Colophon

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Cover illustration: Microscopic image of an intestinal tumor stained for β-catenin (red) and cell nuclei (blue), represented in a dynamic, wind-swept fashion. This symbolizes the Wnt (signaling)-influenced, dynamic process of intestinal tumorigenesis.

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Novel Aspects of Wnt Signaling in Intestinal Development and Cancer

Nieuwe aspecten van Wnt signalering in darmontwikkeling en kanker

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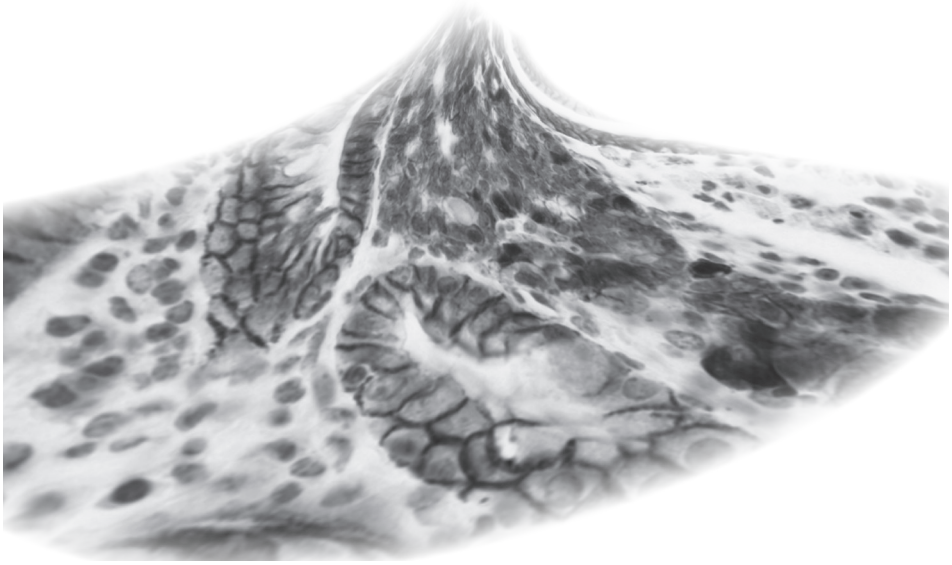
CHAPTER 1

General Introduction



Introduction to the intestinal tract

The gastrointestinal tract is a highly specialized organ system responsible for the processing of ingested food, including the uptake of nutrients and the removal of waste products. Each region of the digestive tract has its own specialized function and associated unique anatomy. The breakdown of food initiates in the oral cavity, after which the components are transferred via the esophagus to the stomach. In the stomach, the food components are processed into chyme, and thereby prepared for entrance into the small intestine. On its turn, the small intestine is responsible for the absorption of nutrients. The anatomy of the small intestine is very suitable for this function, given the enormous absorptive area that is provided by fingerlike protrusions called the villi (Figure 1A). After nutrient uptake in the small intestine, the digestion process continues in the large intestine, where water is absorbed and the remaining parts are prepared for elimination from the body.

The intestinal tract is lined by four layers, the mucosa, submucosa, muscularis and serosa. The mucosa lines the intestinal lumen and consists of the epithelium, the lamina propria (connective tissue, blood and lymph vessels) and the muscularis mucosae. The mucosa represents the most specialized layer of the intestine, since it contains the epithelium that is necessary to execute the digestive function of the intestinal tract but also harbors the cells that become transformed in case of tumorigenesis. In the small intestine, the epithelium is typically organized in crypt-villi structures (Figure 1A). The crypt compartment houses the intestinal stem cells, which give rise to proliferative, uncommitted transient amplifying cells. These transient amplifying cells migrate upwards into the villi while differentiating into specific cell types [1-3]. The four main differentiated cell lineages in the intestine perform their unique function in absorption or secretion (Figure 1B). The absorptive cells are the enterocytes, which harbor an apically located brush border to further increase the absorptive area. In line with the primary function of the intestine in nutrient absorption, enterocytes are the most abundant cells present along the lining of the intestinal tract. Secretory cells are interspersed among the enterocytes, in particular the goblet cells and enteroendocrine cells, which secrete mucins and hormones respectively. Paneth cells form an exception, since these cells do not migrate upwards into the villi while differentiating but rather stay in the crypt, secreting antimicrobial factors and forming a niche for the intestinal stem cells. The structure of the large intestine differs slightly, most importantly since this part of the intestinal tract does not contain villi. Besides that, the overall organization is quite similar.

The main regulatory mechanism to preserve intestinal homeostasis by balancing proliferation from the crypt and differentiation and apoptosis further

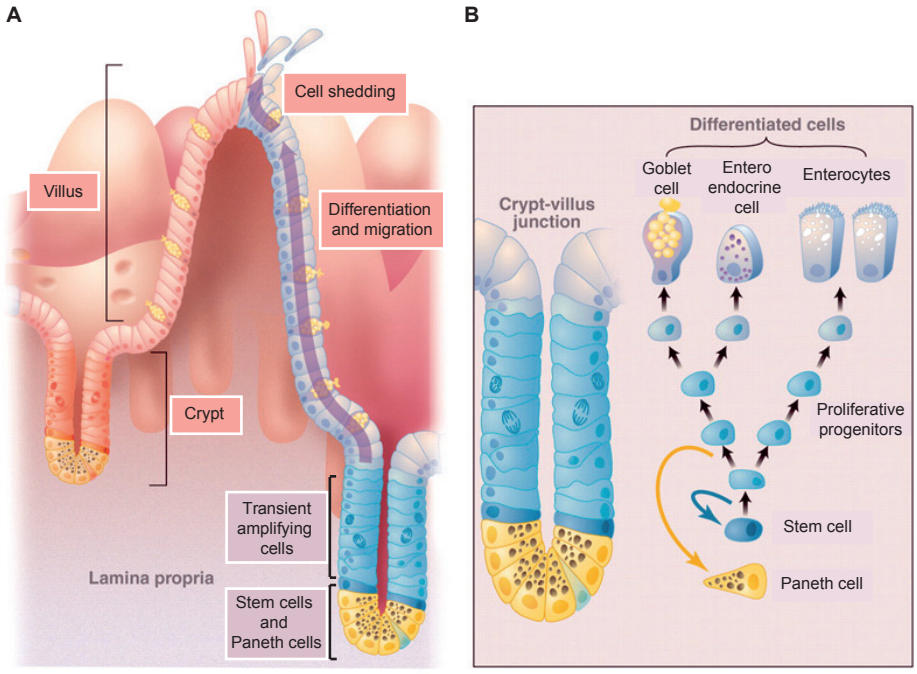


Figure 1. Schematic representation of the small intestinal epithelium. (A) The epithelium is build up by crypts and villi. The crypt compartment houses the intestinal stem cells, from where newly generated cells originate and migrate upwards into the villi while differentiating into specific cell types and finally shedding at the villi tips. Although a single stem cell is depicted in this crypt representation, multiple stem cells alternating with Paneth cells are present per crypt. **(B)** Lineage scheme depicting the stem cell, transient amplifying cells and differentiated cell types positioned in relation to the crypt. The secretory lineage is depicted in the left branch containing goblet cells and enteroendocrine cells, and the absorptive lineage represented by the enterocytes is depicted at the right branch. Figure adapted from Radtke and Clevers [2].

upwards in the villi is represented by the Wnt signaling pathway [1-3]. Underscoring the relevance of balanced Wnt signaling, the majority of colorectal cancer cases results from deregulated Wnt signaling. In addition, embryonic development of the intestine also crucially involves Wnt signaling. Taken together, Wnt signaling is a major player in the normal as well as aberrant regulation of the intestine during different stages of life.

Introduction to Wnt signaling

Canonical Wnt/β-catenin signaling

Wnt signaling is generally subdivided into canonical ('classical') and non-canonical ('alternative') signaling. Canonical Wnt signaling is the best-known pathway and is mediated via β-catenin, which is why this route is also referred

to as Wnt/β-catenin signaling. β-Catenin is involved in cell adhesion through formation of catenin-cadherin complexes at the cell membrane and functions as a co-activator of transcription in the nucleus. Especially this latter function where β-catenin dictates canonical Wnt/β-catenin signaling output by regulating gene transcription in the nucleus is of major relevance in the intestine. As a control mechanism, this canonical Wnt/β-catenin signaling is regulated by β-catenin degradation controlled by adenomatous polyposis coli (APC), which thereby represents the gatekeeper tumor suppressor in the intestine. In the absence of extracellular Wnt ligands, APC participates in a multiprotein degradation complex, further consisting of two kinases GSK3 and CK1, and the scaffold protein Axin (Figure 2A) [4]. This complex catalyses Ser/Thr phosphorylation of β-catenin at specific N-terminal residues, thereby earmarking β-catenin for subsequent ubiquitination and degradation. This is a very important mechanism to restrain canonical Wnt/β-catenin signaling. The formation of this multiprotein degradation complex can be inhibited by canonical Wnt ligands (e.g. Wnt1, Wnt3a), upon binding of their receptors at the cell membrane (e.g. Frizzled and LRP5/6). As a result, β-catenin is stabilized and subsequent translocation of β-catenin to the nucleus is promoted (Figure 2B). In the nucleus, β-catenin binds TCF/LEF as a co-activator of transcription, regulating the expression of target genes including

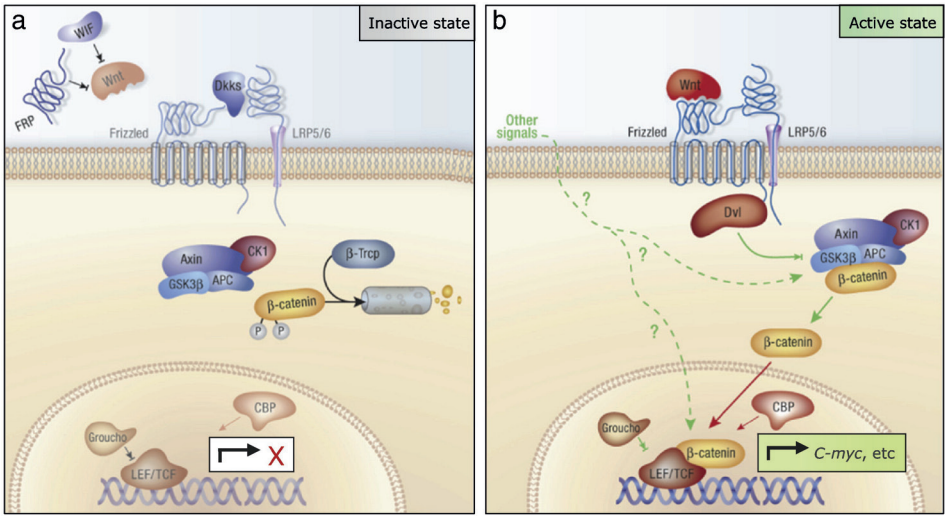


Figure 2. Schematic representation of the canonical Wnt/β-catenin signaling pathway. (A) In the absence of an extracellular canonical Wnt ligand, a multiprotein degradation complex consisting of APC, Axin, GSK3 and CK1, marks β-catenin for degradation by phosphorylation on its N-terminus. Consequently, β-catenin is degraded in the proteasome and no free β-catenin enters the nucleus to activate gene expression. **(B)** When canonical Wnt ligands are present and bind to Frizzled and LRP5/6 receptors at the cell surface, formation of the multiprotein degradation complex is inhibited. Then, β-catenin is stabilized and enters the nucleus where it activates the expression of target genes, e.g. *c-Myc* and *Axin2*. Figure adapted from Albuquerque et al. [4] (Chapter 4).

c-Myc, *Cyclin D1* and *Axin2*, thereby affecting cellular decisions. In addition to agonistic Wnt ligands, the pathways can also be influenced by Wnt antagonists, including Dickkopf (DKK), secreted Frizzled-Related Protein (sFRP) and Wnt Inhibitory Factor (WIF).

Non-canonical Wnt5a signaling

Non-canonical, also referred to as alternative Wnt signaling constitutes of various signaling routes that exist side by side and typically do not require β-catenin. Several Wnt ligands have been described to activate non-canonical Wnt pathways, among which are Wnt4, Wnt5a and Wnt11 [5-6]. The most extensively studied non-canonical Wnt ligand is Wnt5a. Signaling by secreted Wnt5a ligands is initiated by binding receptors at the cell membrane. The best-characterized Wnt5a receptor is receptor tyrosine kinase-like orphan receptor 2 (Ror2), which is a single-pass transmembrane receptor with a tyrosine kinase domain [7-9]. Among the mammalian family of seven-pass transmembrane Frizzled (Fz) receptors, a variety has shown cooperation with Wnt5a, including Fz2 [10], Fz4 [8, 11], Fz7 [12-13], whereas Ror1 [14], receptor-like tyrosine kinase Ryk [15-16] and low-density lipoprotein receptor-related protein 5 (LRP5) [8] have been suggested to act as (co)receptors for Wnt5a as well. Non-canonical Wnt signaling mediated by Wnt5a can activate the Wnt/Ca^{2+} and Wnt/PCP pathways, primarily known to regulate cell migration and polarity during embryogenesis [17-19]. The Wnt/Ca^{2+} pathway involves the action of Disheveled (Dvl), protein kinase C (PKC) and Ca^{2+}/calmodulin-dependent kinase II (CAMKII), whereas the Wnt/PCP route signals through Dvl, small G proteins such as Rac and Rho and Jun N-terminal Kinase (JNK) (Figure 3) [17-19]. Additionally, Wnt5a-mediated non-canonical Wnt signaling can influence canonical Wnt/β-catenin signaling, mostly demonstrated in an inhibiting fashion but promoting effects have also been shown [8, 20-22]. Different mechanisms have been suggested by which Wnt5a can inhibit β-catenin signaling. Work by Topol et al. has indicated that the inhibitory action of Wnt5a is dependent on intact APC and mediated by the upregulation of ubiquitin E3 ligase Siah2, resulting in enhanced breakdown of β-catenin [21]. Another proposed candidate mechanism is the inhibition via CAMKII activation through subsequent activation of Nemo-like Kinase (NLK) which phosphorylates and inhibits TCF [23]. Wnt5a can also conduct its inhibitory action on Wnt/β-catenin signaling by receptor competition [10]. Although Ror2 is the best recognized receptor involved in the inhibitory action of Wnt5a, Fz receptors are also involved and in turn, these participate in canonical Wnt/β-catenin signaling as well, providing a basis for competition [8, 10, 14]. Taken together, Wnt5a likely antagonizes Wnt/β-catenin through multiple mechanisms, possibly occurring at several levels, including the cell membrane, the cytoplasm

and the nucleus. Less clear is how Wnt5a promotes Wnt/β-catenin signaling. This Wnt/β-catenin promoting effect by Wnt5a has been observed when Fz4 and LRP5 were overexpressed in vitro [8], and it was observed in Xenopus animal caps following co-injection of *Xwnt5a* and *Xfz4* RNA [24]. Recently, these data have been further supported by the finding that in developing mice, induced Wnt5a expression causes an increase in Wnt/β-catenin signaling in the developing skull [22]. Although Fz4 is expressed endogenously here, it has not formally been shown to be involved in the observed canonical Wnt/β -catenin signaling promoting effect and a mechanism remains to be elucidated [22].

Importantly, which of the multiple signaling activities is actually activated in a certain situation by Wnt5a is dependent on the molecular and cellular context, especially known to be dictated by receptor availability [8, 25]. This context-dependent character of Wnt5a is nicely illustrated by the finding that ectopic Wnt5a overexpression during mouse embryogenesis causes repression of Wnt/β-catenin signaling in the developing dermis, whereas it activates Wnt/β-catenin signaling in the skull [22]. As a consequence of this complexity, determination of the functions of non-canonical Wnt5a signaling is not straightforward and requires a context-specific approach.

Embryonic development of the intestinal tract

Early in embryogenesis, gastrulation gives rise to three primary germ layers: the ectoderm, mesoderm and endoderm [26-28]. Development of the gastrointestinal tract is conserved among mammalian species and involves the mesodermal and endodermal germ layers. The mesoderm gives rise to the mesenchyme, smooth muscle layer and various additional cell types in the intestine, whereas the endoderm gives rise to the intestinal epithelium. In mice, endodermal cells are specified at embryonic day (E) 6 during gastrulation. During the end of gastrulation around E8.5 the endoderm appears morphologically homogeneous, but the endodermal tube formation is already initiated by folding of the endoderm at the anterior and posterior ends [26-28]. This creates the so-called anterior and caudal intestinal portals (AIP and CIP), representing the precursors of the foregut and hindgut respectively (Figure 4). This regionalization is associated with the early expression of region-specific markers, among which are Sox2 being restricted to the anterior parts and the Caudal type homeobox gene Cdx2 being restricted to posterior parts [28]. Whereas Sox2 is crucial in foregut development, Cdx2 is critically involved in hindgut development [28-30]. Besides the differential gene expression in the endoderm itself, dynamic tissue movements also bring the endoderm into proximity of different mesodermal tissues that secrete

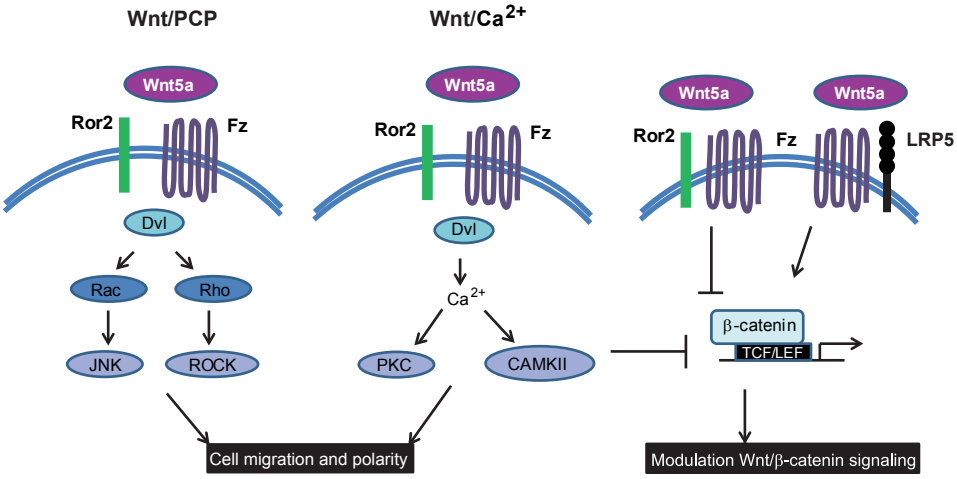


Figure 3. Schematic representation of non-canonical Wnt5a signaling routes. Non-canonical Wnt signaling mediated by Wnt5a can activate the Wnt/PCP route, involving Dvl, small G proteins such as Rac and Rho and the kinases ROCK and JNK (left panel). Wnt5a can also activate the Wnt/Ca^{2+} pathway, depicted in the middle panel, which involves Dvl, PKC and CAMKII. Both Frizzled and Ror2 receptors have been implicated in these pathways [17-19, 92]. The right panel depicts the modulating capacity of Wnt5a on canonical Wnt/β-catenin signaling. Wnt5a exerts contrasting activities on Wnt/β-catenin signaling depending on the context of cell surface receptors [8, 10, 14, 22].

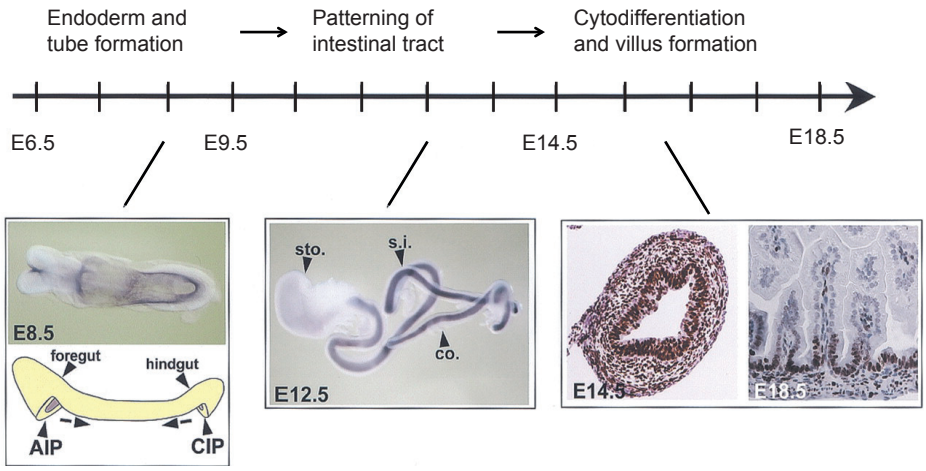


Figure 4. Time line of embryonic development of the mouse intestine. Endodermal cells are specified around E6.5 and endodermal tube formation is initiated around E8.5, by folding of the endoderm at the anterior and posterior ends creating the AIP and CIP respectively (left panel). From ~E9.5 onwards, the gut tube is patterned along the anterior-posterior axis, associated with the expression of specific markers. The middle panel shows a gastrointestinal tract of an E12.5 mouse embryo stained for the intestinal epithelial marker Villin. The formation of villi and cytodifferentiation starts around E14.5. The right panel depicts cross sections of embryonic mouse intestine at E14.5 and E18.5 (stained for a proliferation marker), showing the emergence of villi during late embryogenesis. Figure adapted from Gregorieff and Clevers [102].

instructive patterning factors, among which are Wnt ligands. On the other hand, the mesoderm does not only send instructive signals but also receives signals from the endoderm. In turn, these inductive signals from the endoderm specify and pattern the overlying mesoderm. Thus, a complex interplay of differential gene expression and patterning factors orchestrates regionalization of the gut tube and around E9.5, the foregut, midgut and hindgut regions can be discriminated. The foregut will eventually give rise to the esophagus, stomach and duodenum whereas the midgut is the main precursor of the intestine ranging from the caudal duodenum, through the jejunum, ileum and caecum to the proximal colon. The hindgut develops into the distal regions of the colorectal tract. Throughout its development, the gut tube undergoes dramatic changes in shape, involving planar cell polarity (PCP) and likely convergent extension (CE) [31]. These represent the polarity of cells within their plane or the narrowing (convergence) and lengthening (extension) of a tissue, respectively. Between E9.5 and E13.5, rapid elongation of the gut tube occurs whereby the intestine lengthens with the growing embryo and the circumference of the gut increases along with the size of the lumen [26, 32]. Unfortunately the process of gut elongation is poorly understood. It remains to be clarified where new cells originate, how these newly generated cells are positioned correctly and which tissue layer provides the driving force for elongation. As the gut tube grows, the epithelium reorganizes from a pseudostratified to a columnar epithelium and around E14.5, villus structures emerge and differentiation of characteristic intestinal cell types is initiated. Intestinal cytodifferentiation involves numerous signaling components, among which Wnt signaling, Notch signaling and Cdx2 are often mentioned and believed to interact [26, 28]. A study by Li et al. has indicated that whereas at E14.5 the transcriptomes of stomach and intestinal regions differ only subtly, at E16.5 hundreds of genes are upregulated in the intestinal epithelium [33]. Many of these genes are involved in functions prototypic for the intestine, including metabolism and absorption, distinguishing the digestive function from that of the stomach. As such, the authors referred to this process as intestinalization. Concomitantly to intestinalization, a sharp boundary of epithelial gene expression profiles forms at the pylorus. Development of the intestinal tract completes soon after birth, when the crypts of Lieberkühn containing Paneth cells are formed from which the intestinal epithelium will be renewed and maintained during future life.

Canonical Wnt/β-catenin signaling in embryonic gut development

Wnt signaling is recognized as a master regulator of cell fate decisions by balancing proliferation and differentiation. Enhanced Wnt/β-catenin signaling during development causes axis duplications and drastic phenotypes following

the manipulation of various components of the Wnt/β-catenin signaling cascade have indicated the relevance of this route during fetal development [34-37]. Despite this, the role of canonical Wnt/β-catenin signaling during the development of the intestinal tract remains unclear. In mice, transient Wnt/β-catenin activity has been reported between E7.5-E8.5, ceasing thereafter [38]. Later in gut development, β-catenin transcriptional activity is detected in the intestinal epithelium again only after the emergence of villi around E16, being further increased at E18.5 [39]. Accordingly, expression of the canonical Wnt/β-catenin target gene *Axin2* appears from E16.5 [33, 39]. Kim et al. found that β-catenin activity does not only reappear relatively late in gut development, also the spatial expression was unexpected [39]. Hence, they observed β-catenin activity being restricted to the postmitotic cells lining the villi, rather than presenting in the intervillus regions where the progenitors of the crypts reside. Soon after birth, Wnt/β-catenin activity shifts from the postmitotic villi cells towards the intervillus regions that develop into the crypts of Lieberkühn [39]. The late reappearance of canonical Wnt/β-catenin signaling during gut development from E16 onwards suggests that canonical Wnt/β-catenin signaling possibly contributes to the development of intervillus regions and crypts around birth. Supportively, disruption of Tcf4 (encoded by the *Tcf7l2* gene) in mice causes depletion of the proliferative intervillus compartments and perinatal lethality [40]. Importantly, the first defects in the *Tcf4*$^{-/-}$ embryos emerge late, only from around E16.5 [40], suggesting that Tcf4 might be involved in establishing late fetal and neonatal proliferative intervillus regions and crypts of Lieberkühn.

Taken together, canonical Wnt/β-catenin signaling appears active transiently between E7.5-E8.5, reappearing around E16 in the postmitotic villus cells during final gut development approaching birth. After birth, Wnt/β-catenin activity shifts towards the intervillus regions, i.e. the prospective crypts. A possible role for canonical Wnt/β-catenin signaling in embryonic gut development may lay in orchestrating the establishment of proliferative intervillus regions and crypts around birth.

Non-canonical Wnt5a in embryonic gut development

The indispensable nature of Wnt5a-mediated non-canonical Wnt signaling during embryogenesis is indicated by the perinatal lethality of *Wnt5a* knockout mice [41]. In mice, Wnt5a expression is predominantly detected in structures undergoing extensive outgrowth, such as the limbs, tail and facial structures [41-42]. Expression in these structures is in a graded fashion with highest levels towards the outer tips and most pronounced between E10 and E14 [41-42]. Loss of *Wnt5a* results in a drastic shortening of these structures in whose progenitors Wnt5a is normally expressed [41]. Importantly, the embryonic phenotype of *Ror2*

homozygous knockout mice closely resembles the *Wnt5a* knockout phenotype, although appearing milder [43-44]. Thus, during embryonic development both Wnt5a and its main receptor Ror2 are essential for the proper outgrowth of multiple structures. This also applies to the gastrointestinal tract. Elongation of the mouse midgut region starts around E10 followed by a rapid increase in length. Normal gut development involves temporal and spatial specific expression of Wnt5a and Ror2. Expression of Wnt5a peaks between E10.5 and E13.5 and is restricted to the mesenchyme of the caudal midgut and rostral hindgut (i.e. prospective distal small intestine and proximal large intestine), suggested to correlate with regions of the small intestine that undergo elongation (Figure 5A) [31, 45-46]. Accordingly, homozygous *Wnt5a* knockout embryos display drastically shortened intestines, in addition to an imperforate anus [45]. Cellular differentiation is not grossly affected by *Wnt5a* knockout [45]. Intestinal Ror2 expression is most intense between E10.5 and E13.5 as well and shows a complex region-specific pattern in both the mesenchyme and epithelium (Figure 5B) [31]. Importantly, *Ror2* knockout embryos also display shortened intestines [31]. Thus, Wnt5a and Ror2 show most prominent spatial specific expression in the embryonic intestine during the same period and both *Wnt5a* and *Ror2* depletion disrupt intestinal elongation.

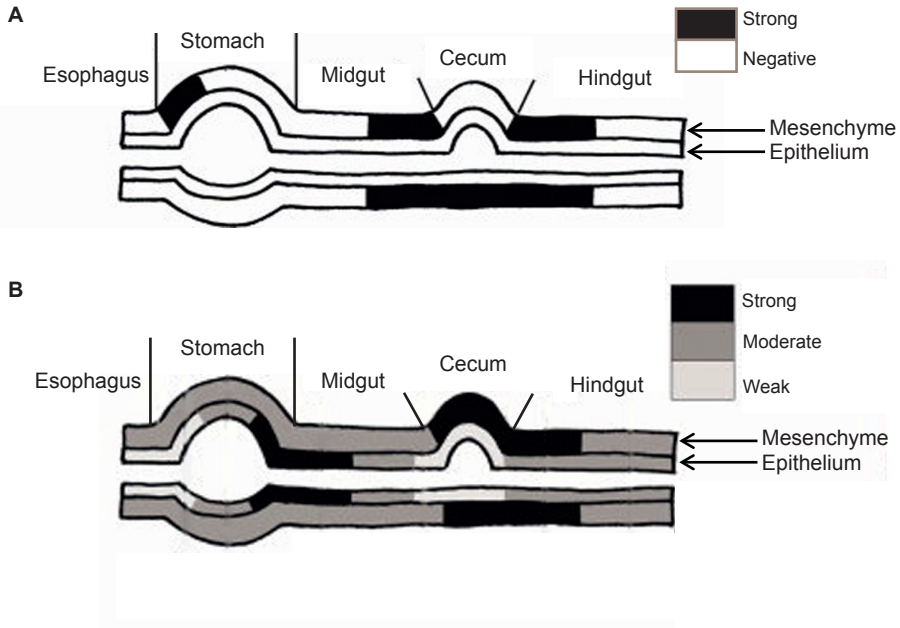


Figure 5. Schematic representation of Wnt5a and Ror2 expression along the developing mouse gastrointestinal tract. (A) At E11.5, Wnt5a expression in the intestine is restricted to the mesenchyme of the caudal midgut and rostral hindgut. **(B)** Ror2 expression from E10.5 to E12.5, showing a complex region-specific pattern in both the mesenchyme and epithelium. Figure adapted from Yamada et al [31].

WNT5A expression has also been reported in human embryos in the hindgut and anorectal regions, most intensely from the 4th-7th week of gestation, suggesting the involvement of WNT5A in human hindgut and anorectal morphogenesis [47]. According to the Carnegie stage comparison, this human time frame of WNT5A expression correlates with the mouse E10-E14 time frame, in which Wnt5a is most abundantly expressed as well in the embryonic gut. These restricted time frames in which Wnt5a is expressed during gut development suggest that also the activities of Wnt5a are dynamic and restricted to a certain period. Unfortunately, not much knowledge exists regarding the dynamics and mechanisms of Wnt5a functioning during the embryonic development of the gastrointestinal tract. We investigated this topic, as addressed in **Chapter 2**.

Adult homeostasis of the intestinal tract

Development of the mouse gastrointestinal tract finalizes in the first week after birth, when the intervillus regions are replaced by the crypts of Lieberkühn. From the crypts, newly generated cells will originate and migrate upwards into the villi while differentiating, thereby contributing to the rapid epithelial cell turnover and the maintenance of intestinal homeostasis. Canonical Wnt/β-catenin signaling is recognized as the dominant force in controlling cell fate by tightly balancing cellular proliferation, differentiation and apoptosis along the crypt-villus axis. Accordingly, Wnt/β-catenin signaling is most active in the proliferative crypt compartment, reflected by the presence of nuclear β-catenin here, while no active Wnt/β-catenin signaling is observed in the differentiated villi. Numerous studies have demonstrated the relevance of Wnt/β-catenin signaling in balancing proliferation versus differentiation of intestinal epithelial cells. Reducing Wnt/β-catenin signaling by either removal of Tcf4 or β-catenin, or induction of the Wnt antagonist Dkk1, results in the loss of proliferative epithelial cells in the intestine of late-embryonic and adult mice [40, 48-51]. Furthermore, deficient cell differentiation was observed, as reduced amounts of goblet cells, enteroendocrine and Paneth cells were seen [40, 48, 50]. On the other hand, promoting Wnt/β-catenin signaling by *APC* mutation causes epithelial hyperproliferation [52-54].

