their migration in the opposite direction, away from higher levels of EphrinB1 expression (**Fig.24 I**).

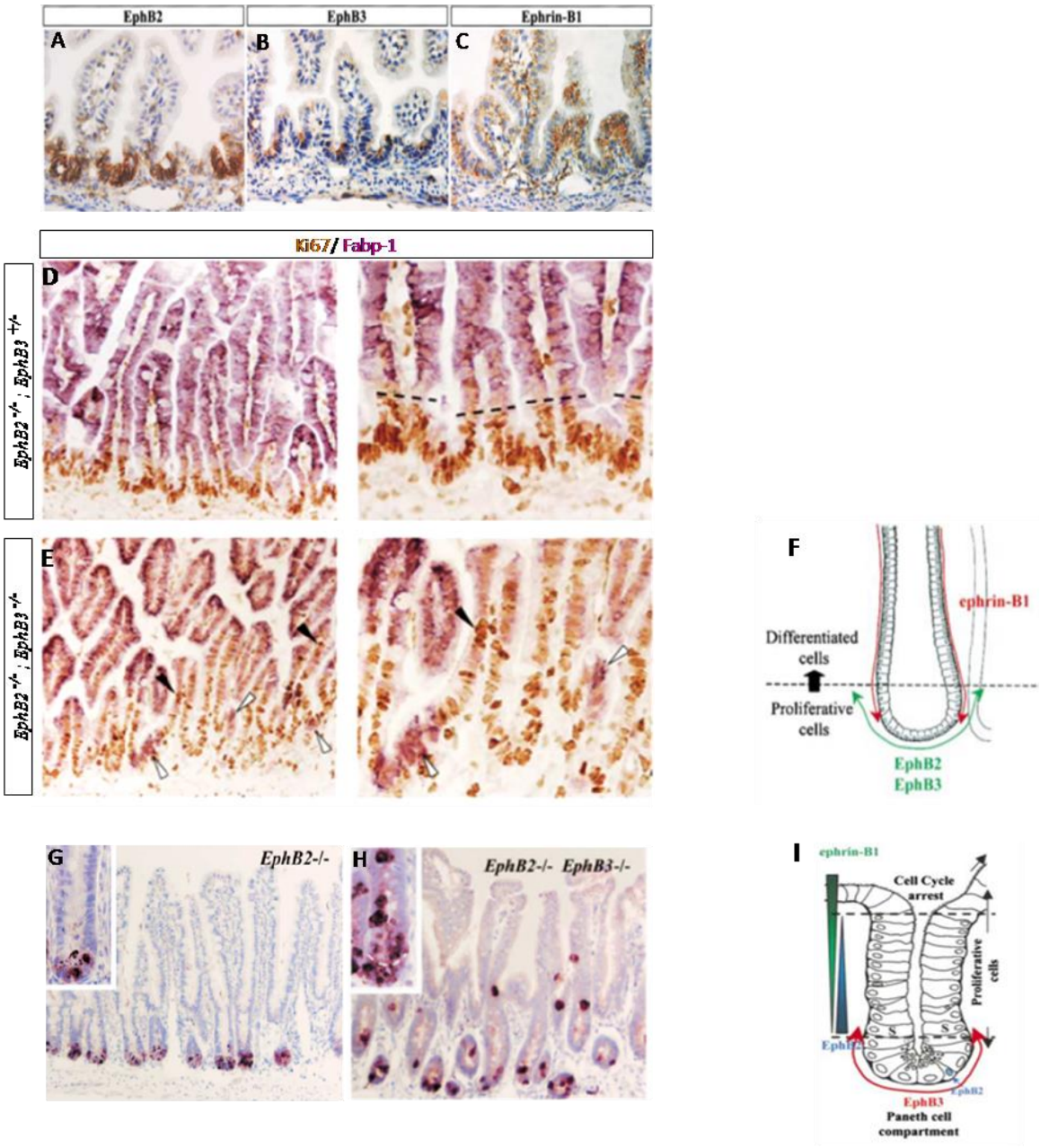

Fig.24. Expression pattern of EphB2, EphB3, and Ephrin-B1 in the small intestine of neonatal and adult mice. A) EphB2 and **B)** EphB3 are expressed in the intervillus pockets of newborn animals. **C)** Ephrin-B1 expression is restricted to differentiated cells. **(D, E)** Double labeling of proliferative cells (Ki67 positive, brown precipitate) and differentiated cells (I-Fabp positive, purple precipitate) in the small intestine of *EphB2-/-;EphB3+/-* **D)** or *EphB2-/-;EphB3-/-* **E)** newborn animals. In *EphB2*$^{-/-}$;*EphB3*$^{-/-}$ animals, differentiated cells (white arrowheads) and proliferative cells (black arrowheads) intermingle. Dotted lines depict the sharp boundary between the proliferative and the differentiated areas in control animals. **F)** Schematic representation of the expression domains of the EphB2, EphB3, and their ligand Ephrin-B1 in neonatal small intestine. EphB2 and EphB3 expression is restricted to the cells in the intervillus regions while ephrin-B1 is expressed in a complementary pattern by the adjacent cells in the villus. Proliferative cells bordering the intervillus pockets coexpress receptors and ligand. **(G, H)** EphB3 restricts the localization of the Paneth Cells to the bottom of the crypts in adult mice, immunodetection of Paneth cells using an anti-lysozyme antibody in intestine sections from *EphB2-/-* **G)** and *EphB2-/-;EphB3-/-* **H)**.
I) Schematic representation of the expression gradients of EphB2, EphB3, and their ephrin ligands in the adult small intestinal crypts. Arrows show the direction of migration flow. S indicates the putative stem cell position. Adapted from (66).

This model implies that graded Ephrin-B and/or Eph receptor activation in intestinal epithelium cells can underlie patterns of tissue organization and cell migration, in which cells position themselves according to the level of expression relative to their neighbors. Therefore, it is not surprising that the disruption of EphB2/EphB3 signaling in the intestine plays an important role in the progression of colorectal cancer.

5.8 Do EPH receptors promote or suppress tumors?

Increasing interest in the Eph/ephrin signaling system stems from its documented importance in a wide range of epithelial and mesenchymal tumors (160). Indeed, most family members were identified in tumor cell lines. EPH cell guidance functions normally active during embryogenesis re-emerge unscheduled and often de-regulated in tumors, modulating cell-cell and cell-matrix attachment, survival during invasion, neoangiogenesis and metastasis (249,250). An example is melanoma, where often expression of EPHs correlates with increased tumor progression and invasive potential (198,251,252). EPHA2 is one of the most abundantly overexpressed EPH receptor in solid tumors (253) and its proto-oncogenic ability was first discovered in melanoma (254,255). Further evidence suggests that EPHA2 is also highly expressed in breast cancer (256) and associated with poor prognosis (257). Moreover, the dichotomy of EPHA2 as an oncoprotein or tumor suppressor is just one example of the complex pleiotropic effects of the EPH receptors in cancer. Overexpression of EPHA2 causes malignant transformation of mammary epithelial cells and decreases ligand binding. These properties appear to be directly linked, since indeed stimulation of EPHA2 reverses the malignant behavior and invasiveness of EPHA2-transformed cells. Ligand-mediated tyrosine phosphorylation of EPHA2 also decreases the growth and invasiveness of malignant breast and prostate cancer cells (212,256). Therefore, the expression level and ligand binding properties work together to allow EPHA2 to differentially regulate tumor cell growth and invasiveness. The model proposed for EPHA2 suggests an oncogenic, ligand-independent role in tumor cells and a tumor suppressing role that involves repression of oncogenic signaling as a result of receptor phosphorylation (253,258) (**Fig.25**). Similarly, EPHB4 in breast cancer has both tumor promoter and tumor suppressor ability (228,259). Despite the substantial levels of EPHB4 expression, EPHB4 tyrosine phosphorylation is much lower in breast cancer cell lines compared with non transformed MCF-10A epithelial cells. Moreover, the silencing of EPHB4 signaling in breast cancer cells is consistent with the low expression of ephrin-B2 in these cells.

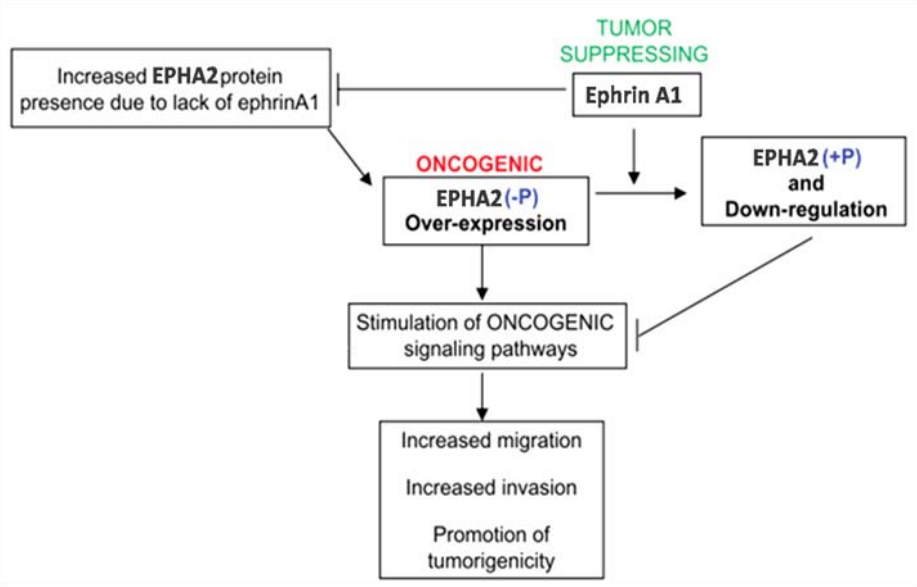

Fig.25. Hypothetical role of the EPHA2/EphrinA1 system in solid tumor cells. EPHA2 becomes overexpressed possibly due to increased gene expression or a lack of ephrinA1-induced receptor down-regulation. Overexpressed EPHA2 is non phosphorylated and stimulates oncogenic processes. EphrinA1 causes receptor phosphorylation and subsequent down-regulation, both of which likely contribute to the tumor-suppressing effects in tumor cells. (-P), no nphosphorylated; (+P), phosphorylated. From (253).

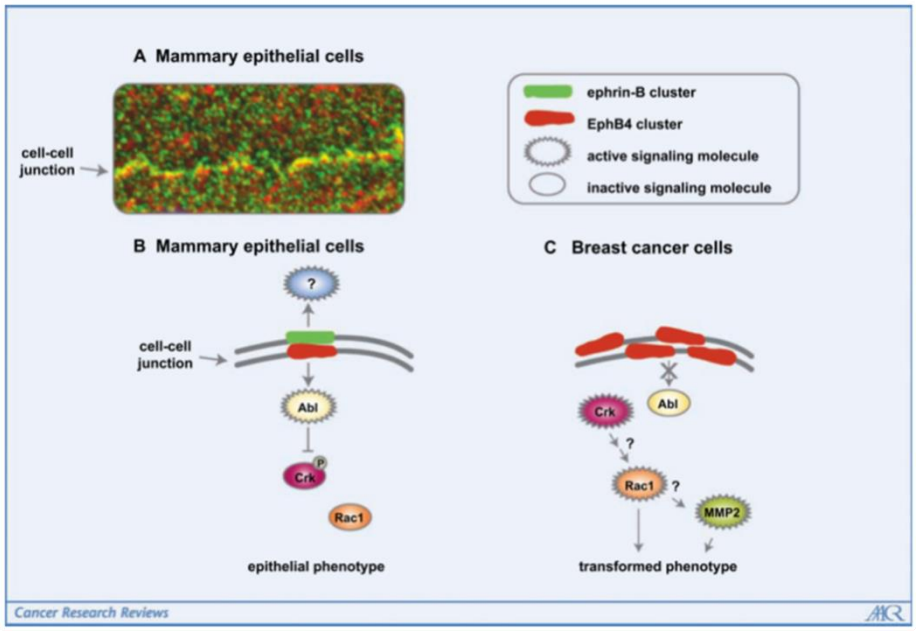

Fig.26. Signaling of EPHB4 receptor in mammary epithelial and breast cancer cells. **A)** MCF-10A nontransformed mammary epithelial cells were stained with EPHB4 antibodies (red) and Ephrin-B antibodies (green). The arrow marks the junction between two cells. **B)** Schematic representation of EPHB4/Ephrin-B2 clusters in MCF-10A cell-cell junctions. The signaling pathways initiated by EPHB4 and Ephrin-B2 in epithelial cells lead to Abl activation and inactivation of Crk adaptor function through phosphorylation by Abl. **C)** Ephrin-B2 expression is low in breast cancer cells, which causes a decrease in EPHB4 and Abl activity, resulting in increased Crk adaptor function and MMP2 expression, presumably through activation of the small GTPase Rac1. Abl represents both Abl and the related kinase Arg. Source (259).

The low EPHB4 tyrosine phosphorylation in mammary tumor cells suggests that ligand-stimulated signaling through the EPHB4 cytoplasmic domain may be detrimental to tumor development. Indeed, treatment of several breast cancer cell lines with ephrin-B2 ligand inhibits proliferation and increases apoptosis. Furthermore, Ephrin-B2 inhibits breast cell motility and invasion concomitant with decreased expression of the matrix metalloprotease MMP2. This tumor suppressor mechanism depends on the activation of the kinase Abl by EPHB4 (**Fig.26**). Paradoxically, in addition to its tumor suppressor activity in breast cancer, EPHB4 can also promote tumorigenesis. EPHB4 knockdown reduces survival, proliferation, migration, and invasion of breast cancer cells where EPHB4 is poorly phosphorylated, suggesting a tumor promoting ability which is independent of ligand-mediated kinase activation. Moreover, the extracellular domain of EPHB4 can also promote tumorigenesis by inducing angiogenic responses through stimulating reverse signaling in cultured endothelial cells (232). Therefore, except those tumors where the overexpression of the EPH receptors and the kinase domain-dependent signaling clearly correlate with increased tumor progression and metastatic potential as occurs in melanoma (198) or lymphoid malignancies (207), growing evidence suggests that tumors with high expression levels of EPH receptors may elude their tumor suppressor activities in more advanced stages by using different strategies: 1) downregulating the Ephrin ligands or additionally upregulating tyrosine phosphatases that dephosphorylate EPH receptors (260), and frequently stimulating downstream oncogenic pathways which are kinase/ligand independent (223,253,259), 2) introducing somatic mutations that impair mainly the kinase or ligand binding domain (261,262), 3) downregulating the EPH receptors through promoter methylation mechanisms (263,264). The epigenetic silencing of the EPHB receptors has been extensively explored in colorectal cancer and perhaps represents the best model to our understanding the complex activity of these membrane binding proteins in cancer.

5.9 EphB/Ephrin-B signaling in colorectal cancer

EPHB2 and EPHB3 signaling impose boundary in colorectal cancer - The expression of EPHB receptors is frequently lost during the progression of colorectal cancer, and this correlates with a poor prognosis (199,263,265). The role of EPHB receptors as suppressors of colorectal cancer progression was initially suspected after analyzing the β-catenin/Tcf target gene program in a collection of human colorectal cancer samples at different stages of malignancy (20). Indeed, dysplastic crypts and small adenomas retained expression of most β-catenin/Tcf targets present in crypt progenitors pinpointing a common tumor initiation mechanism through mutational activation of the Wnt signaling pathway. These initial lesions showed homogenous EPHB2, EPHB3, and EPHB4 expression in all cells at equivalent levels to that of normal crypt progenitors.

Strikingly, the majority of colorectal carcinomas contained >50% EPHB receptor–negative cells despite evident nuclear β-catenin localization. As adenomas represent the benign precursors of carcinomas and tumors of higher grade often behave more aggressively than low-grade ones, the silencing of EPHB expression seems to occur in a subset of tumor cells concomitantly with the acquisition of malignancy. Does loss of EPHB expression confer any advantages to colorectal cancer cells? In the absence of EPHB activity or Ephrin B1 ligand, tumor progression in the large intestine of *Apc*$^{min/+}$ mice is strongly accelerated resulting in the development of aggressive colorectal adenocarcinomas (200). Therefore, whereas constitutive activation of the Wnt signaling pathway is required for the initiation of tumorigenesis (transition from normal epithelium to early adenoma stage), not all the instructions codified within the β-catenin/Tcf crypt progenitor program promote tumorigenesis. Rather, the module that specifies cell positioning seems to block tumor progression beyond the earlier stages (200). *In vitro* and *in vivo* evidence suggests that EPHB receptors suppress CRC progression by mediating the restriction of migration of tumor cells into EphrinB1-expressing territory. Activation of EPHB activity in fully malignant CRC cells enforces compartmentalized growth as opposed to a disseminated distribution. This phenomenon depends on the ability of EPHB signaling to impose cell sorting and E-cadherin-mediated adhesion in cultured CRC cell lines (200,266). Analysis of tumor formation in *Apc*$^{min/+}$ mice confirmed that the expression of EPHB receptors in tumor cells prevented their spreading into the adjacent normal epithelium, which have high levels of EphrinB1 expression (200). The current model suggests that decrease of EPH signaling plays a role during metastatic dissemination (**Fig.27**). Interestingly, EPHB2 and EPHB3 receptors in addition to directing cell migration also regulate proliferation in intestinal crypts (267). The 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 (267). How can the same protein drive proliferation in the normal situation and function as a tumor suppressor in the same tissue? EPHB receptors regulate cell positioning in the intestinal epithelium via PI3K independently of kinase activity. In contrast, intrinsic EPHB tyrosine kinase activity drives proliferation in crypt progenitor cells through Abl, resulting in posttranscriptional regulation of cyclin D1 protein levels. At the progression from adenoma to carcinoma, cyclin D1 expression becomes independent of EPHB signaling, explaining how high proliferation can be maintained and accompanied by invasive growth after loss of EPHB expression. Thus, the fact that EPHB receptors engage separate signaling pathways to regulate proliferation and migration is the basis for the paradoxical proliferative and tumor suppressor functions of the same protein (229,230).