How the gradient of active Wnt/β-catenin signaling in the crypt towards inactive Wnt/β-catenin signaling in the villi is maintained is incompletely understood, although most logically, regulation by Wnt ligands seems involved. In line with active Wnt/β-catenin signaling in the crypt, the agonistic Wnt3 ligand is predominantly found in the deepest part of the crypt [55]. More specifically, Wnt3 is expressed by the Paneth cells, and involved in establishing the niche for intestinal stem cells [55-56]. The non-canonical Wnt5a ligand has a different

expression pattern, strikingly being restricted to the mesenchymal compartment. In the small intestine, Wnt5a is expressed in the mesenchyme of the villi tips and weaker, local expression has been observed along the villi and at the crypt-villus junction [55]. In the colon, Wnt5a is expressed mostly in the mesenchyme beneath the surface epithelium [55]. Wnt5a has been proposed to influence many cellular processes, including cell growth, differentiation and migration, mostly indicated by in vitro studies [17-19, 57]. Regarding these proposed activities of Wnt5a and in line with the villus-located Wnt5a expression, Wnt5a may be expected to inhibit proliferation, induce differentiation or promote oriented cell migration of epithelial cells along the crypt-villus axis. However, it remains to be determined whether Wnt5a indeed has a significant impact on intestinal homeostasis in the context of an adult organism. We address this topic in **Chapter 2**.

Cancer of the intestinal tract

Colorectal cancer is a major cause of morbidity and mortality worldwide, especially prevalent in the United States and Western Europe. In the United States alone, approximately 143,000 new cases are registered each year, and its mortality exceeds 50,000 persons each year. As such, colorectal cancer represents the third most common cancer and it accounts for the third highest number of cancer-related deaths in the United States [58]. In Europe, about 213,000 new cases and 110,000 deaths are reported each year [59].

Colorectal cancer is a multistep process where the accumulation of genetic alterations in oncogenes and tumor suppressor genes in concerted action with various epigenetic changes ultimately leads to the formation of a malignant tumor. Mutation of the *APC* gene is most often the earliest genetic event, initiating the tumorigenic process and leading to the appearance of aberrant crypt foci or early adenoma. Whereas in most cases the *APC* mutation is sporadic, a heterozygous germline *APC* mutation can also be inherited, as is the case in the familial adenomatous polyposis (FAP) syndrome, allowing the development of thousands of adenomatous polyps. Additional mutations lead to further progression of early lesions, which in about half of the cases is represented by an oncogenic mutation in the *KRAS* gene [59-60]. During the subsequent adenoma-to-carcinoma sequence, additional mutations accumulate in other main target genes such as the tumor suppressor genes *TP53* and *SMAD4*, further facilitating tumor progression favoring adenoma to carcinoma transition. In line with Knudson's two-hit model, both alleles of the tumor suppressor genes are affected to inactivate the genes, whereas proto-oncogenes such as *KRAS* or β-catenin require only one activating mutation to become oncogenic. The situation in *Apc*-mutant mouse models is

somewhat different, since the intestinal tumors that develop in these mice rarely show malignant progression. This might be explained by the lack of spontaneous somatic mutations in the *Kras* and *Trp53* genes during the short lifespan of the mouse [61].

Tumor development is generally associated with the acquisition of genetic instability. The two main forms of genetic instability that have been described in colorectal cancers are chromosomal instability (CIN) and microsatellite instability (MIN). CIN is characteristic for the majority of colorectal cancer cases, where tumors exhibit deficient chromosome segregation resulting in variable chromosome numbers among cells [62-63]. MIN tumors account for approximately 15% of colorectal cancers and result from a defect in the mismatch repair (MMR) machinery, being associated with instability of repetitive DNA sequences [64-65]. The genes that are most commonly affected in these microsatellite instable (MSI) colorectal tumors are *MSH2*, *MLH1* and *MSH6*, normally involved in the repair of DNA mismatches. Around 2-3% of all colorectal cancer cases arise in families with heterozygous germline mutations in one of these genes, leading to Lynch syndrome (also referred to as hereditary non-polyposis colorectal carcinoma, HNPCC). However, MMR deficient tumors mostly arise sporadically, representing 10-12% of all colorectal cancer cases [64-65].

Canonical Wnt/β-catenin signaling in intestinal cancer

The overall majority of colorectal cancer cases results from aberrant activation of the Wnt/β-catenin signaling pathway, which is in line with the crucial role of Wnt/β-catenin signaling in fine-tuning stemness, proliferation and differentiation in the intestine. In a recent mutation analysis by the Cancer Genome Atlas Network, researchers detected mutations in one or more members of the Wnt signaling pathway in over 94% of all investigated colorectal tumors [66]. As mentioned previously, most colorectal cancers acquire mutations in both copies of the *APC* gene, resulting in the inefficient degradation of β-catenin and thereby to stabilization and abnormally high transcriptional activity of β-catenin. In approximately 1-2% of colorectal cancer cases, most often MMR deficient cases, the β-catenin proto-oncogene itself is mutated, requiring only one activating mutation to achieve resistance to degradation. In both scenarios, the aberrantly stabilized β-catenin constitutively activates downstream Wnt/β-catenin target genes, triggering a genetic program associated with enhanced stemness, proliferation and tumor initiation. In addition to the clear contribution of *APC* mutation to tumor initiation, it also appears to cause chromosomal instability, thereby contributing to later stages of tumor development as well [67-68]. Enhanced Wnt/β-catenin signaling has also been implicated in tumor progression since heterogeneous patterns

of β-catenin expression are observed in colorectal tumors, despite that *APC* or *CTNNB1* mutations predict constitutive β-catenin signaling in each tumor cell. Hence, the highest levels of nuclear β-catenin expression are observed at the tumor invasion fronts and patches of cells with high nuclear β-catenin levels are associated with invasion and epithelial to mesenchymal transition [69-71]. Enhanced β-catenin signaling has thus been suggested to promote an invasive and metastatic phenotype of colorectal tumors [72-73], in addition to its crucial role in tumor initiation.

APC is a large protein constituting different domains involved in the binding and regulation of other proteins (Figure 6). Among these, four 15 amino acid repeats (AAR) bind β-catenin, whereas seven 20-AARs are involved in both binding and downregulation of β-catenin. Interspersed within those 20-AARs are three binding sites for Axin required for an optimal recruitment of APC into the destruction complex. During intestinal tumorigenesis, the majority of *APC* mutations results in truncated APC proteins that lack all Axin binding motifs while retaining between one and three 20-AARs. Importantly, with these few 20-AARs remaining, APC still retains residual activity in downregulating β-catenin. Accordingly, an inverse correlation is observed between the number of retaining 20-AARs and the resulting level of β-catenin signaling, i.e. more repeats means more β-catenin downregulation and lower β-catenin signaling levels in the nucleus (Figure 6). During tumorigenesis, *APC* mutations do not occur entirely randomly but rather are selected to one another to reach an optimal level of enhanced β-catenin signaling, described as the 'just-right' signaling model [74-77]. According to this model, levels beneath the optimal β-catenin signaling window will not provide cells with sufficient activation of target genes to gain growth advantage and trigger tumor formation, whereas levels exceeding the optimal window will trigger apoptosis instead. Moreover, it appears that different tissues require their own specific dosages of enhanced β-catenin signaling in order to achieve successful tumor formation, as indicated by the different *APC* genotypes that are observed in different tumor types throughout the body. For example, desmoids and duodenal tumors generally contain *APC* mutations retaining 2-3 20AARs, associated with moderate β-catenin signaling activation. On the other hand, most colorectal tumors are associated with shorter APC proteins resulting in higher levels of β-catenin signaling (Figure 6). Correlations are observed even within the colorectal tract, where right-sided colon tumors generally retain more 20-AARs than left-sided ones [78-79]. Based on this observation, we propose a possible explanation for the characteristic distribution of tumors along the colorectal tract in correlation to their genotype in **Chapter 4**.

Comparable *Apc* genotype-tumor phenotype correlations are observed among *Apc*-mutant mouse models (illustrated in Figure 5 of **Chapter 4**). Briefly,

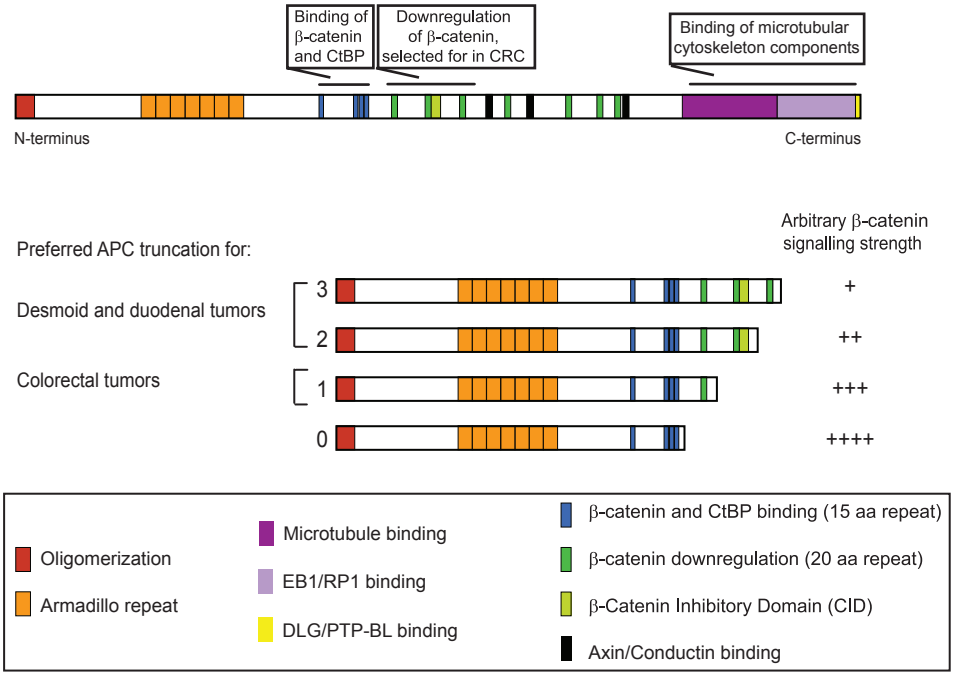


Figure 6. APC, its truncated forms observed during tumorigenesis and associated β-catenin signaling. APC encompasses different domains involved in multiple processes, including the regulation of β-catenin and the binding of CtBP and cytoskeletal components. Most importantly, the majority of intestinal tumors select for APC truncations retaining between one and three 20-AARs and no axin binding repeat. The number of residual 20-AARs determines the resulting β-catenin signaling strength in an inverse fashion. An example is shown of the truncated APCs preferred for tumor formation in different tissues, desmoids and duodenal tumors preferring 2-3 20-AARs versus colorectal tumors mostly selecting for 1 20-AAR.

Apc-mutant mouse models with no 20-AARs left, such as the *Apc*Min mice, exert high β-catenin signaling and develop >100 gastrointestinal tumors. However, mice carrying the hypomorphic *Apc*1638N allele have intermediate β-catenin signaling and develop <10 gastrointestinal tumors and have a high incidence for developing desmoids and cysts. *Apc*1572T mice have three 20-AARs remaining and thereby have a relatively low gain in β-catenin signaling compared to the other models, while interestingly these mice characteristically develop mammary tumors, some desmoids and cysts but no gastrointestinal tumors. Altogether these correlations in mice and men indicate the important role of β-catenin signaling dosage in determining the success of tumor formation in a certain tissue.

Although this concept of β-catenin signaling dosage and its impact on tumor growth among tissues is certainly gaining acceptance, tissue-specific tumor predisposition has not been formally proven to be a direct consequence of β-catenin signaling dosage. APC is a large, multifunctional protein and in addition

to downregulating β-catenin it also functions in other cellular processes, as APC can affect chromosomal segregation, cytoskeletal organization and bind C-terminal binding protein (CtBP) [67-68, 80-83]. CtBP is a transcriptional co-repressor that potentially affects various transcriptional programs [84], and that can bind APC at its 15-AARs [80, 83]. The levels of CtBP appear to increase upon *Apc* loss in early adenomas of FAP patients [82]. Moreover, the relevance of β-catenin signaling for colorectal tumor formation has been challenged by Phelps et al, who rather suggested that adenoma formation is triggered by enhanced levels of CtBP, while according to their observations nuclear β-catenin is only present following the acquisition of oncogenic *KRAS* mutations [82]. We provide direct genetic evidence for the dominant role of β-catenin signaling dosage in dictating tissue-specific tumor predisposition in **Chapter 5**.

Non-canonical Wnt5a in intestinal cancer

In contrast to the well-established contribution of canonical Wnt signaling to colorectal cancer, the role of non-canonical Wnt signaling herein is poorly understood. The non-canonical Wnt5a ligand has gained substantial attention over the last years, since its upregulation is observed in cancer of the colon and other tissues, in addition to inflammatory diseases and metabolic disorders [17, 19, 85-87]. In line with the complex character of Wnt5a reflected by the various pathways which it activates in a context-dependent fashion, the expression patterns and suggested functions of Wnt5a among cancer types are diverse and contradictory [19, 85]. Wnt5a levels are found to be downregulated in high-risk neuroblastoma and leukemia, suggestive of Wnt5a acting as a tumor suppressor [19, 85, 88-89]. On the other hand, Wnt5a is upregulated in other cancer types, among which are gastric cancer and melanoma. Accordingly, Wnt5a has been proposed as an important tumor promoter in these cancer types [18, 85, 90-91]. In these tumor types, Wnt5a enhances tumor cell migration, epithelial to mesenchymal transition and invasion, all of which are important key steps contributing to cancer progression [91-95]. In addition to these critical cancer-related processes, Wnt5a has also been implicated in cell growth and differentiation [17, 19, 57], thereby possibly affecting tumor composition. Furthermore, Wnt5a might be expected to influence canonical Wnt/β-catenin signaling [8, 20, 22]. A possible role of Wnt5a in colorectal cancer is unclear to date.

Colorectal tumors are heterogeneous entities, consisting of mutant epithelial tumor cells but also containing non-mutant supportive cells such as tumor-associated fibroblasts, endothelial cells and immune cells. Interactions between the tumor cells and non-mutant stromal cells are of major importance for a tumor to develop progressively and acquire metastatic potential. Wnt5a is a secreted

ligand and therefore might potentially affect the tumor cells themselves but also the supportive non-mutant cells. *Wnt5a* RNA has been reported to be upregulated in human and mouse intestinal tumors, produced by the stromal cells in the tumor and following an augmenting trend during the progression from normal intestine through adenoma to carcinoma [55, 96-99]. Although upregulation of Wnt5a in intestinal cancer has been shown most consistently, some contradictory findings have been reported, describing reduced Wnt5a protein expression or gene silencing in the epithelial compartment of a subset of colorectal tumors [100-101]. Balancing the currently available data, Wnt5a is mostly found to be upregulated in colorectal cancer and suggested to exhibit a tumor promoting contribution. However, most of the currently available data originate from expression association studies or in vitro studies in cell types not derived from the colon. Most optimally, a significant impact of Wnt5a to intestinal cancer should be investigated in the in vivo context of an adult organism during intestinal tumorigenesis, taking into account the physiological context and cellular interactions. Our findings considering the role of Wnt5a in intestinal cancer are presented in **Chapter 3**.

Scope of this thesis

As described above, Wnt signaling is a main regulatory signaling network crucially involved in embryonic development, maintenance of tissue homeostasis and often deregulated in cancer. Current research was aimed to gain more insight into the complexity of Wnt signaling in the intestinal tract. More specifically, we aimed to investigate the dynamics and activities of non-canonical Wnt5a in the intestine during different stages of life, e.g. embryonic development, adult homeostasis and intestinal tumorigenesis. Furthermore, although canonical Wnt signaling is well known for its relevant role in the intestine, we further explored the impact of β-catenin signaling dosage on tumor formation in the intestinal tract and other organs.

To enable investigation of the activities of non-canonical Wnt5a in vivo, we generated and validated an inducible transgenic Wnt5a mouse model, of which the results are described in **Chapter 2**. Using this model, we find that induced Wnt5a expression during specific timeframes of embryogenesis causes shortening of the gastrointestinal tract as well as the limbs, facial structures and tail. On the other hand, Wnt5a induction during adult homeostasis is well tolerated. Furthermore, we show that Wnt5a downregulates the protein expression of its receptor Ror2 in the intestine. Subsequently, **Chapter 3** describes our findings with regard to the role of Wnt5a in intestinal cancer. We found that Wnt5a promotes directional migration and invasion of human colon cancer cells, although in vivo Wnt5a

induction during *Apc*-driven cancer in mice is not sufficient by itself to enhance tumor malignancy or to cause metastasis in this context.

Chapter 4 shows our review addressing the concept of β-catenin signaling dosage and describing how this can be used to explain the preference for tumor development in the proximal versus distal colon of mismatch repair deficient versus proficient colorectal cancers respectively. The dominant role of β-catenin signaling dosage in dictating tissue-specific tumor predisposition in *Apc*-driven cancer goes beyond the distal or proximal colon. Hence, we showed that by reducing β-catenin levels, the tumor predisposition shifts from the intestinal tract towards mammary glands during Apc-driven cancer in mice, as shown in **Chapter 5**.

In parallel to studying Wnt signaling, we investigated the consequences of induced Sox2 expression during embryonic intestinal development. Our findings are described in **Chapter 6** and indicate that ectopic Sox2 expression in the developing gut drives activation of the foregut transcriptional program, leading to conversion of an intestinal to a premature gastric phenotype. The novel insights that we obtained considering Wnt signaling in the intestine are discussed in **Chapter 7**.

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CHAPTER 2

Induced Wnt5a expression perturbs embryonic outgrowth and intestinal elongation, but is well-tolerated in adult mice

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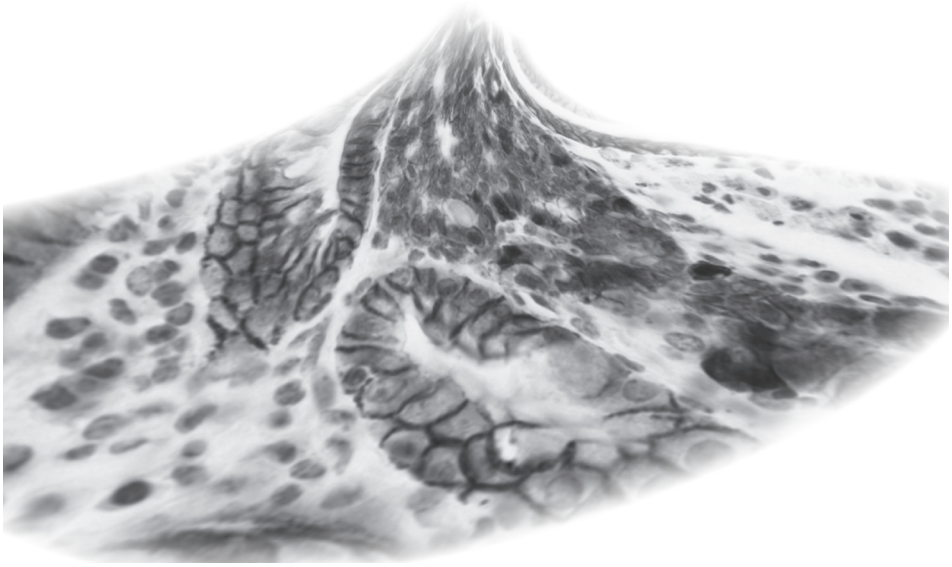

Abstract

Wnt5a is essential during embryonic development, as indicated by mouse *Wnt5a* knockout embryos displaying outgrowth defects of multiple structures including the gut. The dynamics of Wnt5a involvement in these processes is unclear, and perinatal lethality of *Wnt5a* knockout embryos has hampered investigation of Wnt5a during postnatal stages in vivo. Although in vitro studies have suggested a relevant role for Wnt5a postnatally, solid evidence for a significant impact of Wnt5a within the complexity of an adult organism is lacking. We generated a tightly-regulated inducible *Wnt5a* transgenic mouse model and investigated the effects of Wnt5a induction during different time-frames of embryonic development and in adult mice, focusing on the gastrointestinal tract. When induced in embryos from 10.5 dpc onwards, Wnt5a expression led to severe outgrowth defects affecting the gastrointestinal tracts, limbs, facial structures and tails, closely resembling the defects observed in *Wnt5a* knockout mice. However, Wnt5a induction from 13.5 dpc onwards did not cause this phenotype, indicating that the most critical period for Wnt5a in embryonic development is prior to 13.5 dpc. In adult mice, induced Wnt5a expression did not reveal abnormalities, providing the first in vivo evidence that Wnt5a has no major impact on mouse intestinal homeostasis postnatally. Protein expression of Wnt5a receptor Ror2 was strongly reduced in adult intestine compared to embryonic stages. Moreover, we uncovered a regulatory process where induction of Wnt5a causes downregulation of its receptor Ror2. Taken together, our results indicate a role for Wnt5a during a restricted time-frame of embryonic development, but suggest no impact during homeostatic postnatal stages.

Introduction

Whereas the relevance of canonical Wnt/β-catenin signaling in intestinal homeostasis and cancer is well established [1-2], so-called non-canonical Wnt signaling is far less understood. Non-canonical Wnt signaling is represented by multiple alternative signaling routes that act independent of β-catenin transcriptional activity and are typically activated by non-canonical Wnt ligands. Wnt5a is the most extensively studied non-canonical ligand and has gained substantial attention over the last years as it is being implicated in various human diseases including cancer, inflammatory diseases and metabolic disorders [3-5]. Signaling pathways that can be activated by Wnt5a include Wnt/Ca^{2+} and Wnt/JNK pathways, also referred to as the planar cell polarity pathway, are primarily known to mediate oriented cell movements during development [3-4, 6-8]. Additionally, Wnt5a can inhibit β-catenin signaling, although promoting effects have also been demonstrated in receptor overexpression experiments [9]. Importantly, which pathway is actually activated by Wnt5a greatly depends on the context, especially dictated by receptor presence. The best characterized Wnt5a receptor is receptor tyrosine kinase-like orphan receptor 2 (Ror2) [9-12]. Members of the family of Frizzled receptors, Ror1 and receptor-like tyrosine kinase Ryk have also been implicated to act as (co)receptors in Wnt5a signaling [4, 9, 13-14].

The indispensable nature of Wnt5a during embryogenesis is indicated by the perinatal lethality of homozygous *Wnt5a* knockout mice [8]. During development, Wnt5a expression is predominantly detected in structures undergoing extensive outgrowth, including the limbs, tail and facial structures [8, 15]. Expression in these structures is in a graded fashion with highest levels towards the outer tips and most pronounced between 10 and 14 days post coitum (dpc) [8, 15]. Loss of *Wnt5a* results in a drastic shortening of these structures in mouse embryos [8]. Importantly, the embryonic phenotype of *Ror2* homozygous knockout mice closely resembles the *Wnt5a* knockout phenotype, although appearing milder [16-17]. Thus, during embryonic development both Wnt5a and its main receptor Ror2, are essential for the proper outgrowth of multiple structures. This also applies to the gastrointestinal tract. Normal gut development involves temporal and spatial specific expression of Wnt5a and Ror2. Both Wnt5a and Ror2 expression peak between 10.5 and 13.5 dpc, a critical period when midgut elongation occurs. Wnt5a expression is restricted to the mesenchyme of the caudal midgut and rostral hindgut (i.e. future distal small intestine and proximal large intestine), suggested to correlate with regions of the small intestine that undergo elongation [18-20]. Ror2 expression shows a complex region-specific pattern in both the mesenchyme and epithelium [20]. Homozygous knockout of either *Wnt5a* or *Ror2* results in shortened embryonic intestines, in addition to an imperforate anus in *Wnt5a* knockout embryos [18]. The

dynamics of Wnt5a functioning are not completely understood to date. Given that endogenous Wnt5a expression in the gut is most prominent between 10.5-13.5 dpc and diminishes after that might be an indication that Wnt5a is most crucially involved in developmental elongation processes in this specific period. However, it remains to be clarified whether the functioning of Wnt5a extends beyond this stage. Relevant postnatal roles of Wnt5a have been suggested based upon associative Wnt5a expression studies and in vitro studies. Hence, enhanced Wnt5a expression has been observed in a variety of human diseases, including inflammatory conditions [21-22], metabolic disorders [4-5, 23] and a multitude of cancer types, among which colorectal cancer [3, 24-25]. Also, a wide variety of in vitro studies have implicated Wnt5a in multiple cellular processes, including cell growth, differentiation, migration and invasion [3-4, 7, 26]. Despite these supportive data, solid in vivo evidence for a significant postnatal impact of Wnt5a is lacking. To date, it has not been possible to modulate and investigate the activities of Wnt5a in an adult organism. We have generated an inducible *Wnt5a* transgenic mouse model, enabling in vivo determination of the relevance and activities of Wnt5a during desired time-frames. Transgenic Wnt5a expression was induced during specific time-frames of embryonic development and during adulthood, followed by a phenotypic analysis focusing on the intestinal tract.

Materials and methods

Generation of inducible Wnt5a mouse model and in vivo doxycyline administration
To generate the TetO-*Wnt5a* construct, a 1.1 kb XbaI fragment was isolated from a TetO-FLAG-*Wnt5a* vector (kindly provided by Dr. R. Nusse, Stanford University, CA), and inserted into the pTRE-Tight vector containing an additional 0.68 kb β-globin intron. The 2.4 kb expression cassette was XhoI digested from the plasmid backbone, gel-purified and injected into C57Bl/6J oocytes by standard procedures. TetO-*Wnt5a* positive founders were identified by PCR using transgene specific primers SCT2F (5'-CTACACCCTGGTCATCATCC) and mWnt5AFLAG-R (5'-TCGTACCTAGAGACCACCAAG).
TetO-*Wnt5a* mice were interbred with hnRNP-*rtTA2S-M2* mice [27], obtaining double transgenic mice. Wildtype and single transgenic mice were generated simultaneously and served as control animals. Doxycycline (dox) was administered via the drinking water (2 mg/ml dox, 5% sucrose). Induction during embryogenesis was performed by administering dox to timed pregnant females.
All mice were generated, bred and maintained under specific pathogen-free conditions at the animal facility of the Erasmus MC University Medical Center. All animal experiments were approved by the Institute's Animals Ethics Committee and were carried out in accordance with Dutch legislation.

Cell culture and treatments
Tail fibroblasts were isolated from adult mice by incubating tail tips in Dulbecco's modified essential medium (DMEM, Lonza) complemented with 0.14 U/ml Blendzyme (Roche), 1% antibiotics/antimycotics (Gibco) and 0.5% fetal bovine serum (FBS, Sigma Aldrich) for 3 hours at 37°C. Mixture was put on a cell strainer and cells were cultured

in DMEM with 1% antibiotics/antimycotics and 10% FBS at 37°C, 5% CO_2. Mouse embryonic fibroblasts (MEFs) were isolated from embryos of 13.5-15.5 dpc and cultured and transfected as previously described [28]. When indicated, cells were induced with 1 μg/ml dox. HEK293T cells were cultured in DMEM with 10% FBS, 1% p/s at 37°C, 5% CO_2.

β-catenin reporter assay
β-catenin reporter assay in MEFs was performed as previously described [28]. A Ror2 expression vector (kindly provided by Dr. A. Kikuchi, Osaka University, Japan) was co-transfected to assure sufficient receptor availability for the transgenic Wnt5a. Transfected cells were provided with 1 μg/ml dox for 24 hours of which the final 16 hours cells were simultaneously stimulated with either 25% of L-control or L-Wnt3a conditioned medium. Luciferase activities were measured using the dual-luciferase reporter assay system (Promega) and a LUMIstar luminometer. Assays were performed in duplicate and performed twice.

Immunoblotting
Cultured cells and snap-frozen intestinal tissue specimens were lysed in Laemmli sample buffer containing 0.1M DTT and incubated at 95°C for 10 min. Western blotting was performed according to standard chemoluminescent procedures or using fluorescent Odyssey immunoblotting (LI-COR Biosciences, Lincoln, NE, USA) [28]. Antibodies are indicated in Supplementary Table I. Quantification was performed using Odyssey Li-Cor software.

Skeletal preparations and staining
Embryos were skinned, eviscerated, fixed overnight in 96% ethanol containing 1% glacial acetic acid. Cartilage was stained overnight in 0.5 mg/ml Alcian Blue. Soft tissues were dissolved in 1.5% potassium hydroxide for 3 hours followed by staining of bone overnight in Alizarin Red and destaining in 0.5% potassium hydroxide/20% glycerol.

Tissue processing and histology
Adult intestines, whole embryos or embryonic intestines were isolated, washed in PBS and fixed overnight in 4% PBS-buffered paraformaldehyde (4% PFA) at 4°C. Fixed embryonic intestines were pre-embedded in 5% bacto-agar (BD) in PBS and cut into 6-8 pieces enabling proper identification of the different regions. All mouse materials were embedded in paraffin according to routine protocols.
Haemotoxylin eosin (HE), Periodic Acid Schiff (PAS) and Alkaline Phosphatase (AP) stainings were performed according to routine protocols. Immunohistochemistry was performed using antigen retrievals and antibodies as indicated in Supplementary Table II. Visualization was performed using the Envision+ System or StreptABCcomplex/HRP (Dako).

RNA isolation, cDNA synthesis and quantitative PCR
RNA was isolated from snap-frozen embryonic intestines and adult duodenum tissues using Nucleospin RNA II Kit (Machery-Nagel, Düren, Germany), followed by cDNA generation using iScript cDNA Synthesis Kit (Bio-Rad). Quantitative PCR was performed using Sensimix SYBR Green (Bioline) or TaqMan (in case of *Axin2/Actb*) Gene Expression Assays (Applied Biosystems) and run in the IQ5 Real time PCR detection system (Bio-Rad). Primers are shown in Supplementary Table III. Expression levels were corrected for expression of *Actb*, averaged and presented as fold changes.

Results

Tight regulation of functionally active Wnt5a protein in TetO-*Wnt5a* transgenic mouse model

We generated TetO-*Wnt5a* mouse founder lines carrying the mouse *Wnt5a* gene under the control of a Tet-inducible promoter. This promoter is inducible by the rtTA transcription activator in a doxycyline (dox) dependent manner. TetO-*Wnt5a* founders were crossed with the hnRNP-*rtTA* transgenic mouse line, which drives ubiquitous expression of the *rtTA2S-M2* gene [27]. Dox administration allows transgenic Wnt5a induction only in double transgenic mice carrying both TetO-*Wnt5a* and hnRNP-*rtTA* constructs. Wnt5a expressing founders were identified by culturing tail fibroblasts, providing a founder with robust Wnt5a protein expression upon dox administration without displaying Wnt5a expression in controls (Figure 1A). Similar results were observed in mouse embryonic fibroblasts (MEFs) (Figure 1B and S1A). To validate the functionality of the transgenic Wnt5a protein, we determined its capability to inhibit canonical Wnt/β-catenin signaling. MEF lines of control and double transgenic genotypes were stimulated with Wnt3a to enhance β-catenin signaling and doxycyline was added to induce transgenic Wnt5a (Figure 1B, upper panel). β-catenin mediated transcription was significantly decreased upon induction of transgenic Wnt5a (Figure 1B, lower panel). This indicates that upon dox induction in double transgenic MEFs, the cells produce functionally active Wnt5a protein.

In vivo dox administration successfully induced transgenic Wnt5a expression in double transgenic mice exclusively, as shown by immunoblotting and immunohistochemistry (Figure 1C-E). Induction was a rapid process since transgenic Wnt5a was detected already within 24 hours following dox administration (Figure S1B). In adult and embryonic mice, expression of transgenic Wnt5a protein was verified along the entire intestinal tract by immunohistochemistry (data not shown). When induction was performed during embryonic development from 10.5 dpc onwards, transgenic Wnt5a expression was observed in gut mesenchyme and epithelium at 13.5 dpc, shifting towards a predominant epithelial expression pattern at 18.5 dpc (Figure 1D). In the adult intestine, induced Wnt5a was most prominent in the epithelial cells and stromal expression was clearly present although less abundant, displaying an overall diffuse staining in addition to several intensely stained individual cells (Figure 1E). Wnt5a expression was not detected in muscle cells. Although all following experiments were performed with above described TetO-*Wnt5a* founder line unless mentioned differently, we validated a second TetO-*Wnt5a* founder which displayed a mosaic Wnt5a expression pattern (Figure 6C and S1C).