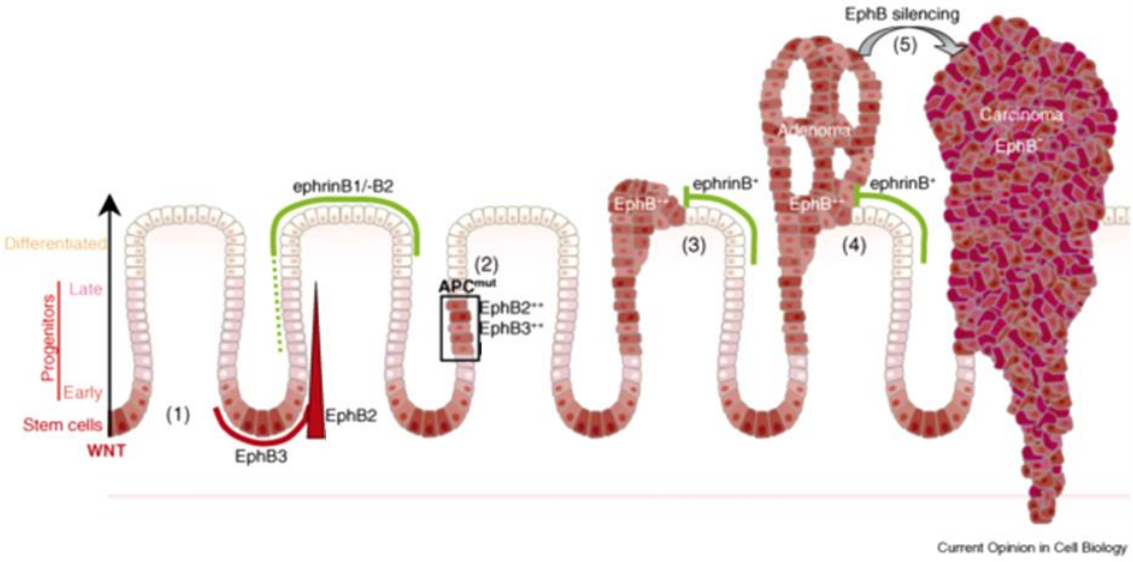

Fig.27. EPHB–ephrinB interactions during CRC progression. (1) Expression domains of EPHB and Ephrin-B proteins in the colon. Wnt signaling occurs at the bottommost positions of the crypts. **(2)** Mutations in the tumor suppressor gene *APC* activate the Wnt pathway and transform intestinal epithelial cells into tumor initiating cells (cells within the square). As a result of constitutive β-catenin/Tcf activity, *APC* mutant cells express high levels of EPHB2, and EPHB3 receptors. **(3)** Tumor initiating cells acquire stem cell properties and repopulate the crypts with their mutant descendants until they reach the surface epithelium. There, tumor cells accumulate and form benign polyp-like outgrowths known as adenomas. Contact of tumor cells with normal differentiated cells that express high levels of EphrinB ligands results in the activation of EPHB signaling. **(4)** Expansion of adenomas is blocked by EPHB repulsive signals which limit the spreading of tumor cells until EPHB expression is silenced **(5)**, coinciding with the acquisition of malignancy. Adapted from (159).

EPHB4 has tumor suppressor activities in intestinal tumorigenesis - EPHB4 expression is reduced in lymph node metastases compared with primary tumors and there is frequent promoter hypermethylation in colorectal cancer (264). In addition, low EPHB4 tumor levels significantly correlate with shorter survival of colorectal cancer patients, suggesting a role as a prognostic marker (**Fig.28**) (264). Overexpression of EPHB4 in colorectal cancer cell line, negatively regulate the tumor growth in a xenograft model. Moreover, inactivation of a single allele of EphB4 in *Apc*$^{min/+}$ mice leads to a 25% shortening of animal survival (**Fig.29**) and this is associated with proliferation and larger tumor size in the small intestine. Importantly, using an *in vitro* assay, loss of EPHB4 in CRC cells results in a significantly increased capacity to invade through a complex extracellular matrix. Together these data confirm an anti-metastatic activity of EPHB4 in CRC disease (265).

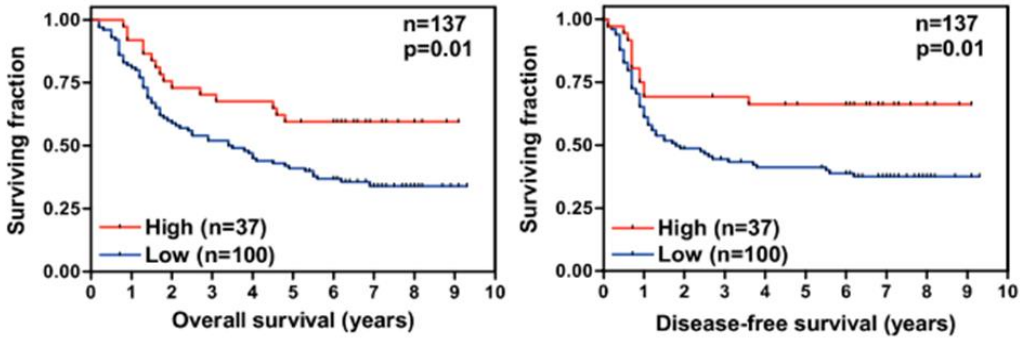

Fig.28. EPHB4 as a prognostic marker in colorectal cancer. Kaplan-Meier plots of overall and disease-free survival in 137 colorectal cancer patients as a function of EPHB4 tumor levels. Patients with low EPHB4 tumor levels had significantly shorter survival than patients with high EPHB4 tumor expression (264).

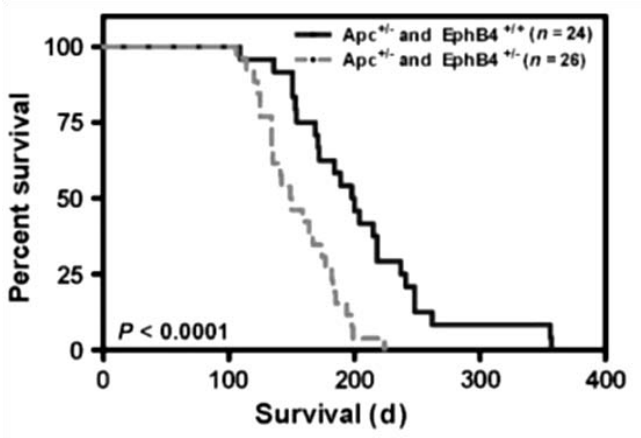

Fig.29. EphB4 inactivation results in shorter survival of *Apc*$^{min/+}$ mice. Heterozygous inactivation of *EphB4* in animals carrying *Apc* mutations (dashed line) results in a significant reduction (25%) of the lifespan of these animals compared with *EphB4* wild-type mice (265).

5.10 EPHA3 receptor

EPHA3 in development - EPHA3 (formerly known as HEK, human EPH-like Kinase) was first identified as a surface antigen on a pre-B lymphoblastic leukemia cell line (LK63) in Andrew Boyd's laboratory by affinity-isolation with a monoclonal antibody (IIIA4) raised against the cells (268). It was then separately identified as an antigen on tumor cells from a melanoma patient (269). Located on the short arm of chromosome 3 (3p11.1), *EPHA3* gene consists of 17 exons and 16 introns and spans 375kb of genomic DNA, thus representing the second largest of the *EPH* genes after *EPHA6*. Its protein, with 983 amino acids, has been found to contribute in different processes during embryonic development. One of the best characterized roles of the EPHA3 receptor thus far is in axon guidance in the developing nervous system, where this receptor plays a repulsive role that causes axons to avoid regions of strong Ephrin ligand expression (270). In fact, knockdown of EphA3 resulted in disorganized segregation of the callosal axons and disrupted axon pathfinding *in vivo* (271). Contrary to predictions made based on high EphA3 expression in the developing medial motor column (272), constitutive loss of murine EphA3 receptor did not lead to abnormal motor axon topography (273). Moreover, overlapping patterns of expression of *EphA3*, *EphA4*, and *EphA7* were detected in the developing palate and nasal structures, however, no overt midfacial phenotype was observed in either $EphA3^{-/-}$ or $EphA3^{-/-}$; $EphA4^{-/-}$ mice, suggesting redundant function of multiple Eph receptors during palate development (274). Instead, more direct evidence of non-redundant function comes from phenotypic analysis of the heart in *EphA3* Knockout mice. Approximately 70-75% of *EphA3* null mice died within 48h of birth due to cardiac abnormalities caused by defective endothelial-to-mesenchymal transition (EndMT), a specific form of mesenchymal conversion that generates endocardial cushions and atrioventricular septa (275) (**Fig.30**).

Similar to other EPH proteins, EPHA3 functions during embryogenesis modulating many cellular mechanisms including cell adhesion, movement and cell shape. All of these are important aspects of cancer progression. Thus, it is predictable that this receptor could re-emerge in cancer.

5.11 EPHA3 in cancer

As mentioned previously, EPHA3 was identified as a tumor antigen on cancer cells from a melanoma patient and high EPHA3 levels were found in a range of melanomas, particularly metastatic tumors (269).

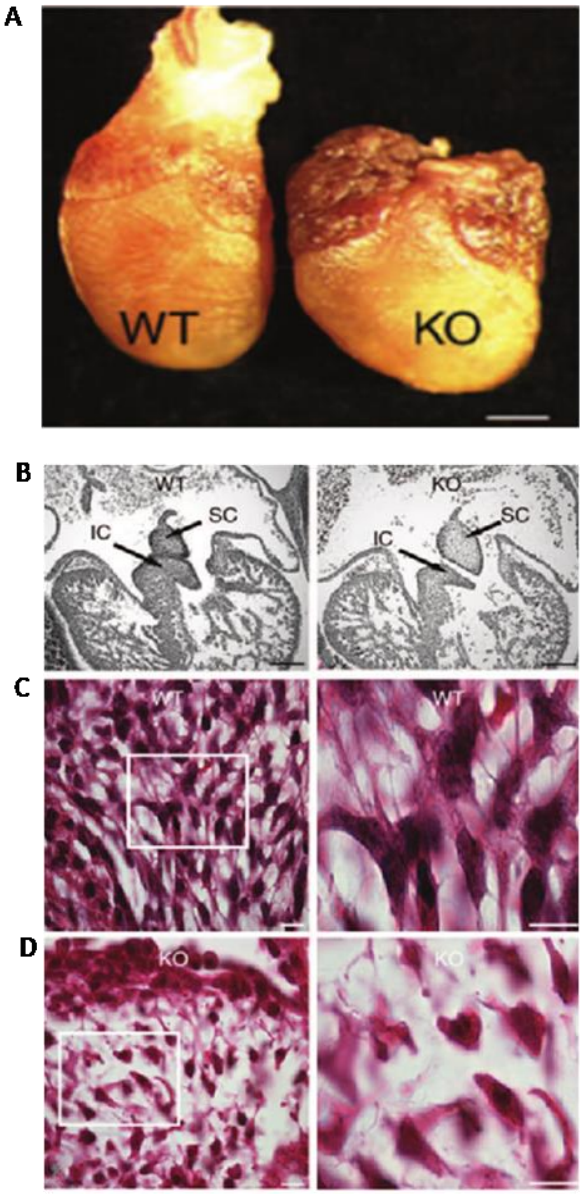

Fig.30. Examination of wild type and *EphA3*$^{-/-}$ P0 hearts in mice. **A)** At postmortem examination, *EphA3*$^{-/-}$ P0 hearts have grossly enlarged, blood-filled atria. **(B, C, D)** Atrioventricular endocardial cushion fusion and cellular morphology is abnormal in embryo *EphA3*$^{-/-}$. **B)** Hematoxylin and eosin-stained wild type and *EphA3*$^{-/-}$ cross sections demonstrate that there is a delay in atrioventricular endocardial cushion fusion in approximately 75% *EphA3*$^{-/-}$ embryos. **(C, D)** Hematoxylin and eosin-stained wild type and *EphA3*$^{-/-}$ endocardial cushion cells. **C)** Wild type endocardial cushion cells have a flattened morphology with many cellular protrusions and extensions that appear to connect neighboring cells. **D)** In *EphA3*$^{-/-}$ embryos, the endocardial cushion cells appear rounded-up and to have few cellular extensions. IC, inferior endocardial cushion tissue; SC, superior endocardial cushion tissue; WT, wild type; KO, *EphA3* knockout. Adapted from (275).

More recent genomic screening of somatic copy number alterations in melanoma cell lines also identified EPHA3 as both amplified and overexpressed (276). *In vitro* studies demonstrated that EPHA3 activation on human melanoma cells under Ephrin-A5 stimulation induces rapid Rho-dependent cytoskeletal re-organization, cell retraction and increased migration, which may promote tumor metastasis (198). In contrast, rhabdomyosarcoma (RMS) cell lines expressing EPHA3, when stimulated with Ephrin-A5 ligand display increased receptor phosphorylation and Rho GTPase activity, loss of adhesion to fibronectin and decreased migration. In addition, the downregulation of EPHA3 increases cell motility, suggesting that this receptor may act in rhabdomyosarcoma cells as a tumor suppressor in a kinase/ligand dependent fashion (277). Thus, depending on the tumor types, the kinase/ligand dependent signaling of EPHA3 receptor can have both, tumor suppressor and tumor promoting functions. The role of EPH proteins in tumor etiology and progression has remained controversial. A growing body of studies suggests a tumor promoting role of EPHA3 that often coincides with loss of the preferred ligand and kinase independent functions, whereas its activation by ligand stimulation reverts the malignant phenotype. As mentioned before, another case in point is EPHA2 receptor. It is overexpressed in a variety of human malignancies and is associated with poor prognosis in several different tumor types. In various studies, EPHA2 overexpression has been linked to malignant progression. Paradoxically, activation of EPHA2 kinase on tumor cells can trigger signaling events that are more consistent with a tumor suppressor activity (258). High levels of EPHA3 expression are associated with more invasive capacity and poor overall survival in hepatocellular carcinoma (278) and with angiogenesis and poor prognosis in gastric cancer (279). However, the exact role of EPHA3 in both cancers is still unclear. Although undetectable in mature hematopoietic cells, EPHA3 protein is frequently overexpressed in hematopoietic malignancies (280). Recent data showed that EPHA3 is widely expressed and kinase inactive on tumor vasculature and supporting stromal tissue (281). In this context EPHA3 is also overexpressed and kinase dormant in glioblastoma (GBM) (223,281). Similar to the ligand Ephrin-A5, pre-clustered IIIA4 agonistic anti-EPHA3 antibody effectively triggers EPHA3 activation and rapid internalization into EPHA3-positive cells (282). Stimulation with IIIA4 antibody results in partial differentiation and decreased proliferation of glioblastoma cell lines (GBM) (223). Despite a couple of studies have reported a tumor promoting activity of EPHA3 that is ephrin- and/or kinase-dependent such as in some hematopoietic tumors (207) and melanomas (198), other evidences suggest a tumor promoting ability that is dependent on the crosstalk with other signaling molecules and that does not require Ephrin binding or kinase activity (223,283). When this occurs, EPHA3 is overexpressed and kinase inactive as consequence of low or absent levels of ligand, however, its activation typically

suppresses the tumor. In keeping with the notion that the kinase/ligand dependent signaling of EPHs suppresses tumorigenesis, somatic mutations likely to affect this signaling have been reported for a number of EPH receptors, and particularly for EPHA3. In fact, genetic screens of tumor specimens from cancer patients have revealed *EPHA3* as the most highly mutated within the EPH receptor family (261). Importantly, many EPHA3 mutations identified in lung, colorectal and hepatocellular cancers impair kinase activity or ephrin ligand binding and/or decrease the level of receptor cell surface localization (261). These results suggest that EPHA3 has ephrin- and kinase-dependent tumor suppressing activities, which are disrupted by somatic cancer mutations.

EPHA3 and cancer somatic mutations: a look to colorectal cancer - EPHA3 is considered the EPH receptor most frequently mutated in a wide range of tumors, including colorectal cancer (261) (**Fig.31**). Kinase domain mutations in EPHA3 were first identified by genetic screening of the tyrosine kinome in a panel of 182 colorectal cancers (284). In addition, a number of other mutations were independently identified, again in CRC, where *EPHA3* was classified the sixth most recurrently mutated gene. This study was based on a high-throughput screening of 13,023 genes in a series of 11 colorectal cancer samples (285) and further validation of the genes with higher mutation rates in an independent cohort of 96 colorectal tumors (132). To distinguish genes likely to contribute to tumorigenesis from those in which passenger mutations occurred by chance, novel statistical methods were developed to identify genes with a number of mutations greater than expected from the background mutation rate. For each gene, this analysis incorporated the number of somatic alterations observed, the number of tumors studied, and the number of nucleotides successfully analyzed. The output of this analysis was a cancer mutation prevalence (CaMP) score for each gene analyzed (286). Validated genes with CaMP scores greater than 1.0 were considered to be candidate cancer genes (CAN genes), identifying 69 of the >13,000 genes investigated as likely drivers of the tumorigenic process. Surprisingly, *EPHA3* mutations were observed in >12% of the tumors sequenced, resulting in a CaMP score >4 (Table 2). Only *APC*, *TP53*, *KRAS*, *FBXW7* and *SMAD4* showed CaMP scores higher than *EPHA3* and mutation frequency in this EPH receptor was higher than the incidence observed in other well known tumor suppressor genes in this organ, such as *SMAD2* and *TGFBRII* (285) (**Table 2**). The *EPHA3* mutations identified in these studies were then validated *in vitro* confirming the predicted effects (261) (**Table 3**). Indeed, most of them impair the kinase activity or the Ephrin ligand binding and/or decrease the level of receptor cell surface localization (261). Moreover, additional genetic screenings have confirmed the presence of recurrent *EPHA3* mutations in colorectal tumors that are distributed throughout the coding sequence of this gene (287–289) (**Fig.32**). However, there are no studies that

clarify the role of EPHA3 in intestinal tumorigenesis. Thus, the high mutation frequency together with previous studies that confirm the inactivating effects of these genetic alterations on EPH receptor activity, robustly suggest a potential tumor suppressor role of EPHA3 in colorectal cancer.

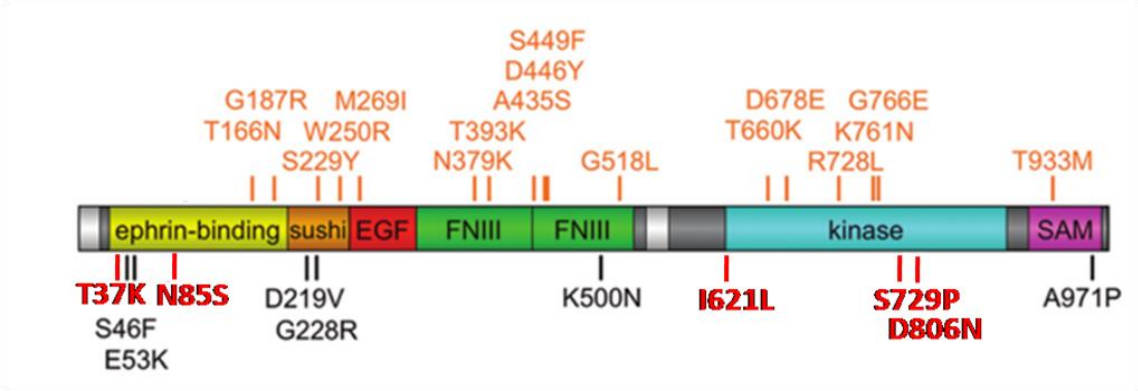

Fig.31. Location of the cancer somatic mutations studied in EPHA3 domain structure. Mutations identified in lung (orange) and colorectal cancer (red). Mutation identified in other cancers (black) (261).

CAN Gene	CCDS accession	CaMP Score	Estimate fraction of tumors containing mutation	Total number of mutations	Homo-zigous	Hetero-zigous
APC	CCDS4107.1	> 10	90%	31	16	15
KRAS	CCDS8702.1	> 10	44%	16	4	12
TP53	CCDS11118.1	> 10	51%	18	15	3
FBXW7	CCDS3777.1	5.07	14%	4	1	3
SMAD4	CCDS11950.1	4.56	13%	4	4	0
EPHA3	CCDS2922.1	4.22	13%	4	0	4
MLL3	CCDS5931.1	3.69	21%	6	0	6
GUCY1A2	CCDS8335.1	3.51	12%	3	0	3
EPHB6	CCDS5873.1	3.50	13%	4	0	4
PKHD1	CCDS4935.1	3.46	16%	5	0	5
TBX22	CCDS14445.1	3.27	12%	3	1	2
SMAD2	CCDS11934.1	3.05	10%	3	3	0

Table 2. CAN genes in colorectal cancer. A high-throughput screening of 13,023 genes in a fraction of 11 colorectal cancer tumors identified 69 candidate genes (CAN genes) with a cancer mutation prevalence (CaMP) score >1. Genes with CaMP score >1 are predictive to have mutation frequency higher than the background mutation frequency. The table shows only those genes with a CaMP score >3. Of note, *EPHA3* is shown in red. From supporting online material, Table S6 – Colorectal CAN genes, (285).

Mutation	Domain	Ptyr in cells[1]	In vitro Kinase Activity	Ephrin-A5 binding[2]	Cell Surface Localization	Ref.
Wilde Type		=	=	=	=	
T37K	EB	<	nd	=	=	(285)
N85S	EB	=	=	<	=	(285)
I621L	Kinase	=	=	nd	<	(285)
S792P	Kinase	=	=	nd	=	(284)
D806N	Kinase	<<	<<	nd	=	(284,285)

Table 3. Effects of EPHA3 mutations in HEK-293T cells. Legend: =, similar to that of the wild type (HEK 293T overexpressing wild type form of EPHA3); <, less than that of the wilde type; ≪, much less than that of the wild type. [1]Tyrosine phosphorylation of EPHA3 overexpressed in HEK-293T cells. [2]Ephrin-A5 binding domain. nd, not determined. Adapted from (261).

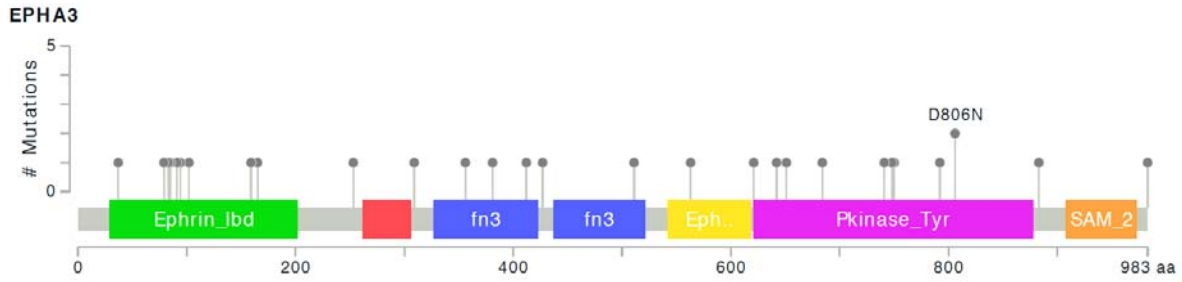

Fig.32. EPHA3 mutations reported in colorectal tumors. Representation along the EPHA3 protein of all *EPHA3* mutations found in the 558 colorectal tumors investigated in these studies: (284,285,287–289).

Aims of study

The loss of EPHB receptors activity has been shown to play a causal role in colorectal tumorigenesis, mainly in adenoma-carcinoma transition. EPHA3 signaling is frequently dysregulated in cancer and different studies identified this receptor as one of the most recurrently mutated in colorectal cancer. The present study set out to investigate the role of the EPHA3 in colorectal tumorigenesis. The specific aims of this thesis were:

- to study the functional relevance of EPHA3 overexpression in colorectal cancer cell lines using inducible *in vitro* and *in vivo* xenografts and lung metastasis models.

- to investigate the effects of EPHA3 inactivation in colorectal tumorigenesis employing a model of *EphA3* Knockout mice.

- to evaluate the possible association between EPHA3 tumor levels and survival or clinicopathological features of Dukes C colorrectal cancer patients.

Material and Methods

Cell Lines and Transfections. DLD1 and LS174T colon cancer cell lines were cultured on RPMI medium with 10% fetal bovine serum (Sigma) and 1x antibiotic antimycotic (Life Technologies) at 37^0C and 5% CO_2. LS174T and DLD1 cells carrying the tet-repressor plasmid (TR1 and TR7, respectively) were generated using the T-rex system (Invitrogen) as described previously (120) and were a kind gift of Dr. Hans Clevers (Hubrecht laboratory and Center for Biomedical Genetics, Utrecht, Netherlands). To engineer EPHA3-inducible clones, DLD1-TR7 and LS174T-TR1 were transfected with pLenti/TO-EPHA3 and the corresponding empty vector (pLenti-CMV/TO Neo DEST, Addgene 17292) using Lipofectamine 2000. Transfectants were selected in medium containing G418 (1 or 0.5 mg/ml for DLD1 and LS174T respectively; Invitrogen). Resistant clones were picked and expanded. After doxycycline treatment (1μg/ml; Sigma) for 48h, the overexpression of EPHA3 was tested by RT-PCR and western blot. Cells transfected with pLenti-CMV/TO (empty vector) were used to control for possible non-specific effects of doxycycline treatment.

Clinical Samples. Samples from colorectal cancer patients with locally advanced disease (Dukes C) were collected at collaborating medical institutions in Spain and Finland as previously described (264,290). Informed consent for genetic analysis of the tumor sample was obtained from each patient, according to protocols approved by the human investigations and Ethical Committee in the appropriate Institution. For tissue microarray preparation, areas containing a high proportion of tumor cells were selected after histological examination of hematoxylin and eosin stained tumor sections. Triplicate 0.6-mm cores from every sample were arrayed in a fresh paraffin block using a Beecher Instrument tissue arrayer (Silver Spring, MD). Unstained 4-mm sections from the tissue microarray were mounted on slides coated with 3-aminopropyl-triethoxy-silane (Sigma, St Louis, MO). A total of 159 patients with Duke C colorectal tumors were used for immunohistochemical assessment of EPHA3 levels. The mean follow up of the patients was 7.3 years (range from 3.1 to 9.5 years).

RNA Extraction and Quantitative Real Time PCR (qPCR). Total RNA was extracted from liver and epithelial cells of small intestine using the TRI Reagent® (Molecular Research Center) according to the manufacturer's instructions. The RNA (500ng) was reverse transcribed using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems). Relative *EphA3* mRNA levels were assessed using SYBR Green Master Mix (Applied Biosystems). Real time PCR reactions were performed in triplicate on a ABI PRISM 7500 Real-Time System (Applied Biosystems). *18s* rRNA was used as a standardization control for the $2^{-\Delta\Delta Ct}$ method (291). The primers used were *EphA3*-qPCR-F: 5'-CAGCCTTCCAACGAAGTTAAT-3'; *EphA3*-qPCR-R: 5'-CCATGGGATGGGTAGGAG-3';*18s*

rRNA-F: 5'-AGTCCCTGCCCTTTGTACACA-3'; *18s* rRNA-R: 5'-GATCCGAGGGCCTCACTAAAC-3'.

Protein Extraction and Quantification. To obtain whole protein cell lysates, cell cultures were 70% confluence when washed once with ice-cold PBS and harvested with a rubber sterile scraper. Cell pellets were collected in a micro tube by centrifugation for 5min at 3000g at 4°C and resuspended in 30µl of radioimmunoprecipitation (RIPA) lysis buffer (0.1% SDS, 1% NP40 and 0.5% Na-deoxycholate in PBS) complemented with protease inhibitors (Pepstatine 5µg/µl, PMSF 0.3mM, Aprotinine 1µg/µl and Sodium orthovanadate 100µM). After 30min of incubation on ice, cells were sonicated for 10sec 3 times at 20-50 kHz on ice, then the lysate was centrifuged for 20min at 16000g at 4°C, and the supernatant was transferred into a new microtube and stored at -80°C. In order to estimate protein yields, protein concentrations were quantified with a BCA™ Protein Assay Kit (Thermo Scientific). The BCA (bicinchoninic acid) protein assay reagent kit is based on the biuret reaction where Cu^{2+} is reduced to Cu^{1+} by proteins in an alkaline medium. Addition of bicinchoninic acid leads to a purple-coloured end product which can be detected colometrically at 562 or 620nm. The protocol is the following: 2µl of test sample diluted in distilled water (final volume 25µl) were mixed with 200µl of BCA mixture in a 96 well plate. A series of protein standards with BSA diluted in distilled water was run alongside with the protein lysates to establish a standard curve. The plate was dark-incubated at 37°C for 30min prior to the measurement. The absorbance was read at 620nm on a plate reader (Sunrise™ model, TECAN Group Ltd.). Protein concentrations were determined using the BSA standard curve method.

Western blot:

Gel separation **-** Separation of proteins was performed by one dimensional SDS-PAGE electrophoresis assay as follows. Proteins were thawed on ice and 50-70µg were mixed with loading dye (25mM Tris pH 6.8, 8% SDS, 40% Glycerine, 0.02% bromphenol blue, 400 mM mercaptoethanol) and denatured at 100°C for 5min before being loaded into a polyacrylamide gel (4% stacking gel, 10% running gel). The electrophoresis chamber was filled with 1x running buffer (0.025M Tris, 0.19M Glycine, 0.1% SDS pH 8.3). The current was set to 120mA and proteins were left to run until the loading dye reached the edge of the gel. ***Transfer*** **-** After the protein separation step, the gels were removed from the glass plates and equilibrated in cold 1x transfer buffer (0.023M Tris, 0.19M Glycine) for 10min at room temperature. To transfer the proteins from the gel to a PVDF (PolyVinyliDene Fluoride) membrane, a wet blotting system was used. For this, the membrane and filters were stacked as a "sandwich" together with filter papers and sponges in the following order: sponge, filter paper, membrane, gel, filter paper and sponge. This setup was run at 100V for 60-100min in a chamber filled with ice-cold 1x

transfer buffer. ***Blocking and Blotting*** **-** After protein transfer, the membrane was blocked with a blocking buffer (5% skim milk in PBS-0.1% Tween) for 1h in order to prevent unspecific binding of the antibodies. The membrane was then incubated overnight at 4°C with the primary antibody. *Primary Antibodies used*: rabbit anti-EPHA3 (1:200; L18, Santa Cruz), rabbit anti-EphrinA5 (1:500; Novus Biological), mouse anti-phosphotyrosine (1:2000, PY20, BD Transduction Laboratories. Mouse anti-β-Tubulin (1:2500; TUB 2.1, Sigma) was used as loading control. Unbound antibody was removed by washing with PBS-0.1%Tween. The membrane was then incubated for 1h at room temperature with a secondary antibody conjugated with horseradish peroxidase: anti-mouse (1:5000; polyclonal goat, Dako), anti-rabbit (1:5000; polyclonal swine, Dako). ***Detection*** **-** Detection of proteins by western blotting was achieved using Enhanced Chemiluminescence system (ECL, GE Healthcare), a light-emitting non-radioactive substrate for the horseradish peroxidase. Briefly, membranes were incubated with an equal volume of detection reagent A and reagent B for 1min. Then, AGFA (CP-BU) films were exposed to the membrane to detect the chemiluminescent signal, and after automated film development the bands were visualized.

Immunoprecipitation. Cells were lysed with RIPA buffer complemented with protease and phosphatase inhibitors and briefly sonicated. Total protein (1mg) was pre-incubated with mouse anti-EPHA3 (IIIA4, (223); 1µg per 1mg of protein) or mouse anti-GAPDH (4µg per 1mg of protein; 6C5, Santa Cruz Biotechnology) in immunoprecipitation (IP) buffer (50mM Tris-HCl pH7.5, 150mM NaCl, 1.5mM $MgCl_2$, 1% Triton X-100, 5% Glycerol) at 4^0C overnight. Prewashed protein G-Agarose beads (Santa Cruz Biotechnology) were added with further incubation for 1h at 4^0C. After incubation with G protein beads, samples were washed five times in IP buffer and analyzed by western blot with the indicated antibodies.