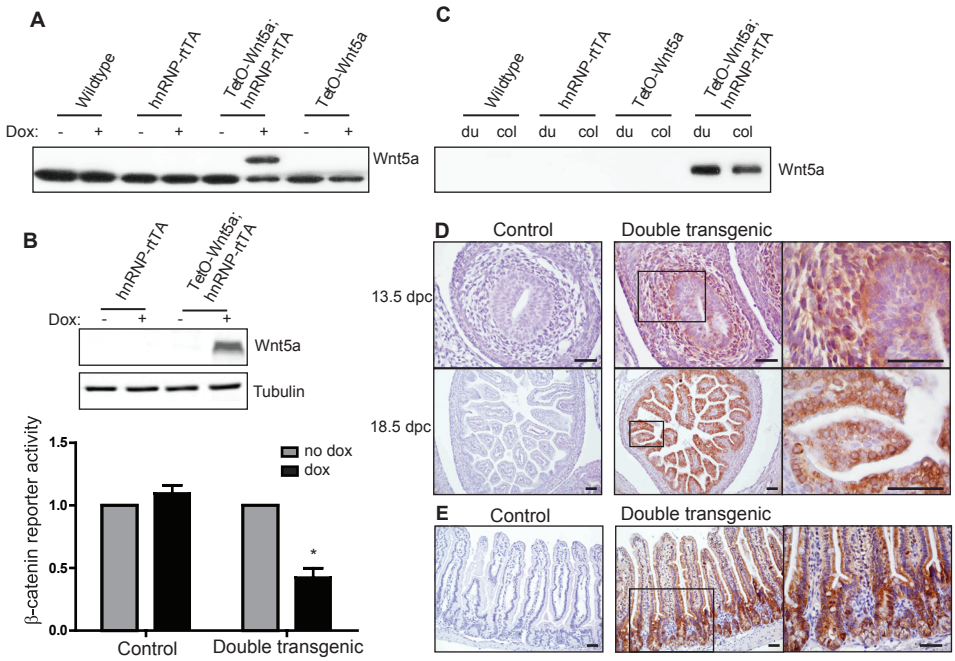

Figure 1. Tight regulation of functionally active Wnt5a protein in TetO-*Wnt5a* transgenic mouse model. (A) Wnt5a immunoblot of tail fibroblasts and **(B)** MEFs. Wnt5a antibody only detects overexpressed Wnt5a because of low sensitivity. β-catenin reporter assay showing that Wnt3a-induced β-catenin signaling is reduced following transgenic Wnt5a induction, * p=0.015. Two individual MEF lines were used for each genotype. **(C)** Intestine of adult mice induced for 1 week analyzed by Wnt5a immunoblot (du=duodenum, col=colon) and by **(D)** immunohistochemical Wnt5a staining. Abundant Wnt5a expression is revealed in double transgenic embryos (dox from 10.5 dpc onwards) and **(E)** adult intestine.

Induced Wnt5a expression deregulates embryonic development

Wnt5a is indispensable during mouse development, since its absence results in defective embryonic outgrowth and perinatal lethality [8]. We induced Wnt5a expression during several time-frames of embryogenesis to investigate the developmental consequences of overexpressing Wnt5a. Dox-induced double transgenic mice will be further referred to as Wnt5a^{ind} and compared to dox-induced control mice. Wnt5a induction from 6.5 dpc to 13.5 dpc resulted in severe developmental defects (Figure 2A). Externally, primarily the head, limbs and tail showed outgrowth defects. As the Wnt5a^{ind} embryos had already died in utero at 13.5 dpc, necrosis precluded a more detailed examination of these embryos. Starting induction of transgenic Wnt5a later, i.e. at 10.5 dpc, did not result in embryonic lethality but led to facial deformities and shortened limbs and tail (Figure 2B-C). Similar defects were observed using our mosaic Wnt5a founder (Figure S2A). Later dox inductions, from 13.5 dpc to 18.5 dpc, did not lead to apparent developmental defects in Wnt5a^{ind} embryos (Figure S2B). Examination

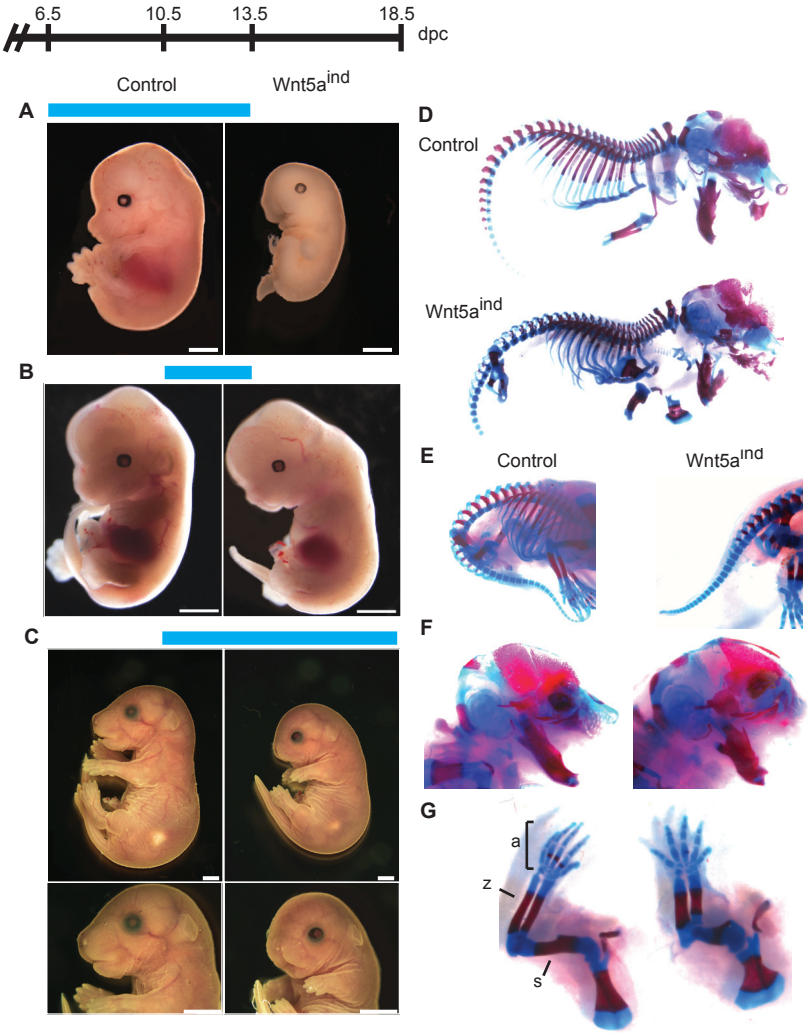

Figure 2. Induced Wnt5a expression deregulates embryonic development. Macroscopic appearance of dox-induced control versus double transgenic embryos. Time-frames of dox administration are indicated by blue bars against timeline. **(A)** Wnt5a^{ind} embryos at 13.5 dpc, induced from 6.5 dpc onwards, were already necrotic. **(B,C)** Embryos at respectively 13.5 and 18.5 dpc, induced from 10.5 dpc onwards, showing deficient outgrowth of face, limbs and tail. **(D-G)** Skeletal stainings of 17.5-18.5 dpc embryos, comparing similarly aged embryonic structures. **(D)** Overview of control (upper) and Wnt5a^{ind} (lower) phenotype. Limbs and tails are not complete in this figure panel. Clear shortening is observed affecting **(E)** tail **(F)** head **(G)** forelimb (a=autopod, z=zeugopod, s=stylopod).

of skeletal stainings provided insight regarding the basis of the externally visible defects in late-stage embryos that had been induced from 10.5 dpc onwards (Figure 2D). The tails of Wnt5a^{ind} embryos were clearly shortened, caused by a decreased number of vertebrae (Figure 2E). In the head, the most severely shortened bones are those in the upper jaw, while lower jaw elements were

mildly shortened (Figure 2F). Limb shortening was chiefly caused by deficient outgrowth of individual skeletal elements, including stylopodal and zeugopodal bones which appear thicker than normal as well (Figure 2G). Autopods appeared underdeveloped with shorter and thicker digits and a delay of ossification events, but no obvious loss of skeletal elements as has been reported for *Ror2* and *Wnt5a* knockout embryos was observed. Together, these experiments revealed that Wnt5a overexpression causes embryonic defects resembling those observed in *Wnt5a* KO and *Ror2* KO embryos, affecting the structures whose progenitors normally express Wnt5a in a graded fashion. Time dependency, i.e. before 13.5 dpc, of the Wnt5a overexpression phenotype matches the period in which endogenous Wnt5a is normally expressed most abundantly [8, 15, 18-20].

Induced Wnt5a expression disturbs embryonic intestinal elongation

In the developing gastrointestinal tract, Wnt5a expression is detected in the mesenchyme of the caudal midgut, rostral hindgut and stomach fundus [18-20]. Loss of *Wnt5a* leads to severely shortened gastrointestinal tracts [18]. Interestingly, we found that time-specific Wnt5a overexpression from 10.5 dpc until 18.5 dpc also resulted in drastic shortening of the small and large intestine, cecum and stomach (Figure 3A-C). Furthermore, Wnt5a^{ind} embryos displayed an imperforate anus (Figure 3D), as has also been observed in *Wnt5a* KO embryos [18]. A similar phenotype was revealed using the mosaic TetO-*Wnt5a* founder line, albeit less drastic (Figure S2C). The phenotype was not observed when Wnt5a expression was induced from 13.5 dpc onwards (Figure S2D). This indicates that the activities by which induced Wnt5a interferes with intestinal elongation mainly take place between 10.5 and 13.5 dpc, which is the phase of intestinal elongation when a rapid increase in length occurs and when Wnt5a is normally expressed most abundantly.

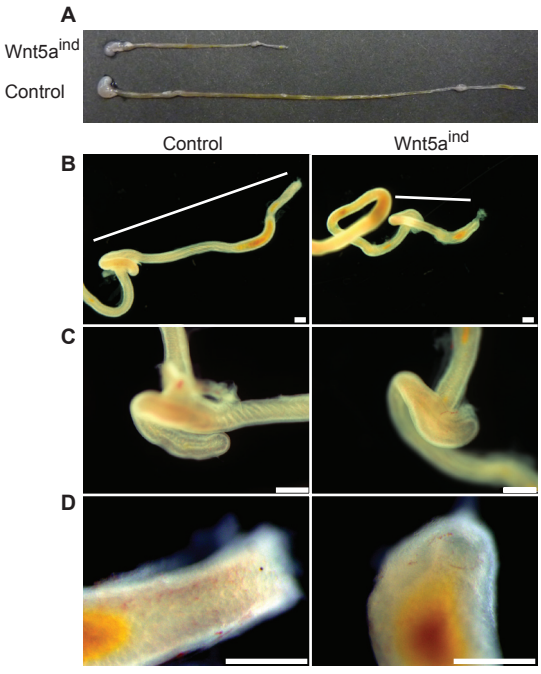

Figure 3. Induced Wnt5a expression disturbs embryonic intestinal elongation. Gastrointestinal structures of 18.5 dpc embryos induced from 10.5 dpc onwards. **(A)** Overview of gastrointestinal tracts **(B)** Higher magnification of the large intestine and **(C)** cecum. **(D)** The Wnt5a^{ind} embryonic anus is imperforated.

Together, our data demonstrate that ubiquitous Wnt5a overexpression causes gastrointestinal elongation defects resembling those observed in *Wnt5a* KO and *Ror2* KO embryos, when induced from 10.5 dpc but not from 13.5 dpc onwards, reflecting the time-frame when Wnt5a is normally expressed at highest levels [8, 15, 18-20].

Wnt5a does not alter cell fate during intestinal development

To investigate whether the differentiation state of the embryonic Wnt5a^{ind} shortened intestines was altered, intestines of embryos induced from 10.5 dpc until 18.5 dpc were isolated and analyzed extensively. Hematoxylin eosin (HE) staining revealed no histological aberrations in Wnt5a^{ind} embryonic intestines (Figure 4A). Presence of goblet and enteroendocrine cells was not influenced by Wnt5a induction as determined by Periodic Acid Schiff (PAS), Muc2 and synaptophysin staining (Figure 4B-C and S3A). Smooth muscle actin (SMA) staining showed normal smooth muscle layer development and expression of homeobox protein Cdx2 was unaffected (Figure 4D-E). Furthermore, no abnormal localization of either β-catenin or E-cadherin expression was observed in the intestines of Wnt5a^{ind} embryonic intestines (Figure 4F and S3B). Proliferation of intestinal cells determined by phospho-histone H3 staining revealed no gross differences in proliferation rate among the different genotypes (Figure 4G). Altogether this indicated that Wnt5a has no impact on embryonic intestinal cell fate, in line with similar findings in *Wnt5a* knockout embryos [18].

Induced Wnt5a expression is well-tolerated in the adult mouse intestine

To determine the consequences of increased Wnt5a expression postnatally, we performed doxycycline inductions in adult mice for multiple durations, including 1 day, 1 week and 3-5 months. During the induction period, the overall appearance of the mice remained unaffected. Following the inductions, mice were examined for abnormalities with a specific focus on the intestinal tract. No gross abnormalities in length and macroscopic appearance were observed as a consequence of transgenic Wnt5a induction. HE staining revealed no histological abnormalities in the intestines of Wnt5a^{ind} mice (Figure 5A). Staining for specific intestinal cell types including PAS for goblet cells, synaptophysin for enteroendocrine cells, lysozyme for Paneth cells, SMA for muscle and stromal cells and alkaline phosphatase (AP) for enterocytes all showed no changes upon transgenic Wnt5a expression in adult mice (Figure 5B-E and S3C). Immunohistochemical staining of β-catenin revealed no aberrant expression, displaying membranous and nuclear β-catenin in Wnt5a^{ind} intestine comparable to corresponding control mice (Figure 5F). E-cadherin staining was unaffected (Figure S3D) and also proliferation of intestinal cells was unaltered (Figure 5G).

Altogether, induced Wnt5a expression is tolerated well by adult mice and does not affect postnatal intestinal homeostasis and cell fate.

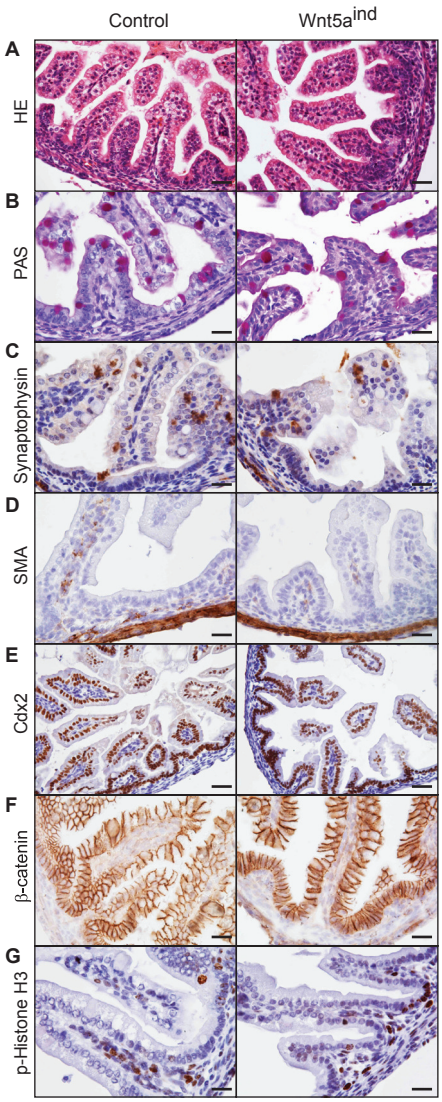

Figure 4. Wnt5a does not alter cell fate during intestinal development. Stainings of intestinal cross-sections of 18.5 dpc embryos of control versus double transgenic genotypes, all induced from 10.5 dpc onwards. **(A)** HE and **(B)** PAS staining show unaffected histology and goblet cell presence. **(C)** Staining for synaptophysin and **(D)** SMA reveal no differences in enteroendocrine cells and smooth muscle cells respectively. **(E-G)** Cdx2, β-catenin and phospho-Histone H3 appear unaffected.

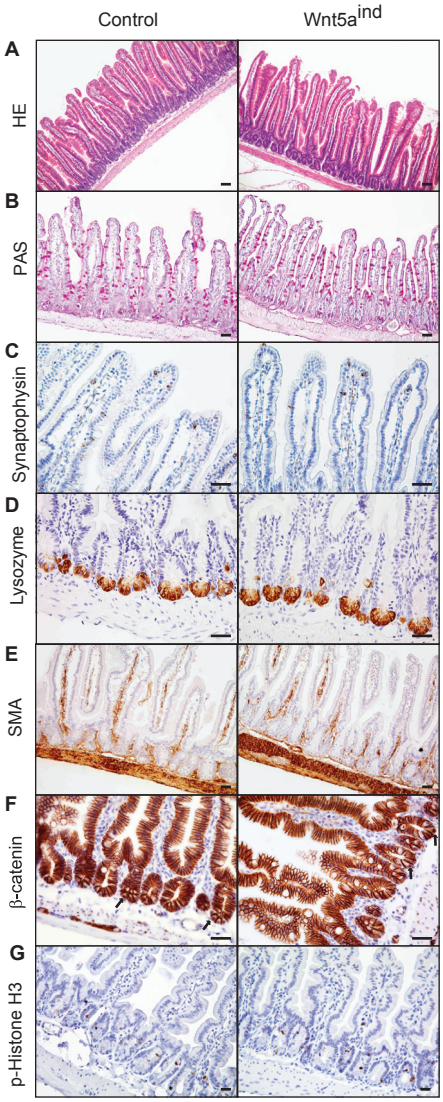

Figure 5. Induced Wnt5a expression is well-tolerated in the adult mouse intestine. Stainings of intestinal sections of adult mice induced for 1 week. **(A)** HE and **(B)** PAS staining show unaffected histology and goblet cell presence. **(C)** Staining for synaptophysin and **(D)** lysozyme reveal no differences in enteroendocrine cells and paneth cell presence respectively. **(E)** SMA staining reveals no obvious differences. **(F)** β-catenin staining shows equal membranous and nuclear (arrowheads) expression. **(G)** phospho-Histone H3 staining indicates unaffected proliferation.

Wnt5a downregulates Ror2 protein in embryonic and adult intestine

As Ror2 is an important receptor mediating Wnt5a signaling, we determined Ror2 protein expression by immunohistochemistry. All embryos were subjected to dox induction from 10.5 dpc onwards and adult mice were induced for 1 week. Examination of Ror2 protein expression in 13.5 and 18.5 dpc embryonic intestines of control genotypes revealed abundant expression in the mesenchyme and muscle layers, and membranous Ror2 in the epithelium (Figure 6A). In the adult intestine of control genotypes, Ror2 protein levels were strongly reduced

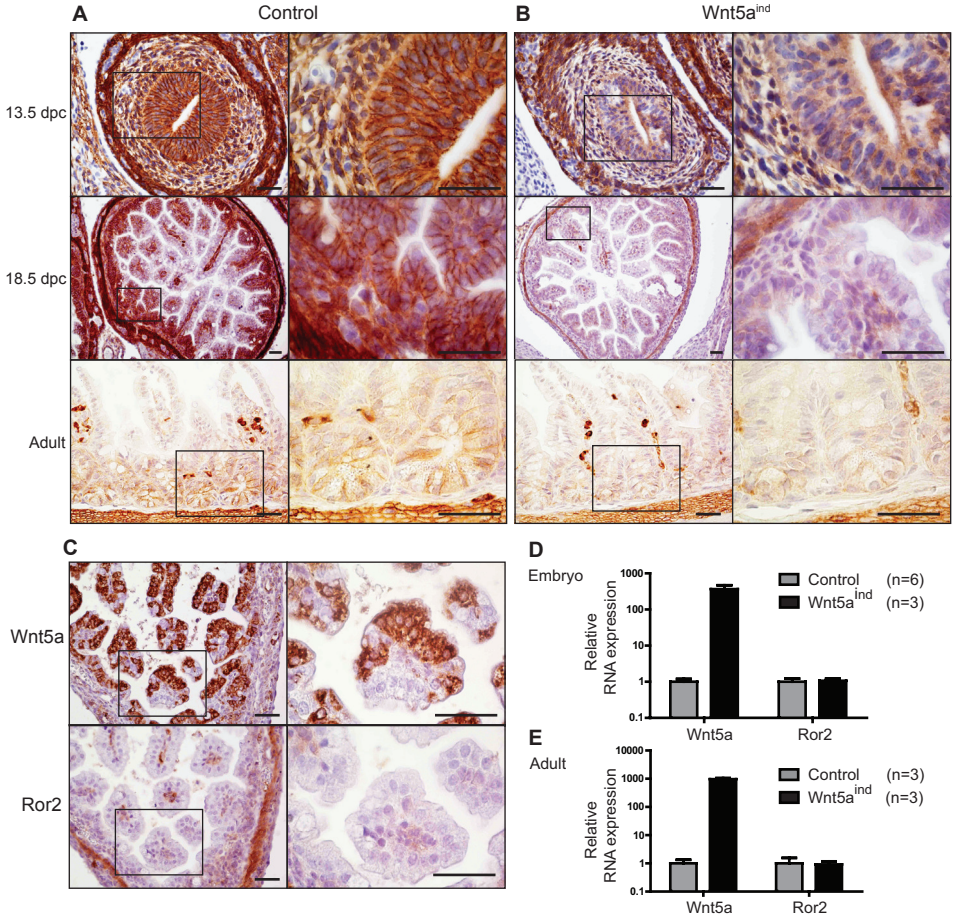

Figure 6. Wnt5a downregulates Ror2 protein in embryonic and adult intestine. All embryos were subjected to dox induction from 10.5 dpc onwards and adult mice were induced for 1 week. In adults, intensely stained stromal cells assumingly represent IgG producing cells, unintentionally detected by our secondary anti-mouse antibodies. **(A)** Immunohistochemical Ror2 staining of cross-sections of 13.5 and 18.5 dpc embryonic intestines of control genotypes. Relatively low Ror2 expression is observed in adult intestine of control genotype (lower panel). **(B)** Drastically reduced Ror2 expression in Wnt5a^{ind} embryonic and adult intestines. **(C)** Mosaic Wnt5a expression in 18.5 dpc Wnt5a^{ind} embryonic intestine using the alternative TetO-*Wnt5a* founder (upper panel). Ror2 expression is reduced in a general fashion. **(D,E)** Relative *Wnt5a* and *Ror2* RNA expression in 17.5 dpc embryonic and adult intestine respectively.

compared to embryonic stages, presenting mainly in the muscle layers while along the crypt-villus structures only low level expression was observed in crypt compartments (figure 6A, lower panel). Reduced *Ror2* expression in adult compared to embryonic intestine was confirmed on RNA level (data not shown). Interestingly, we found that intestinal Ror2 protein expression was strongly reduced in corresponding Wnt5a^{ind} mice (Figure 6B). This Ror2 downregulation was particularly clear at embryonic stages, when a major reduction in all gut compartments, including muscle, epithelium and mesenchyme was observed. In the adult intestine, Wnt5a induction reduced the expression of Ror2 in muscle cells and to barely detectable levels in crypt cells. Notably, using the alternative founder with a mosaic Wnt5a expression pattern we also observed an overall downregulation of Ror2 protein in 18.5 dpc Wnt5a^{ind} intestines, even in regions that do not overexpress Wnt5a. This indicates that secreted transgenic Wnt5a ligands acted in a paracrine fashion (Figure 6C). *Ror2* RNA levels were not affected in the Wnt5a^{ind} intestines of both the embryonic and adult stages, despite the clear increase in *Wnt5a* RNA production (Figure 6D and E), demonstrating that Wnt5a does not regulate Ror2 expression at the level of RNA production. In contrast to the reduced Ror2 protein levels that we observed in Wnt5aind embryonic intestines, we did not observe altered Ror2 protein expression in the intestines of homozygous *Wnt5a* knockout mice compared to wildtype or heterozygous littermates (Figure S4A-B). We did however observe downregulation of endogenous Ror2 protein on cultured HEK293T cells and MEFs upon Wnt5a stimulation (Figure S5). Taken together, our data show that Wnt5a reduces its receptor Ror2 on the protein level in a paracrine fashion.

Induced Wnt5a expression enhances downstream signaling in embryonic intestine

The finding that induced Wnt5a expression reduces Ror2 protein levels raised the question what the consequences are for downstream Wnt5a signaling. We determined the levels of Wnt5a downstream targets in embryonic intestines (induced 10.5-17.5 dpc) by immunoblotting. Ror2 protein levels were strongly reduced in Wnt5a^{ind} mice, confirming the immunohistochemical analyses (Figure 7A). Several studies have shown that Wnt5a can induce Dvl2 phosphorylation, presumably in a Ror2 dependent manner [13, 29-30]. In line with this, we observed a reproducible 1.4-fold increase in phosphorylated Dvl2 levels (upper bands). Moreover, levels of the Wnt5a downstream target pJNK were clearly increased in Wnt5a^{ind} embryonic intestines, showing a 1.8 fold increase. Thus, although Ror2 levels are strongly reduced in Wn5a^{ind} embryos, Wnt5a signaling is enhanced. Despite possible modulation of Wnt/β-catenin signaling by Wnt5a, we observed no changes in RNA expression levels of the β-catenin target gene *Axin2* in

Wnt5a^{ind} embryonic intestines (Figure 7B). Similar analyses were performed on duodenal tissues of adult animals induced for 1 week. Immunoblotting for Ror2 revealed a 2-fold downregulation in Wnt5a^{ind} adult intestines, and confirmed the relatively low expression compared to the embryonic situation (Figure 7C). No significant differences in pDvl2 were observed upon Wnt5a induction,

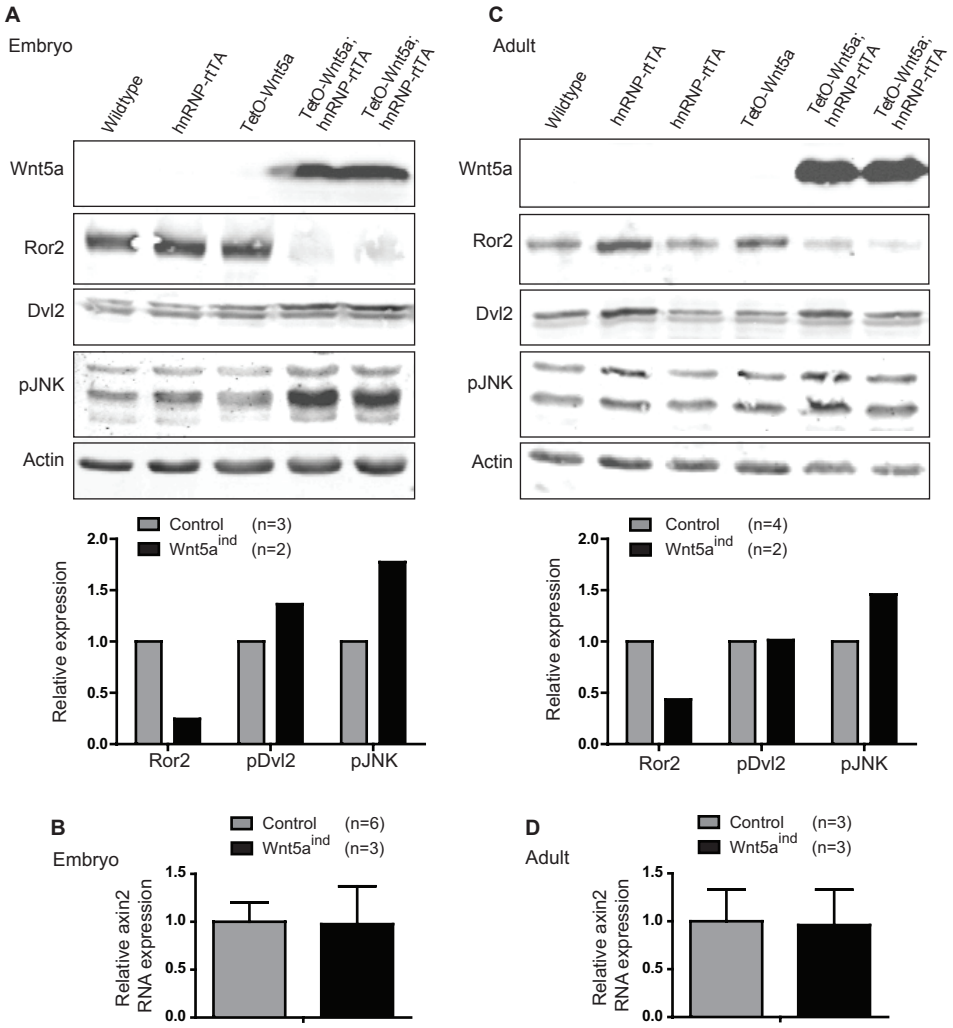

Figure 7. Induced Wnt5a expression enhances downstream signaling in embryonic intestine despite Ror2 downregulation. Immunoblotting and quantification. Expression levels were corrected for loading controls or in the case of pDvl2, presented as the proportion of pDvl2 of total Dvl2, and averaged. Representative blots are shown. **(A)** Immunoblotting of 17.5 dpc embryonic intestines induced from 10.5 dpc onwards, showing Ror2 downregulation in Wnt5a^{ind} intestines as well as slightly enhanced levels of pDvl2, represented by the upper band, and increased pJNK levels. **(B)** Relative *Axin2* RNA expression levels in 17.5 dpc embryonic intestine induced from 10.5 dpc onwards. **(C)** Immunoblotting of duodenum tissues of adult mice induced for 1 week, showing Ror2 downregulation in Wnt5a^{ind} intestines whereas pDvl2 and pJNK appear less consistently affected. **(D)** Relative *Axin2* RNA expression levels in duodenum tissues of adult mice.

whereas pJNK levels were at most slightly increased. In accordance with the embryonic intestines, *Axin2* RNA expression levels were not changed in adult Wnt5a^{ind} duodenum (Figure 7D). Taken together, despite the drastic downregulation of Ror2 protein in Wnt5a^{ind} intestines, embryonic levels of pDvl2 and pJNK were enhanced, indicating a net gain of Wnt5a signaling. In the adult Wnt5a^{ind} intestine, enhanced Wnt5a signaling was not clearly observed.

Discussion

Wnt5a undoubtedly plays a significant role during embryonic development, as indicated by multiple outgrowth defects affecting limbs, tail, face and the gastrointestinal tract in *Wnt5a* knockout mice [8, 18]. However, not much is known on the dynamics of Wnt5a functioning to date. Although endogenous Wnt5a expression in the gut is most prominent before 13.5 dpc and diminishes after that period, it is not known whether Wnt5a is involved beyond this time-frame of development. In addition, relevant postnatal roles of Wnt5a have been suggested based upon associative Wnt5a expression studies and in vitro studies. Unfortunately, it has not been possible to date to modulate and investigate the activities of Wnt5a in an adult organism as a consequence of the perinatal lethality of *Wnt5a* knockout mice. We have generated an inducible *Wnt5a* transgenic mouse model, and induced transgenic Wnt5a expression during specific time-frames of embryonic development and during adulthood, followed by a phenotypic analysis focusing on the intestinal tract.

Regarding the consequences of Wnt5a induction in embryos from 10.5 dpc onwards, the observed defects including shortened snout, limbs and tail resemble those seen in *Wnt5a* and *Ror2* knockouts and correspond to structures in whose progenitors Wnt5a is normally expressed [8, 15-17, 20]. Our results demonstrate that embryonic Wnt5a gain of function results in a phenotype resembling a Wnt5a loss of function situation, a phenomenon reported more often [31-32]. Importantly, these phenotypic defects were not observed when Wnt5a was induced from 13.5 dpc onwards. Together with the knowledge that Wnt5a is most prominently expressed before 13.5 dpc, this suggests that developmental outgrowth processes involving Wnt5a mainly occur before 13.5 dpc. Similar phenotypical resemblance and time-dependency of the phenotype apply to the gastrointestinal tract. When we induced transgenic Wnt5a from 10.5 dpc onwards, intestinal length was severely reduced, as is observed in *Wnt5a* knockout embryos [18]. Intestinal elongation was not affected when Wnt5a was induced from 13.5 dpc onwards. During normal embryogenesis, intestinal Wnt5a expression is restricted to the mesenchyme of the caudal midgut and rostral hindgut, thereby providing positional information

directing intestinal elongation [18-20]. However, both in the Wnt5a overexpression and *Wnt5a* knockout situation, such a regionalized endogenous Wnt5a gradient is lost, resulting in lack of positional cues required for directional elongation, likely explaining the comparable elongation defects. Supportively, time dependency of our Wnt5a-mediated gut phenotype coincided with the period during which endogenous Wnt5a is normally expressed most prominently, i.e. between 10.5 and 13.5 dpc. Together, our data indicate that Wnt5a is involved in intestinal elongation processes that occur before 13.5 dpc and that proper regionalized Wnt5a expression between 10.5-13.5 dpc is required for complete elongation [18-20]. An alternative explanation for the observed intestinal shortening might be presented by the strongly downregulated Ror2 protein levels in our Wnt5a^{ind} embryos, thereby phenocopying a *Ror2* knockout situation. However, we do not consider this a solid explanation, given the residual Ror2 protein expression and increased levels of pDvl2 and pJNK in Wnt5a^{ind} embryonic intestines, indicating a net gain of Wnt5a signaling. Although we cannot exclude that the enhanced signaling is mediated via alternative Wnt5a receptors, such as the Frizzleds, it suggests that Ror2 reduction has not hampered Wnt5a signaling. Ror2 has been shown to undergo clathrin-mediated internalization upon Wnt5a stimulation and to present in early endosomes [33-34]. Subsequent routes taken by Ror2 remain elusive. Other Wnt5a-related receptors including Fz2, Fz4 and Fz5 may undergo lysosomal degradation following internalization [35-37]. Our results support the possibility that Ror2 follows this route as well, involving a process more often observed after receptor-ligand endocytosis where receptors are either recycled to the membrane or directed to lysosomes for degradation [38]. As shown for the EGF receptor, high ligand exposure directs more receptors towards lysosomal degradation as a mechanism to restrict signaling. This seems a likely scenario for Ror2 to follow the high Wnt5a levels induced in our mice.