FACS Analysis. Cell surface expression of EPHA3 on LS174T-EPHA3 and DLD1-EPHA3 cell lines was confirmed by FACS. Cells ($5x10^5$ per sample) pretreated with Doxycycline for 48h were suspended in 100µl of PBS with mouse anti-EPHA3 (IIIA4; 5ug/ml) or mouse anti-GAPDH (1:200; 6C5; Santa Cruz Biotechnology) for 1h. Cells were washed with cold PBS and incubated with a FITC-labeled secondary antibody specific for mouse IgG (1:200; Jackson ImmunoResearch Laboratories). After incubation for 30min at 4^0C, cells were again washed and resuspended in propidium iodide solution (2mg/ml). Fluorescence was quantified by flow cytometry with a BD FACSCalibur™ instrument and CellQuest Software (BD Biosciences).

Mouse Knockout Strains and Azoxymethane Treatment. $Apc^{min/+}$mice on a 129/Sv background have been generated previously (292). These mice carry a heterozygous Apc mutation which converts codon 850 from a leucine (TTG) to a stop (TAG) codon inducing

formation of multiple polyps in the small and large intestine associated with loss of the remaining wild type gene (293). The *EphA3* knockout mouse has been previously described and is on a 129/Sv genetic background (273). The first exon of *EphA3*, encoding its signal sequence, was removed and replaced with a PGK-neo cassette by homologous recombination. Male *Apc$^{min/+}$;EphA3$^{+/+}$* mice were crossed with female *Apc$^{+/+}$;EphA3$^{-/-}$* mice to obtain *Apc$^{min/+}$;EphA3$^{+/-}$* males and *Apc$^{+/+}$;EphA3$^{+/-}$* females that were subsequently crossed to obtain the *Apc$^{min/+}$;EphA3$^{+/+}$, Apc$^{min/+}$;EphA3$^{+/-}$*and *Apc$^{min/+}$;EphA3$^{-/-}$.* In addition, nine-week old *EphA3$^{+/+}$* and *EphA3$^{-/-}$* mice (both *Apc$^{+/+}$*) were injected intraperitoneally with the intestine-specific carcinogen azoxymethane (AOM; 10mg/kg; Sigma) once a week for 9 weeks and sacrificed 7 weeks after the last AOM injection.

Xenograft and Lung Metastasis Mouse Models. Twelve NOD/SCID mice (Harlan Laboratory) 7-8 weeks old were injected subcutaneously with 2.8x10^6 DLD1-EPHA3 (right flank) and the DLD1-EV (left flank) resuspended in 100µl PBS. The animals were randomized in a group receiving doxycycline *ad libitum* in drinking water (1mg/ml doxycycline, Sigma and 2.5% sucrose, Sigma) or a control group (2.5% sucrose). The same experimental set-up was carried out for LS174T-EPHA3 and the corresponding empty vector (2.8x10^{6}cells). Tumor size was measured using a caliper three times per week. Tumor volume was calculated with the formula: $V = (L \times W^2) \times 0.5$, where L is the length and W is the width of a xenograft. For the model of experimental lung metastasis**,** LS174T-EPHA3 (3x10^{6}cells) or DLD1-EPHA3 (2x10^{6}cells) resuspended in 100µl PBS were injected in the lateral tail vein of 20 NOD/SCID mice (Harlan Laboratory) 8-9 weeks old. The animals were randomized in a group receiving *ad libitum* doxycycline via drinking water (1mg/ml doxycycline, Sigma and 2.5% sucrose, Sigma) or a control group (2.5% sucrose) and sacrificed at the indicated time. The number of lung metastasis foci was scored and then the lungs were formalin-fixed, paraffin-embedded, sectioned and stained with hematoxylin and eosin. All animal experiments were carried out under protocols approved by the Vall d'Hebron Ethical Committee and the appropriate governmental agency.

Histology. Eighty-six-week-old mice (*EphA3$^{+/+}$* and *EphA3$^{-/-}$*) or forty-two-week-old mice (*Apc$^{min/+}$;EphA3$^{+/+}$*, *Apc$^{min/+}$;EphA3$^{+/-}$ and Apc$^{min/+}$;EphA3$^{-/-}$)* were sacrificed, the small and large intestines were dissected, opened longitudinally and fixed with 4% formalin. Tumor size and number were scored under a dissecting microscope (OLYMPUS SZH stereo-zoom microscope, magnification X 7.5) before paraffin inclusion. Next, the intestine was rolled longitudinally using the 'Swiss roll' technique (294), with the mucosa side inwards and the distal part of the intestine toward the center of the roll. The preparation was transferred to a tissue cassette and dehydrated by serial

immersion in 50%, 70%, 96% and 100% ethanol. Excess ethanol was removed by incubation in xylene three times for 1h at room temperature and the cassettes then immersed in 65^0C paraffin overnight. Tissues were then included in paraffin blocks. Tissue sections (4μm) were cut and placed on poly-L-Lysine coated microscope slides, incubated at 54^0C for 1h and de-waxed by immersion in xylene (2x5min) and hydrated by serial immersion in 100% EtOH (2x5min), 96% EtOH (5min), 70% EtOH (5min), 50% EtOH (5 min) and distilled water.

Immunohistochemistry. The NovoLink polymer detection system (Novocastra Laboratories) was used according to the instructions of the manufacturer. For human EPHA3 staining, antigen retrieval was done in 10mM citrate buffer (pH 6.0) in a pressure cooker (4min at 120ºC). Sections were incubated with rabbit anti-EPHA3 (1:200; L18, Santa Cruz) at 4ºC overnight. Slides were counterstained with Mayer's haematoxylin dehydrated and mounted with DPX mounting medium (Panreac Quimica).

Tissue Microarrays (TMAs) Analysis. EPHA3 staining levels were scored using a semiquantitative scale from 0 (absence of EPHA3 Immunostaining) to 3 (highest immunostaining). EPHA3 expression was evaluated blinded from the clinical data. For Kaplan-Meyer plots, EPHA3 levels were dichotomized as low or high EPHA3 using an average score cutoff value of 1.5. Importantly, no significant survival differences between high/low EPHA3 groups were observed with any other possible cutoff value. EPHA3 was considered as a continuous variable for Cox multivariate regression analysis of prognostic factors (covariates: EPHA3 levels, histologic grade, sex, age, and tumor location) as shown in **Table 4**.

Clonogenicity Assay. LS174T-EPHA3 or DLD1-EPHA3 cells and the corresponding empty vector control cells were seeded ($5x10^2$) into 6-well plates and allowed to attach overnight. The medium was then replaced with complete medium with or without doxycycline (1μg/ml) as indicated; the cells were then allowed to grow for 10 days. The colonies were stained with crystal violet 0.1% and the number of macroscopically visible colonies was scored blinded from the sample identity. Three independent experiments were carried out in triplicate.

Proliferation Assay. Cells were seeded into 24-well plates in triplicate and allowed to attach overnight ($5x10^5$ for LS174T-EPHA3 or $3x10^5$for DLD1-EPHA3 and the corresponding empty vector cells). Doxycycline (1μg/ml) was added as indicated. Cell counting was performed by cell trypsinization and staining with trypan blue. Viable cells were counted using a hemocytometer at times 0, 24, 48, 72 and 96h. Growth curves presented are the average of three independent experiments carried out in triplicate.

Wound-Healing Assay. Cells were seeded into 6-well plates ($2x10^6$cells per well) and allowed to grow until they reached 90% confluence with or without doxycycline (1ug/ml) as indicated. The cell monolayer was scratched with a sterile micropipette tip and the wound region was allowed to heal by cell migration. The area that remained clear of cells after 4, 8, 12, 24 and 48h was quantified blinded from sample identity with Image J (National Institutes of Health, NIH) and compared with the area of the wound at time zero. The average of three independent experiments in triplicate is shown.

Matrigel Invasion Assay. The ability of cells to invade through matrigel-coated filters was determined using a 24-well Boyden chamber (Beckton Dickinson; 8µm pore size) covered with 100µl of 1mg/mL Matrigel (Beckton Dickinson). Cells ($6x10^5$ of LS174T-EPHA3 or $3x10^5$ of DLD1-EPHA3) were seeded in 100µl of RPMI medium containing 1%FBS in the upper compartment of the transwell. Where indicated doxycycline (1µg/ml) was added. The lower compartment was filled with RPMI medium (with or without doxycycline) containing 10% FBS, acting as an attractant. After incubation for 48h at 37^0C in 5% CO_2, the cells that did not penetrate the filter were wiped out with a cotton swab, and the cells that had invaded into the lower surface of the filter were fixed and stained with 5% crystal violet. Filters were mounted on microscope slides to enable cell counting under the microscope (10X) blinded from the sample identity. The total number of invading cells was determined and the average of three independent experiments run in triplicate is shown.

Soft-Agar Colony Formation Assay. LS174T-EPHA3 or DLD1-EPHA3 cells were resuspended ($1×10^5$) in complete RPMI medium containing 0.3% agar with or without doxycycline (1µg/ml) and then plated into 6-well plates on top of 0.6% agar in RPMI medium. Cultures were maintained at 37^0C in a 5% CO_2 incubator for 2-3 weeks depending on the cell line. Fresh complete RPMI medium was added with or without doxycycline (1µg/ml) every 2-3 days. The colonies were stained with nitro blue tetrazolium chloride (1mg/ml; Sigma) and the number of macroscopically visible colonies was scored blinded from the sample identity. Three independent experiments were carried out in triplicate.

Results

1. Generation of colon cancer cell lines with inducible EPHA3 activity

Several EPH receptors and their Ephrin ligands have been found to be aberrantly expressed in multiple cancer types (160) and to significantly contribute to colorectal tumorigenesis (199,265). To explore the functional role of EPHA3 in colorectal cancer, we engineered cell line systems with doxycycline-dependent inducible EPHA3 expression. As recipients, we chose two colorectal cancer cell lines, LS174T expressing mutant β-catenin protein and DLD1 which is mutant for *P53* and *APC* (120). Both cell lines show low endogenous levels of EPHA3 (**Fig.33 A**) and high expression levels of the preferred ligand, Ephrin-A5 (295) (**Fig.33 B**). Moreover, sequencing of the full coding region of *EPHA3* in these cells discovered that DLD1 carry two different point mutations localized in the kinase domain that are expected to disrupt the kinase activity of EPHA3 receptor, whereas LS174 cells are wild type. Clones of DLD1 and LS174T constitutively overexpressing the tetracycline repressor (T-rex system), were stably transfected with a vector expressing human EPHA3 under the control of the doxycycline-inducible CMV/TO promoter (pLenti-CMV/TO-EPHA3) or the corresponding control empty vector. Doxycycline-dependent-expression of EPHA3 was confirmed on individual clones by Western blot (**Fig.34 A, B**) and membrane localization was assessed by flow cytometry analysis (**Fig.34 C, D**). The tumor suppressor role of EPHA3 has been discovered to be kinase-dependent in some types of cancer (277,283). Therefore we checked the phosphorylation of EPHA3 receptor upon doxycycline treatment by an immunoprecipitation assay, confirming the activation of EPHA3 signaling in these cells (**Fig.34 E, F**).

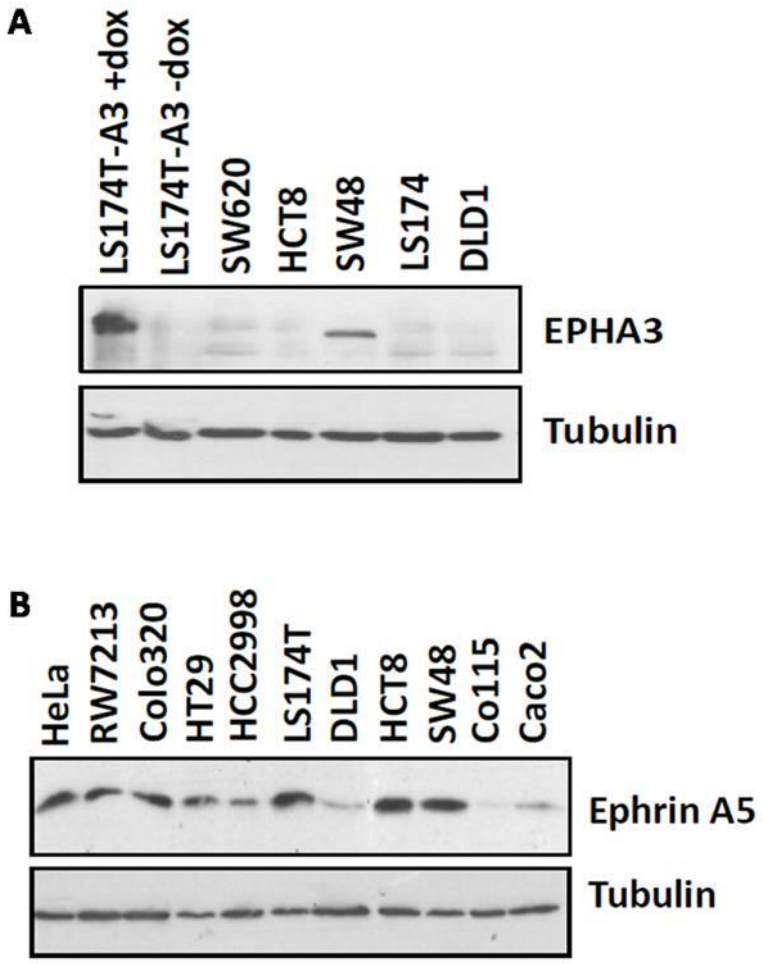

Fig.33. EPHA3 and Ephrin A5 levels in colorectal cancer cell lines. The levels of EPHA3 **A)** and its preferred ligand Ephrin A5 **B)** were assessed in a panel of colon cancer cell lines by Western blotting. Tubulin levels were used as a loading control.

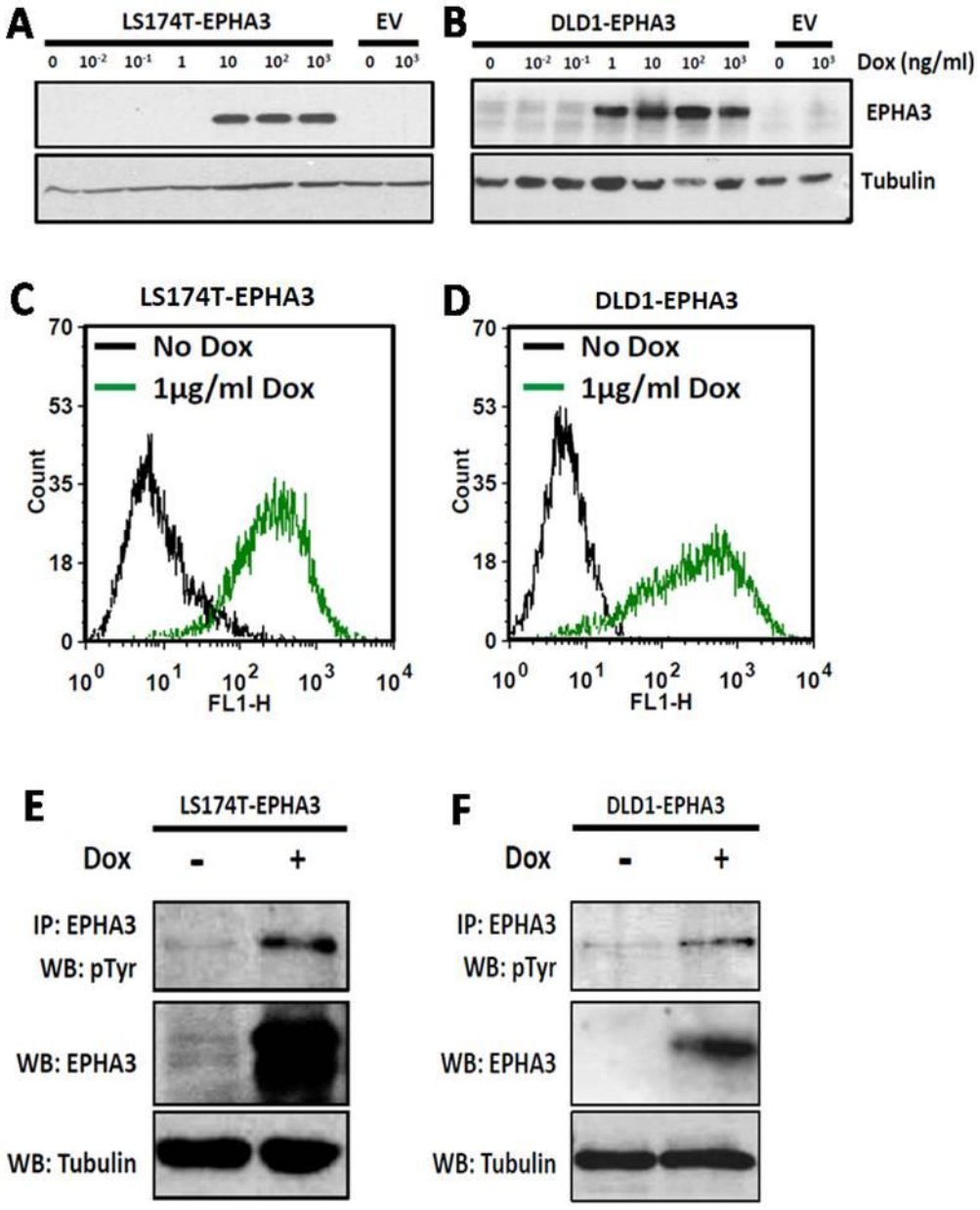

Fig.34. Inducible EPHA3 activity in colon cancer cell lines. (A, B) Western blot showing the levels of EPHA3 protein in LS174T **A)** and DLD1 **B)** cells stably transfected with plenti/TO-EPHA3 or the control empty vector (EV) after treatment with the indicated concentrations of doxycycline for 48h. Tubulin levels are shown as a loading control. **(C, D)** Cell surface levels of EPHA3 following induction with doxycycline (1µg/ml) were assessed by flow cytometry analysis in LS174T-EPHA3 **C)** and DLD1-EPHA3 **D)**. **(E, F)** The levels of EPHA3 phosphorylation after doxycycline treatment (1µg/ml) for 48h were determined by immunoprecipitation with anti-EPHA3 and Western blotting with anti-phospho-Tyrosine. Total input levels of EPHA3 and tubulin are also shown for LS174T-EPHA3 **E)** and DLD1-EPHA3 **F)**.