Beyond the involvement of Wnt5a in embryonic development, relevant postnatal roles of Wnt5a have been suggested based upon associative Wnt5a expression studies and in vitro studies. However, our data show that the adult mouse intestine is not affected by induced Wnt5a expression, neither showing histological aberrations nor changes in β-catenin signaling. This might appear surprising since Wnt5a can potentially interact with multiple signaling pathways and has been attributed modulating capacities with regard to cell proliferation, migration and differentiation [3-4, 7, 26]. On the other hand, our results imply that the main function of Wnt5a exists during embryonic outgrowth events, which obviously do not occur during the maintenance of adult intestinal homeostasis. Supportively, levels of endogenous Wnt5a expression in adult intestine are relatively low compared to levels in developing gut [18-20, 24], also applying to Ror2 expression levels (Figure 6A-C). Considering this, it might be speculated

that sufficient Ror2 expression is needed to acquire an effect following Wnt5a induction. However, embryonic Wnt5a induction from 13.5 dpc onwards did not result in a phenotype despite abundant Ror2 presence, showing that the phenotypic consequences do not entirely depend on the presence of Ror2. Extra-intestinal aberrations resulting from postnatal hnRNP-*rtTA*-driven Wnt5a expression were not observed, although other adult organs were not investigated in sufficient detail to allow firm conclusions.

Taken together, our results indicate a role for Wnt5a during a restricted time-frame of embryonic development, but suggest no impact during homeostatic postnatal stages. Conditional *Wnt5a* knockout studies in adult animals could ideally further support our findings. Furthermore, as increased Wnt5a expression is typically observed in diseased situations, additional triggers may be needed to reveal postnatal activities of Wnt5a. In the future, this can be investigated by exposing our newly generated inducible Wnt5a mouse model to specific stress conditions or combining it with disease models.

Acknowledgements

The authors are grateful to John Kong-a-San for oocyte injections and to the animal caretakers.

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Supplementary Figures

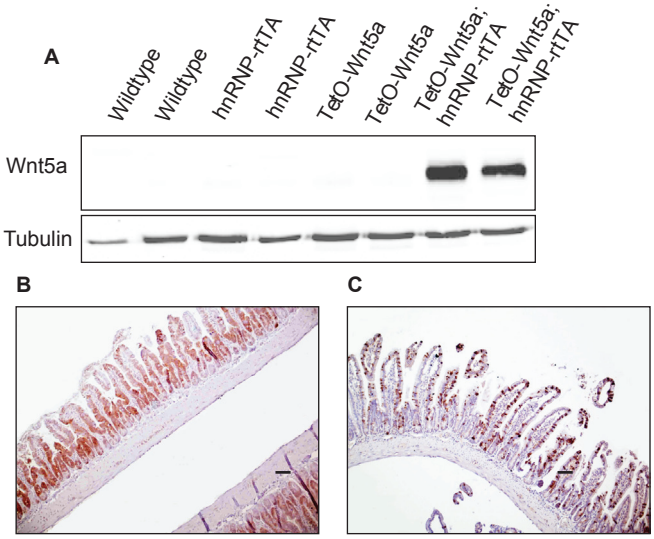

Figure S1. Further validation of the TetO-*Wnt5a* mouse model. (A) Wnt5a immunoblot showing multiple MEF lines derived from all different genotypes, all induced in vitro with dox. Wnt5a is detected in double transgenic MEF lines exclusively. **(B)** Immunohistochemical staining reveals induced Wnt5a expression in double transgenic adult mouse intestine already within 24 hours after dox administration. **(C)** Double transgenic adult intestine of the alternative TetO-*Wnt5a* founder induced for 1 week, displaying a mosaic expression pattern of induced Wnt5a protein.

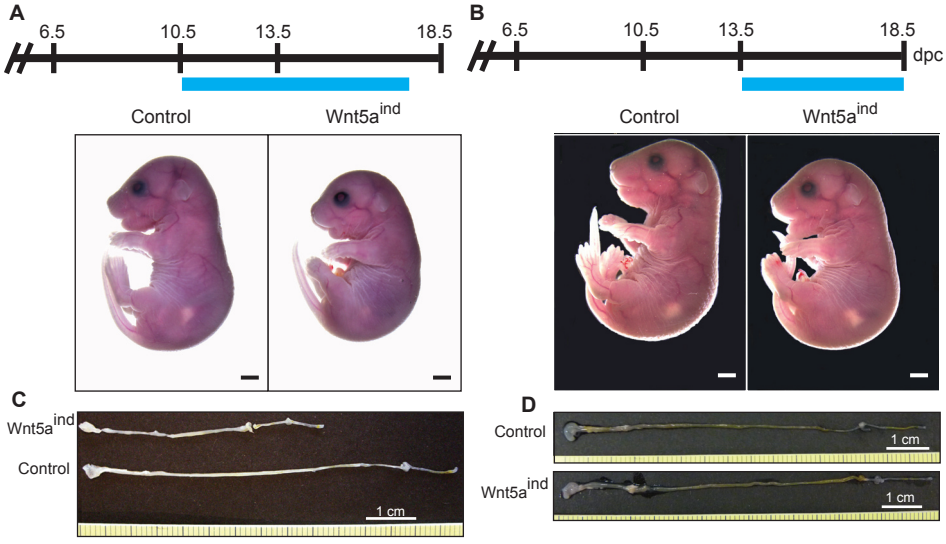

Figure S2. Induced Wnt5a deregulates embryonic development and intestinal elongation. (A) 17.5 dpc embryos of control versus double transgenic genotypes using the alternative mosaic TetO-*Wnt5a* founder, dox-induced from 10.5 dpc onwards. Developmental defects are similar to the defects observed using the regular TetO-*Wnt5a* founder **(B)** 18.5 dpc embryos of control and double transgenic genotypes (regular founder) induced from 13.5 dpc do not reveal gross differences in development. **(C)** Gastrointestinal tracts isolated from 17.5 dpc control and double transgenic embryos using the mosaic TetO-*Wnt5a* founder, induced from 10.5 dpc onwards. **(D)** Gastrointestinal tracts isolated from 18.5 dpc control and double transgenic embryos (regular founder), induced from 13.5 dpc onwards.

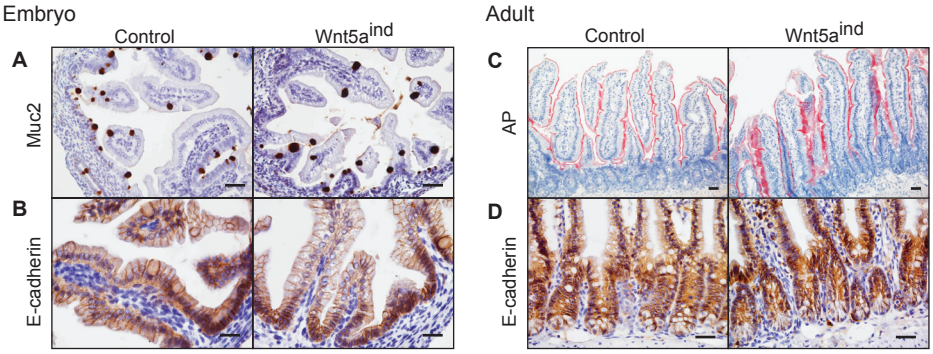

Figure S3. Induced Wnt5a does not alter cell fate during intestinal development and adult homeostasis. Immunohistochemical stainings of intestinal sections of 18.5 dpc embryos induced from 10.5 dpc onwards and adult mice induced for 1 week. Representative images are shown from similar regions of the gastrointestinal tract when comparing control and Wnt5a^{ind} intestine within a staining. **(A)** Muc2 staining indicating unchanged goblet cell presence in Wnt5a^{ind} embryonic intestine. **(B)** E-cadherin staining showing no effect upon Wnt5a induction in embryonic intestine. **(C)** Alkaline phosphatase (AP) staining of adult intestine indicating similar appearance of enterocytes in control and Wnt5a^{ind} intestines **(D)** E-cadherin staining of adult intestines reveals no differences between control and Wnt5a^{ind} intestines.

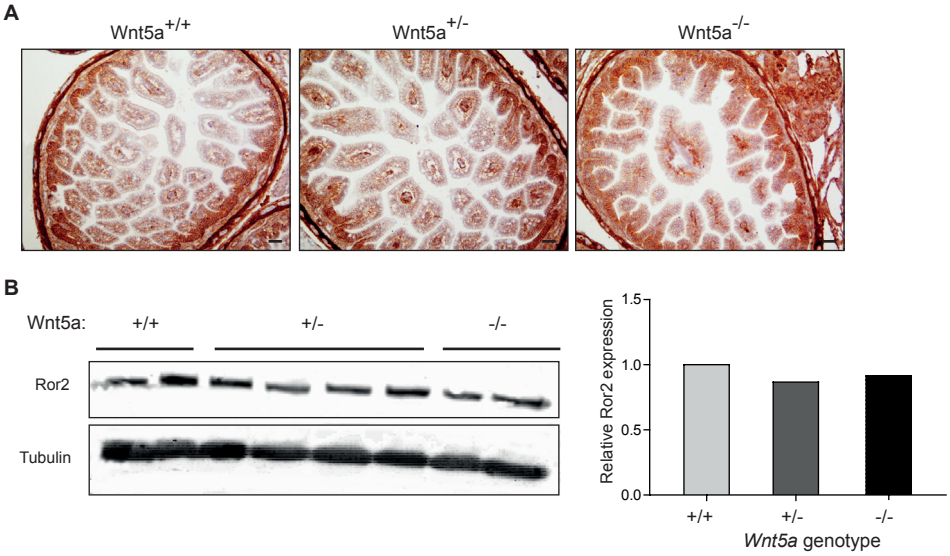

Figure S4. Ror2 protein expression is not altered in the intestines of *Wnt5a* knockout embryos. **(A)** Immunohistochemical Ror2 staining on the intestines of Wnt5a$^{+/+}$, Wnt5a$^{+/-}$ and Wnt5a$^{-/-}$ 17.5 dpc embryos. **(B)** Immunoblot and quantification of Ror2 protein in intestines of Wnt5a$^{+/+}$, Wnt5a$^{+/-}$ and Wnt5a$^{-/-}$ 17.5 dpc embryos, corrected for corresponding tubulin levels and averaged.

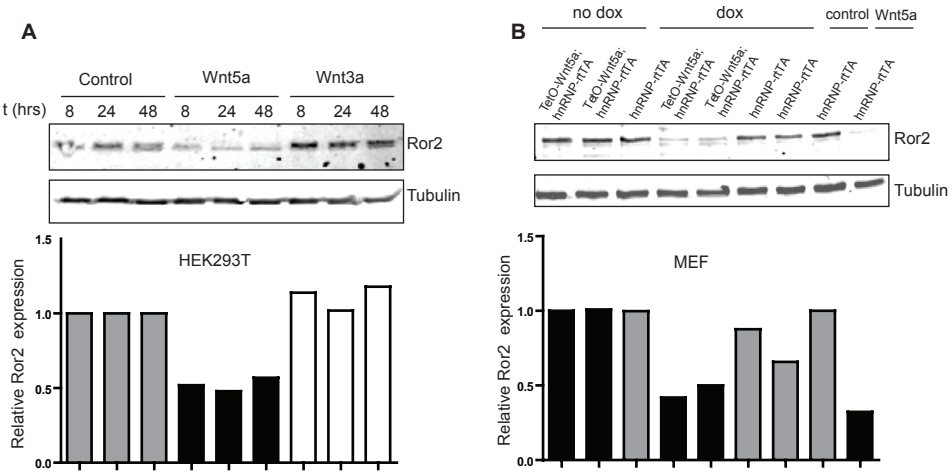

Figure S5. Wnt5a downregulates Ror2 protein in HEK293T and MEF cells in vitro. (A) Ror2 immunoblot of HEK293T cells stimulated with control, Wnt5a or Wnt3a conditioned medium for indicated durations. Endogenous Ror2 protein levels are clearly reduced following Wnt5a stimulation but not in response to control or Wnt3a conditioned medium. Graph shows quantification of corresponding individual lanes. **(B)** Ror2 immunoblot of MEF lines of hnRNP-*rtTA* and TetO-*Wnt5a*;hnRNP-*rtTA* genotypes with/without dox induction, next to a hnRNP-*rtTA* MEF line stimulated exogenously with control or Wnt5a conditioned medium. Dox induction in TetO-*Wnt5a*;hnRNP-*rtTA* lines and exogenous Wnt5a stimulation on hnRNP-*rtTA* cells reduce endogenous Ror2 protein levels. Graph shows quantification of corresponding individual lanes.

Supplementary Tables

Table I Antibodies immunoblotting

Primary antibodies

Antibody	*Dilution*	*Company*
Goat-anti-Wnt5a	1:500	R&D
Mouse-anti-Ror2	1:200	gift R. Nusse (DSHB)
Rabbit-anti-Tubulin	1:10000	Abcam
Rabbit-anti-Dvl2	1:200	Santa Cruz
Rabbit-anti-phospho-SAPK/JNK	1:500	Cell Signaling
Mouse-anti-Actin	1:2500	Santa Cruz

Secondary antibodies

Antibody	*Dilution*	*Company*
Rabbit-anti-goat-HRP	1:10000	DAKO
Goat-anti-mouse IgG IRDye 680	1:5000	Westburg
Goat-anti-rabbit IgG IRDye 800CW	1:5000	Westburg
Donkey-anti-goat IgG IRDye 680	1:5000	Westburg

Table II Antibodies and antigen retrievals immunohistochemistry

Primary antibodies

Antibody	*Dilution*	*Company*	*Antigen retrieval*
Goat-anti-Wnt5a	1:200	R&D	Citrate pH6
Mouse-anti-Ror2	1:200-1:1000	DSHB (gift R. Nusse)	TE pH9
Mouse-anti-E-cadherin	1:1000	BD Transduction Lab.	Citrate pH6
Rabbit-anti-β-catenin 1247-1	1:2000	Epitomics	TE pH9
Rabbit-anti-lysozyme	1:12500	Dako	0.1% Pronase
Mouse-anti-SMA	1:250	Dako	Citrate pH6
Mouse-anti-Cdx2	1:20	Biogenex	TE pH9
Rabbit-anti-Mucin2	1:400	Santa Cruz	TE pH9
Rabbit-anti-p-Histone H3	1:800	Upstate	TE pH9
Rabbit-anti-Synaptophysin	1:250-1:750	Dako	Citrate pH6

Secondary antibodies

Antibody	*Dilution*	*Company*
HRP-conjugated goat-anti-mouse IgG (EnVisionTM)	n/a	Dako
HRP-conjugated goat-anti-rabbit IgG (EnVisionTM)	n/a	Dako
HRP-conjugated rabbit-anti-goat	1:250	Dako

Table III Primers quantitative PCR

Wnt5a F	5'cctatgagagcgcacgcatc 3'
Wnt5a R	5'ggagccagacactccatgac 3'
Ror2 F	5'gaaggcccgtggtgctttac 3'
Ror2 R	5'cccatcttgctgccatctcg 3'
Actb F	5'agacctctatgccaacacag 3'
Actb R	5'cacagagtacttgcgctcag 3'

CHAPTER 3

Wnt5a promotes human colon cancer cell migration and invasion, but does not augment intestinal tumorigenesis in *Apc*1638N mice

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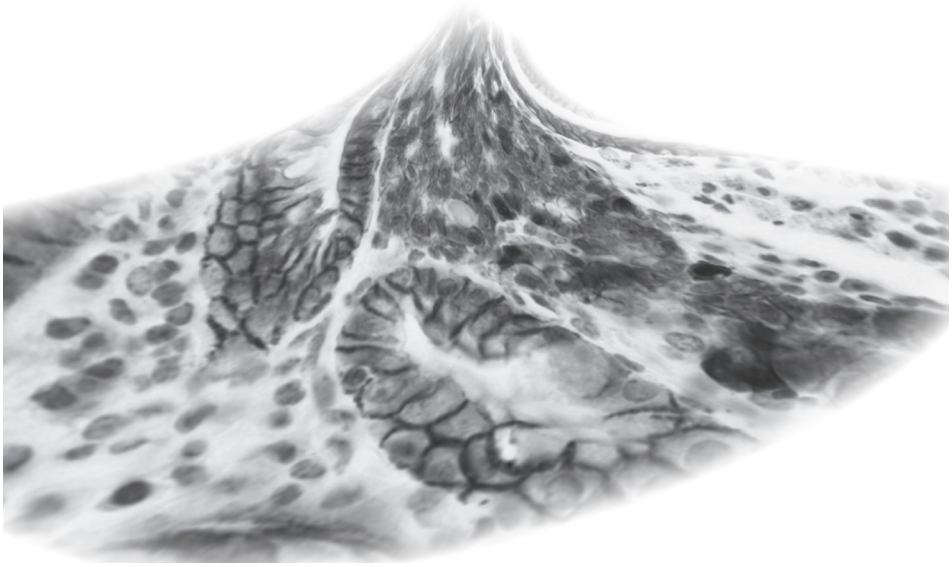
Under review



Abstract

Whereas aberrant activation of canonical Wnt/β-catenin signaling underlies the majority of colorectal cancer cases, the contribution of non-canonical Wnt signaling is unclear. As enhanced expression of the most extensively studied non-canonical Wnt ligand WNT5A is observed in various diseases including colon cancer, WNT5A is gaining attention nowadays. Numerous in vitro studies suggest modulating capacities of WNT5A on proliferation, differentiation, migration and invasion, affecting tumor and non-mutant cells. However, a possible contribution of WNT5A to colorectal cancer remains to be elucidated. We have analyzed WNT5A expression in colorectal cancer profiling datasets, generated WNT5A knockdown colon cancer cells and used our inducible Wnt5a transgenic mouse model to gain more insight into the role of WNT5A in intestinal cancer. We observed that increased *WNT5A* expression is associated with poor prognosis of colorectal cancer patients. Human colon cancer cells with WNT5A knockdown showed more evenly distributed adhesion sites throughout the cells as compared to the focal adhesion sites observed in corresponding control cells. Accordingly, WNT5A knockdown colon cancer cells exhibited reduced directional migration and invasion. Despite these observed pro-tumorigenic activities of WNT5A, the induction of Wnt5a expression in intestinal tumors of *Apc*1638N mice was not sufficient to augment malignancy or metastasis by itself. In conclusion, WNT5A promotes adhesion sites to form in a focal fashion, and promotes the directional migration and invasion of colon cancer cells. Although these activities appear insufficient by themselves to augment malignancy or metastasis in *Apc*1638N mice, they might explain the poor colon cancer prognosis associated with enhanced *WNT5A* expression.

Introduction

Whereas canonical Wnt/β-catenin signaling is well known to be a major player in intestinal cancer [1-2], the role of non-canonical Wnt signaling herein is poorly understood. Wnt5a is the most extensively studied non-canonical Wnt ligand, as it has gained substantial attention over the last years, being implicated in various human diseases including a multitude of cancer types, inflammatory diseases and metabolic disorders [3-7]. Wnt5a is especially known to be an important regulator of oriented cell movements and structure outgrowth during embryonic development, however, a possible contribution to postnatal processes remains unclear [8-10]. A wide variety of in vitro studies have suggested tumor promoting activities of Wnt5a during multiple cellular processes, including growth, differentiation, epithelial-to-mesenchymal transition (EMT), angiogenesis, migration and invasion thereby affecting both tumorigenic and non-mutant cells [3, 5, 7, 11-13]. Clear pro-tumorigenic roles for Wnt5a have been shown for melanoma and gastric cancer, in which Wnt5a enhances migration and invasion of tumor cells [13-17]. On the other hand, loss of Wnt5a protein is associated with high-risk neuroblastoma and leukemia, suggesting a tumor-suppressing role of Wnt5a in these tumor types [4, 7, 18-19]. The contribution of Wnt5a expression to colon cancer however, is poorly understood. Enhanced expression of *Wnt5a* RNA in human and mouse intestinal tumors has been reported consistently, notably produced by the stromal cells of the tumor and following an augmenting trend during progression from normal intestine through adenoma to carcinoma [20-24]. However, contradictory results have been reported describing reduced expression of Wnt5a protein or gene silencing in the epithelial compartment of a subset of colorectal tumors [25-26]. Despite the relevance of Wnt5a as suggested by associative Wnt5a expression and in vitro studies, solid evidence for a significant impact of Wnt5a on intestinal cancer is lacking. We aimed to gain insight into the contribution of Wnt5a to intestinal cancer. Therefore, we analyzed *WNT5A* expression profiling data of colon cancer patients and colon cancer cell lines. Next, we generated stable WNT5A knockdown cell lines in the human SW480 colorectal cancer cell line, and examined the consequences for its migratory and invasive properties. Moreover, we investigated the impact of increased Wnt5a expression during *Apc*-driven intestinal tumor development in vivo, using our previously generated inducible Wnt5a transgenic mouse model [8]. Our data suggest that enhanced WNT5A expression is associated with poor prognosis in colorectal cancer patients. We found that in SW480 colon cancer cells, WNT5A promotes directional cell migration and invasion, probably through regulating the focal formation of adhesion sites. However, we observed no discernible consequences on intestinal tumorigenesis when enhanced Wnt5a expression was induced in *Apc*1638N mice, providing evidence that increased

Wnt5a by itself is not sufficient to promote invasion and metastasis of adenomas that develop in a genetically modified mouse model like *Apc*1638N.

Materials and methods

RNA expression profiles
Expression profiles from a Dutch cohort of 90 stage II colorectal cancer patients were used from GEO dataset GSE33113 [27] and p-values were calculated per probe using Student t-tests. Expression profiles from colorectal cancer cell lines were obtained from two publically available datasets, i.e. GSE10843 and woost-00041, the latter available from the caArray data portal [28].

Cell culture and treatments
Colon cancer cell lines were cultured in DMEM (Lonza) supplemented with 10% fetal bovine serum (FBS, Sigma Aldrich) and 1% p/s (Gibco) at 37°C, 5% CO_2. Stable human WNT5A-knockdown and non-targeting control SW480 cells were generated using Sigma Mission shRNA pLKO-puro lentiviral vectors; *WNT5A* shRNA-1 CGTGGACCAGTTTGTGTGCAA and *WNT5A* shRNA-2 CACATGCAGTACATCGGAGAA. A non-targeting shRNA CAACAAGATGAAGAGCACCAA was used as control. Cells were selected and maintained in DMEM with 10% FBS, 1% p/s and 2 ug/ml puromycin (Sigma) at 37°C, 5% CO_2.

Immunoblotting
Subconfluent cells were lysed in Laemmli sample buffer containing 0.1M DTT and incubated 10 min at 95°C. Immunoblotting was performed using fluorescent Odyssey immunoblotting (LI-COR Biosciences, Lincoln, NE, USA) [29]. Antibodies are indicated in Supplementary Table I. Quantification was performed using Odyssey LI-COR software.

RNA isolation, cDNA synthesis and quantitative PCR
RNA was isolated from cultured cells followed by cDNA generation using iScript cDNA Synthesis Kit (Bio-Rad). Quantitative PCR was performed using Sensimix SYBR Green (Bioline) and an IQ5 iCycler PCR machine (Bio-Rad). Primers used are *WNT5A* forward 5'-CTACGAGAGTGCTCGCATCC-3' and reverse 5'-AGGCCACATCAGCCAGGTTG-3', and *GAPDH* forward 5'-GCATTGCCCTCAACGACCAC-3' and reverse 5'-CCACCACCCTGTTGCTGTAG-3'. Expression levels were corrected for expression of *GAPDH*, averaged and presented as fold changes. Assay was performed three times in duplicate and p-values were calculated using the Student t-test.

MTT proliferation assay
SW480 cells were seeded $6x10^4$ cells per M24 well and measured at indicated time-points by adding 100 μl 5 mg/ml MTT per well, 30 min incubation at 37°C and replacement of the medium by 100 μl DMSO. Absorbance (550 nm) was measured and averaged. Assays were performed three times in duplicate.

β-catenin reporter assay
β-catenin reporter assay was performed as previously described [29], using the Wnt-responsive element reporter kindly provided by Dr Georges Rawadi (Galapagos, Paris, France). This β-catenin-responsive construct encodes the firefly luciferase reporter gene under the control of a minimal collagenase promoter preceded by six tandem repeats of the TCF/LEF transcriptional response element, for which we also generated a mutant-responsive element-luciferase variant. Luciferase activities were measured using the

dual-luciferase reporter assay system (Promega). Assays were performed three times in triplicate.

Migration assay

The migration assay was conducted as previously described [30], with minor modifications. Briefly, a coverslip was inserted into an Attofluor incubation chamber (Molecular Probes) and sterilized. Coverslips were coated with gelatin (1 mg/ml) or fibronectin (10 μg/ml) and incubated for 1 hour at 37ºC, prior to cell seeding. A removable circular migration barrier was inserted into the chamber, which averts cell growth in the center of the coated coverslip. $2x10^5$ cells were seeded in DMEM, 10% FBS, 1% p/s and 2 μg/ml puromycin around the barrier and the rings were incubated at 37ºC for 24 h, thereby generating a confluent monolayer in the periphery and a cell-free area in the center of the coverslip. Post 24 h, the migration barrier was removed, the cells were washed twice and medium wash refreshed. Images of migrating cells were captured every 12 min, for the duration of 24 h, using a 10X/0.30 Plan-Neofluar objective (Carl Zeiss). Migration was monitored using time-lapse microscopy on Axiovert 100 M inverted microscopes, equipped with AxioCam MRC digital cameras (Carl Zeiss B.V., Sliedrecht, Netherlands). Time-lapse movies were used to establish and quantify parameters of cell migration. The total, or absolute movement of the cells in 24 h was termed 'total distance of migration' and includes random cell movement. The net, directional movement of cells to the cell-free center of the coverslip was termed 'effective distance of migration', representing directed migration rather than random migration. Migration efficiency was calculated as the percentage of 'effective distance of migration' over the 'total distance of migration'. Migration velocity was calculated by dividing total distance of migration by time. Parameters are also indicated in Supplementary Figure 1. Three independent migration assays were performed and 10 cells were tracked, per assay and condition, and p-values were calculated with Student t-test.All cell tracking measurements were conducted using AxioVision 4.5 software. Track diagram images were processed in Adobe Illustrator CS3 (Adobe Systems Inc, San Jose, CA).

Cell dispersion assay

Cytodex-3 microcarrier beads (Sigma-Aldrich) were mixed with cells considering a density of 25 cells per bead. The suspension was incubated at 37°C for 24h with gentle mixing every two hours to ensure complete coating of the beads. Coated beads were embedded in 1,6 mg/ml collagen gel (collagen:MEM:7.5% w/v NaHCO3 in the ratio 8:1:1) in a 12 well plate, such that each well had approximately 300 beads. Plates were incubated at 37°C for 2h for the beads to settle in the gel and the polymerized gels were covered with 500 μl DMEM, 10% FBS, 1% p/s and 2 μg/ml puromycin. Cell dispersion was followed with a 10X/0.30 PLAN-NEOFLUAR objective lens (Carl Zeiss) and measured as the maximum migrated distance from the surface of the bead into the collagen gel. All measurements were performed using AxioVision 4.5 software and assays were performed three times in duplicate. Two-way ANOVA analysis was performed to calculate p-values.

Immunofluorescence

Subconfluent cells cultured on gelatin-coated glass coverslips were fixed 15 minutes in 4% PBS-buffered paraformaldehyde (4% PFA), permeabilized in 0.15% Triton-X100 in PBS and blocked in 1% BSA, 0.05% Tween-20 in PBS. Primary antibodies were incubated O/N at 4°C followed by visualization using secondary antibody and Vectashield (Vector Laboratories) containing DAPI 1:1000 (Invitrogen). Antibodies are indicated in Supplementary Table II. Immunofluorescent images were taken using an Axiovert 100M microscope with 63X Oil-FLUAR lens (Carl Zeiss) and a ORCA II ER camera (Hamamatsu Photonics Systems).

Adhesion assay
Cells were seeded at a density of $5x10^3$ per well in 96 well plates. At each time point cells were fixed with 10% trichloroacetic acid and stained with sulphorhodamine B (SRB). After washing with 1% acetic acid, plates were dried at 50°C and the dye was solubilized in 10mM Tris buffer (Sigma-Aldrich). The absorbance was measured using a microplate reader (Victor 1420, Wallac, Turku, Finland) at 510 nm.

Transgenic mouse models and in vivo doxycyline administration
TetO-*Wnt5a* mice [8] were interbred with hnRNP-*rtTA2S-M2* mice [31], obtaining double transgenic TetO-*Wnt5a*$^{+/-}$;hnRNP-*rtTA*$^{+/-}$ mice. Subsequently, TetO-*Wnt5a*$^{+/-}$;hnRNP-*rtTA*$^{+/-}$ mice were crossed with *Apc*1638N mice, generating *Apc*$^{1638N/+}$;TetO-*Wnt5a*$^{+/-}$;hnRNP-*rtTA*$^{+/-}$ mice and corresponding control animals [32-33]. From 3-8 months of age, 0.2 mg/ml doxycycline with 5% sucrose was administered via drinking water, directly followed by examination of the mice. Animal experiments were approved by the Institute's Animals Ethics Committee and performed according to Dutch legislation.

Tissue processing and histology
Mouse intestinal tumor tissues were washed in PBS and fixed overnight in 4% PFA at 4°C. Paraffin embedding, HE and PAS stainings and immunohistochemistry were performed according to routine protocols. More details with regard to antigen retrieval and antibodies used in immunohistochemistry are shown in Supplementary Table III.

Results

WNT5A expression is associated with poor prognosis of colon cancer

Although the contribution of WNT5A expression to colon cancer is poorly understood, enhanced expression of WNT5A has been reported consistently in human and mouse intestinal tumors, following an augmenting trend during progression from normal intestine through adenoma to carcinoma [20-24]. We analyzed *WNT5A* expression in a publically available RNA expression dataset of human colorectal tumors [27]. This analysis revealed an increased *WNT5A* expression in cases showing early recurrence or metastasis, indicating that WNT5A associates with poor prognosis in colon cancer patients (Figure 1A). Subsequently we analyzed *WNT5A* RNA expression in human colon cancer cell lines from two publically available expression profiling datasets [28]. This revealed that SW480 cells show strongly enhanced levels of *WNT5A* compared to other cell lines (Figure 1B). In parallel, we analyzed WNT5A protein levels of the different colon cancer cell lines, and we were able to detect WNT5A protein in SW480 cells only (Figure 1C, upper band). Notably, SW480 cells have been derived from a primary adenocarcinoma of a patient that suffered from recurrence with widespread metastasis [34]. Together these findings indicate that enhanced WNT5A expression is associated with colon cancer malignancy.