2. EPHA3 does not regulate the growth of colon cancer cells

The sustained and uncontrolled cell growth represents one of the most critical events of the cancer development. The balance between the proliferation and differentiation, tightly regulated in normal condition, is often disrupted at the expense of cell differentiation in a tumorigenic context (69). EPHB signaling has been shown to play an important role in maintaining active proliferation rates found in the intestinal epithelial cells as well as in intestinal tumors (199,265,267). Recent data, suggested that the activation of EPHA3 signaling in glioblastoma cells reduces their growth (223). Thus, we used the cell line systems engineered to study whether EPHA3 signaling regulates the proliferative activity of colon cancer cells.

In vitro: First, we investigated whether the reintroduction of EPHA3 into LS174T and DLD1 cells modulated their growth by directly counting the number of cells at different times post-seeding, and found no differences after EPHA3 signaling activation by doxycycline treatment (**Fig.35 A,B**). It is well known that the capability of a cancer cell to form colonies is a powerful indicator of its tumorigenicity. However, we observed that the ectopic expression of EPHA3 did not affect the ability of colon cancer cells to growth as colonies either in a solid or semisolid soft-agar substrate (**Fig.35 C, D**).

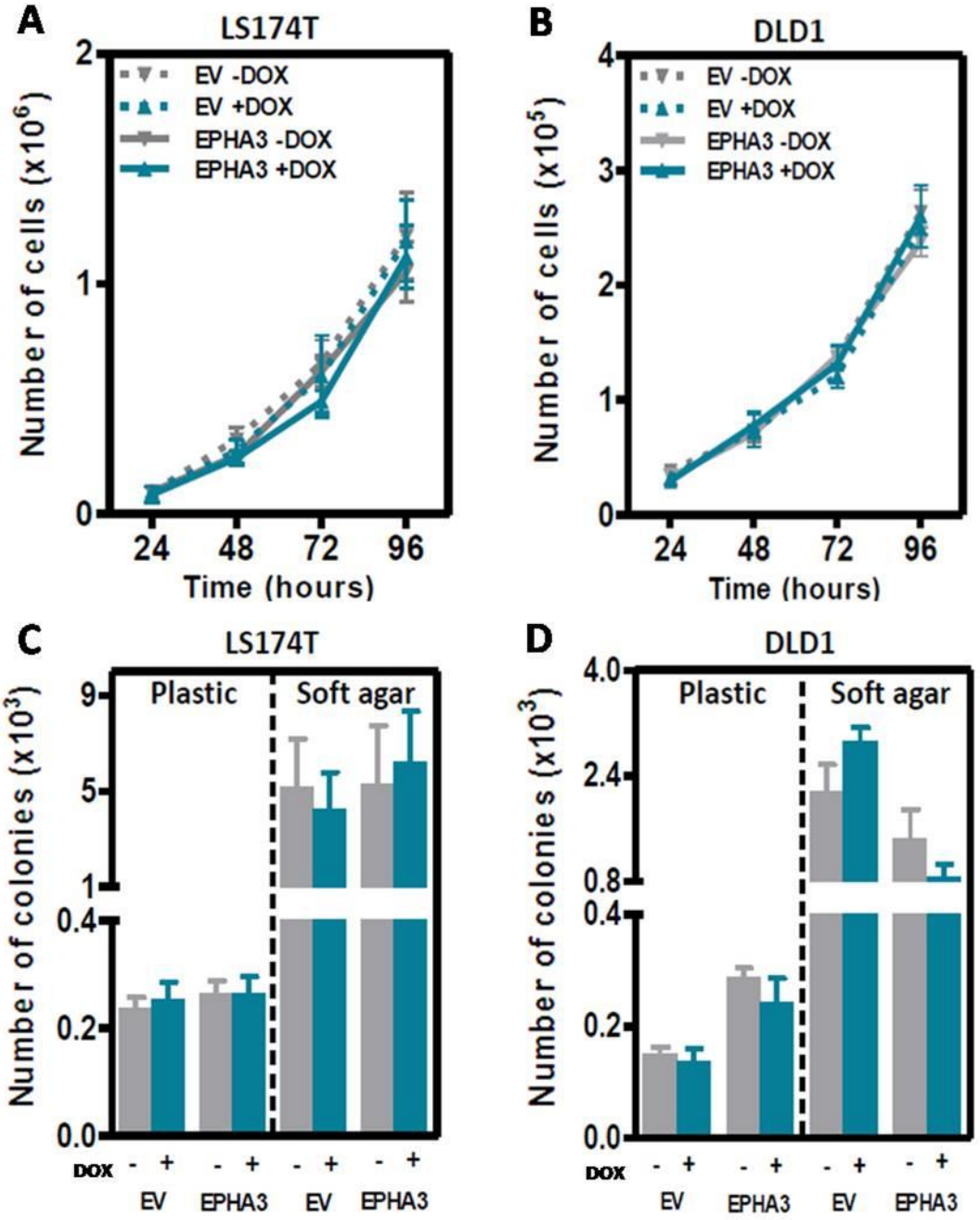

Fig.35. Effects of EPHA3 overexpression on the growth of colon cancer cells *in vitro*. The average number of LS174T-EPHA3 **A)** and DLD1-EPHA3 **B)** cells and the corresponding empty vector control (EV) at the indicated times after seeding is shown (mean ±SEM of three independent experiments). Panels **(C, D)** show the number of colonies of LS174T-EPHA3 **C)** and DLD1-EPHA3 **D)** cells and the corresponding empty vector control (EV) grown on a solid plastic substrate (left), or on soft agar (right) with or without doxycycline treatment (1µg/ml). The mean (±SEM) of three independent experiments run in triplicate is shown.

In vivo - The effects of EPHA3 on tumor growth were further investigated using a xenograft model. LS174T cells stably transfected with the EPHA3 receptor or the control empty vector were injected subcutaneously in the right and left flank of NOD/SCID immunodeficient mice, respectively. Animals were then randomized to receive doxycycline in the drinking water or a control group, and xenograft growth was monitored over time. The same experimental layout was carried out with the DLD1 derivative lines. No differences were observed in the growth of the xenografts formed by these cell lines in the control mice or the animals treated with doxycycline (**Fig.36 A, B**). Furthermore, we confirmed by immunohistochemistry the overexpression and cell surface localization of EPHA3 in the tumors from doxycycline-treated mice at the end of the experiment (**Fig.36 C, D**).

Collectively, these results show that the activation of EPHA3 into deficient colon cancer cells does not affect their growth whether *in vitro* or *in vivo* using a xenograft tumor model.

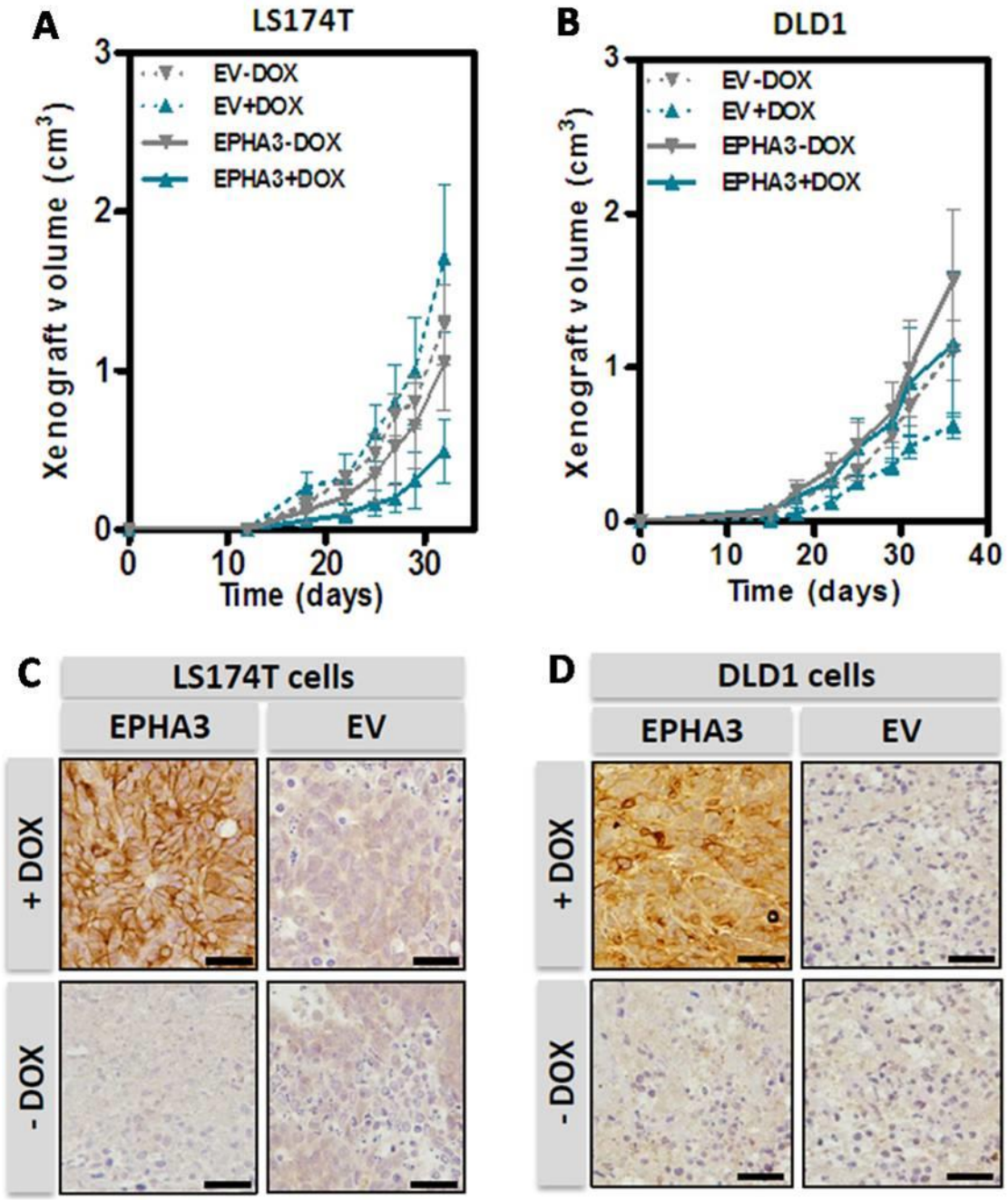

Fig.36. (A, B) Effects of EPHA3 overexpression on the tumor growth *in vivo*. Growth of LS174T-EPHA3 **A)** and DLD1-EPHA3 **B)** cells or the corresponding empty vector when injected subcutaneously in immunodeficient NOD/SCID mice. Doxycycline was administered to the indicated groups of animals in the drinking water. The average tumor size (±SEM) is shown. Not significant differences were observed on tumor growth at any time point (days). N=6 animals per group. **(C, D) EPHA3 levels in xenografts of colon cancer cell lines.** The levels of EPHA3 protein expression were determined by immunohistochemistry in formalin-fixed, paraffin-embedded samples from the subcutaneous xenografts of LS174T-EPHA3 **C)** and DLD1-EPHA3 **D)** cells. EPHA3 levels were also assessed in the corresponding derivative cell lines transfected with the control empty vector. Scale bar: 50µm.

3. Characterization of the role of EphA3 in intestinal tumorigenesis using a knockout mouse

In order to investigate the role of EphA3 *in vivo*, we used a knockout mouse model where the first exon of *EphA3* has been deleted by homologous recombination (273). As previously reported (273,275), we observed perinatal mortality of approximately two thirds of the *EphA3*$^{-/-}$ mice due to cardiac defects, although the remaining 34% of the knockout mice were viable with no obvious cardiac or other abnormalities. We, firstly, confirmed loss of expression in the intestine and liver of adult *EphA3* KO mice by quantitative real-time RT-PCR (**Fig.37 A, B**).

Two groups of mice, wild type (n=18) and KO (n=16) for *EphA3,* were monitored and then scarified at 600 days of age. The intestine of each animal was removed and then the tumors were scored under the microscopy. Importantly, we found that the loss of EphA3 does not affect the survival of adult mice (**Fig.38 A**) or the incidence of intestinal tumors at 20 months of age (**Fig.38 B, C**), indicating that targeted inactivation of EphA3 does not promote tumor initiation.

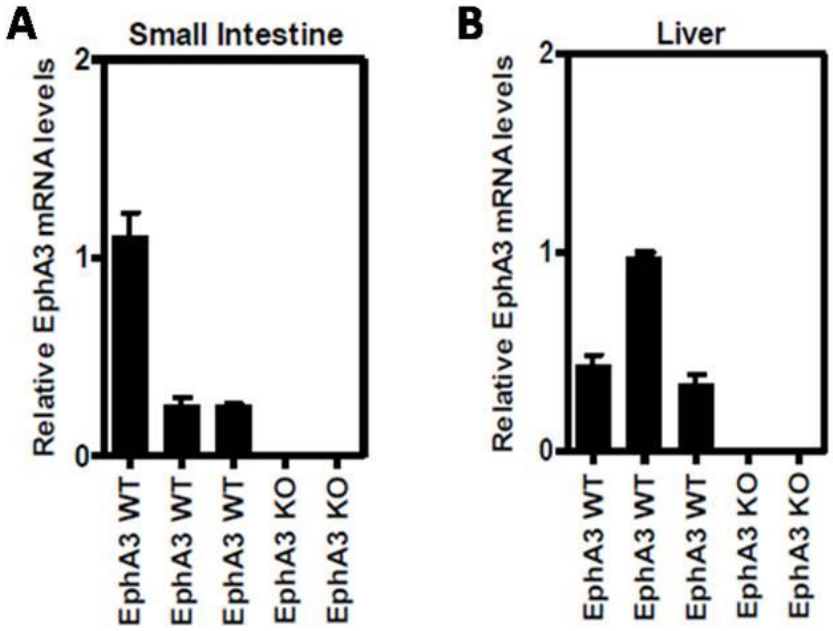

Fig.37. EphA3 levels in wild type and knockout mice. The levels of EphA3 mRNA were assessed in the small intestine **A)** and the liver **B)** of wild type and knockout *EphA3* mice by quantitative Real-Time RT-PCR.

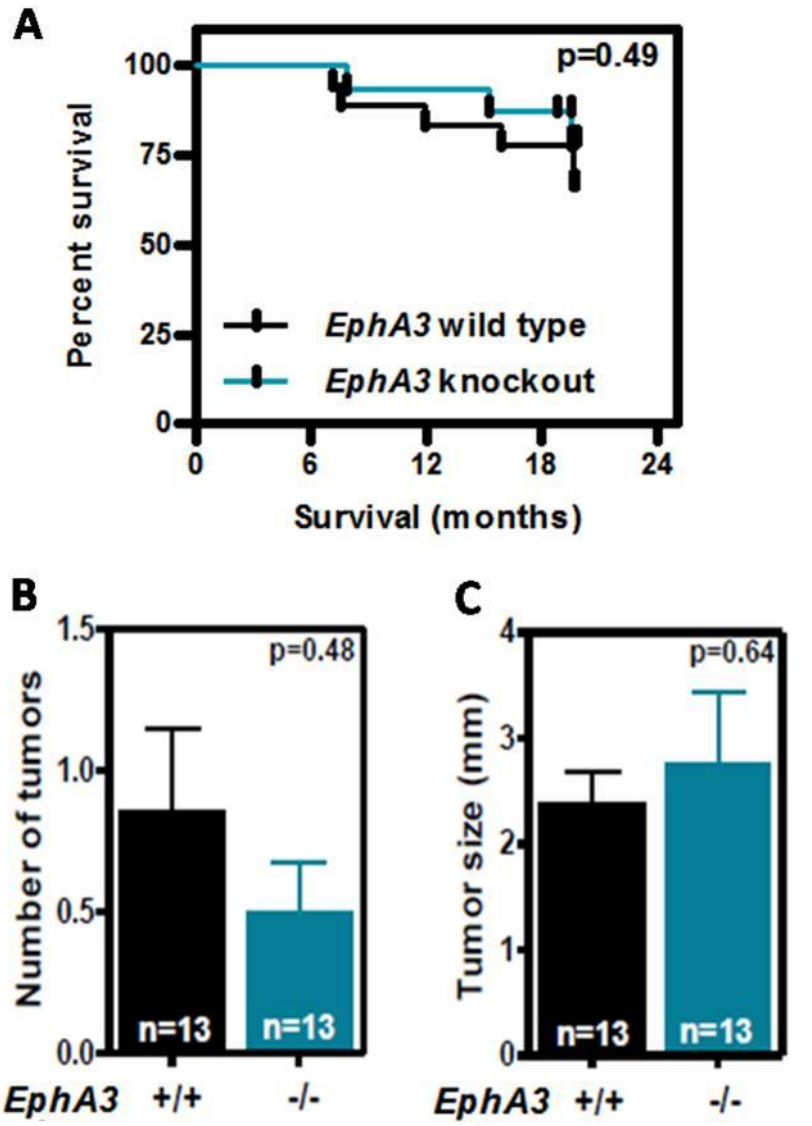

Fig.38. Effects of targeted inactivation of EphA3 in adult mice. **A)** Survival of *EphA3* wild type (n=18) and knockout mice (n=16)**:** the percentage of mice alive at different times is shown for both genotypes. The p value was calculated with the Logrank test. **(D, E)** Histograms showing the number **B)** and size **C)** of intestinal tumors in *EphA3* wild type and knockout mice at the age of 20 months; n= number of animals.

APC inactivation is considered the initiating event in most CRCs. *Apc*$^{min/+}$ (Multiple Intestinal Neoplasia) mice carry a single mutant *Apc* allele and develop multiple benign adenomas when the remaining wild type copy is lost (293). As observed here for EphA3, the loss of other Eph receptors on its own does not cause intestinal tumors (66). However, reduction of EPHB activity has been shown to strongly accelerate tumorigenesis in the intestine of *Apc*$^{min/+}$ mice, resulting in the formation of aggressive adeno-carcinomas (199,265). Therefore, we decided to initiate intestinal tumorigenesis genetically by crossing the *EphA3* model with *Apc*$^{min/+}$mice carrying heterozygous mutations in the *Apc* tumor suppressor gene (both on a pure 129/Sv background). First, we observed that the lifespan of *Apc*$^{min/+}$ mice was not affected by the loss of one or two copies of *EphA3* (**Fig.39 A**).

Furthermore, a separate cohort of animals was scarified at 42 weeks old age, when the tumor burden was maximal before having significant mortality. The intestine was removed and the number of tumors microscopically visible was counted under stereo-zoom microscope. In good agreement with the lifespan observed, the number and size of intestinal tumors at 42 weeks of age was not different in *Apc*$^{min/+}$ mice that are either wild type, heterozygous or homozygous for the *EphA3* knockout allele (**Fig.39 B, C**). Moreover, histological analysis on paraffin sections confirmed that the majority of the intestinal tumors were adenomas (54 of 83; 65.1%). In addition, some adenocarcinomas that invaded through the mucosa (20.5%), submucosa (4.8%) or the muscularis propria (6.0%) were observed and the histological tumor type was confirmed to be independent of the mice genotype.

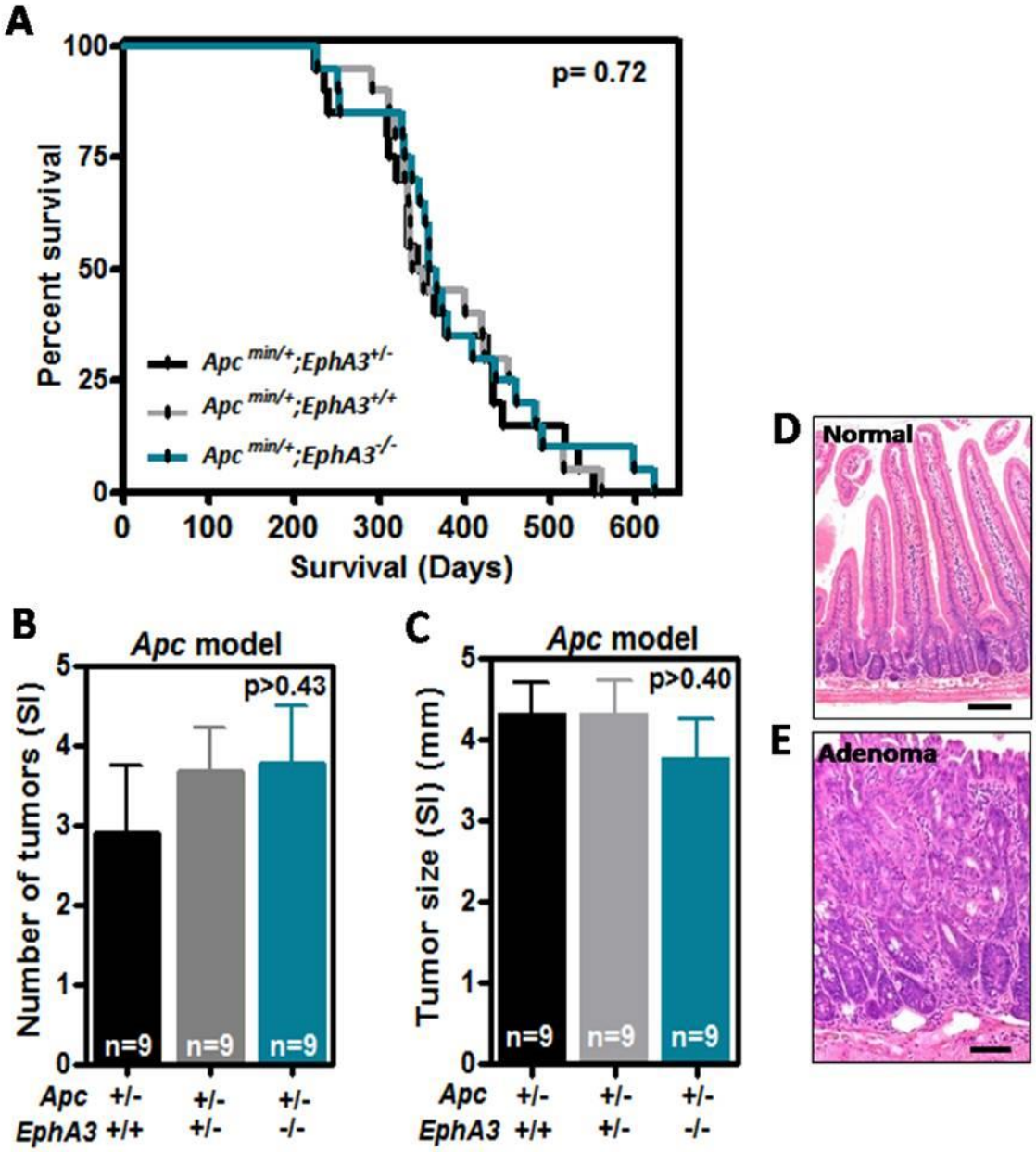

Fig.39. Colorectal tumorigenesis in *Apc*$^{min/+}$mice bearing *EphA3*-null alleles. **A)** Survival of *Apc*$^{min/+}$mice that are either wild type, heterozygous or homozygous for *EphA3* inactivation (n=20 animals per group). Number **B)** and size **C)** of small intestinal tumors observed in 42-week-old *Apc*$^{min/+}$mice that are either wild type, heterozygous or homozygous for *EphA3* inactivation; n= number of animals per group. **(D, E)** Representative hematoxylin-eosin sections of the normal small intestine **D)** and intestinal adenoma **E)**, scale bar 100µm.

In addition, intestinal tumorigenesis was induced pharmacologically in an independent cohort of mice with the intestinal-specific carcinogen azoxymethane (AOM) (296). However, consistent with the findings of the *Apc*$^{min/+}$ model, no differences were observed in the number, size or histology of large intestinal tumors in *EphA3* wild type and knockout mice (**Fig.40 A, B**). Most tumors found were adenomas (25 of 36; 69.4%). Some adenocarcinomas infiltrating the mucosa (25.0%) or the submucosa (5.6%) were also observed in the large intestine, while no tumors were found in the small intestine of AOM-treated mice.

Overall, these experiments indicate that, unlike the loss of EphB receptors, EphA3 inactivation does not significantly contribute to tumor initiation or progression during the early stages of intestinal tumorigenesis in murine models.

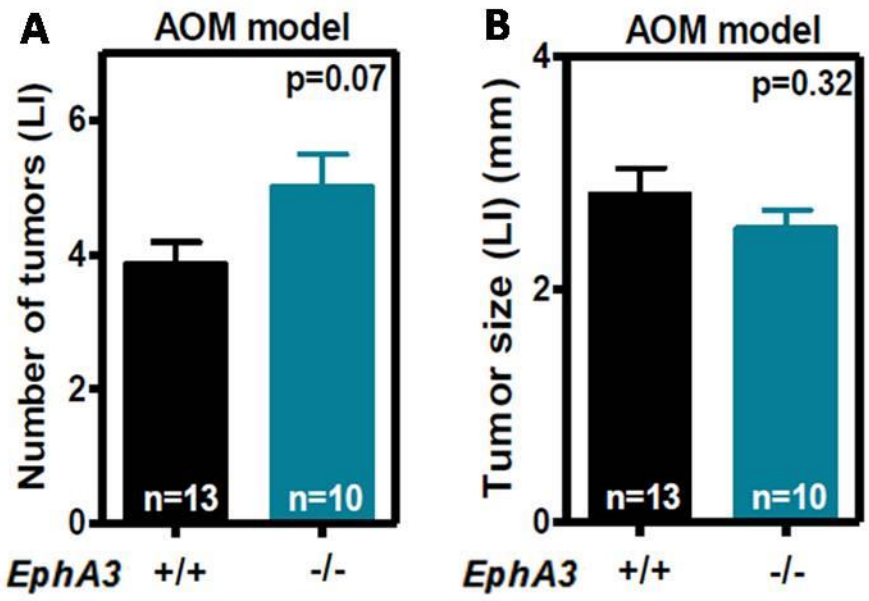

Fig.40. Effects of EphA3 inactivation in the mice intestine upon AOM induced carcinogenesis. Number **A)** and size **B)** of large intestinal tumors observed in 25- week-old wild type or *EphA3* knockout mice after azoxymethane (AOM) treatment. n=number of animals per group. All histograms show average values ±SEM. P values shown are from Student's T-test.

4. Reintroduction of EPHA3 into deficient colon cancer cells does not affect their metastatic potential

Although our results with the *EphA3* knockout mouse model indicate that the loss of this receptor is not an important event in the early stages of colon cancer development, it remained possible that EPHA3 may be involved in the metastatic process. Moreover, given its essential function in regulating cell motility and adhesion during normal development, EPHA3 signaling often reemerges in human cancer playing a key role in cell dislodgement and invasion. In fact, in melanoma cells activated EPHA3 triggers Rho-mediated cytoskeletal changes and detachment with effects on the melanoma progression and metastasis (198). On the other hand, the activation of EPHA3 signaling in rhabdomyosarcoma cells decreases adhesion to fibronectin and migration, acting as a suppressor of motile and metastatic phenotype (277). Thus, we first investigated the effects of EPHA3 activation on the cell motility/migration capacity of colon cancer cells *in vitro*.

In vitro - LS174 and DLD1 overexpressing EPHA3 receptor under doxycycline treatment as well as the corresponding empty vector cells were used to perform a wound healing assay. As shown in the **Fig.41 (A-D)**, the reintroduction of EPHA3 in colon cancer cells did not change significantly their migration capacity. Furthermore, we investigated whether restoration of EPHA3 signaling affected the invasive potential of these cells though matrigel. A Boyden chamber assay demonstrated that EPHA3 function in LS174T and DLD1 cells did not affect their potential to invade through a complex extracellular matrix (**Fig.41 E, F**).

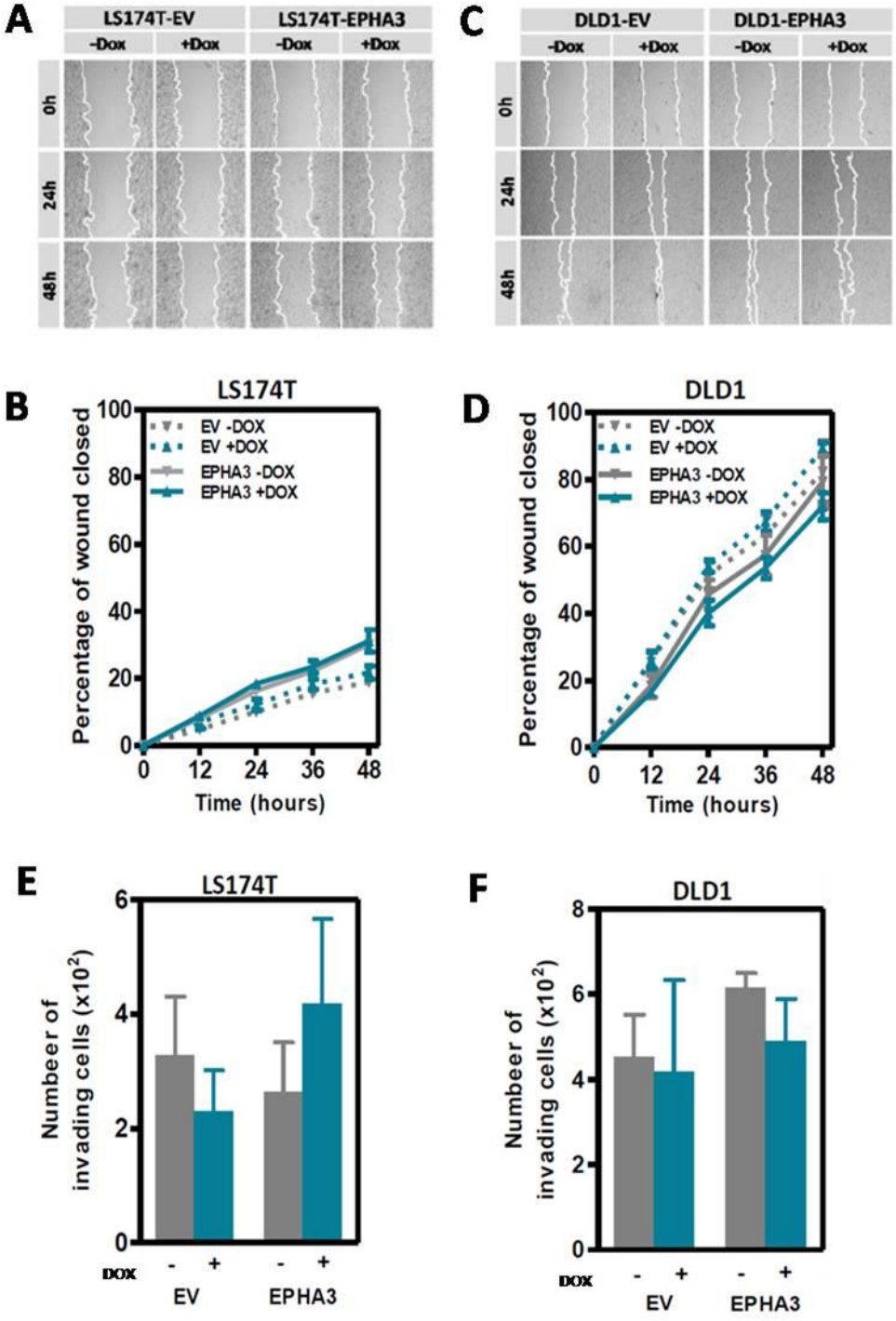

Fig.41. Effects of EPHA3 on colon cancer cell motility and invasion. Changes in the motility of LS174T **(A** and **C)** and DLD1 **(B** and **D)** cells after EPHA3 overexpression were assessed using a wound healing assay. Cells transfected with the control empty vector (EV) were used along with cells expressing EPHA3 after doxycycline (Dox) treatment (1μg/ml). Panels **(A, B)** show representative images and panels **(C, D)** show the average (±SEM) percentage of the initial wound closed after the indicated times in three independent experiments carried out in triplicate. **(E, F)** Matrigel Invasion capacity (Boyden chamber invasion assay) of LS174T-EPHA3 **E)** and DLD1-EPHA3 **F)** with and without doxycycline (Dox)-dependent induction of EPHA3 overexpression. The corresponding empty vector (EV) derivative lines were used to control for possible effects of doxycycline on the invasion of these cells. The average (±SEM) of three independent experiments carried out in triplicate is shown.