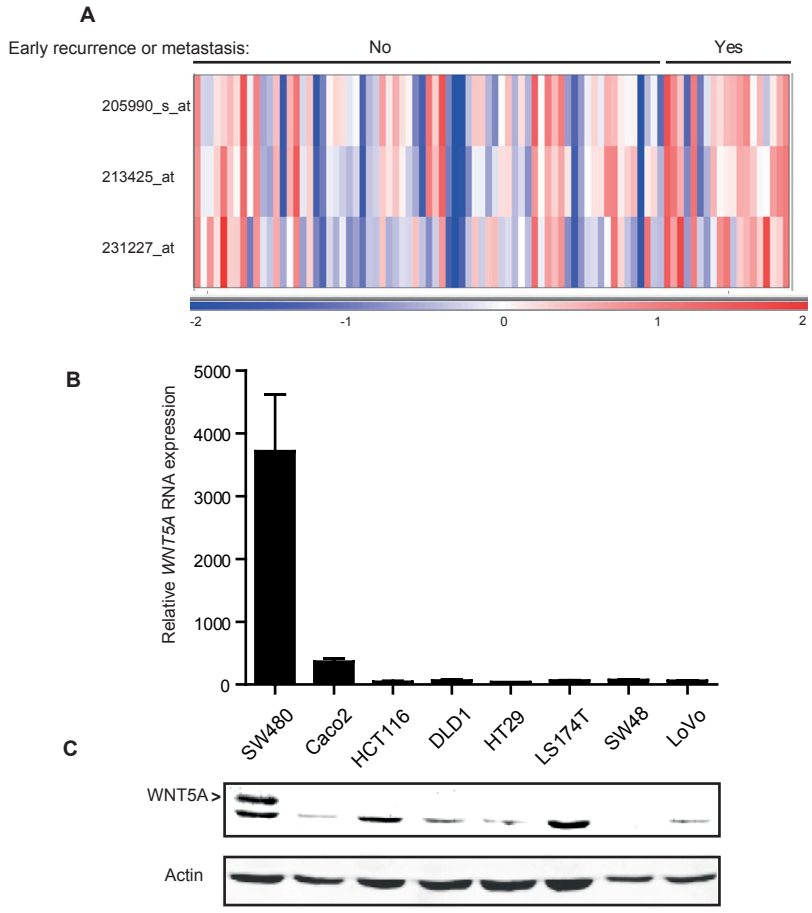


Figure 1. WNT5A expression is associated with poor prognosis of human colon cancer. (A) Treescape indicating *WNT5A* RNA expression in human colorectal tumors, showing higher *WNT5A* expression in patients with poor prognosis (231227_at p=0.003, 213425_at p=0.048, 205990_s_at p=0.071). **(B)** *WNT5A* RNA expression in human colon cancer cell lines. **(C)** Wnt5a immunoblot showing detectable WNT5A protein in SW480 cells only. Wnt5a antibody is known to detect high or overexpression Wnt5a levels only, given its low sensitivity. Upper band represents WNT5A protein.

WNT5A promotes migration and invasion but does not influence proliferation of colon cancer cells

To dissect the mechanisms through which WNT5A might contribute to colon cancer, we generated stable WNT5A knockdown SW480 cell lines. Two different *WNT5A* shRNA constructs were used and cells were compared with simultaneously generated control non-targeting shRNA SW480 cells. Successful knockdown of endogenous WNT5A was verified on protein and RNA level in both *WNT5A* shRNA lines (Figure 2A,B). Cell proliferation was determined using an MTT assay, revealing no gross differences in SW480 cells induced by WNT5A

knockdown (Figure 2C). Subsequently, β-catenin luciferase assay showed that WNT5A knockdown does not influence the intrinsic Wnt/β-catenin signaling of SW480 cells (Figure 2D). Together this suggests that WNT5A does not influence growth-related properties of SW480 cells.

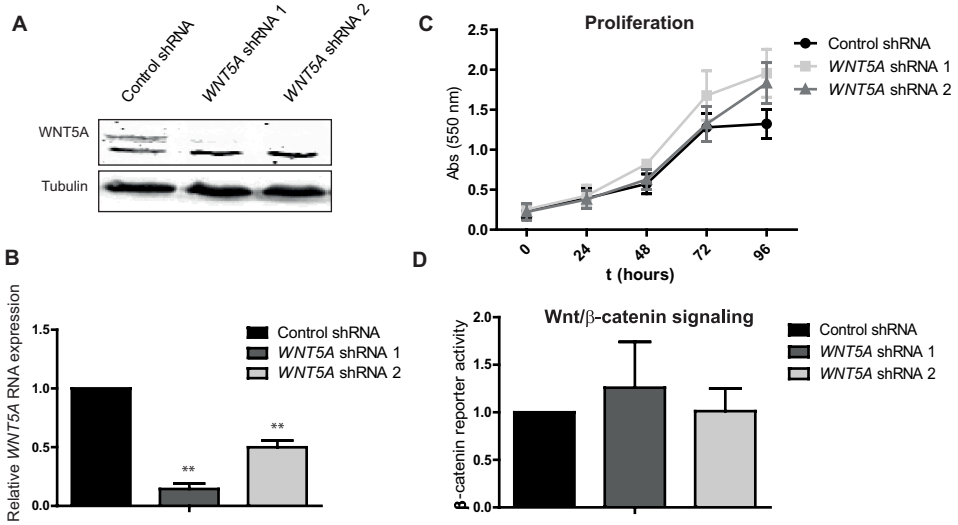


Figure 2. (A) Wnt5a immunoblot verifying efficient knockdown of WNT5A protein and **(B)** *WNT5A* qPCR indicating reduced *WNT5A* RNA production in both SW480 *WNT5A* shRNA 1 and 2 stable knockdown lines. ** p< 0.01 **(C)** MTT assay showing no gross differences in proliferation and **(D)** β-catenin reporter assay revealing unaffected β-catenin transcriptional activity in SW480 WNT5A knockdown lines compared to control SW480 cells.

We investigated the role of WNT5A in migration of colon cancer cells using a previously described ring-barrier system [30]. In short, cells migrate to a reproducibly sized cell-free area in the center of the well and time-lapse photography enables precise tracking of individual cells. We analyzed cellular migration tracks of SW480 cells on either gelatin or fibronectin coating, showing reduced migration towards the cell-free area by WNT5A knockdown SW480 cells on both coatings (Figure 3A,B). Quantification revealed that WNT5A knockdown slightly reduced total migration and migration velocity (Figure 3C,D). Moreover, the effective migration and thereby migration efficiency were drastically reduced by WNT5A knockdown in SW480 cells (Figure 3C,D). Effective migration is defined as the directional movement of the cells to the cell-free center, distinguishable from random cell movement (Figure S1A). This indicates that WNT5A is especially important for directional cell migration. Next, we examined the three-dimensional cell dispersion, representing the invasive capacity. Beads coated with SW480 cells and settled in collagen gel were tracked at multiple time points (Figure 3E).

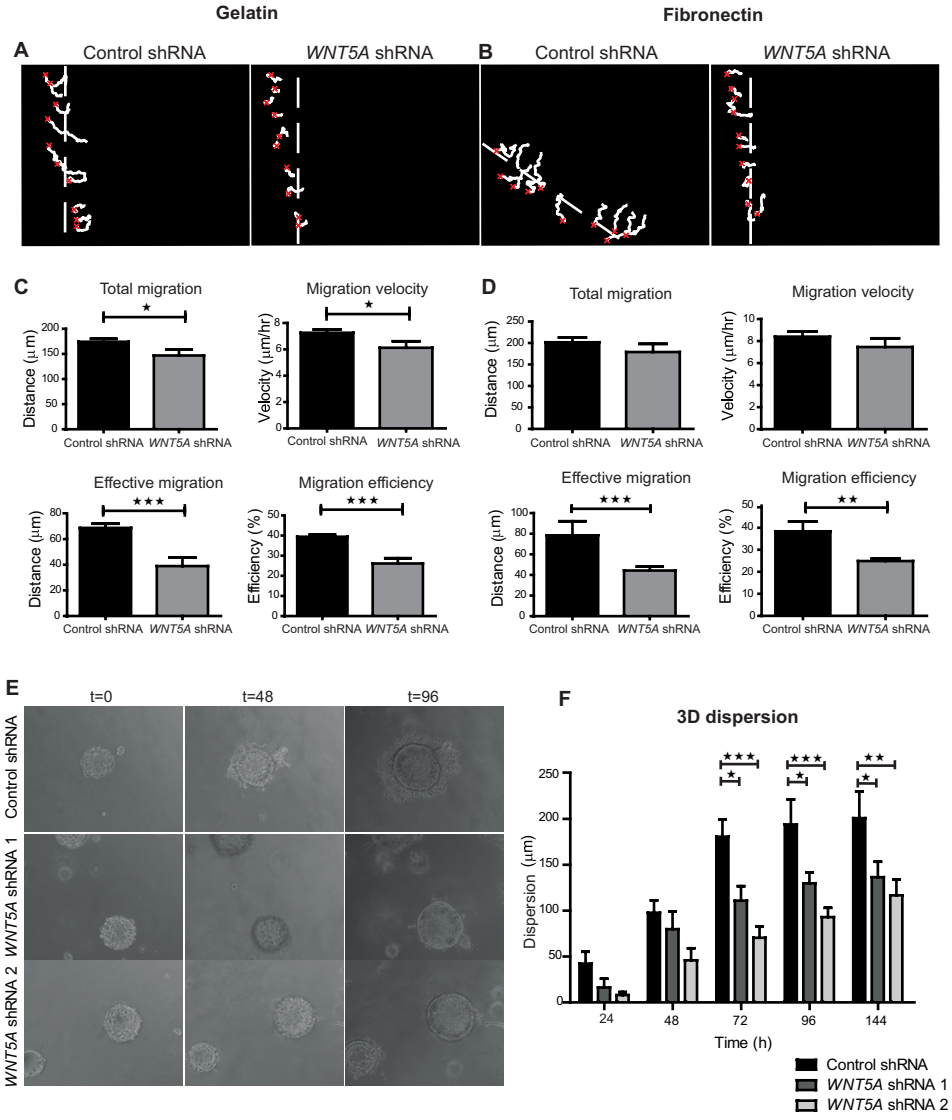


Figure 3. Two- and three dimensional migration of WNT5A knockdown versus control SW480 cells. (A,B) Overview of the 2D migratory tracks of individual cells on gelatin and fibronectin during 24 hours, locations being captured every 12 min (**x**= start position, cell free areas are at the right sides of dotted lines). WNT5A knockdown (shRNA 1 is shown) cells show decreased migration towards the cell free area. **(C,D)** Quantification of 2D cellular migration parameters on gelatin and fibronectin. In addition to a slight decrease in total migration and migration velocity, especially the effective migration and migration efficiency are reduced in WNT5A knockdown SW480 cells. **(E)** Pictures of representative beads from which cells disperse into surrounding collagen gel and dispersion was tracked at indicated time points. **(F)** Quantification of 3D cell dispersion through collagen gel. WNT5A knockdown SW480 cells reveal less distance migrated compared to control cells. * p<0.05, ** p< 0.01, *** p<0.001

The distance migrated by the cells from the surface of the bead into the surrounding matrix was quantified (Figure 3F). WNT5A knockdown SW480 cells were clearly reduced in their capacity to migrate through the collagen gel, indicating reduced invasive capacities. Together these data indicate that WNT5A promotes directional migration and invasion of SW480 cells.

WNT5A regulates focal adhesion of colon cancer cells

Cellular migration requires tightly regulated dynamics of assembly and disassembly of focal adhesions sites, as these serve as traction sites during migration. This involves the local activation of focal adhesion kinase (FAK) and its downstream partner paxillin by phosphorylation. To gain more insight into the involvement of WNT5A in the migration of colon cancer cells we examined the adhesion characteristics of the SW480 cells. First we envisaged the cellular adhesions sites by staining with phalloidin (detecting F-actin). Control SW480 cells displayed clear focal phalloidin staining reflecting focal adhesion sites (Figure 4A). However, SW480 cells with WNT5A knockdown revealed phalloidin staining that was more evenly distributed along the entire cell membrane instead of the focal expression observed in control cells (Figure 4A). Staining for p-FAK and p-Paxillin further supported this observation. Hence, more focally located expression of these proteins was observed at the cell membrane in control SW480 cells than in WNT5A knockdown SW480 cells (Figure 4B,C). Overall levels of p-FAK appear to show a minor increase whereas p-Paxillin is more clearly (1.5-2 fold) increased in WNT5A knockdown cells (Figure 4D). This increase in p-FAK and p-Paxillin levels is even more pronounced (2.5-fold for both) when SW480 cells are subjected to serum starvation (Figure S1B). Altogether, these data show that SW480 WNT5A knockdown cells exhibit less focal and more enlarged regions of adhesion, compared to control SW480 cells. We assessed whether this has consequences for the initial adhesion of cells to their substrate. As shown in Figure 4E, the amount of cells that adhered at indicated time points is not significantly affected by knockdown of WNT5A either without coating or on fibronectin. Altogether, these data indicate that WNT5A is not involved in cell-substrate adhesion in a quantitative manner, but rather regulates adhesion in a spatial manner by promoting focal adhesion above randomly distributed adhesion.

Induced Wnt5a expression does not affect intestinal tumorigenesis in *Apc*1638N mice

As our data indicate that WNT5A is associated with colon cancer cell migration, invasion and malignancy, we aimed to investigate the effects of Wnt5a modulation in a genetically modified mouse model for intestinal cancer. *Apc*1638N mice develop gastrointestinal tumors spontaneously and extra-intestinal lesions

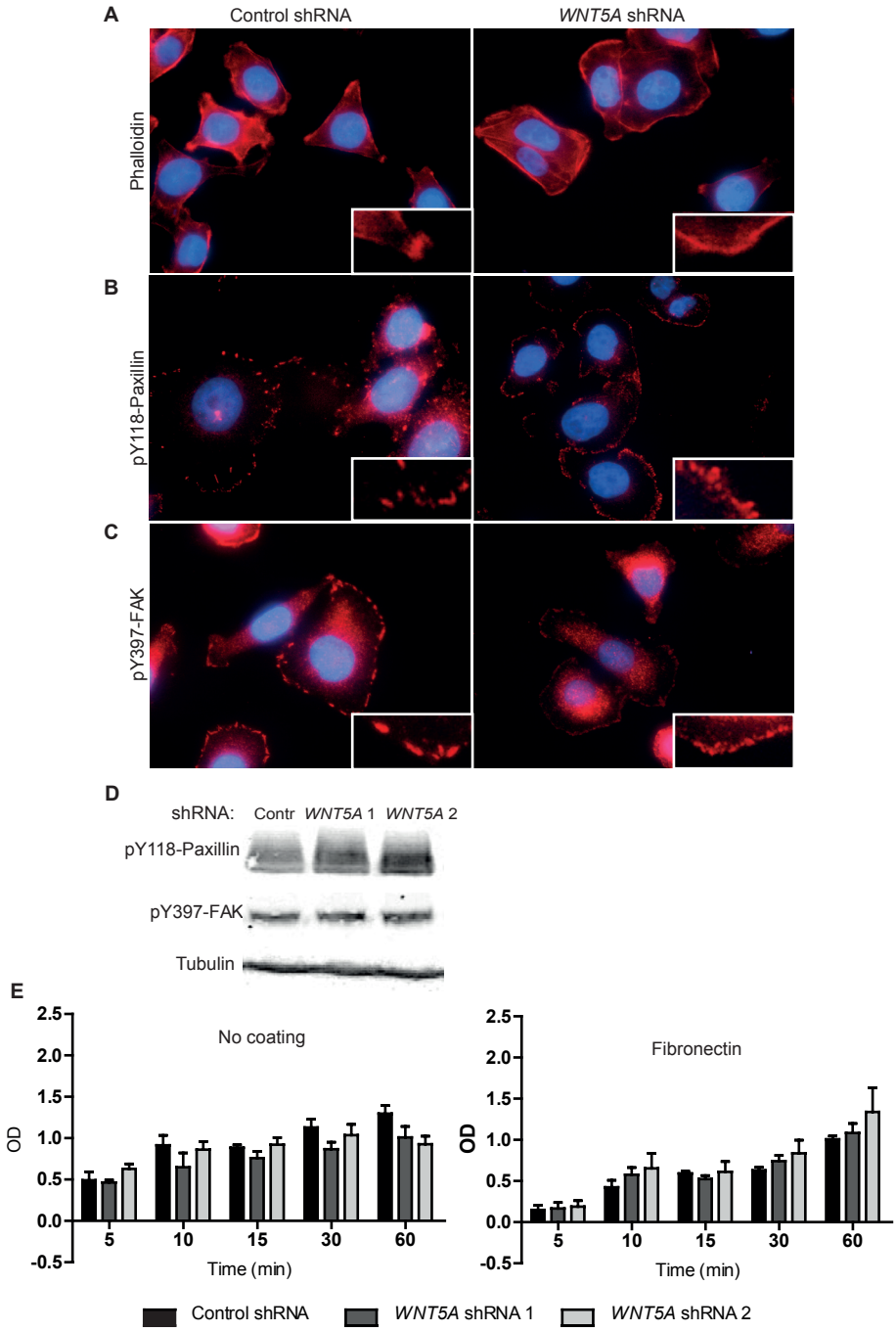


Figure 4. Analysis of focal adhesions and adhesive properties of SW480 cells. (A) Immunofluorescent Phalloidin **(B)** p-Paxillin and **(C)** p-FAK staining of WNT5A knockdown (shRNA 1 is shown) versus control SW480 cells. Stainings indicate less focal but more evenly distributed adhesion of WNT5A knockdown SW480 cells. **(D)** Immunoblot of p-Paxillin and p-FAK of SW480 cells. **(E)** Adhesion assay of SW480 cells without or with fibronectin coating.

including desmoids and cutaneous cysts, without exhibiting metastasis to distant organs [33]. In mouse and human intestinal tumors, enhanced *Wnt5a* RNA expression has been observed [20-24]. We performed Wnt5a immunohistochemical stainings on *Apc*1638N intestinal tumors. The Wnt5a antibody is known to detect Wnt5a protein only when overexpressed and we have shown previously that no Wnt5a protein can be detected in the non-tumorigenic, healthy mouse intestine [8]. In *Apc*1638N mice, we were able to confirm enhanced stromal Wnt5a expression in a subset of the intestinal tumors on protein level (Figure 5A). To investigate the in vivo effects of increased Wnt5a expression on intestinal tumorigenesis, we used the inducible transgenic Wnt5a mouse model that we generated previously [8]. TetO-*Wnt5a*;hnRNP-*rtTA* (Wnt5a^{ind}) mice were crossbred with *Apc*1638N mice and transgenic Wnt5a expression was induced during tumor development. Abundant transgenic Wnt5a expression was confirmed in the developing tumors (Figure 5B).

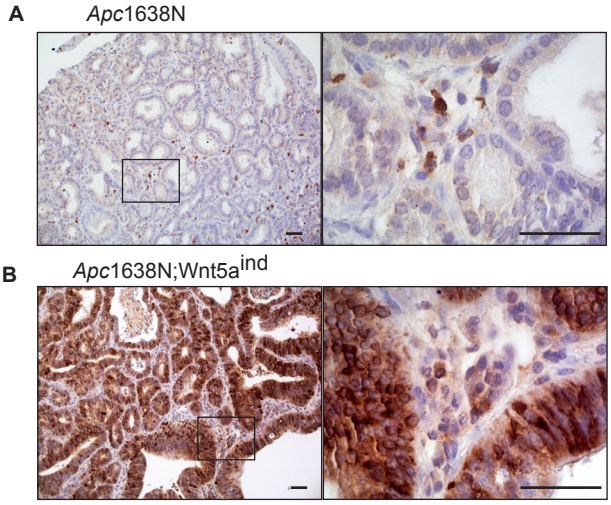
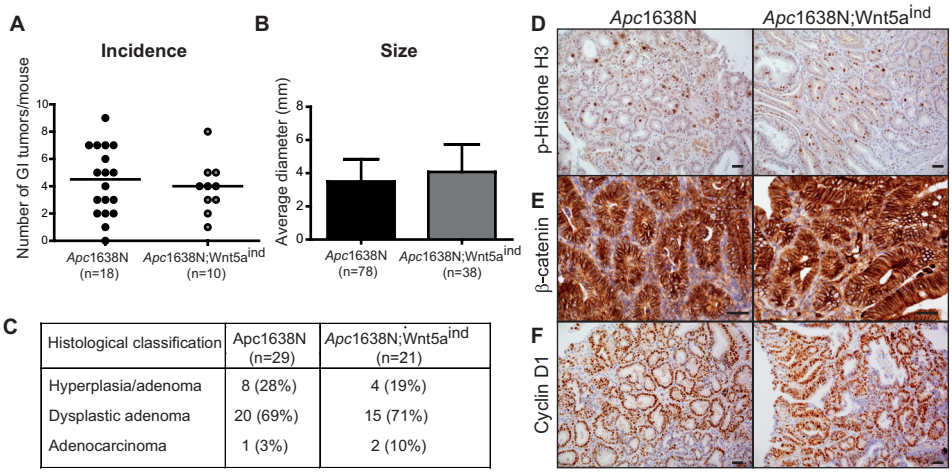


Figure 5. (A) Wnt5a immunohistochemistry showing endogenous Wnt5a protein expression in the stroma of an *Apc*1638N intestinal tumor. **(B)** Abundant induction of transgenic Wnt5a expression in *Apc*1638N; Wnt5a^{ind} intestinal tumor.

Functionality of the transgenic Wnt5a protein and its paracrine activity have been demonstrated in our previous report [8]. In addition, the availability of Wnt5a receptor Ror2 was confirmed on protein level in the tumors (data not shown). Characteristic for the *Apc*1638N mouse model, the overall majority of tumors was identified in the proximal small intestine of *Apc*1638N as well as *Apc*1638N;Wnt5a^{ind} mice. Despite the abundance of Wnt5a in *Apc*1638N;Wnt5a^{ind} mice, the number of gastrointestinal tumors that developed was not altered compared with *Apc*1638N mice (Figure 6A), nor did we observe an effect on

the size of the gastrointestinal tumors (Figure 6B). Histological tumor grading as hyperplasia/adenoma, dysplastic adenoma or adenocarcinoma revealed that induced Wnt5a did not affect tumor malignancy (Figure 6C). Hence, in *Apc*1638N as well as *Apc*1638N;Wnt5a^{ind} mice the majority of the tumors was identified as dysplastic adenoma. Concomitantly, no metastasis to distant organs was found in both groups. In parallel, extra-intestinal lesions associated with *Apc*1638N mice were examined and the incidence of desmoids and cysts was found unaltered upon transgenic Wnt5a induction (Figure S2).

*Apc*1638N;Wnt5a^{ind} intestinal tumors showed no difference in size compared to corresponding *Apc*1638N intestinal tumors, suggesting no influence of Wnt5a on tumor cell growth or proliferation. We further confirmed this by phospho-Histone H3 staining, detecting no altered proliferation upon induction of transgenic Wnt5a protein (Figure 6D). Although β-catenin signaling can be influenced by Wnt5a [35-36], we observed unaltered membrane-associated and nuclear β-catenin following transgenic Wnt5a induction (Figure 6E). Also the presence of the β-catenin target Cyclin D1 was unaffected (Figure 6F). These data are in line with the in vitro findings that WNT5A knockdown in SW480 cells does not affect proliferation and β-catenin signaling of these *APC*-mutant tumor cells. Altogether, our data indicate that Wnt5a does not contribute significantly to formation and proliferation of *Apc*-driven intestinal tumors.



C

Histological classification	Apc1638N (n=29)	*Apc*1638N;Wnt5a^{ind} (n=21)
Hyperplasia/adenoma	8 (28%)	4 (19%)
Dysplastic adenoma	20 (69%)	15 (71%)
Adenocarcinoma	1 (3%)	2 (10%)

Figure 6. Characteristics of intestinal tumorigenesis in *Apc*1638N versus *Apc*1638N;Wnt5a^{ind} mice. (A) Number of intestinal tumors detected per mouse. Medians are indicated. **(B)** Average diameter (mm) of all intestinal tumors per group. **(C)** Histological grading of intestinal tumors. **(D-F)** Immunohistochemical stainings of gastrointestinal tumors of *Apc*1638N and *Apc*1638N;Wnt5a^{ind} mice. **(D)** Phospho-Histone H3 staining revealed unaffected proliferation of tumor cells. **(E)** β-catenin staining shows equal membranous and nuclear expression. **(F)** High and equal expression of the β-catenin target Cyclin D1.

Wnt5a has been suggested to influence cell types of tumorigenic but also non-mutant nature, which might have consequences for the composition of tumors. We assessed the presence of different intestinal cell types by staining for goblet cells by PAS, enteroendocrine cells by Synaptophysin and Paneth cells by Lysozyme, all revealing no differences between both groups (Figure 7A-C). Staining for smooth muscle actin (SMA) indicated no alteration in the stromal composition of the intestinal tumors upon transgenic Wnt5a induction (Figure 7D). Also, epithelial E-cadherin was unaltered by induced Wnt5a, not suggesting any effect on EMT (Figure 7E).

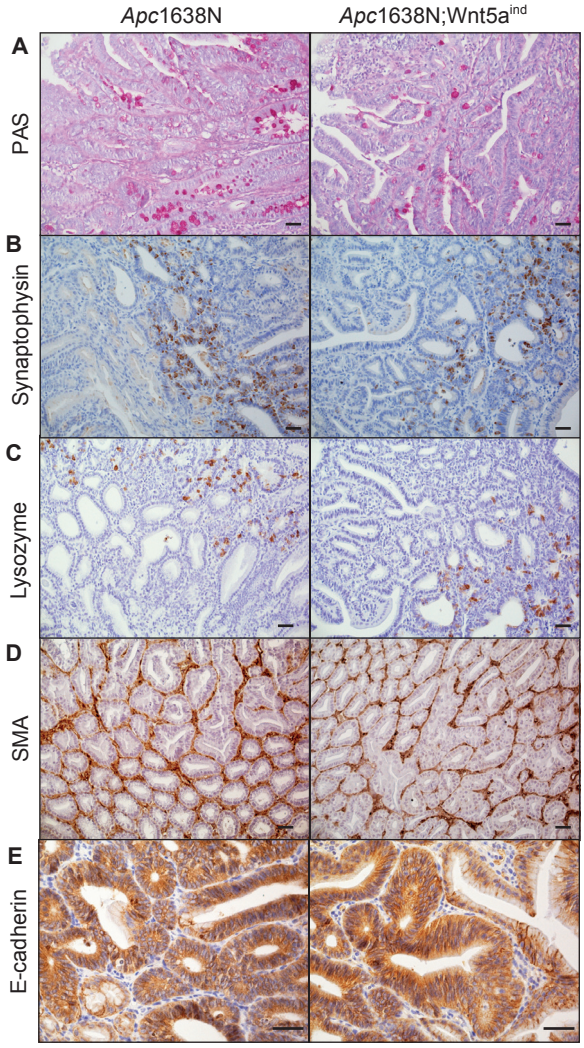


Figure 7. Induced Wnt5a expression does not alter tumor composition. Stainings of gastrointestinal tumors of *Apc*1638N and *Apc*1638N;Wnt5a^{ind} mice. **(A)** PAS, **(B)** Synaptophysin and **(C)** Lysozyme staining indicating unaltered presence of goblet cells, enteroendocrine cells and Paneth cells respectively. Staining for **(D)** SMA and **(E)** E-cadherin revealed no obvious differences in stromal composition or epithelial-cell adhesive properties induced by Wnt5a.

Taken together, despite the many processes in which Wnt5a has been implicated, our data demonstrate that Wnt5a does not affect the formation, growth, cellular characteristics or malignancy of *Apc*-driven intestinal tumors in mice, showing that Wnt5a by itself is not capable to initiate metastasis to distant organs.

Discussion

Given the controversial indications with regard to a possible role for WNT5A in colon cancer, we aimed to gain more insight herein. We investigated this using expression profiling datasets, human WNT5A knockdown colon cancer cells and our previously generated inducible Wnt5a transgenic mouse model. Our data suggested that enhanced *WNT5A* expression is associated with poor prognosis in human colorectal cancer patients. We found that in SW480 colon cancer cells, WNT5A promotes directional cell migration and invasion, possibly through regulating the formation of focal adhesion sites. However, no discernible consequences on intestinal tumorigenesis were observed when enhanced Wnt5a expression was induced in adenomas of *Apc*1638N mice, providing evidence that increased Wnt5a by itself is not sufficient to promote invasion and metastasis in a genetically modified mouse model like *Apc*1638N.

Our *WNT5A* expression analysis indicated that enhanced *WNT5A* expression is associated with poor prognosis in human colorectal cancer patients. The location of WNT5A expression in these tumors is undefined and discussion exists considering the main source of WNT5A in colorectal tumors. Most reports described an increase in *WNT5A* RNA expression produced by the stromal cells of the tumor, following an augmenting trend during progression from normal intestine through adenoma to carcinoma [20-24]. These studies suggest a tumor promoting role for WNT5A. On the other hand, Dejmek et al. described expression of WNT5A protein in the epithelial compartment of colorectal tumors and suggested that reduced epithelial WNT5A expression is associated with tumor progression [25]. In this report, epithelial expression was detected using a home-made polyclonal antibody. However, epithelial WNT5A expression has not been validated by an independent report using alternative antibodies. We did not succeed in detecting WNT5A expression in human colorectal tumors using a generally used commercial Wnt5a antibody (data not shown). Supporting the reports indicating enhanced stromal WNT5A expression, we were able to verify enhanced endogenous Wnt5a protein expression in the stroma of Apc1638N mouse intestinal tumors (Figure 5A). Furthermore, the majority of the human colorectal cancer cell lines, all derived from the epithelial compartment, do

not express WNT5A (Figure 1B,C) and during mouse gut development Wnt5a expression is typically restricted to the mesenchyme [37]. Altogether, most indication exists for the upregulation of Wnt5a expression in the stromal compartment of intestinal tumors.

The majority of colorectal tumors acquire mutations resulting in aberrant activation of the Wnt/β-catenin signaling pathway [1]. WNT5A has been attributed the capacity to modulate canonical Wnt/β-catenin signaling, mostly in an inhibiting manner [35-36, 38-39]. Although the exact mechanism is incompletely understood, Topol et al. have indicated that the inhibitory action by WNT5A is dependent on intact APC [36]. As most colorectal cancers acquire loss of function mutations in both copies of the *APC* gene, WNT5A is not expected to grossly affect intestinal tumor growth through modulation of β-catenin signaling within colorectal tumor cells. Accordingly, we observed that WNT5A knockdown in SW480 cells does not affect intrinsic Wnt/β-catenin signaling, nor did we observe changes in staining for β-catenin and its target Cyclin D1 in *Apc*1638N intestinal tumors following transgenic Wnt5a expression. Our data are in line with those of Topol et al, who observed unaffected Wnt/β-catenin signaling following ectopic Wnt5a expression in SW480 cells [36]. Few exceptions have been reported demonstrating that WNT5A inhibits intrinsic β-catenin signaling in colorectal cancer cell lines. However, these cell lines express either full length APC (HCT116 and SW48) or a long truncated APC protein with a considerable level of residual activity (HT29) [26, 36, 40]. Wnt/β-catenin signaling is an important determinant of intestinal cell proliferation and tumor initiation. Corresponding with the observed unaffected Wnt/β-catenin signaling in intestinal cancer cells, we observed no discernible alterations in intestinal tumor cell proliferation following WNT5A knockdown in SW480 cells or Wnt5a induction in intestinal tumors in vivo. Accordingly, tumor initiation in the gastrointestinal tract and extra-intestinal tissues were not affected by induced Wnt5a expression.

In addition to unaltered tumor formation, induced Wnt5a caused no gross differences in tumor composition or in the degree of malignancy of the *Apc*1638N intestinal tumors. Moreover, no metastases to distant organs were observed. Although we clearly demonstrated that in vitro, WNT5A promotes the formation of focal adhesions, directional migration and invasion of SW480 colon cancer cells, these activities of Wnt5a appear not sufficient to augment tumor malignancy or metastasis by itself in *Apc*1638N mice. Despite the relevance of *Apc*-mutant mouse models to study intestinal tumorigenesis in a representative in vivo context, to date no genetically modified mouse models for intestinal cancer have been reported that show robust metastasis to distant organs. One possible explanation for this lack of metastases, is the absence of spontaneous somatic mutations in the *Ras* and *P53* genes in *Apc*1638N intestinal tumors [41-42], which

typically contribute to the progression of human colorectal cancers [43], and which are also present in SW480. We postulate that these additional mutations might be required to cooperate in inducing the tumor-promoting effect of enhanced Wnt5a expression.

WNT5A has been implicated in regulating cellular migration and invasion in various cell types [3, 7, 11-13, 15-17]. However, the role of WNT5A in colon cancer cell migration had been clarified insufficiently to date. We have now used reproducible migration and invasion assays that allow specific tracking of individual cells and refined quantification. With this approach, we show that when SW480 colon cancer cells are deprived from their endogenous WNT5A, their efficient directional migration is clearly reduced. Moreover, also their 3D dispersion into collagen matrix is decreased. Together this provides evidence that WNT5A promotes migration and invasion of colon cancer cells. Comparable tumor promoting activities of WNT5A have also been reported for gastric cancer cells and melanoma cells [13, 15-17]. To elucidate how WNT5A regulates directional migration, we investigated adhesion characteristics of the cells. Although initial cell-substrate adhesion directly after seeding appears not grossly affected by WNT5A knockdown, our data show that WNT5A is needed for the local formation of cellular focal adhesion sites. Hence, in WNT5A knockdown SW480 cells, F-actin, p-FAK and p-Paxillin present in a more diffusely distributed pattern, instead of focal adhesion sites. It has been indicated in other cell types that enlargement of cellular adhesion sites is associated with reduced turnover of adhesion sites and concomitantly, with reduced cellular migration [15, 44-45]. Since we observed that WNT5A promotes the focal formation of adhesion sites, this activity of WNT5A likely explains the reduced directional migration of Wnt5a knockdown colon cancer cells.

In conclusion, our study has confirmed enhanced stromal Wnt5a expression in mouse intestinal tumors and has shown an association between increased WNT5A expression and poor colon cancer prognosis. Induction of increased Wnt5a expression in *Apc*1638N mice did not alter intestinal tumorigenesis, indicating that induced Wnt5a is not sufficient by itself to augment tumor malignancy or induce metastasis during *Apc*-driven intestinal cancer in mice. However, our data demonstrated that WNT5A promotes adhesion sites to form focally and stimulates directional migration and invasion of colon cancer cells. These properties can obviously contribute to local invasion and metastasis and thereby to colon cancer progression. Above all, these WNT5A activities can explain the poor prognosis associated with increased WNT5A expression and propose WNT5A as a potential candidate target for therapy in colon cancer. A genetically modified mouse model of intestinal cancer that exhibits metastasis to distant organs would be very useful to further unravel the capacities of Wnt5a in intestinal cancer.

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Supplementary Figures

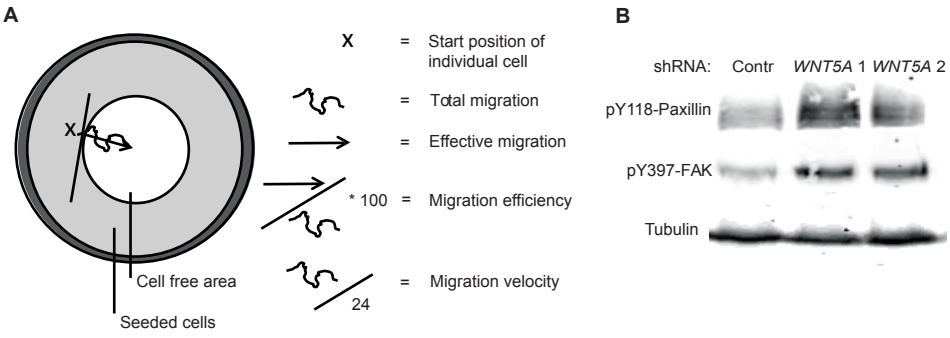

Figure S1. (A) Schematic overview of the migration assay and associated parameters used during the analysis. **(B)** Immunoblot of p-Paxillin and p-FAK of SW480 cells with/without WNT5A knockdown that were serum starved for 24 hours.

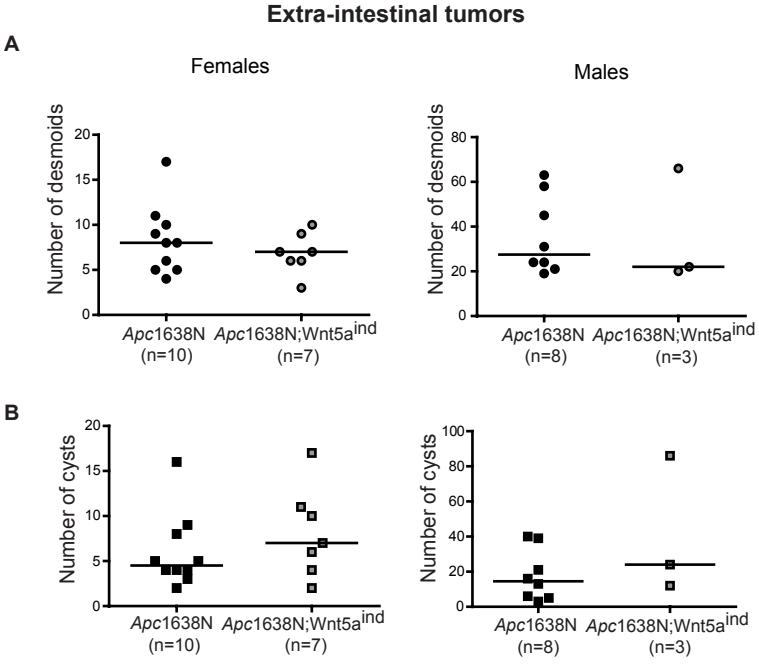

Figure S2. Incidence of extra-intestinal tumors in *Apc*1638N versus *Apc*1638N; Wnt5a^{ind} mice. (A) Number of desmoids per mouse and **(B)** number of cysts per mouse, both unaltered by induced Wnt5a expression.

Supplementary Tables

Table I Antibodies immunoblotting

Primary antibodies

Antibody	*Dilution*	*Company*
Goat-anti-Wnt5a	1:500	R&D
Rabbit-anti-Tubulin	1:10000	Abcam
Mouse-anti-Actin	1:2500	Santa Cruz
Rabbit-anti-phospho-Paxillin (Y118)	1:1000	Cell Signaling
Rabbit-anti-phospho-FAK (Y397)	1:1000	Invitrogen

Secondary antibodies

Antibody	*Dilution*	*Company*
Rabbit-anti-goat-HRP	1:10000	DAKO
Goat-anti-rabbit IgG IRDye 800CW	1:5000	Westburg
Donkey-anti-goat IgG IRDye 680	1:5000	Westburg

Table II Antibodies immunofluorescent stainings

Primary antibodies

Antibody	*Dilution*	*Company*
Phalloidin-Rhodamin	1:200	Invitrogen
Rabbit-anti-phospho-Paxillin (Y118)	1:50	Cell Signaling
Rabbit-anti-phospho-FAK (Y397)	1:1000	Invitrogen

Secondary antibody

Antibody	*Dilution*	*Company*
Donkey-anti-rabbit-Alexa Fluor 555	1:500	Molecular Probes, Invitrogen

Table III Antibodies and antigen retrievals immunohistochemistry

Primary antibodies

Antibody	*Dilution*	*Company*	*Antigen retrieval*
Goat-anti-Wnt5a	1:200	R&D	Citrate pH6
Mouse-anti-E-cadherin	1:1000	BD Transd. Lab.	Citrate pH6
Rabbit-anti-β-catenin 1247-1	1:2000	Epitomics	TE pH9
Rabbit-anti-lysozyme	1:12500	Dako	5' 0.1% Pronase
Mouse-anti-SMA	1:250	Dako	Citrate pH6
Rabbit-anti-pHistone H3	1:800	Upstate	TE pH9
Rabbit-anti-Synaptophysin	1:250-1:750	Dako	Citrate pH6
Rabbit-anti-Cyclin-D1	1:200	Vector Laboratories	TE pH9

Secondary antibodies

Antibody	*Dilution*	*Company*
HRP-conjugated goat-anti-mouse IgG (EnVision™)	n/a	Dako
HRP-conjugated goat-anti-rabbit IgG (EnVision™)	n/a	Dako
HRP-conjugated rabbit-anti-goat	1:250	Dako

CHAPTER 4

Colorectal cancers choosing sides

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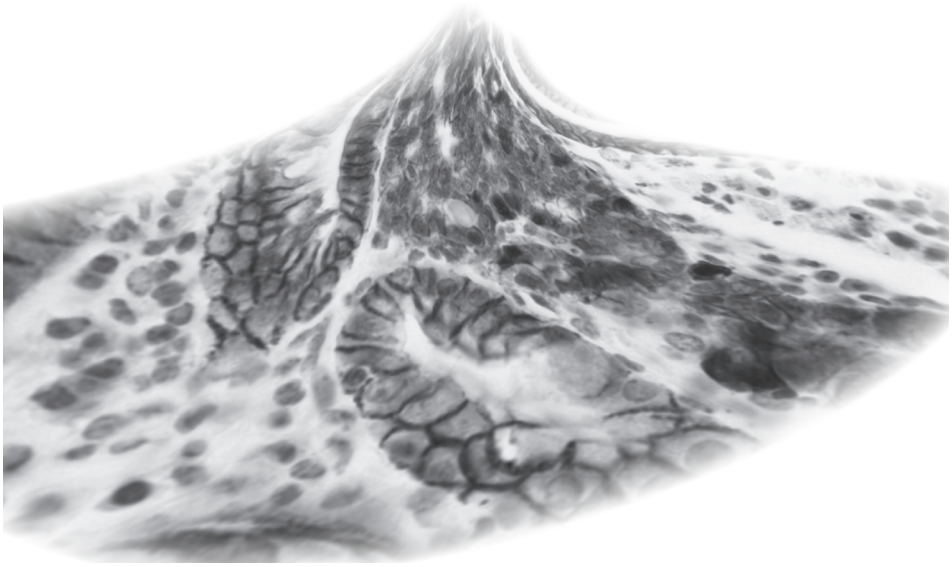


Abstract

In contrast to the majority of sporadic colorectal cancer which predominantly occur in the distal colon, most mismatch repair deficient tumours arise at the proximal side. At present, these regional preferences have not been explained properly. Recently, we have screened colorectal tumours for mutations in Wnt-related genes focusing specifically on colorectal location. Combining this analysis with published data, we propose a mechanism underlying the side-related preferences of colorectal cancers, based on the specific acquired genetic defects in β-catenin signalling.

Introduction

Epidemiological data show that 55-70% of all colorectal cancers develop in the distal half of the colon, i.e. distal to the splenic flexure (also referred to as "left-sided colon") [1]. However, colorectal tumours harbouring a DNA mismatch repair (MMR) deficiency form an exception as they are predominantly located in the proximal part of the colon (also referred to as "right-sided colon") (Figure 1) [2-6]. About 15% of all colorectal tumours are characterized by a defect in the MMR machinery, which consists of a handful of proteins involved in the recognition and repair of DNA mismatches that occur during replication [5-6]. These can be either base substitution mutations incorporated during DNA synthesis or, occurring more frequently, slippage of the DNA polymerase at repetitive sequences resulting in small insertions/deletions of base pairs. In case of a defective MMR system these mistakes are not repaired correctly, resulting in a mutator phenotype at the nucleotide level. The MMR genes most commonly affected in colorectal cancer are *MSH2*, *MLH1*, and *MSH6*. About 2-3% of all colorectal cancers arise in families carrying heterozygous germline mutations in either one of these genes, leading to the autosomal dominant disease referred to as Lynch syndrome. MMR deficient tumours may also arise in a sporadic context, corresponding to 10-12% of all colorectal cancers. In contrast to most colorectal tumours, the ones with a MMR defect usually present with low levels of chromosomal instability and loss of heterozygosity (LOH), are more likely to be diploid, and are preferentially located in the proximal colon [5-6].

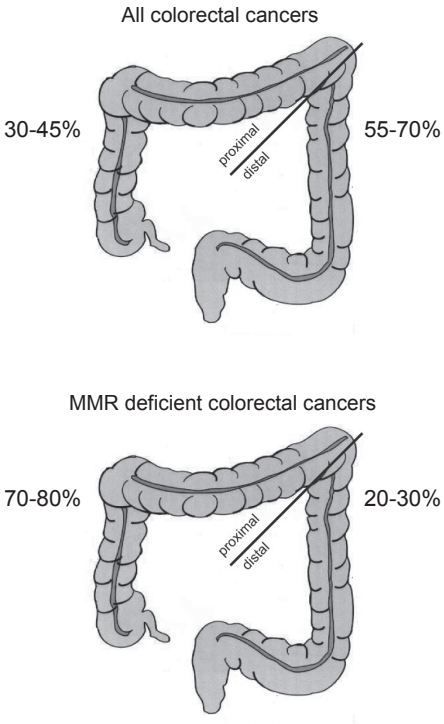


Figure 1. Distribution of cancers along the colorectal tract. Looking at the distribution of tumours along the colorectal tract, the majority is identified in the distal colon. However, tumours harbouring a mismatch repair (MMR) defect form an exception, as they are predominantly located in the proximal part of the colon. The colon is divided in proximal and distal side at the splenic flexure here marked by a diagonal line.

At present it is unknown why different types of colorectal cancer occur predominantly at one side of the colorectal tract. Both sides of the colon differ in various aspects [7]. For example, whereas the proximal colon originates from the embryonic midgut,

the distal colon is formed by the hindgut. Both sides also differ in their bile acid metabolism, water resorption, luminal content, bacterial colonization, short-chain fatty acid production, and various other features. None of these aspects can however easily explain the side preferences of colorectal tumours. Here, we present data supporting our hypothesis that these side preferences may in part be explained by specific acquired genetic defects in β-catenin signalling along the colorectal tract. First, we will review the literature related to β-catenin signalling dosage and its importance in tumour formation.

Wnt/β-catenin signalling defects in colorectal cancer

The majority of colorectal tumours, including those with a mismatch repair defect, acquire mutations resulting in aberrant activation of the Wnt/β-catenin signalling pathway [8-10]. This pathway normally represents one of the main regulatory mechanisms to preserve tissue homeostasis in the adult organism by regulating the balance between self-renewal, differentiation and apoptosis in several adult stem cell niches[9]. As depicted in Figure 2, in the absence of signalling from an extracellular Wnt ligand, β-catenin is degraded by an intracellular multi-protein complex. This so-called destruction complex encompasses two kinases, GSK3 and CKI-α, the Adenomatous Polyposis Coli (APC) tumour suppressor, β-catenin (official gene name *CTNNB1*), and the scaffold proteins AXIN1 and AXIN2.

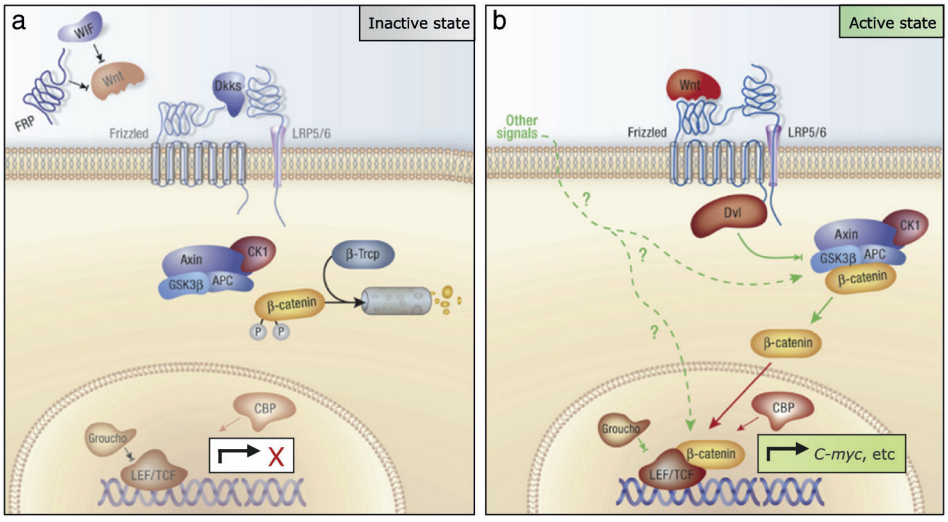


Figure 2. Schematic representation of the Wnt/β-catenin signalling pathway. (A) In the absence of an extracellular Wnt ligand, β-catenin is recruited into a destruction complex consisting of APC, Axin, GSK3 and CK1, and marked for degradation by phosphorylation at its N-terminus. The phosporylated β-catenin binds to β-Trcp triggering ubiquitin-mediated degradation in the proteasome. As a result, no free β-catenin enters the nucleus to form transcriptional complexes with LEF/TCF and activate downstream gene expression. **(B)** When Wnt ligand binds to the frizzled and LRP5/6 receptors, formation of the destruction complex is inhibited. β-catenin can enter the nucleus and regulate the expression of target genes such as c-Myc. (Figure adapted from Reference 13 with permission from Lab. Invest., courtesy of Dr. Tong-Chuan He, University of Chicago Medical Center, Chicago, USA).

Formation of this complex catalyzes Ser/Thr phosphorylation of β-catenin at specific N-terminal residues, thereby triggering its subsequent ubiquitination and proteolytic degradation. In the presence of the ligand, Wnt molecules bind to the frizzled and LRP5/6 receptors and inhibit the formation of the destruction complex. β-Catenin is now free to accumulate intracellularly and translocate into the nucleus where it associates with members of the TCF/LEF family of transcription factors, thus regulating the expression of specific downstream Wnt target genes and affecting subsequent cellular decisions.

In a large number of tumour types enhanced β-catenin signalling strongly contributes to tumour growth, underscoring its importance in maintaining tissue homeostasis throughout the organism [11-12]. Most colorectal cancers acquire 'loss of function' mutations in both copies of the *APC* gene, resulting in inefficient breakdown of intracellular β-catenin and enhanced nuclear signalling. In a subset of tumours, oncogenic β-catenin mutations within exon 3 are observed at N-terminal phosphorylation residues, making the protein more resistant to proteolytic degradation [8-9, 11, 13]. In both scenarios, the aberrantly stabilized β-catenin constitutively activates downstream Wnt/β-catenin target genes, triggering a genetic program that initiates tumour formation.

Enhanced β-catenin signalling, however, is not only involved in tumour initiation but has also been implicated in tumour progression. Although the presence of *APC* or *CTNNB1* mutations predicts constitutive β-catenin signalling in each tumour cell, heterogeneous patterns of nuclear, cytoplasmatic, and membrane-bound β-catenin are observed within colorectal tumours [14]. Patches of cells with high levels of nuclear β-catenin are associated with tubular branching, invasion, and epithelial to mesenchymal transitions [15]. Nuclear β-catenin staining is also strongly correlated with tumour size and grade of dysplasia, and the highest levels of nuclear β-catenin are observed at the invasion fronts of adenocarcinomas [16-17]. Based on these observations, enhanced β-catenin signalling has been implicated in the induction of an invasive and metastatic phenotype of colorectal tumours[18-19].

Up to 85% of colorectal cancers with a functional MMR system are characterized by *APC* mutations, whereas *CTNNB1* mutations in this subset of tumours are rare [20-21]. In colorectal tumours with a MMR defect, *APC* mutations are detected in about 50% of the cases, whereas the rate of *CTNNB1* mutations is 10% in sporadic MMR deficient tumours and reaches 30% in Lynch syndrome associated colorectal cancers [10, 20, 22-26]. In a significant proportion of MMR deficient tumours, mutations have also been reported in the Wnt-related *AXIN1*, *AXIN2*, and *TCF7L2* (also known as *TCF4*) genes, although the relevance of these mutations for tumour growth is unclear as they can coincide with mutations in either *APC* or *CTNNB1* [10, 27-30]. These data show that the majority of colorectal tumours, irrespective of the underlying form of genetic instability, acquire

mutations resulting in aberrant activation of the Wnt/β-catenin signalling pathway.

Level of β-catenin signalling associated with *APC* and *CTNNB1* mutations

The *APC* gene consists of an open reading frame of over 8500 basepairs encoding a 312 kDa protein (Figure 3). Several motifs in the central domain are responsible for regulating intracellular β-catenin levels. Four 15 aa repeats bind β-catenin, whereas seven 20 aa motifs are involved in both binding and downregulation of β-catenin. Interspersed within those 20 aa repeats are three binding sites for AXIN1/AXIN2 required for an optimal recruitment of APC into the destruction complex. More recently, an additional motif, referred to as the β-catenin inhibitory domain (CID), was identified directly following the second 20 aa repeat, which appears to contribute to APC's ability to regulate β-catenin signalling [31].

The vast majority of *APC* mutations result in truncated proteins that lack all AXIN1/AXIN2 binding motifs while retaining between one and three 20 aa repeats associated with the down-regulation of intracellular β-catenin levels. Originally, all mutations within the *APC* gene were considered to fully impair its β-catenin downregulating activity. However, subsequent studies have shown that most truncated proteins observed in tumours, have retained residual activity in regulating β-catenin signalling, which turns out to be of great importance for successful tumour formation. Truncated proteins with two or three 20 aa repeats inhibit signalling almost as efficiently as full-length APC when overexpressed in vitro [31-35]. Moreover, even when expressed at endogenous levels, a truncated Apc protein encompassing three 20 aa repeats shows considerably more β-catenin downregulating activity than cells only expressing truncated proteins where all β-catenin regulating domains are lacking [36-38]. Additionally, a truncated APC protein with only one repeat is still capable of exporting β-catenin out of the nucleus, and may therefore reduce β-catenin signalling activity [39-41]. Thus, although nearly all of the truncated APC proteins observed in colorectal tumorigenesis are impaired in their β-catenin downregulating activity, they do not represent null alleles and code for some residual β-catenin regulating activity.

Compiling the results of above-mentioned studies, we have categorized the mutant APC proteins in groups based on their residual activity (Figure 3). An inverse correlation is observed between the number of 20 aa repeats and the resulting level of β-catenin signalling, i.e. more repeats means a lower β-catenin signalling level to the nucleus. As the second 20 aa repeat by itself cannot bind β-catenin efficiently [42], truncated APC proteins with two repeats require the CID domain directly following this repeat, in order to retain additional breakdown activity [31]. Therefore, truncated APC proteins including two 20 aa repeats are at least 1430 residues in length to give them a distinctive increased

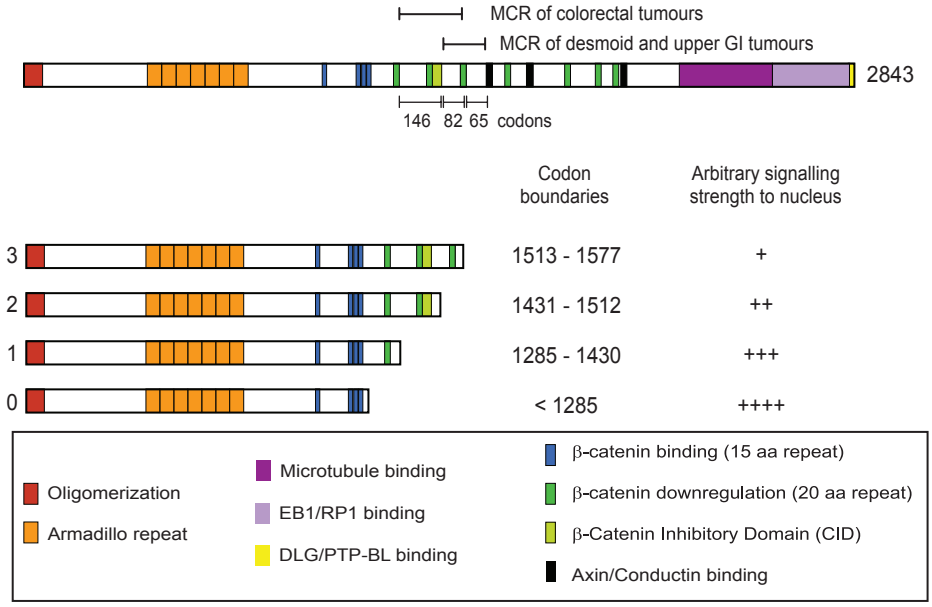


Figure 3. APC truncations and residual β-catenin regulating activity. Compiling various studies [31-38], we have subdivided the APC truncations observed in tumours in four categories, based on the arbitrary β-catenin signalling level associated with their residual β-catenin regulating activity. The main determining factor is the number of 20 aa repeats depicted on the left. Truncated proteins with 2 repeats have to include the CID domain to acquire additional regulating activity. Codon boundaries are mentioned for each category. The subdivision shown here is used throughout the paper. Truncated proteins with 1, 2, or 3 repeats are specifically encoded by resp., 146, 82, and 65 codons. On top of the full length APC protein we have demarcated the mutation cluster regions (MCR) for APC mutations observed in colorectal, desmoid and upper GI tumours.

β-catenin regulating activity compared with truncated proteins with one repeat. The codon boundaries shown in Figure 3 are used throughout the paper.

How do oncogenic *CTNNB1* mutations fit within this signalling spectrum? In most cases only one *CTNNB1* allele is affected, meaning that only half the β-catenin produced is resistant to degradation, whereas the entire β-catenin signalling pool is affected in case of a full-blown APC inactivation. Furthermore, Wang et al. have shown that phosphorylation of β-catenin at S33/S37/T41 still occurs efficiently in the presence of a mutant S45 residue, suggesting that one of the prevailing mutant forms of β-catenin in CRC is not fully resistant to proteolytic degradation [43]. Accordingly, as depicted in Figure 4, measuring the signalling activity in various colorectal cell lines harbouring specific *APC* or *CTNNB1* mutations, showed that oncogenic *CTNNB1* mutations confer a low induction of a β-catenin reporter construct, similar to cell lines expressing truncated APC proteins retaining three 20 aa repeats [44]. As such, these observations suggest that oncogenic *CTNNB1* mutations result in a modest activation of the β-catenin signalling pathway.

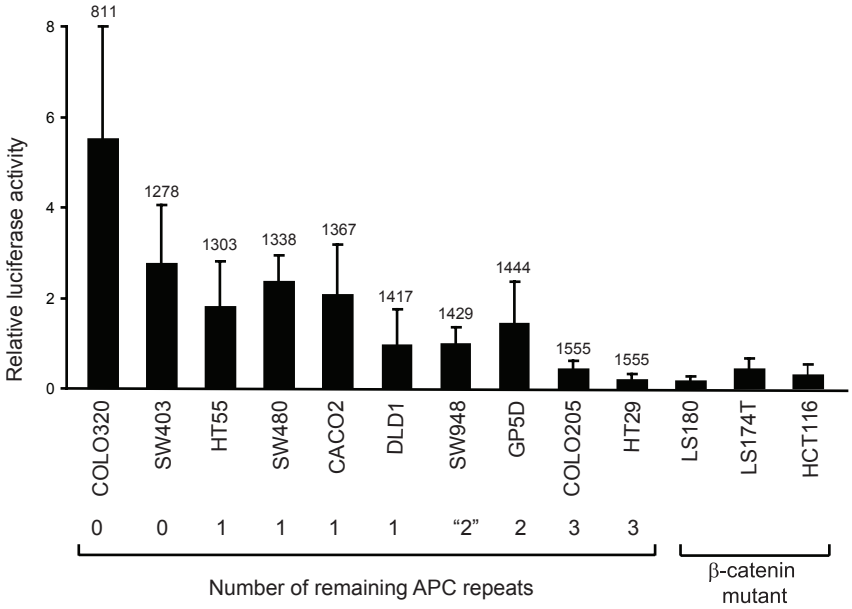


Figure 4. Using a β-catenin reporter assay, Rosin-Arbesfeld et al. showed that colorectal cancer cell lines expressing oncogenic variants of β-catenin, induce reporter activity to the same extent as cell lines expressing truncated APC proteins retaining three 20 aa repeats. For the other cell lines, the inverse correlation between the β-catenin signalling strength and the number of 20 aa repeats remaining in the truncated APC protein can be seen. (Figure adapted from Reference 44 with permission from EMBO J, courtesy of Dr. Mariann Bienz, MRC Laboratory of Molecular Biology, Cambridge, UK).

Tissue-specific selection for β-catenin signalling defects

Tumour formation is considered an evolutionary process where clonal expansion occurs through spontaneously acquired somatic mutations, which are subsequently selected upon the growth advantage they provide to the tumour cell. Mutations that do not provide the cell with sufficient growth advantage will not allow clonal expansion and tumour formation. On the other hand, mutations which have too much impact on the cellular phenotype will not be compatible with cell survival and lead to cell death. In both cases, these mutations will not contribute to the somatic mutation spectrum that is finally observed in tumours. The Wnt/β-catenin signalling pathway represents a prime example for this concept, as we and others have shown that during tumour initiation specific *APC* genotypes are selected on the basis of the specific level of residual β-catenin down-regulating activity of the resulting truncated proteins, rather than on the complete inactivation of this signal transduction regulatory function of APC [45-51]. According to this "just-right" signalling model, a specific degree of APC impairment is required to allow sufficient accumulation

of nuclear β-catenin and activation of the downstream target genes relevant for tumour formation [48]. However, cells characterized by β-catenin signalling levels above a given threshold undergo apoptosis and hence will not contribute to tumour formation. At the molecular level, low levels of β-catenin signalling mainly modulate the expression of the most accessible and responsive genes, whereas high doses of nuclear β-catenin will also affect the expression of less responsive ones [37]. Activation of an apoptotic response requires higher levels of β-catenin signalling [52-54]. Similar results have been reported for the proto-oncogene *c-Myc*, one of the main target genes activated by the Wnt/β-catenin signalling pathway [55], and required for the majority of Wnt/β-catenin target gene activation following Apc loss [56]. A tissue-specific increase in proliferation was observed in a transgenic mouse model expressing c-Myc at modest elevated levels, whereas the expression of pro-apoptotic genes was robustly induced when c-Myc was expressed at high levels [57]. Thus, eliminating cells with too much signalling of proto-oncogenes such as β-catenin or c-Myc, appears to be a mechanism to safeguard against tumour formation. This mechanism also applies to *APC*-driven tumour formation.

The *APC* gene is a large gene, subject to a variety of mutations that could theoretically impair its function. Nevertheless, the mutations observed in colorectal as well as other tumour types converge to a small region termed the mutation cluster region (MCR), coinciding with the region in between the first 20 aa repeat and the AXIN binding domain (Figure 3) [11]. Different *APC* genotypes have been observed in different tumour types throughout the body, suggesting that tissue-specific dosages of β-catenin signalling are selected to efficiently trigger tumorigenesis. In about 80% of colorectal tumours with *APC* mutations, at least one of the mutated alleles code for truncated APC proteins retaining one or, less frequently, two 20 aa repeats, resulting in relatively high to moderate β-catenin signalling levels [31, 45, 47-49]. Colorectal tumours solely expressing truncated APC proteins lacking all 20 aa repeats or expressing longer truncated proteins, are less frequently observed. According to the “just-right” signalling hypothesis, this can be explained by assuming that these truncated APC proteins do not provide the optimal level of β-catenin signalling to efficiently initiate colorectal tumour growth.

Desmoid tumours (also named aggressive fibromatosis) are rare mesenchymal tumours likely selecting for low to moderate β-catenin signalling defects. This is either accomplished by acquiring truncated APC proteins retaining two or three 20 aa repeats (~5-10% of cases), or more frequently an oncogenic *CTNNB1* mutation (~80%) [58-60]. These truncated APC proteins with 2-3 repeats, or oncogenic *CTNNB1* mutations, are also the most frequently observed alterations in polyps arising in the gastric and duodenal epithelium [61-64]. The spectrum of

mutations in the β-catenin signalling pathway observed in these tumour types, is consistent with our hypothesis that oncogenic *CTNNB1* mutations provide an equivalent induction of β-catenin signalling as truncated APC proteins retaining 2-3 repeats (Figure 4).

The selection for specific dosages of β-catenin signalling is corroborated by the phenotypes of familial adenomatous polyposis (FAP) patients carrying specific germline mutations in the *APC* gene. Patients carrying a germline mutation resulting in a truncated APC protein retaining one 20 aa repeat are associated with the most severe form of polyposis, whereas patients carrying mutations resulting in truncated proteins retaining two or three 20 aa repeats are at highest risk for the formation of desmoid tumours and polyps in the upper GI-tract, in addition to a reduced risk for severe colorectal polyposis [65-66].