In vivo - The capability of colon cancer cells to spread out from the primary tumor and colonize via extravasation distant organs is the most common cause of death in patients with colorectal cancer. Interestingly, a study reported that high EPHA3 protein levels in colorectal carcinoma positively correlated with histological grade, depth of invasion, lymph node metastasis and distant metastasis (297). However, no data are available to understand the role of this receptor during the late stages of colorectal cancer progression. Therefore, we decided to use an experimental mouse model of lung metastasis where LS174T-EPHA3 or DLD1-EPHA3 cells were injected in the tail vain of immunodeficient NOD/SCID mice that were then randomized to a control group or a group receiving doxycycline in the drinking water to induce EPHA3 expression. No differences were observed in the number of lung metastases formed by LS174T or DLD1 cells after EPHA3 signaling activation (**Fig.42 A, B**). The presence of metastatic lesions in the lungs of these animals was confirmed on histological sections (**Fig.42 C, D**).

When considered together, these results demonstrate that the overexpression of EPHA3 as well as the kinase-dependent signaling of this receptor in colon cancer cells do not interfere with their metastatic potential both *in vitro* and *in vivo.*

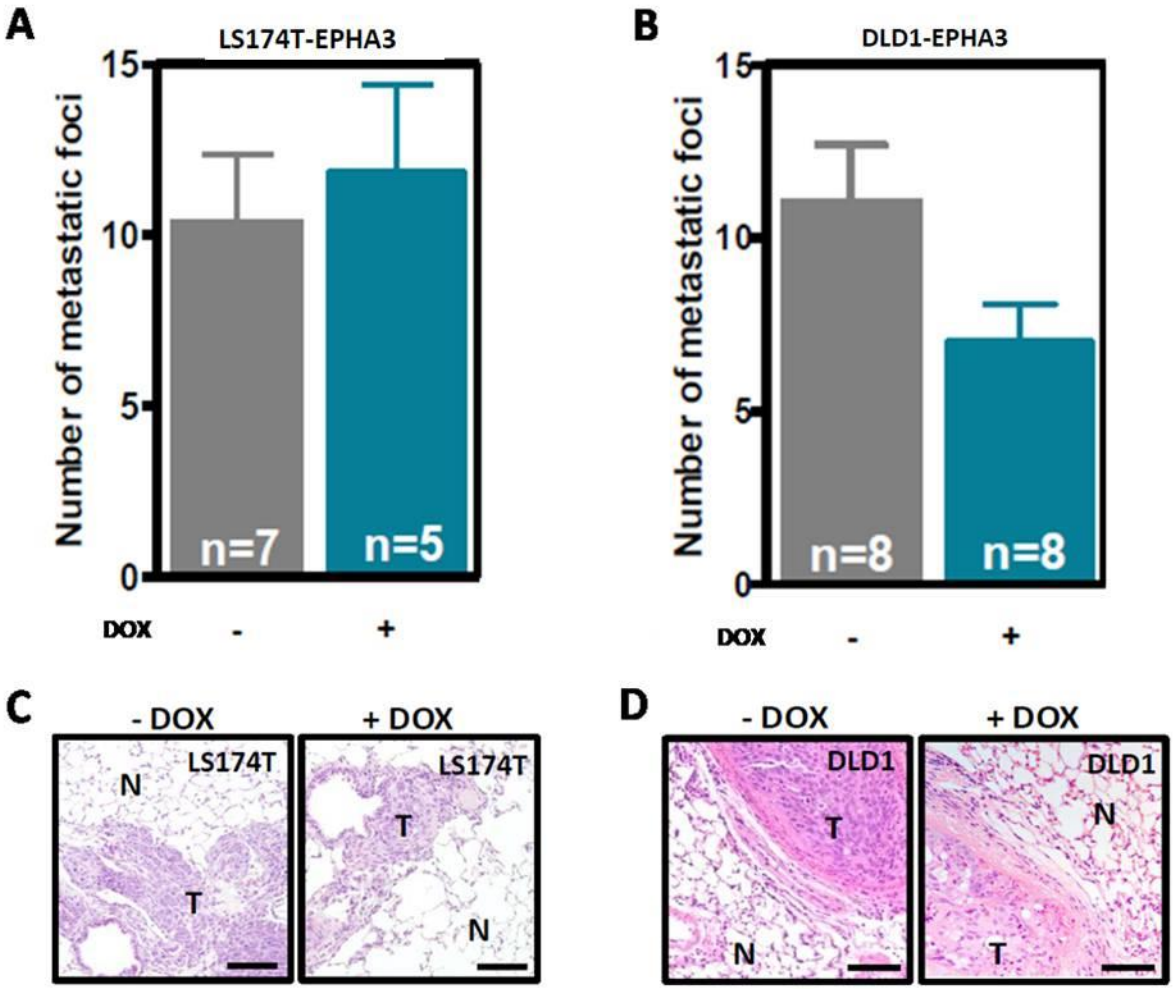

Fig.42. Effects of EPHA3 overexpression on the metastatic potential of colon cancer cells. (A, B) Average number (±SEM)of macroscopically visible metastases formed by LS174T-EPHA3 **A)** or DLD1-EPHA3 **B)** cells 6 or 10weeks, respectively, after tail vein injection in NOD/SCID immunodeficient animals receiving doxycycline (Dox) in the drinking water or control animals. n=number of animals. **(C, D)** Representative images of hematoxylin and eosin stained histological lung sections of the mice in **(A, B)**. N: normal; T: Tumor. Scale bar: 100μm.

5. Survival of colorectal cancer patients as a function of EPHA3 tumor levels

Different studies have suggested an association between EPHA3 levels and the survival of patients with various tumor types, including colorectal cancer (278,279,297). Here, we used a tissue microarray containing triplicate tumor samples from 159 patients with Dukes C colorectal cancer to investigate possible associations between EPHA3 levels and patient survival. The levels of EPHA3 protein in these tumors were determined by immunohistochemistry and the specificity of the antibody used was confirmed on formalin-fixed, paraffin-embedded samples from the xenografts generated with the cell lines engineered to overexpress EPHA3 (**Fig.31 C, D**). The staining levels of each sample were scored using a semiquantitative scale from 0 (absence of EPHA3 immunostaining) to 3 (highest immunostaining), and were evaluated blinded from the clinical data of the patients (**Fig.43 A, B**).

For Kaplan-Meyer plots, EPHA3 levels were dichotomized as low or high EPHA3 using an average score cutoff value of 1.5. However, no associations were observed between EPHA3 expression and disease-free (Logrank test $p>0.39$) or overall survival (Logrank test $p>0.63$) (**Fig.43 C, D**). Importantly, no significant survival differences between high/low EPHA3 groups were found with any other possible cutoff values. Moreover, when the tumor levels of EPHA3 were considered as a continuous variable by using Cox multivariate regression we confirmed no correlation with other clinicopathological features of Dukes C colorectal cancer patients (covariates: EPHA3 levels, histologic grade, sex, age, and tumor location) (**Table 4**).

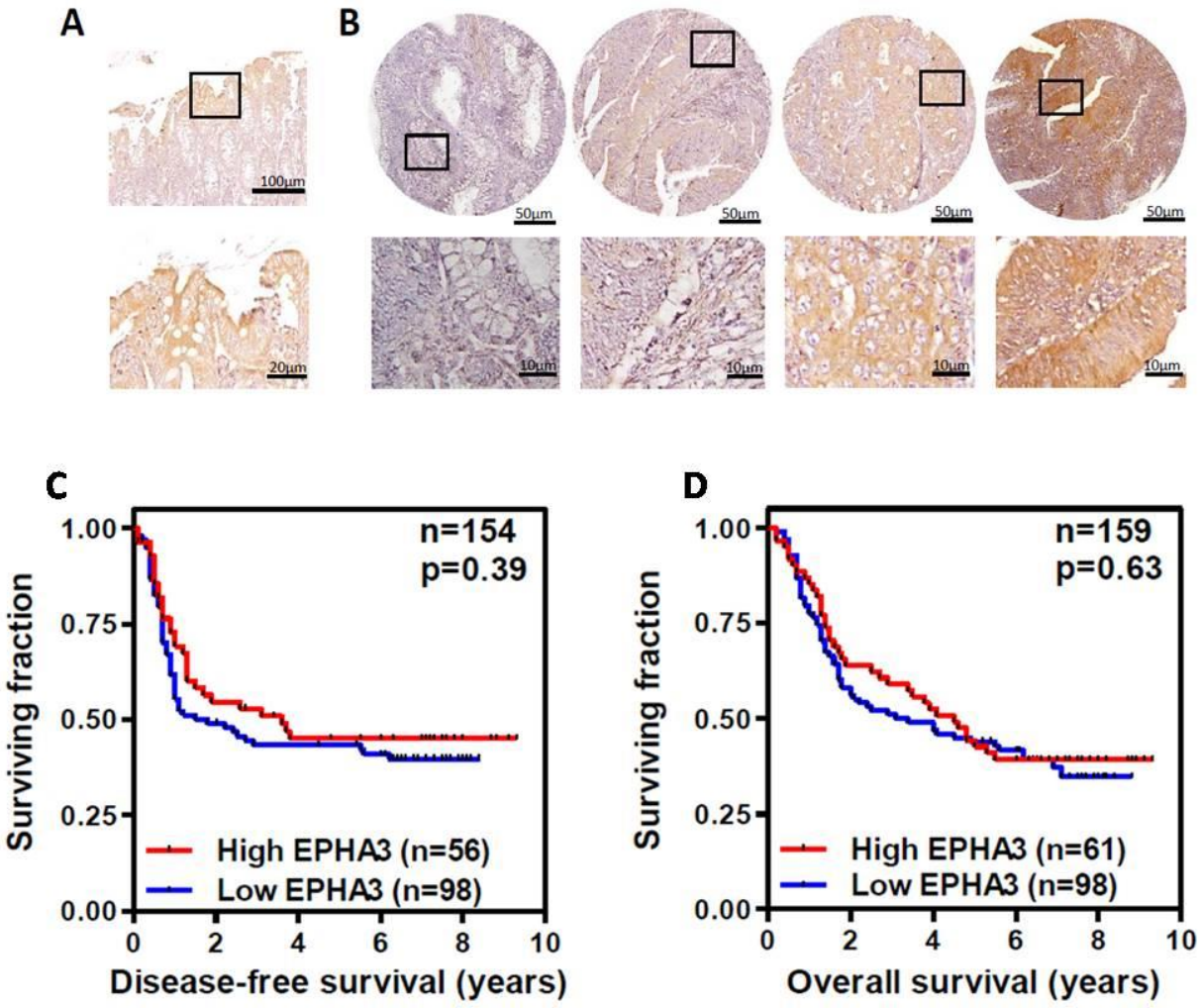

Fig.43. Survival of colorectal cancer patients with high and low EPHA3 levels in their tumors. The levels of EPHA3 protein were assessed by immunohistochemistry with a specific antibody in normal colonic mucosa **A)** and colorectal tumors **B)**. The disease-free **C)** and overall survival **D)** of 159 Dukes C colorectal cancer patients as a function of tumor EPHA3 levels was studied. P values are from the Logrank test.

Table 4. Clinicopathological features of Duke C colorectal cancer patients.

	All cases	Low EPHA3	High EPHA3	p Value
Sex, n (%)				
Female	99	53 (54.1)	25 (43.1)	0.24[1]
Male	58	45 (45.9)	33 (56.9)	
Age (years), mean±SD	64.69±12.93	65.2±13.6	63.8±11.9	0.36[2]
Site, n (%)				
Colon	39	57 (58.1)	38 (64.4)	0.78[1]
Rectum	29	41 (41.9)	21 (35.6)	
Degree of differentiation, n (%)				
Good	19	12 (12.4)	7 (12.3)	0.99[1]
Moderate	114	72 (74.2)	42 (73.7)	
Poor	21	13 (13.4)	8 (14.0)	
Mean Follow up, mean±SD	7.3±1.1	7.2±1	7.5±1.2	0.17[3]
Adjuvant treatment, n (%)				
Yes	70	41 (41.8)	29 (49.2)	0.4[1]
No	87	57 (58.2)	30 (50.8)	
5- year overall survival, n (%)				
Alive	61	38 (38.4)	23 (38.3)	1[1]
Dead	98	61 (61.6)	37 (61.7)	
5- year disease-free survival, n (%)				
Alive	67	40 (41.7)	27 (46.6)	0.61[1]
Dead	87	56 (58.3)	31 (53.4)	

[1]Fisher test; [2]Mann Whitney test; [3]Student's T-test.

Finally, for 16 of these primary Dukes C tumors, the paired lymph node metastasis from the same patient was also available. Consistent with our *in vitro* data and the animal model of experimental metastasis, no significant differences in the levels of EPHA3 were observed between matched primary and metastatic lesions (**Fig.44**), further indicating that EPHA3 does not regulate the metastatic dissemination of colon cancer cells.

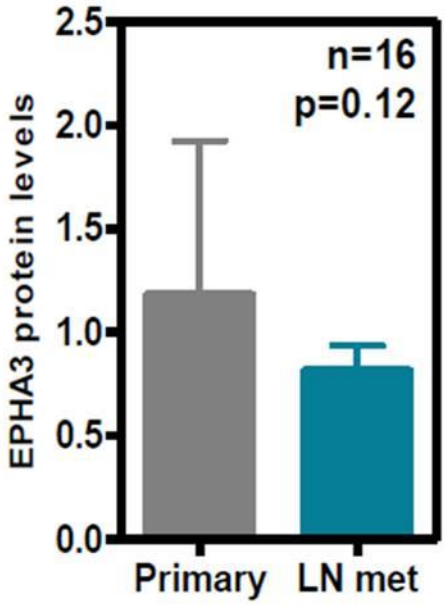

Fig.44. EPHA3 levels in paired lymph node metastasis and primary tumors of Duke C colorectal cancer patients. Average protein expression levels in 16 paired primary Dukes C tumors and lymph node metastases (LN met) from the same patients (p value shown is from a paired Student's T-test).

Discussion

EPHs, which make up the largest family of mammalian receptor tyrosine kinases, bind cell surface–associated Ephrins that have either a glycosylphosphatidylinositol (GPI) membrane anchor (A-type Ephrins) or a transmembrane region followed by a conserved cytoplasmatic tail (B-type Ephrins). Clustering of EPH receptors and their ligand at the surface of adjacent cells activates downstream signaling in both the EPH- and Ephrin-expressing cells, termed forward and reverse signaling, respectively (298). EPHs and Ephrins are not only numerous but their relationship is also complex. Eph–ephrin interactions can trigger a wide array of cellular responses including cell adhesion, repulsion and cell sorting/positioning during tissue boundary and the exact mechanisms leading to these different responses are complicated and appear highly dependent on the cell type and tissue context (181,299). Indeed, seemingly contradictory responses have been described in different cells even from the same EPH–Ephrin interaction (160,299,300). Targeted deletions of EPHs and ephrins have been shown to cause severe embryonic defects in different organs, thus highlighting their crucial roles during all phases of early development. However, EPHs and ephrins also have continued crucial roles in adult tissue and organ maintenance (301), regeneration and pathogenesis, including cancer (160). Not surprisingly, the activities of these molecules in adult life reflect their dichotomous functions and signaling activities during development. This is especially prevalent in oncogenesis, in which, depending on the tumor type, disease stage and kinase function, EPH overexpression can promote or inhibit tumor progression, and most importantly can even perform both functions within the same tumor type (258,259).

The EPHA3 receptor, originally recognized and isolated from membranes of pre-B acute lymphoblastic leukemia cells, is widely expressed during embryonic development, with the highest levels occurring in the nervous system and heart (272,275). As expected from its first identification, it was then implicated as having a key role in many cancers. However, understanding the function of EPHA3 in cancer, as often occurs with other EPH receptors, is most intricate.

Potential kinase/ligand-dependent tumor suppressor activity of EPHA3 in colorectal cancer cell lines - The inactivating effects of many somatic mutations found in different cancers, strongly suggest that wilde-type EPHA3 receptor suppresses the malignant properties of cancer cells in an ephrin-and kinase-dependent manner (261). Lung cancer is the cancer type with highest rates of somatic mutations in *EPHA3* (302,303). Importantly, reexpression of wild-type EPHA3 in lung cancer cells increases apoptosis and inhibits tumor growth *in vivo* (262). EPHA3-induced tumor suppression is mediated through enhanced apoptosis via inhibition of AKT signaling (262). The products of the somatically mutated *EPHA3* genes discovered in lung cancer, however, form complexes

with wild-type EPHA3 in a dominant negative manner and inactivate its tumor suppressive function (262). As described in the introduction, two independent studies of next-generation DNA sequencing using large cohorts of human colorectal cancer samples showed unexpected high mutation frequency in *EPHA3* (132,284). However, the biological impact of somatically mutated variants of *EPHA3* and role of this receptor in intestinal tumorigenesis are poorly defined. Here, we asked why *EPHA3* is mutated at such high frequency in colorectal cancer and importantly why the mutations mainly impair its kinase/ligand dependent activity. Are *EPHA3* mutations drivers of the tumorigenic process or simply passenger genetic alterations?