Even more dramatic differences in tumour phenotype are observed in *Apc* mutant mouse models generated by us and others (Figure 5) [67-69]. Mice with *Apc* mutations resulting in high β-catenin signalling levels, such as *Apc*Min and *Apc*Δ716, mainly develop intestinal tumours at high multiplicity (>100) with only a low penetrance of extra-intestinal manifestations. On the other hand, age-matched animals carrying the hypomorphic *Apc*1638N mutation resulting in intermediate β-catenin signalling, are characterized by a reduced incidence of intestinal tumours (on average 5-6) combined with a high susceptibility for several extra-intestinal tumour types such as cutaneous cysts and desmoid tumours [70]. More recently, the *Apc*1572T mouse model, characterized by a mild defect in β-catenin regulation, has been shown to develop aggressive and metastasizing mammary tumours associated with nuclear accumulation of β-catenin [38], and a histological appearance similar to the mammary tumours observed in transgenic models overexpressing Wnt1 or β-catenin [71-74]. Surprisingly, *Apc*1572T mice do not develop intestinal tumours, even when followed up to old age, suggesting that the mild induction of β-catenin signalling is not sufficient to induce tumour formation in the mouse intestine [38]. Further support for a dominant role of β-catenin signalling dosages in establishing these different tumour phenotypes was provided by Buchert et al. [50]. By combining different *Apc* mutant models with a heterozygous knock-out of *Ctnnb1* resulting in reduced β-catenin signalling, they observed specific β-catenin signalling thresholds for successful intestinal and liver tumour formation.

How to explain those phenotypic differences in more detail? APC is a tumour suppressor protein, meaning that both copies have to acquire a mutation before tumour formation ensues. All cells in the body of FAP patients and *Apc* mutant mouse models carry the same heterozygous *APC/Apc* germline mutation in which the remaining wild type copy will be randomly hit by a number of somatic mutational mechanisms including allelic loss, insertions, deletions, and single-

base substitutions. Of these, allelic loss by means of mitotic recombination occurs at highest spontaneous frequency, especially in the mouse (more than 90%) [75-77]. Cells acquiring allelic loss through this mechanism will solely express the truncated protein originally encoded by the germline mutation, and hence the β-catenin signalling strength associated with that mutation (Figure 5). In case of the *Apc*1572T model, this implies that in the majority of emerging tumour cells, β-catenin signalling is mildly activated. According to the "just-right" signalling model this level is ideal for outgrowth of the mammary tumour, but not sufficient for intestinal tumour development in the mouse. Likewise, most nascent tumour cells in the *Apc*Min and *Apc*Δ716 models acquire high levels of β-catenin signalling, ideal for the intestine but too high for mammary cells. Further support for the

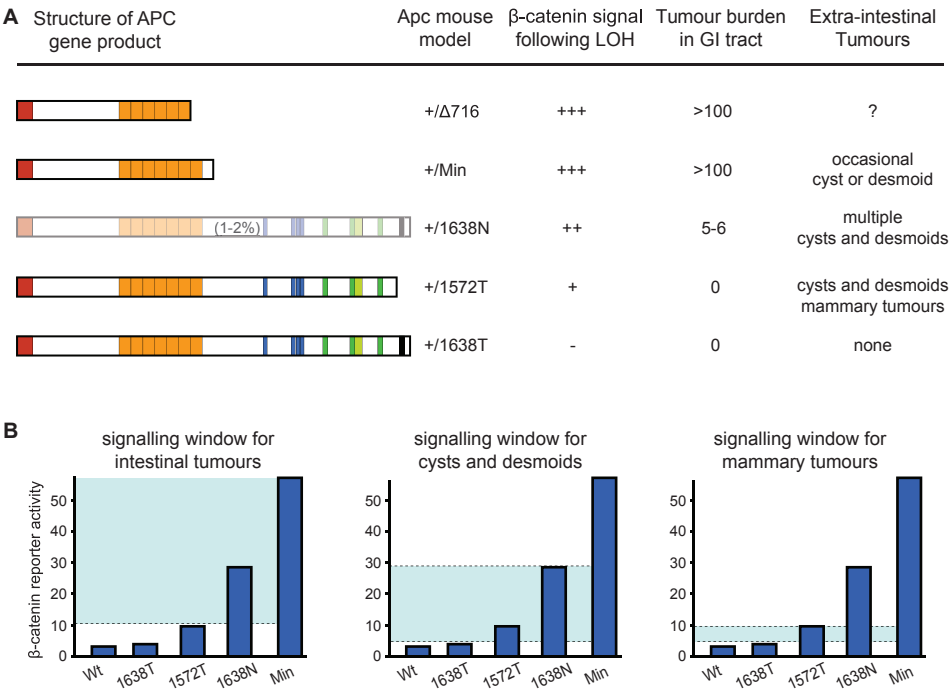


Figure 5. Genotype-phenotype correlations of *Apc* mutant mouse models. (A) For each model the truncated Apc proteins encoded by the mutation are depicted, as well as the expected β-catenin signalling strength emerging in a nascent tumour cell following LOH of the wild type *Apc* allele. LOH is by far the most common spontaneous second hit in mouse models [75-76]. Please note that *Apc*1638N and *Apc*1638T truncated proteins are virtually identical as far as the position of the termination codon is concerned. However, whereas *Apc*1638T is present in a 1:1 ratio with wild-type *Apc*, in *Apc*1638N only 'leaky' amounts (1–2%) of the predicted protein are generated. Tumour multiplicity in the gastro-intestinal tract and elsewhere are indicated. Extra-intestinal tumour types have not been reported for *Apc*Δ716. **(B)** Windows of β-catenin signalling associated with specific tumour types in *Apc* mutant mouse models. Intestinal tumours are only observed in *Apc* models associated with high to moderate levels of β-catenin signalling. Desmoid tumours and cutaneous cysts prefer lower levels of signalling, while mammary tumours readily occur with the low signalling level observed in *Apc*1572T. The level of β-catenin signalling is derived from β-catenin reporter assays performed on embryonic stem cells homozygous for each mutation [36-38].

strong selective pressure on acquiring specific *Apc* mutations, was provided by mutation analysis of mammary tumours developing in an *Apc* model associated with high levels of signalling [78]. These tumours select for specific somatic point mutations clustering in the codon 1521–1570 region, expected to result in a low level of signalling similar to the truncated protein expressed by the *Apc*1572T model.

Importantly, oncogenic *CTNNB1* mutations are also selected during tumour formation. Although the S33, S37, and S45 residues are all specified by the same TCT codon within the human *CTNNB1* gene, several tumour types show a strong preference for mutations in either one of these residues. For example, whereas colorectal cancers mainly show T41 and S45 mutations (Figure 6) [10-11, 23, 25-27, 79-81], tumours of the endometrium, ovarium, and pilomatricomas (of hair follicle origin) select for mutations confined to residues S33 and S37 [11, 24, 58-59, 82]. Overexpression experiments suggest that different oncogenic *CTNNB1* mutations result in different levels of downstream signalling [83], likely explaining the preference for specific mutated residues providing the optimal growth advantage for each cell type.

In summary, not all mutations in *APC* or *CTNNB1* result in tumour formation. According to the “just-right” signalling hypothesis, only those mutations that provide sufficient growth advantage for a certain cell type, while simultaneously avoiding the induction of apoptosis, will successfully induce tumour formation.

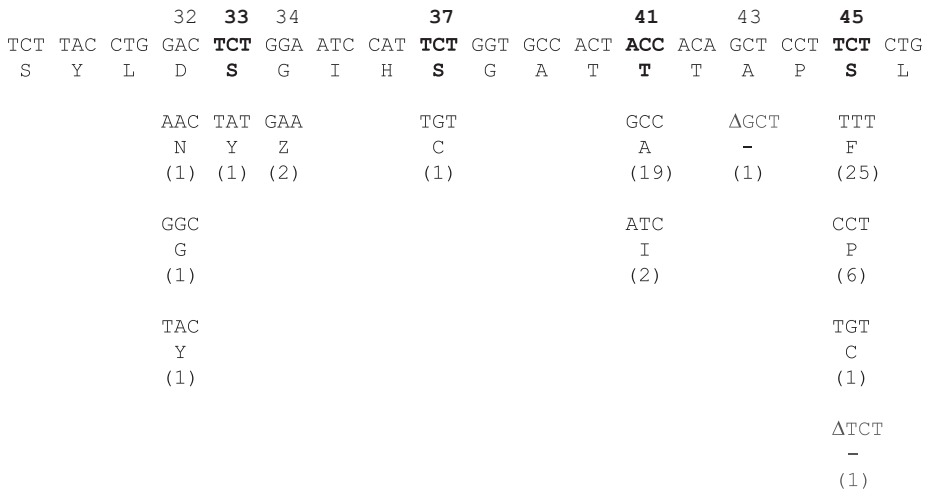


Figure 6. Selection of *CTNNB1* mutations in colorectal tumours. Depicted is the type and location of *CTNNB1* mutations reported for colorectal tumours. N-terminal serine and threonine phosphorylation residues are indicated bold. Numbers in brackets are absolute number of tumours reported with given mutation. Although the S33, S37, and S45 residues are all specified by the same TCT codon within the human *CTNNB1* gene, colorectal tumours mainly acquire mutations of S45 (33/62) or T41 (21/62), strongly suggesting that specific *CTNNB1* mutations are selected during tumour formation.

Possible alternative explanations for *Apc*-driven tumour initiation

Although the concept of β-catenin signalling dosage and its impact on tumour growth is gaining acceptance, alternative explanations for *APC*-driven tumour formation have been presented. The *APC* gene encodes a large multifunctional protein implicated in various other cellular processes in addition to regulating β-catenin signalling. At its C-terminus, APC binds to components of the microtubular skeleton. Loss of these domains has been implicated in defects in cell migration [84] and chromosomal segregation [85-86], both aspects of relevance for tumorigenesis. However, the *Apc*1638T mouse model expressing a truncated Apc protein retaining normal β-catenin regulation but lacking these C-terminal domains is tumour-free, showing that loss of these domains by itself is not sufficient to induce tumour formation and requires simultaneous activation of β-catenin signalling [36]. Nevertheless, the debate is still open to what extent loss of these microtubular functions contributes to tumour progression.

More recently, the β-catenin dogma has been challenged by Phelps et al. who presented data suggesting that *Apc*-driven tumour formation is not initiated by nuclear accumulation of β-catenin, but instead depends on the transcriptional corepressor C-terminal binding protein-1 (Ctbp1) [87]. Ctbp1 physically interacts with Apc and its levels appear to increase upon Apc loss in early adenomas [87-88]. As such, changes in Ctbp1 activity may represent a plausible alternative explanation for tumour initiation. The main argument against a role for β-catenin put forward by Phelps et al., was the lack of its nuclear detection in early adenomas using immunofluorescence. As discussed by Fodde and Tomlinson, while nuclear staining for β-catenin is a reliable indicator of Wnt activation, its absence does not exclude the robust activation of β-catenin target genes [89]. Detection of nuclear β-catenin is more challenging than generally appreciated. For example, nuclear β-catenin is not detectable in frozen colorectal tumour sections [90], is not easy to discern using immunofluorescence on paraffin sections while this is the case using immunoperoxidase-based methods (unpublished observations), and is absent in most colorectal cancer cell lines despite strong evidence of enhanced Wnt signalling by using more sensitive approaches such as β-catenin reporter assays and target gene activation. Using immunoperoxidase-based methods, most investigators detect nuclear accumulation of β-catenin in early adenomas [89, 91]. Moreover, whereas oncogenic *CTNNB1* mutations have been detected in a large number of tumour types and expression of oncogenic β-catenin leads to the development of numerous tumours in the mouse intestine [92], similar results have not yet been reported for Ctbp1.

To date, the C-terminal microtubular functions and Ctbp1 have not been linked to the selection for specific truncated APC proteins retaining between one and three 20 aa repeats that is observed in tumours. Therefore, we believe that

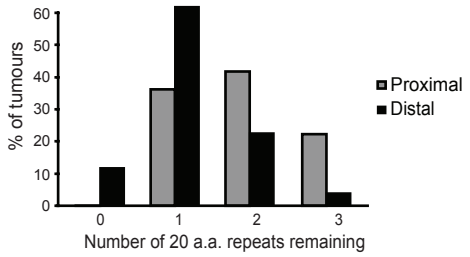
selection for β-catenin signalling dosage currently represents the best explanation for the tissue-specific selection of truncated APC proteins.

Selection for β-catenin dosage along the colorectal tract

So far, we have for simplicity considered the colorectum as one uniform organ with an overall equal selection for a specific level of β-catenin signalling, favouring truncated APC proteins with one or, less frequently, two 20 aa repeats. However, regional differences in β-catenin signalling preference affecting tumour formation also exist along the GI-tract. Recently, we have screened a number of colorectal tumours for mutations in Wnt-related genes with a specific focus on colorectal location, and combined these results with a meta-analysis of publications in which tumour location, defects in mismatch repair, and somatic mutations were reported [10]. Focussing on *APC* mutations in tumours with a functional MMR machinery, we noticed a different mutation spectrum between both sides of the colon (see Table 1) [10, 47, 93-98]. Distal colonic tumours preferentially acquire mutations retaining one 20 aa repeat (63/102 tumours, i.e. 62%), while long truncated APC proteins

Table 1. Distribution of *APC* mutations within the proximal and distal colon. *APC* mutations were grouped according to the number of 20 aa repeats present in the longest reported *APC* mutation present in that tumour, as this will largely determine the residual β-catenin regulating activity. Divisions were made according to the codon boundaries shown in Figure 3. Studies were only included when tumour location was properly recorded, and when the mutation analysis minimally included the entire MCR of *APC* up to the first AXIN binding domain. Furthermore, tumours with an underlying MMR defect were excluded. A graphical representation of these data is depicted below the table. Differences in number of repeats between both groups are highly significant (p< 0.001; ordinal logistic regression model fitted with SAS version 9.2, proc glimmix).

Number of 20 aa repeats	Proximal tumours (n=36)				Distal tumours (n=102)			
	0	1	2	3	0	1	2	3
Aoki et al. [93]	0	0	1	0	0	1	0	0
Smith et al. [94]	0	6	5	1	3	29	13	2
Miyaki et al. [95]	0	2	3	1				
Aust et al. [96]	0	1	2	0	0	9	0	0
Rowan et al. [47]	0	1	1	3	2	3	4	0
Scholtka et al. [97]	0	3	1	2	0	8	3	2
Ku et al. [98]	0	0	0	1	0	4	1	0
Albuquerque et al.[10]	0	0	2	0	7	9	2	0
	0	13	15	8	12	63	23	4



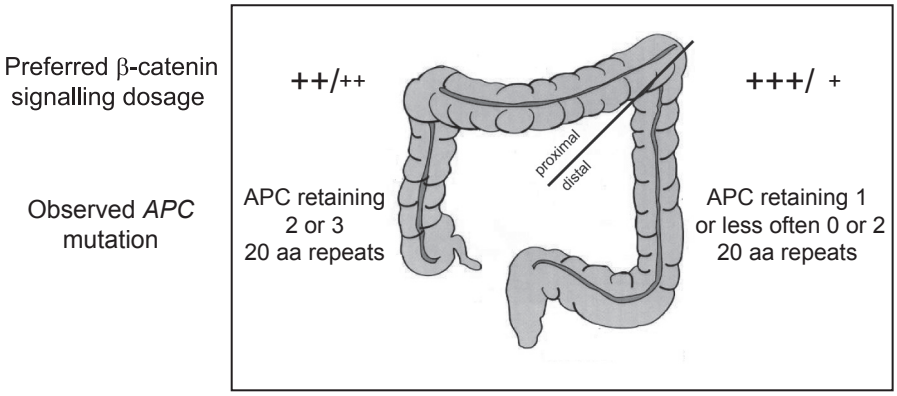


Figure 7. Selection of *APC* mutations and preferred β-catenin signalling strength along the colon. Focussing on tumours with a functional MMR machinery, we noticed that most tumours in the proximal colon are characterized by *APC* mutations resulting in a truncated protein retaining two or three 20 aa repeats associated with mild (+) to moderate (++) levels of β-catenin signalling [10, 47, 93-98]. This strongly suggests that the proximal colon for successful tumour initiation, prefers lower β-catenin signalling levels than the distal side. These mainly develop with APC truncated proteins retaining one or less frequently no or two repeats, resulting in a moderate (++) to high (+++) β-catenin signalling level. See Table 1 for details.

with two or three 20 aa repeats are less common in this segment of the colon (27/102 tumours, i.e. 27%). On the other hand, the latter mutations resulting in a moderate signalling level are selected in tumours arising in the proximal colon (23/36 tumours, i.e. 64%). Recently, our observation was supported by data of Leedham et al. [99]. Analysing *APC* mutations in proximal and distal polyps of FAP patients and a set of sporadic CRCs, the same prevalence for specific *APC* mutations along the colorectal tract was observed [99]. Proximal tumours also present with less nuclear accumulation of β-catenin than their distal counterparts [100]. Overall, these results suggest that a gradient of β-catenin signalling dosage for tumour initiation exists along the colorectal tract with moderate levels being preferred proximally, and higher levels towards the rectum (Figure 7).

This phenomenon is also observed for the location of intestinal tumours in *Apc* mutant mouse models. *Apc* mutant mice to a large extent develop tumours located in the small intestine, and select for higher signalling levels associated with short truncated proteins lacking all 20 aa domains [46, 78, 101]. Despite these differences between human and mouse intestinal tumour formation, clear effects on tumour location associated with β-catenin signalling levels have been observed. The *Apc*1638N model, characterized by only a moderate increase in β-catenin signalling, develops intestinal tumours almost exclusively in the duodenum and first half of the jejunum with a characteristic clustering at the stomach to duodenum transition [102-104]. A proximal preference for tumour formation has also been reported for the *Apc*1322T mouse model associated with a submaximal level of β-catenin signalling [105]. In contrast, tumours developing in the *Apc*Min

and *Apc*Δ716 models, both resulting in a high dosage of β-catenin signalling, are preferentially selected in the distal half of the small intestine [101, 104-106]. *Apc* mutant models associated with high levels of β-catenin signalling also develop some colonic tumours, especially when expression of the mutant allele is restricted to the distal part of the GI-tract using Cre-LoxP technology [107-109]. Interestingly, these tumours almost exclusively develop in the distal part of the colon. Thus, both in the colon as well as the small intestine of the mouse, the proximal region seems to prefer lower levels of β-catenin signalling to initiate tumour growth than its distal counterpart.

At present, the mechanisms underlying these side preferences for the optimal level of β-catenin signalling remain largely elusive. Although β-catenin signalling in the intestine is an important determinant in establishing cell fate, it will operate in concerted action with many other cellular inputs. Several differences exist between the proximal and distal colon that may cooperate with the defect in β-catenin signalling to successfully initiate tumour growth [7]. For example, short-chain fatty acids such as butyrate, are produced at considerably higher levels by bacterial fermentation reactions in the proximal colon [110]. In vitro, these fatty acids have been shown to induce an apoptotic response when administered to colorectal cancer cell lines [111], suggesting that the higher fatty acid levels present in the proximal colon reduces the apoptotic threshold, which in turn may lead to a preference for a moderate activation of β-catenin signalling to initiate tumour growth. A similar line of reasoning can be postulated for bile acids of which various structural variants also differ in their concentrations along the colon [112-113]. Moreover, both sides of the colon differ significantly in their gene expression profiles [114]. For example, *CDX2* is expressed at higher levels in the proximal colon [115]. Interestingly, Aoki et al. have shown that a 50% reduction in Cdx2 levels results in a strong shift from mainly small intestinal tumours towards a large intestinal tumour phenotype in *Apc*Δ716 mice [106]. With respect to β-catenin signalling, Leedham et al. presented data suggesting that the proximal small intestine as well as the proximal colon present with higher basal levels of β-catenin signalling [99]. Based on this observation, they postulated that this might explain the selection for mutations leading to a mild increase in β-catenin signalling on the proximal side, as a lower increase in signalling is required to reach a hypothetical threshold for tumour initiation.

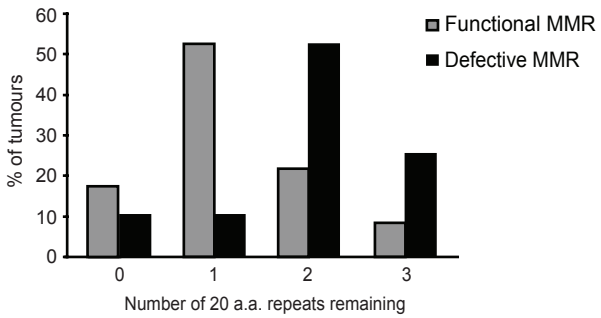
Colorectal cancers choosing sides: Tumours having a functional MMR system

As mentioned previously, tumours characterized by a deficient MMR system are more frequently observed at the proximal side of the colon, whereas most others occur at the distal side. We propose that these side preferences may

in part be explained by the specific acquired genetic defects in β-catenin signalling along the colorectal tract. As outlined below, MMR deficient tumours more frequently acquire mutations in *APC* or *CTNNB1* that are ideal for the proximal colon, whereas the ones with a functional MMR more often acquire *APC* mutations optimal for the distal colon. As depicted in Table 2 (left section), mutations leading to truncated APC proteins retaining one 20 aa repeat are the most commonly observed mutations in tumours with a functional MMR system (181/345, i.e. 53%) [10, 20, 22, 47, 94-95, 97-98, 116]. In part, the high frequency of these mutations is likely to be explained by the fact that the region between the first and second repeat contains an (AAAAG)2 direct repeat around codon 1309 that is prone to a deletion of 5 bps, representing one of the most common somatic *APC* mutations [117]. Secondly, the *APC* sequence between the first and second repeat codes for 146 amino acid residues, which is approximately twice the number of codons between repeats two and three and between repeat three and the first AXIN binding motif, 82 and 65 codons respectively (Figure 3). Thus, based on

Table 2. MMR deficient tumours acquire significantly more often truncated APC proteins retaining two or three repeats than their MMR proficient counterparts, which mainly develop with truncated proteins retaining one such repeat. Tumours were only included if the MMR status was clear. Tumours were included irrespective of location. Differences in number of repeats between both groups are highly significant (p< 0.0001; ordinal logistic regression model fitted with SAS version 9.2, proc glimmix).

Number of 20 aa repeats	**Functional mismatch repair (n=345)**				**Defective mismatch repair (n=63)**			
	0	**1**	**2**	**3**	**0**	**1**	**2**	**3**
Huang et al. [22]	13	24	13	5	6	4	14	2
Konishi et al. [122]					0	1	2	1
Olschwang et al.[116]	21	25	8	4	0	0	1	0
Rowan et al. [47]	2	9	7	4	0	1	3	2
Lovig et al. [20]	14	62	17	7	1	0	2	4
Smith et al. [94]	3	35	18	3				
Miyaki et al. [95]	0	2	3	1				
Domingo et al. [123]					0	1	5	3
Scholtka et al. [97]	0	11	4	4	0	0	0	1
Ku et al. [98]	0	4	1	1	0	0	0	3
Albuquerque et al.[10]	7	9	4	0	0	0	6	0
	60	181	75	29	7	7	33	16



sequence length, the region in between the first and second repeat is expected to acquire half of the mutations occurring within the mutation cluster region. Overall, in colorectal cells with a functional MMR system this leads to the generation of tumour cells preferentially expressing truncated APC proteins with one repeat, which provides the ideal β-catenin signalling level for the distal colon. According to the "just-right" signalling hypothesis, the same type of mutation is believed to induce an apoptotic response resulting from the high level of β-catenin signalling when occurring in the proximal colon, thereby preventing tumour initiation in this part of the colon. Therefore, we propose that the higher chance of obtaining a mutation leading to a truncated APC protein retaining one 20 aa repeat, shifts the balance of tumours towards the distal colon.

Colorectal cancers choosing sides: Mismatch repair deficient tumours

In MMR deficient intestinal cells the predominant mutational mechanism consists of mutations at the nucleotide level. Overall, a defective MMR system leads to a 10-30 fold higher induction of single basepair substitutions [118-120]. However, sequences most prone to mutations are the ones containing stretches of mono- or dinucleotide repeats, of which the mutation frequency can be increased up to 100-fold, depending on the length of the repeat. As such, genes containing these repetitive sequences in their reading frame will be most prone to mutation in a MMR deficient background [5-6, 121]. The *APC* gene contains several of these repetitive sequence features, which are indeed more frequently mutated in MMR deficient tumours [20, 22]. In Figure 8 we have highlighted in red the sequences prone to mutation in a MMR deficient background within the region harbouring the first three 20 aa repeats. As depicted in Table 3, mutations in these repetitive sequences, i.e. codons 1455 (A5), 1462 (AG5) or 1554 (A6), are observed in about 37% (23/63) of MMR deficient colorectal tumours carrying *APC* mutations, whereas these mutations are observed in less than 5% (16/345) of tumours with a functional MMR system [10, 20, 22, 47, 95, 97-98, 116, 122-123]. Mutations at one of these hotspots lead to the generation of a truncated APC protein retaining two or three 20 aa repeats. The high chance of acquiring mutations at one of these hotspots superimposed on the normal mutation spectrum, likely explains the high proportion of MMR deficient tumours (49/63 tumours, i.e. 78%) that obtain truncated APC proteins retaining two or three repeats (see Table 2), which is considerably higher than observed in tumours with a functional MMR system (104/345 tumours, i.e. 30%).

In about 50% of MMR deficient tumours *APC* mutations are observed [10, 20, 22-26]. An additional 10% obtain oncogenic *CTNNB1* mutations, resulting in a moderate signalling activity equivalent to the truncated APC proteins with 2-3 repeats. As such, these mutations are expected to be selected in the proximal colon, which

is in accordance with over 80% (47/58) of tumours carrying *CTNNB1* mutations being detected on this side [10, 25-27, 80-81]. Recently, we have obtained additional evidence for this observation by screening 54 MMR deficient tumours for *CTNNB1* mutations. We confirmed that these mutations occur more frequently in proximal tumours (15/36, i.e. 42%) than in their distal counterparts (1/18, i.e. 6%), irrespective of sporadic or familial origin (p< 0.01, Chi-square test, unpublished observations).

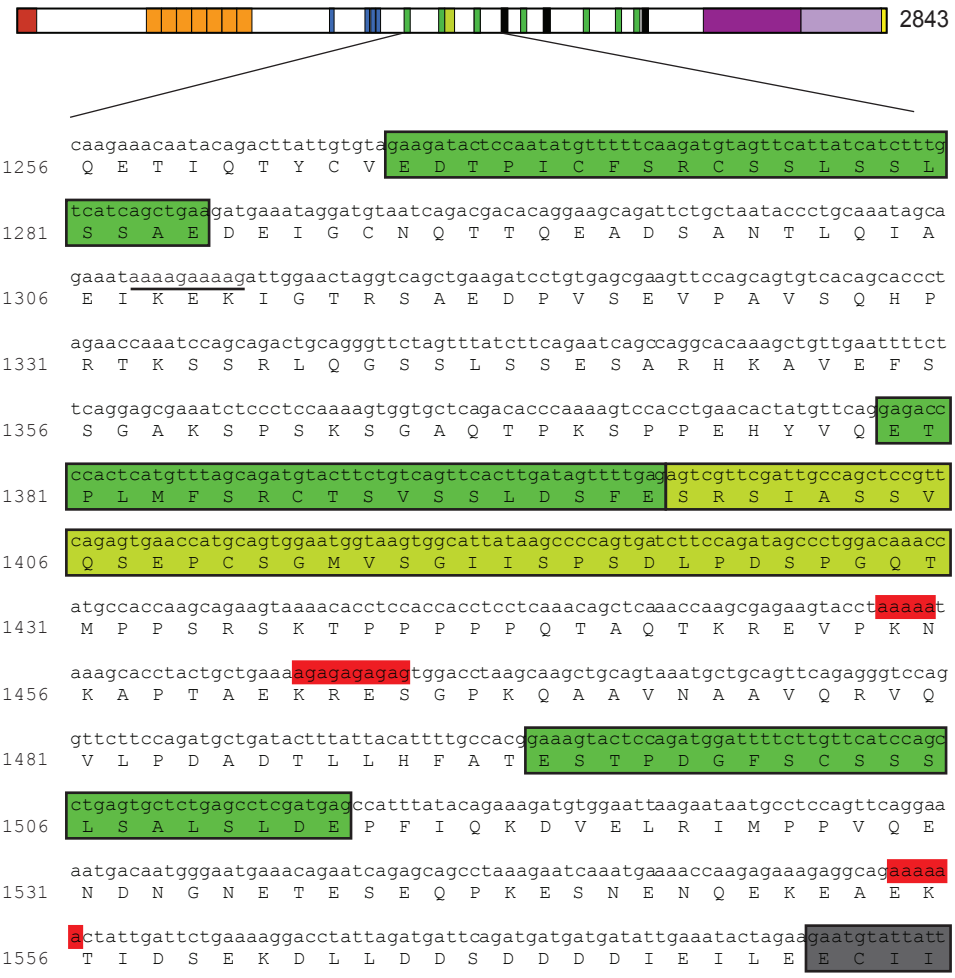
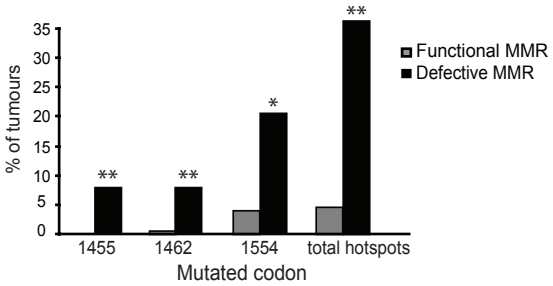


Figure 8. Expected hotspots for mutation within *APC* in MMR deficient tumours. The *APC* gene contains several mono- and dinucleotide stretches that are prone to mutation in a MMR deficient background. Here, we have highlighted in red the mononucleotide stretches of 5 or more of the same kind as well as the dinucleotide repeats with at least 4 repeats, in the region between the first 20 aa repeat and the first AXIN binding domain. Mutations at one of these hotspots lead to the generation of a truncated APC protein retaining two or three 20 aa repeats. Colour codes for structural domains are as in Figure 3. The (AAAAG)2 direct repeat around codon 1309, often mutated in MMR proficient CRCs, is underlined.

Table 3. The potential mutational hotspots, i.e. codons 1455, 1462, and 1554, marked in Figure 8, are mutated in about 37% (23/63) of MMR deficient colorectal tumours carrying *APC* mutations, whereas this is only the case in less than 5% (16/345) of tumours with a functional MMR system. These differences are highly significant (Fisher exact test; * p<0.01; ** p<0.001).

Codons	Functional mismatch repair (n=345)			Defective mismatch repair (n=63)		
	1455	1462	1554	1455	1462	1554
Huang et al. [22]	0	1	1	2	1	2
Konishi et al. [122]				0	1	1
Olschwang et al. [116]	0	0	1	0	0	0
Rowan et al. [47]	0	0	4	0	0	1
Lovig et al. [20]	0	0	6	0	1	3
Miyaki et al. [95]	0	0	0			
Domingo et al. [123]				2	2	2
Scholtka et al. [97]	0	1	1	0	0	1
Ku et al. [98]	0	0	1	0	0	3
Albuquerque et al. [10]	0	0	0	1	0	0
	0	2	14	5	5	13



In summary, due to the underlying mutational mechanism under MMR deficient conditions, a significant proportion of cells acquire either inactivating mutations in the *APC* gene following the second and third repeat, or oncogenic *CTNNB1* mutations. Both types of mutations are associated with a low to moderate activation of β-catenin signalling. According to the "just-right" signalling model, this provides the ideal level for successful tumour initiation in the proximal colon, whereas it will not supply sufficient growth advantage in the distal colon. As a result, MMR deficient cells are more successful in forming a detectable tumour on the proximal side.