To directly address these questions, we generated a doxycycline-dependent EPHA3 overexpression system in two different colon cancer cell lines and confirmed the membrane expression of the ectopic EPHA3 protein and the conditional activation of the kinase activity. Direct sequencing of the full coding region of *EPHA3* in these cell lines, revealed that LS174T cells are wild type, while DLD1 cells have two different point mutations localized in the kinase domain of both alleles that are expected to impair the kinase activity of EPHA3. Unlike the observations made in other cancer types such as lung tumors (262) and irrespectively of the presence of EPHA3 mutations, reintroduction of wild type EPHA3 into colon cancer cells, had no effects on their growth, whether on a solid substrate, semisolid substrate or when injected subcutaneously in immunodeficient NOD/SCID mice. Thus, employing *in vitro* and *in vivo* experimental models here we show that EPHA3 kinase dependent-signaling does not regulate the proliferation of colorectal cancer cells. As mentioned before, the biological outcome of EPH receptors is strongly dependent on the cellular context and frequently the same receptor can induce different responses in cells from different tissues. To further investigate the functional role of EPHA3 receptor in colorectal cancer, we decided to extend our study by using a mouse model with targeted inactivation of EphA3.

Effects of targeted inactivation of EphA3 in murine intestinal tumorigenesis - Mice carrying homozygous *EphA3* null mutant alleles show about 75% of perinatal lethality due to cardiac failure as a consequence of abnormalities in the development of their atrial septa and atrioventricular endocardial cushions (275). Despite the high mutation frequency of *EPHA3* in human colorectal cancer, we observed that targeted inactivation of this receptor in adult mice does not affect their lifespan and does not initiate the tumorigenic process in the intestine. Previously studies showed that the intestine of *EphB2* deficient mice matures normally and, mainly, does not develop tumors (66). Moreover, despite the disordered epithelial organization observed in the intestine of *EphB3* null mice, inactivation of this gene is not strong enough to initiate intestinal

tumorigenesis (66). However, the loss of EphB2 or EphB3 has been show to rapidly accelerate the tumorigenesis in *Apc*$^{min/+}$ mice (199). In fact, EphB expression or function suppress the tumor expansion and transition from adenoma to carcinoma (199,200,247). We then investigated whether EphA3 inactivation in the murine intestine could modulate the oncogenic process once initiated either genetically or pharmacologically. In this study, we demonstrate that loss of either one or both alleles of *EphA3* does not affect the animal lifespan or the number/size of intestinal tumors initiated by heterozygous *Apc* mutations (*Apc*$^{min/+}$mice). Consistently, these data were confirmed using a pharmacological mouse model where intestinal tumors are induced by administration of the intestine-specific carcinogen azoxymethane (AOM). Thus, despite the reported loss of function of type-B EPH receptors in the progression of intestinal tumors and the reported mutations that disrupt EPHA3 activity in colorectal cancer, our results indicate that the inactivation of EPHA3 does not affect the intestinal tumorigenesis from small to large adenoma or from adenoma to carcinoma. A recent study shows that the putative tumor suppressor gene *EPHA3* failed to demonstrate a crucial role in murine lung tumorigenesis (304). The lack of an intestinal phenotype in *EphA3* null mice can imply that a) this receptor is not a tumor suppressor gene of colorectal cancer; alternatively may indicate b) a partial penetrance of the EphA3 null genotype (275), or c) functional redundancy between murine *EphA* receptors expressed in adult intestine. Interestingly, a recent study suggested functional compensation of *EphA3* loss by *EphA7* co-expression during palate development, as *EphA3* knockout animals failed to show defective midfacial development (274). Furthermore, EPHA7 is frequently silenced in gastric, colon and prostate cancer (160) and, a truncated form of this receptor has been reported to act as a tumor suppressor in follicular lymphoma (305).

Role of EPHA3 in colorectal cancer metastasis and patient survival - Inactivation of most tumor suppressor genes significantly contribute to the increased tumor growth, either by enhancing the proliferation of cancer cells such as *APC* (117), or preventing their death through, for example, apoptotic pathways like *P53* (306). However, other genes that contribute to the overall tumorigenic process do not modulate the growth of the tumor, but rather contribute to the metastatic spread of the malignant cells. This is indeed an important process during tumorigenesis and is ultimately responsible for the death of most cancer patients. The transcriptional factor Twist, a master regulator of embryonic morphogenesis, has been shown to promote tumor cell metastasis by inducing an epithelial-mesenchymal transition (EMT), with no effect on the growth of the primary tumor (307). Similarly, Snail promotes tumor progression but attenuates the proliferation, rather than promoting growth (308).

The function of EPH receptors in regulating cell motility and adhesion during normal development as well as in cancer progression has been widely explored; members of the Ras/Rho families of GTPases are essential regulators of these processes. In normal tissue, EPHA3 activity plays a critical role in mediating endothelial-mesenchimal transition (EndMT) during heart development. EndMT also provides a major source of cancer-associated fibroblasts that contribute to carcinoma progression (309). Moreover, the kinase/ligand dependent signaling of EPHA3 has been reported, depending on the tumor type, to suppress or promote cell motility and invasiveness of cancer cell. EPH overexpression in tumors such as malignant melanoma has been shown to correlate with tumor progression and metastasis. Melanomas oroginate from neural crest-derived skin melanocytes, and although EPH signaling is crucial for neural crest migration, EPH receptors are not expressed in mature melanocyte. The unscheduled expression of EPHA3 receptor in melanoma cells lines has been suggested to contribute to malignant phenotype (269). Activation of EPHA3 receptor through a mechanism that is kinase and ligand dependent promotes cell rounding, membrane blebbing and de-adhesion of melanoma cells. Functional and biochemical analysis of the underlying mechanisms revealed that these morphological changes are mediated through recruitment of CRKII to ligand-activated EPHA3 and a concurrent, transient activation of RHOA. Importantly, the activation of EPHA3 in a kinase dependent fashion does not affect cell viability, rather it confers to the melanoma cells a more metastatic phenotype (198). Similarly, EPHA3 is expressed in neoplastic but not in normal T-cells. Again, stimulation of EPHA3 receptor on Jurkat cells inhibits cell adhesion to fibronectin and promotes CRKII recruitment. Signals from either CD28 or IGF-1R induce EPHA3 expression. The ability of EPHA3 to regulate cell adhesion in Jurkat cells suggests that it has a role in the motility and cancer progression of malignant T cells that depends on its kinase-ligand activity (207). In this study we observed low or undetectable levels of EPHA3 expression in all the colon cancer cells analyzed, expect for SW48 cells that carry a deletion of a single nucleotide that leads to a premature stop codon in the coding region of EPHA3, resulting in a truncated form of the protein that lacks the kinase domain. Moreover, most of cancer somatic mutations of *EPHA3* identified in colon and rectum, map to the ligand-binding, receptor clustering or kinase domains and affect the kinase-active signaling (261). Thus, these data support a tumor suppressor role of EPHA3 which is kinase-dependent. In agreement with this role, cell lines of rhabdomyosarcoma (RBM) expressing EPHA3, upon Ephrin-A5 stimulation, show loss of adhesion to fibronectin, decreased migration, and consequently a phenotype less metastatic (277). Similarly, activation of EPHB2 and EPHB3 receptors in LS174T results in rapid morphology changes such as loss of lamellipodia and cell extensions, and decreased migration (66).

Therefore, we wandered if the reintroduction of EPHA3 could affect the invasive and metastatic potential of colorectal cancer and several *in vitro* assays were carried out in this study with the cell lines engineered to conditionally overexpress EPHA3. Reintroduction of EPHA3 in colon cancer cells did not affect their growth in soft agar, their motility on a solid substrate or their capacity to invade through a complex extracellular matrix. However, these assays do not closely reproduce the host environment and often poorly correlate with the true metastatic potential of cancer cells. The experiments carried out with the *EphA3* knockout mouse models, did not show any changes in the capacity of intestinal tumors to invade locally. However, the $Apc^{min/+}$ and AOM mouse models of intestinal carcinogenesis are not ideally suited to investigate the possible role of EPHA3 in the capacity of intestinal cancer cells to form distant metastases as their tumors very rarely metastasize.

The metastatic process consists of a number of distinct steps requiring the concerted actions of multiple genes. Specific genes allow tumor cells to overcome barriers to local invasion, intravasation, survival while circulating in blood or lymph vessels, arrest in capillaries, extravasation and finally outgrowth to produce macrometastases in distant organs (310). Although all these events are difficulty to reproduce *in vitro*, experimental animal models that rely on the introduction of tumor cells directly into the systemic circulation have been successfully used to identify those genes involved in the late stages of cancer progression. For this purpose, we investigated the metastatic potential of EPHA3 using a mouse model of lung metastasis. However, ectopic overexpression of EPHA3 in colon cancer cells does not affect their capacity to infiltrate distant organs when injected intravenously into immunocompromised mice. These results are consistent with our IHC data showing no EPHA3 expression differences in primary Dukes C tumors and matched lymph node metastases. Collectively, these results demonstrate that inducible overexpression of EPHA3 does not modulate the invasiveness and metastatic potential of colon cancer cell lines both *in vitro* and *in vivo.*

On the other hand, a previous study reported that EPHA3 regulates the metastatic spread of colorectal tumors to lymph nodes and distant organs, and its overexpression correlates with poor prognosis (297). Here, we assessed the levels of EPHA3 in the tumors of a cohort of 159 patients with locally advanced (Dukes C) colorectal cancer by immunohistochemistry with a rabbit polyclonal antibody that specifically detects human EPHA3 on formalin-fixed, paraffin embedded samples. Our analysis did not find any associations between EPHA3 tumor levels and disease-free survival, overall survival or any clinicopathological features of Dukes C colorectal cancer patients. This apparent discrepancy with the previous study could be due to the use of different antibodies to detect EPHA3. Importantly, the specificity of the antibody used by Xi et al., was not

investigated casting doubts on the accuracy of these findings. Alternatively, EPHA3 levels could be associated with tumor stage, but have no prognostic value when the analysis is restricted to patients with the same disease stage. Furthermore, recent findings suggest an oncogenic activity of EPHA3 when overexpressed and kinase dormant. In keeping with this, if EPHA3 is overexpressed in patients with advanced Dukes D colorectal cancer as suggested by Xi et al., it would be useful to check in these patients the levels of EPHA3 activity and/or Ephrin-A5 ligand.

Kinase/ligand–independent tumor promoting and kinase/ligand dependent suppressive functions - As reviewed in the introduction, an unresolved paradox in EPH field is whether EPHA3 is an oncogene or a tumor suppressor gene. Upon examination of accumulating studies an emerging model suggests that EPHA3 expression and function in the absence of kinase activity is tumor promoting and often linked to low or absent levels of the cognate ligand. In fact, EPHA3 receptor is frequently overexpressed in glioblastoma multiforme (GBM) and, particularly, in the most aggressive mesenchymal subtype (223). Importantly, EPHA3 is highly expressed and kinase dormant on the tumor-initiating cell population in glioma and appears critically involved in maintaining tumor cells in a less differentiated state by negatively regulating mitogen-activated protein kinase signaling (ERK/MAPK) (223). Previous studies showed that EPHA3 activation drives neural cell differentiation through increased MAPK pathway activation (222). However, EPHA3 is present and functional in GBM but it is most likely functioning in a kinase-independent fashion to decrease MAPK signaling. The EPHA3-specific monoclonal antibody, IIIA4, binds and activates human and mouse EPHA3 with similar affinities. The binding is followed by internalization of receptor-antibody complexes. Upon stimulation with IIIA4, glioblastoma cell lines overexpressing EPHA3 display rapid differentiation and reduced proliferation (223). Moreover, the upregulation of EPHA3 in different hematopoietic malignancies has been shown to correlate with more aggressive and invasive disease (280). EPHA3 is highly expressed in LK63 pre-B ALL (acute lymphocytic leukemia) cells whereas, it is not expressed in the Reh cells, a similar pre-B ALL cell line. In a LK63 xenograft model, administration of the IIIA4 antibody leads to inhibition of tumor growth, decreases the spreading from the bone morrow to the spleen and other organs and increases the latency of the disease. Similar effects were observed in LK63 EPHA3 knock down xenografts. Importantly, IIIA4 treatment of a xenograft model using EPHA3-transfected Reh, shows reduction in the bone marrow engraftment and increases the latency of the disease (283). Together these data strongly suggest that EPHA3 receptor when overexpressed, not mutated, and mainly kinase dormant has prevalently a tumor promoting function. However, its activation in the same tumor type is typically tumor suppressive. As previously

suggested for other EPH receptors (258,259), both functions seem to be mutually exclusive. The detailed molecular mechanisms and signaling pathways that regulate opposite responses in the same context are still unclear and further investigations should be directed to answer these unresolved questions. Here we report that the kinase dependent signaling of EPHA3, despite being frequently inactivated in colorectal cancer, does not play a tumor suppressive function in any stage of intestinal tumorigenesis by using *in vitro* and *in vivo* models. Thus, our results are not consistent with the hypothesis that EPHA3 could have in colorectal cancer both functions, tumor suppressive and promoting. However, the role of the reported mutations found in colon and rectum tumor specimens still remains an important open question. As suggested by their high frequency, it is tempting to speculate that these genetic alterations could confer EPHA3 an oncogenic activity which functions in a kinase/ligand independent manner. Alternatively, or in addition, the mutant products may form with wild-type EPHA3 kinase-defective complexes that impose to the cells a tumor promoting phenotype. If this hypothesis is correct, the EPHA3 mutations discovered in colorectal cancer do not impose a loss of function but rather a gain of function. To test this hypothesis, we planned to use DLD1 and LS174 colorectal cancer cell lines as recipients to overexpress in both systems the mutant form of EPHA3 which carry the pathogenic mutation D806N discovered in two different colorectal cancer samples, and known to strongly affect the kinase domain activity as well as tyrosine phosphorylation (261). On the other hand, we are performing experiments to knockout the truncated form of EPHA3 found in SW48 colon cancer cells by using a CRISPR/Cas9 approach. These engineered cellular systems will be used to perform *in vitro* experiments and will contribute to elucidate the function of mutant EPHA3 and, finally, the role of this receptor in colorectal cancer.

Recent data reported unexpected EPHA3 overexpression within the microenvironment of a range of human cancers and mouse tumor xenografts (281). The role of EPH receptors and their Ephrin ligands have been extensively studied in vascularization during development as well as in adults (231), including tumor angiogenesis (232). These recent findings suggest an oncogenic activity of EPHA3 receptor in maintaining the stroma and tumor vasculature through a mechanism which is kinase/ligand independent. Consistent with EPHA3 expression on mesenchymal stromal cells (MSCs) and tumor vasculature, IIIA4 treatment of prostate xenografts rapidly activates the receptor kinase-dependent signaling and promotes disruption of newly developing tumor vessels and supporting stroma (281). To test this novel possible role of EPHA3 in the tumorigenic process, we are currently performing experiments by using mouse allografts models. Murine colon cancer cells (MC-38) (311) have been injected

subcutaneously in animals wild-type or knockout for *EphA3,* thus differences in the growth of the murine tumor cells would reflect the role of EphA3 on mesenchymal stromal cells (MSCs) and tumor vasculature.

Conclusions

Although most extensively characterized for their roles in development, Eph receptors and ephrin ligands are re-expressed in a variety of diseases including a number of human malignancies (160). Dysregulated cellular adhesion and abnormal cytoskeletal functions affecting cell shape and motility are key features of advanced cancers. Eph and ephrins control many of these key cellular processes, enabling them to promote tumor invasion and metastasis. EPHB receptors are involved in the homeostasis of the normal intestinal epithelium and loss of function is a key event of colorectal cancer progression. EPHA3 was reported to be highly mutated in tumor specimens from colorectal cancer patients and importantly many mutations impair kinase activity or ligand binding domain, thus suggesting that EPHA3 receptor has Ephrin and kinase-dependent tumor suppressor role. In this study we have performed a functional validation of the role of EPHA3 in colorectal cancer. Concluding, the main findings of this work are as follows:

- The reintroduction of EPHA3 activity in two different colorectal cancer cell lines does not affect their growth *in vitro* or *in vivo* by using a xenografts model.
- The targeted inactivation of EphA3 in a mouse model does not contribute to intestinal tumor initiation.
- Loss of either one or both alleles of *EphA3* in $Apc^{min/+}$ and AOM-treated mice does not play a causal role in intestinal tumor progression.
- EPHA3 signaling does not have a major role in the metastatic potential of colorectal cancer cells.
- Immunohistochemical analysis of EPHA3 tumor levels revealed no association with disease-free or overall survival in Duke C colorectal cancer patients.

Here, we show for the first time that EPHA3 signaling does not have a tumor suppressor role in colorectal cancer and highlight the importance of functional validation to confirm the relevance of putative cancer driver genes identified in sequencing efforts of the cancer genome.

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