Concluding remarks

We propose that the side preference of colorectal tumours may in part be explained by their particular mutational mechanism, resulting in *APC* and *CTNNB1* mutations leading to a specific level of β-catenin signalling that is ideal for either side of the colon. Obviously, our hypothesis only applies to colorectal tumours initiated by enhanced β-catenin signalling, which is the case for the great majority of tumours with a functional MMR system. However, among the MMR deficient

tumours, a mutation in *CTNNB1* or *APC* has not been reported in about 40% of the tumours. There are several potential explanations for this apparent lower involvement of the β-catenin signalling pathway in MMR deficient tumours. The first one is of technical nature, as a characteristic feature of these tumours is the high level of lymphocytic infiltration [5-6]. Since none of the quoted investigations have enriched their samples for tumour cell content, e.g. by laser capture microdissection, mutations may have been obscured by the DNA isolated from the large numbers of contaminating normal cells. Secondly, in 30-40% of MMR tumours a mutation or inactivation of either *AXIN1* or *AXIN2* is identified, some of which appear to be the sole defect in the Wnt/β-catenin signalling pathway in these tumours [10, 27-30]. Given the redundant nature of both Axin homologues [124], a modest increase in β-catenin signalling is to be expected, which is in line with a proximal preference for MMR deficient tumours. Lastly, a subset of colorectal tumours arises without an underlying defect in the Wnt/β-catenin signalling pathway. In support of this, the proximal colon shows more tumours with no apparent nuclear accumulation of β-catenin [100], although β-catenin IHC should be interpreted with caution. Alternative explanations have to be provided to explain the side preferences of these tumours. Most MMR deficient tumours carry mutations in the *TGFBR2* gene leading to resistance to the anti-proliferative effects of TGF-β [121, 125]. The proximal colon might be more sensitive to loss of this inhibitory effect for thus far unknown reasons, resulting in the outgrowth of more MMR deficient tumours on this side. Also, activating *BRAF* mutations have strongly been correlated with proximal sporadic MMR deficient tumours [123, 126-127]. BRAF is a member of the RAF family of kinases acting upstream of MEK1/2 kinases in response to RAS signals. As such, *BRAF* mutations are considered as an alternative to *KRAS2* mutations to activate the same downstream MEK-MAPK signalling pathways [128]. In line with the “just-right” signalling hypothesis, it may be postulated that activating *BRAF* mutations provide the optimal level of downstream signalling ideal for the proximal colon, and therefore support tumour growth on this side. However, experimental support for this hypothesis is currently lacking.

In our manuscript, we have highlighted the importance of β-catenin signalling dosages for the tumorigenic process, and presented data supporting our hypothesis that selection for specific dosages along the colorectal tract may in part explain the side preferences of colorectal tumours. It shows that we should not perceive mutations in signalling pathways merely as on/off switches, but that we should appreciate the nuances of signalling. The dosage-dependence as described here for β-catenin signalling will also be of relevance for other signalling pathways commonly involved in cancer development.

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Final note

Following acceptance of our manuscript, the DDW 2011 conference abstract of Leedham et al. quoted here as reference no. 99, has been submitted as full paper (personal communication). In this paper they describe the existence of a physiological Wnt signalling gradient along the intestinal tract. Based on this observation, they independently propose a mechanism for the side preferences of colorectal tumours, similar to ours.

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CHAPTER 5

β-Catenin signaling dosage dictates tissue-specific tumor predisposition in *Apc*-driven cancer

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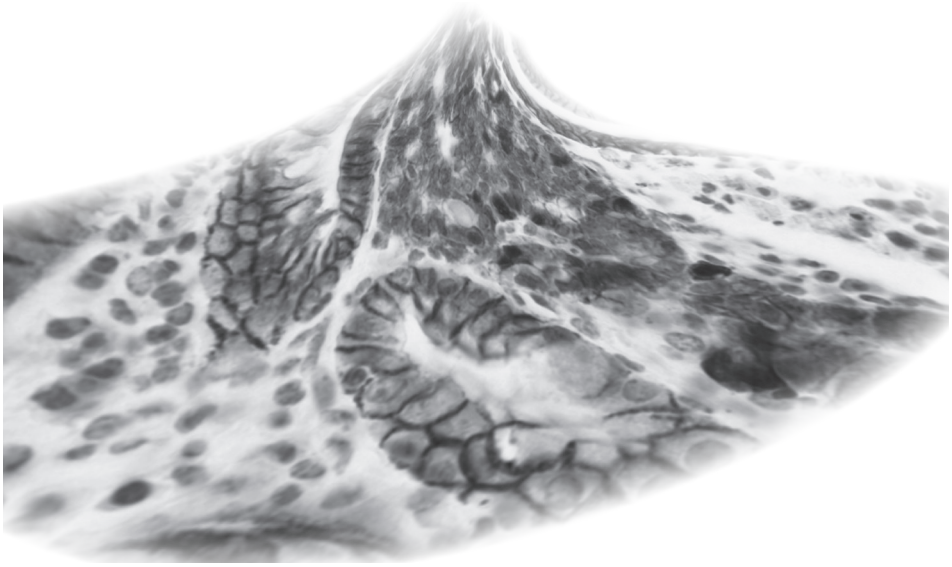

Abstract

***Apc*-driven tumor formation in patients and *Apc*-mutant mouse models is generally attributed to increased levels of β-catenin signaling. We and others have proposed that a specific level of β-catenin signaling is required to successfully initiate tumor formation, and that each tissue prefers different dosages of signaling. This is illustrated by *APC* genotype - tumor phenotype correlations in cancer patients, and by the different tumor phenotypes displayed by different *Apc*-mutant mouse models. *Apc*1638N mice, associated with intermediate β-catenin signaling, characteristically develop intestinal tumors (<10) and extra-intestinal tumors, including cysts and desmoids. *Apc*1572T mice associated with lower levels of β-catenin signaling are free of intestinal tumors, but instead develop mammary tumors. Although the concept of β-catenin signaling dosage and its impact on tumor growth among tissues is gaining acceptance, it has not been formally proven. Additionally, alternative explanations for *Apc*-driven tumor formation have been proposed. To obtain direct evidence for the dominant role of β-catenin dosage in tumor formation and tissue-specific tumor predisposition, we crossed *Apc*1638N mice with heterozygous β-catenin knockout mice, thereby reducing β-catenin levels. Whereas all the *Apc*1638N;*Ctnnb1*$^{+/+}$ mice developed gastrointestinal tumors, none were present in the *Apc*1638N;*Ctnnb1*$^{-/+}$ mice. Incidence of other *Apc*1638N-associated lesions, including desmoids and cysts, was strongly reduced as well. Interestingly, *Apc*1638N;*Ctnnb1*$^{-/+}$ females showed an increased incidence of mammary tumors, which are normally rarely observed in *Apc*1638N mice, and the histological composition of the tumors resembled that of *Apc*1572T-related tumors. Hereby, we provide in vivo genetic evidence confirming the dominant role of β-catenin dosage in tumor formation and in dictating tumor predisposition among tissues in *Apc*-driven cancer.**

Introduction

The Wnt/β-catenin signaling pathway represents one of the main regulatory mechanisms to retain tissue homeostasis in the adult organism by balancing self-renewal, differentiation and apoptosis in several adult stem cell niches [1]. Underscoring the relevance of this pathway, many tumor types exhibit enhanced Wnt/β-catenin signaling that strongly contributes to tumor growth. In the Wnt/β-catenin signaling pathway, the adenomatous polyposis coli (APC) protein is a central component regulating the degradation and concomitantly the transcriptional activity of β-catenin in the nucleus. As depicted in Figure 1A, several motifs in the central domain of APC are responsible for regulating intracellular β-catenin levels. Four 15 amino acid repeats (AAR) bind β-catenin, whereas seven 20-AARs are involved in both binding and downregulation. Interspersed within those 20-AARs are three binding sites for Axin required for an optimal recruitment of APC into the destruction complex. Inactivation of APC perturbs the formation of the β-catenin degradation complex, leading to increased nuclear translocation and target gene expression, thereby affecting important cellular decisions and favoring a genetic program that initiates tumor formation. In case of colorectal cancer, a small subset (~1-2%) of tumors are acquired by activating mutations in β-catenin itself, whereas most others result from inactivating biallelic *APC* mutations [2]. The vast majority of these *APC* mutations result in truncated proteins that lack all Axin binding motifs while retaining between one and three 20-AARs. As a result, these truncated proteins still have residual activity in downregulating β-catenin signaling. Accordingly, an inverse correlation is observed between the number of retaining 20-AARs and the resulting level of β-catenin signaling, that is, more repeats means a lower β-catenin signaling level to the nucleus. Based on these observations, we and others have proposed that *APC* mutations do not occur entirely in a random manner, but rather in respect to one another to reach an optimal level of enhanced β-catenin signaling, described as the 'just-right' signaling model [2-6]. According to this model, levels beneath the optimal β-catenin signaling window will not provide cells with sufficient activation of target genes to gain growth advantage and trigger tumor formation, whereas levels exceeding the optimal window will trigger apoptosis instead. As reviewed in Albuquerque et al, optimal β-catenin signaling dosages favoring tumorigenesis differ throughout the body, as indicated by different *APC* genotypes that are observed in tumors on different locations [2]. Both sporadic as well as familial forms of desmoid and duodenal tumors contain *APC* mutations retaining 2-3 20AARs associated with moderate β-catenin signaling activation. On the other hand, most colorectal tumors are associated with shorter truncating proteins resulting in higher levels of β-catenin signaling. Interestingly, correlations are observed even within the

colorectal tract, where right-sided colon tumors generally retain more 20-AARs than the left-sided ones [2, 7-8]. Although in human breast cancer patients mutations in β-catenin or *APC* are rarely found, aberrant activation of β-catenin signaling is observed frequently [9].

Phenotypes of *Apc*-mutant mouse models strongly support *Apc* genotype-tumor phenotype correlations (Figure 1B). *Apc*$^{Min/+}$ mice have high levels of β-catenin signaling and develop intestinal tumors at high multiplicity (>100). Animals carrying the hypomorphic *Apc*1638N mutation, associated with intermediate β-catenin signaling, characteristically develop intestinal tumors at

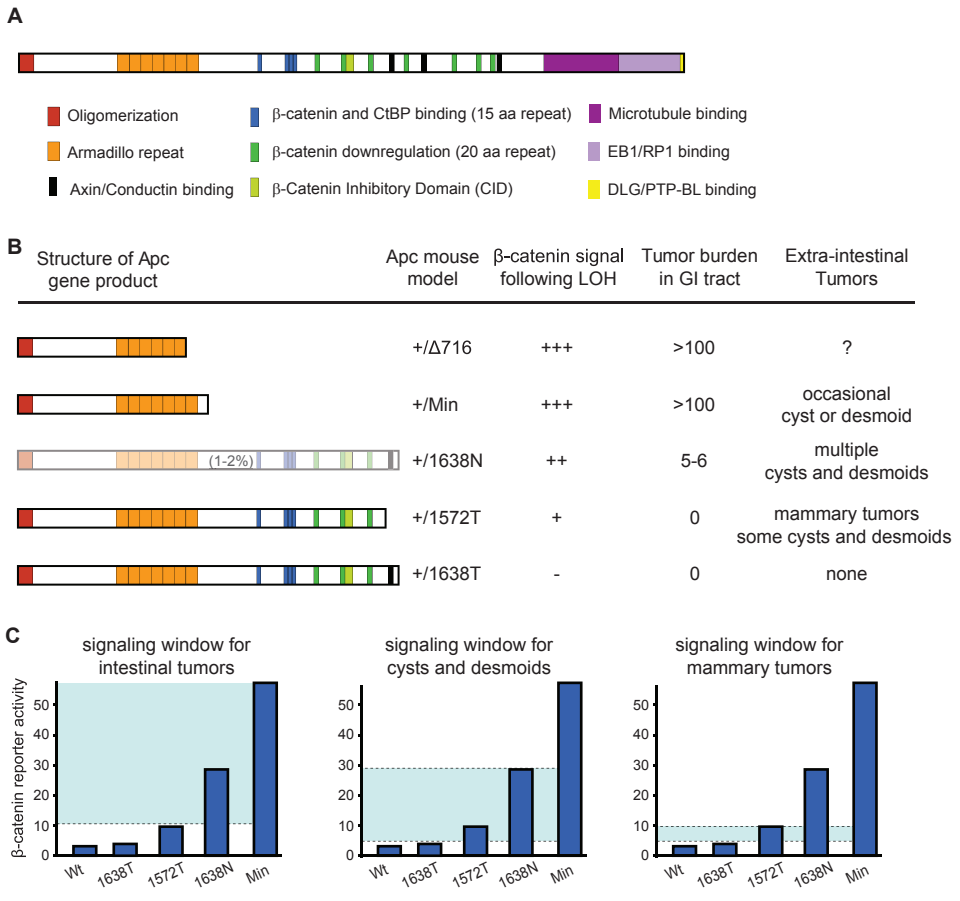

Figure 1. APC structure and associated β-catenin signaling dosage. (A) Structure of the Apc protein containing multiple regulatory domains. **(B)** Truncated Apc in different *Apc*-mutant mouse models. The number of remaining 20-AARs is inversely correlated with the β-catenin signaling dosage and associated with tumor development in different tissues. **(C)** Windows of β-catenin signaling dosages associated with specific tumor types in *Apc*-mutant mouse models. Whereas intestinal tumorigenesis requires high to moderate β-catenin signaling, cysts and desmoid development is associated with moderate β-catenin signaling and mammary tumors occur with the low level of β-catenin signaling as observed in *Apc*1572T mice. Figure modified from [2] with permission of BBA Reviews on Cancer.

lower multiplicity (<10) and in parallel show a high susceptibility for extra-intestinal tumor types such as cutaneous cysts and desmoid tumors [10]. The *Apc*1572T mouse model, associated with lower levels of β-catenin signaling, is free of intestinal tumors but instead develops mammary tumors with high penetrance, in addition to cysts and desmoids albeit with reduced numbers compared to *Apc*$^{1638N/+}$ mice [11]. Taken together, this indicates that tissue-specific dosages of β-catenin signaling are required to efficiently trigger tumorigenesis, where intestinal tumors are associated with higher levels of β-catenin signaling than cysts and desmoids, which in turn are associated with higher β-catenin signaling than mammary tumors (Figure 1C).

Although the concept of β-catenin signaling dosage and its impact on tumor growth among tissues is gaining acceptance, tissue-specific tumor predisposition has not been formally proven to be a direct consequence of β-catenin signaling dosage. Furthermore, alternative explanations for *APC*-driven tumor formation have been proposed. APC is a large, multifunctional protein and in addition to downregulating β-catenin signaling it is implicated in various other cellular processes, as APC can affect chromosomal segregation, cytoskeletal organization and bind C-terminal binding protein (CtBP) [12-16]. Here, we provide direct genetic evidence for the dominant role of β-catenin in tumor formation and establish the impact of β-catenin signaling dosage in dictating tissue-specific tumor predisposition. To this aim, we reduced the pool of available β-catenin in *Apc*$^{1638N/+}$ (*Apc*1638N) mice by heterozygous β-catenin (*Ctnnb1*) knockout. Consequently, gastrointestinal tumor formation was completely prevented while mammary tumor predisposition was enhanced, shifting the phenotype towards the *Apc*1572T-related tumor phenotype.

Materials and methods

Cells and β-catenin reporter assay

Mouse embryonic fibroblasts (MEFs) were isolated from embryos of embryonic day (E)13.5-15.5, cultured and transfected as described previously [33]. The β-catenin reporter assay was performed as described previously [33]. Assays were performed in duplicate three times.

Mouse strains

Mouse strains (C57BL/6J) used in this study were: *Apc*$^{+/1638N}$, *CAG-Cre* and *Ctnnb1*$^{-/+}$ [10, 18]. *Ctnnb1*$^{fl/+}$ males (gift from Dr. J. Huelsken) had been crossed previously with *CAG-Cre* females, obtaining *Ctnnb1*$^{-/+}$ knockout mice. Subsequently, these *Ctnnb1*$^{-/+}$ mice were crossed with *Apc*$^{+/1638N}$, resulting in compound heterozygous *Apc*$^{+/1638N}$/*Ctnnb1*$^{-/+}$ animals and corresponding single transgenic controls *Apc*$^{+/1638N}$/*Ctnnb1*$^{+/+}$. All mice were maintained under specific pathogen-free conditions at the animal facility of the Erasmus Medical Centre. Mice were examined for tumor formation at the age of 8 months. All experiments were approved by the Animal Ethics Committee and carried out in accordance with Dutch

and international legislation.

Histology and immunohistochemistry
Tissues were washed in PBS and fixed overnight in 4% PBS-buffered paraformaldehyde at 4°C. Paraffin embedding and Haematoxylin Eosin (HE) staining were performed according to routine protocols. For immunohistochemistry, antigen retrieval citrate pH6 preceded staining for SMA (1:200, DAKO), Cytokeratin-14 (1:10000, Covance) and CD44 (1:1000, BD Biosciences), Tris-EDTA pH9 for β-catenin (1:2000, Epitomics), Cyclin D1 (1:200, Vector Laboratories) and Ki67 (1:200, DAKO) and 0.1% pronase for Cytokeratin-8 (1:800, DSHB).

RNA isolation, cDNA synthesis and quantitative PCR
RNA was isolated from cultured MEFs followed by cDNA generation using iScript cDNA Synthesis Kit (Bio-Rad). Quantitative PCR was performed with TaqMan Gene Expression Assays (Applied Biosystems) and run in the IQ5 Real time PCR detection system (Bio-Rad). Expression levels were corrected for expression of *Actb*. Assay was performed in duplicate three times.

Results and discussion

The *Apc*1638N mouse model is a representative model to investigate intestinal cancer, where mice characteristically develop about 1-7 gastrointestinal tumors [17]. In addition, these mice are highly susceptible for extra-intestinal tumor types including desmoids and cutaneous cysts [10]. To reduce their dosage of β-catenin, we crossed *Apc*$^{1638N/+}$ mice with *Ctnnb1*$^{-/+}$ knockout mice [18]. First, non-tumorigenic intestinal tissues were characterized of both *Apc*$^{1638N/+}$/*Ctnnb1*$^{+/+}$ and *Apc*$^{1638N/+}$/*Ctnnb1*$^{-/+}$ mice. Haematoxylin Eosin (HE) staining showed normal intestinal histology in both groups (Figure 2A). Moreover, no discernible alterations in β-catenin protein expression were detected using β-catenin immunohistochemistry (Figure 2A). These results were in line with expectations, as normal tissues only harbor the heterozygous germline *Apc*-mutation and intestinal tissue homeostasis of mice harboring the *Apc*1638N mutation is also unaltered compared to that of wild-type mice [19]. Furthermore, β-catenin immunohistochemistry is not a very sensitive technique to detect differences in β-catenin protein expression and to determine associated alterations in β-catenin signaling. To verify reduced β-catenin signaling as a consequence of heterozygous *Ctnnb1* knockout, we used a more sensitive approach. For this, β-catenin reporter assays were performed to measure the intrinsic β-catenin signaling of mouse embryonic fibroblasts (MEFs) that we generated of embryos of the different genotypes. In the absence of *Apc* mutation, levels of β-catenin signaling were low and not detectably reduced by heterozygous β-catenin knockout (Figure 2B). *Apc*$^{+/1638N}$ MEFs showed slightly enhanced β-catenin signaling, and here, heterozygous β-catenin knockout clearly resulted in reduced β-catenin signaling. Moreover, *Apc*$^{+/1638N}$ MEFs

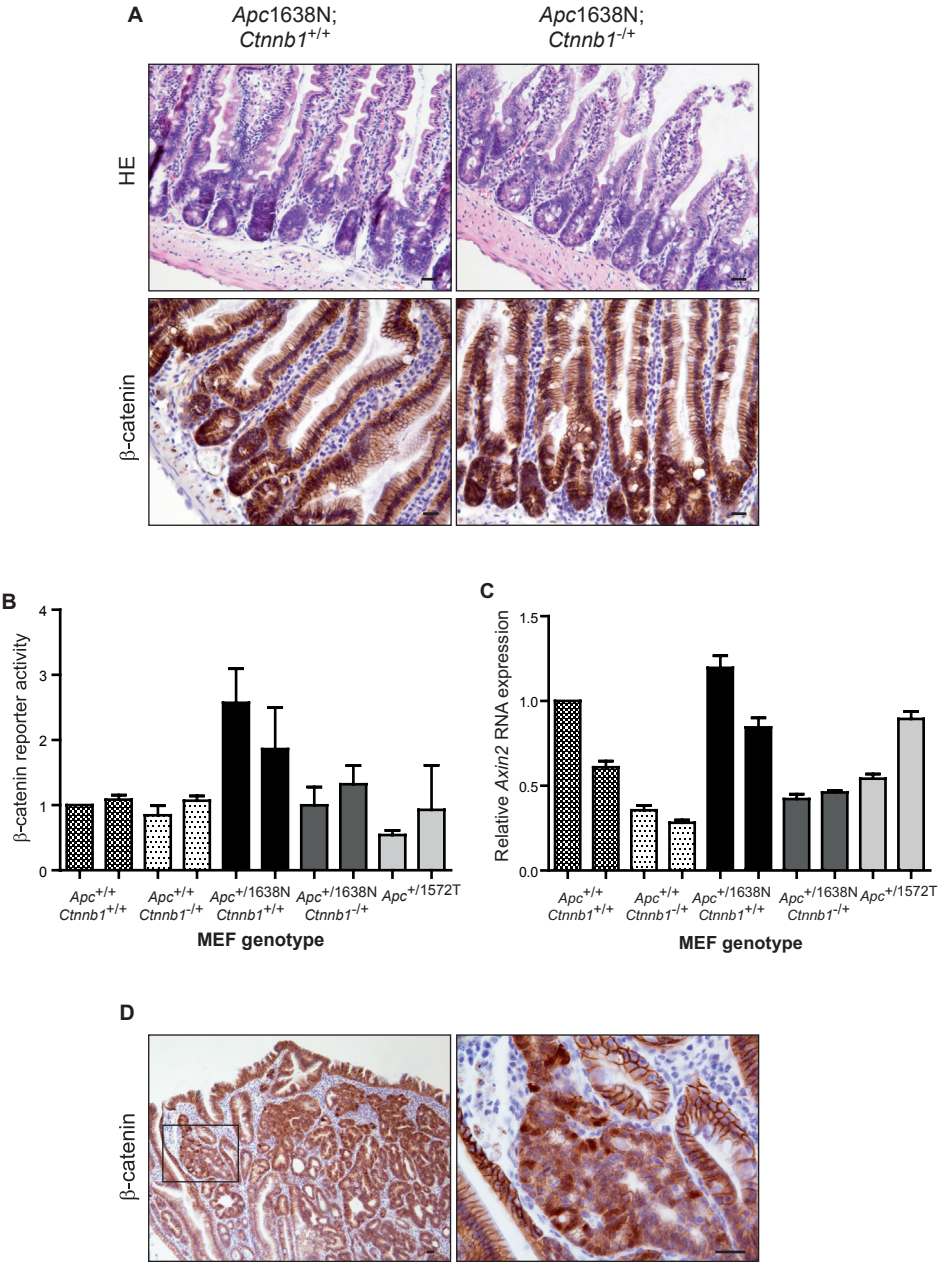

Figure 2. Heterozygous β-catenin knockout reduces β-catenin signaling. (A) HE and β-catenin staining of non-tumorigenic intestine, showing no gross differences between groups. **(B)** β-catenin reporter assay of MEFs of *Apc*$^{+/+}$ or *Apc*$^{+/1638N}$ genotype, each in combination with *Ctnnb1*$^{+/+}$ or *Ctnnb1*$^{-/+}$, in addition to *Apc*$^{+/1572T}$, and **(C)** *Axin2* mRNA expression in these MEFs determined by quantitative PCR. Two bars per genotype indicate two individual MEF lines that were used. **(D)** β-catenin staining of an intestinal tumor of an *Apc*$^{+/1638N}$ mouse. Nuclear β-catenin staining is clearly detected.

with heterozygous β-catenin knockout showed intrinsic β-catenin signaling levels that approached the β-catenin signaling dosage as observed in *Apc*$^{+/1572T}$ MEFs. Comparable results were obtained when we assessed β-catenin signaling by determining RNA levels of the β-catenin target gene *Axin2* in these MEFs (Figure 2C). In the absence of *Apc* mutation, *Axin2* levels were slightly reduced by heterozygous β-catenin knockout. *Apc*$^{+/1638N}$ MEFs showed enhanced *Axin2* levels, which again were reduced by heterozygous β-catenin knockout, reaching levels comparable to those observed in *Apc*$^{+/1572T}$ MEFs. Together these data verify that heterozygous β-catenin knockout indeed substantially reduces β-catenin signaling in *Apc*1638N mice, moreover, in such a way that β-catenin signaling levels approach the β-catenin dosage of *Apc*1572T mice. These observations are in line with those of Buchert et al, who showed that halving β-catenin levels consistently reduced β-catenin signaling in various *Apc*-mutant MEFs [20].

We then determined β-catenin expression in the intestinal tumors that developed in the *Apc*1638N mice, since Phelps et al. have proposed that following *Apc* loss, activation of oncogenic KRAS is required to impose nuclear accumulation of β-catenin [14]. In the *Apc*1638N intestinal tumors, we clearly detected nuclear β-catenin staining in the epithelial tumor cells in addition to membranous β-catenin expression (Figure 2D), in accordance with previous results [21]. Importantly, *Apc*1638N intestinal tumors and those of other *Apc*-mutant models do not spontaneously acquire Ras mutations [22-24]. This shows that oncogenic Ras is not required for nuclear accumulation in mouse intestinal tumors, which recently has also been confirmed for human colorectal tumors [25-26].

Subsequently, we examined tumor phenotypes of 8-months old *Apc*1638N/*Ctnnb1*$^{+/+}$ and *Apc*1638N/*Ctnnb1*$^{-/+}$ mice. Strikingly, although all the 19 *Apc*1638N/*Ctnnb1*$^{+/+}$ mice developed gastrointestinal tumors as characteristic for *Apc*1638N mice, none of the 21 *Apc*1638N/*Ctnnb1*$^{-/+}$ mice developed any gastrointestinal tumor nor was any microlesion detected (Figure 3A). This provides direct evidence for the absolute requirement of a sufficiently enhanced β-catenin level for intestinal tumorigenesis. The complete absence of intestinal tumors in mice with heterozygous β-catenin knockout precluded us to compare β-catenin and associated signaling characteristics in intestinal tumors between groups following second-hit *Apc* mutation. *Apc*1638N-associated extra-intestinal lesions were still observed in *Apc*1638N/*Ctnnb1*$^{-/+}$ mice with characteristic gender-specific distribution, although with a clearly reduced incidence (Figure 3B,C). Desmoid numbers were reduced from 8.6 ± 3.0 to 0.2 ± 0.4 in females and from 61.4 ± 14.4 to 19.1 ± 8.1 in males (Figure 3B). Histological appearance of the desmoids was similar in both groups, showing abundant collagen fibers interspersed by fibroblast-like cells, as has been described for *Apc*1638N mice previously [10]. Cyst numbers were lowered from 5.6 ± 3.8 to 0.4 ± 0.6 in females and from 29.8 ± 19.5

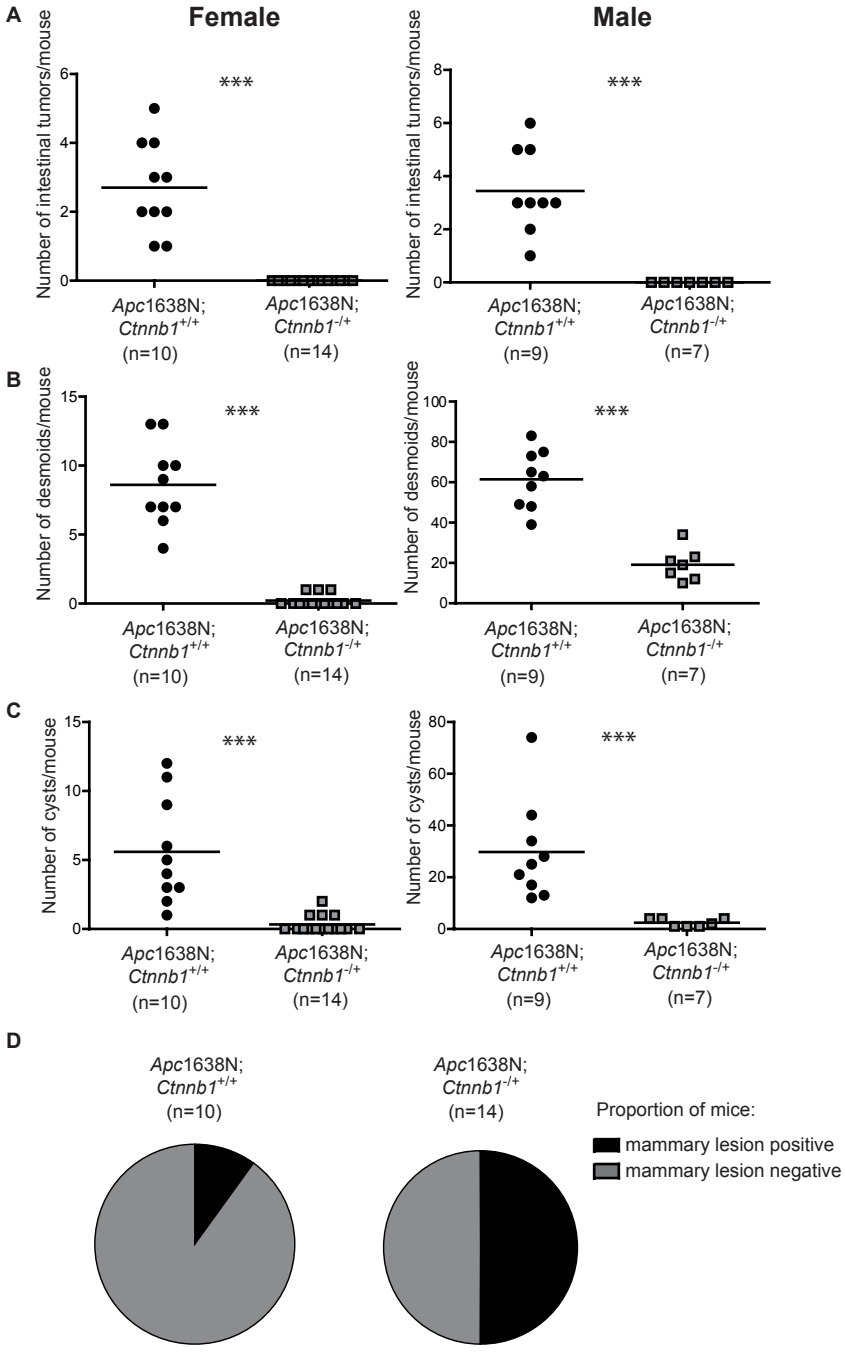

Figure 3. Heterozygous β-catenin knockout prevents gastrointestinal tumor formation but predisposes for mammary tumors in *Apc*1638N mice. Compound heterozygous *Apc*$^{+/1638N}$; *Ctnnb1*$^{-/+}$ animals and corresponding single transgenic *Apc*$^{+/1638N}$ control mice were examined for tumor formation at the age of 8 months. Number of **(A)** gastrointestinal tumors **(B)** desmoids and **(C)** cysts per mouse, distinguishing distribution in females (left) and males (right). ***p<0.001 (Student t-test) **(D)** Proportion of females with/without mammary tumor development. *p<0.05 (Chi-square test).

to 2.4 ± 1.5 in males (Figure 3C). Thus, reducing β-catenin levels in *Apc*1638N mice prevented gastrointestinal tumor formation and significantly reduced the incidence of other lesions associated with the *Apc*1638N mouse model. Most strikingly, we observed that half of the *Apc*1638N/*Ctnnb1*$^{-/+}$ females developed mammary lesions, reflecting a strongly enhanced incidence of mammary lesions following heterozygous β-catenin knockout (Figure 3D). Normally, mammary lesions are rarely observed in *Apc*1638N mice but are characteristic for *Apc*1572T mice. Our findings nicely illustrate that by reducing β-catenin in *Apc*1638N mice, the tumor phenotype shifts towards an *Apc*1572T-related phenotype, which is associated with a relatively lower activation level of β-catenin signaling. This confirms that β-catenin signaling dosage by itself dictates tissue-specific tumor predisposition in *Apc*-mutant mice.

The mammary lesions we observed in *Apc*1638N/*Ctnnb1*$^{-/+}$ mice were relatively small, showing an average diameter of 2.6 ± 1.4 mm, compared to those generally observed in *Apc*1572T mice, reaching 2 cm^3 by 4-5 months of age [11]. In accordance with this relatively mild mammary tumor phenotype in our *Apc*1638N/*Ctnnb1*$^{-/+}$ mice, metastases were not observed. Microscopic characterization of the identified mammary lesions revealed a heterogeneous histology, displaying glandular and squamous regions, keratinizing components and inflammatory cells (Figure 4A). Immunohistochemical analyses further established the heterogeneity of the mammary tumors found in *Apc*1638N/*Ctnnb1*$^{-/+}$ mice. Hence, staining for cytokeratin-8 confirmed luminal epithelial differentiation, cytokeratin-14 indicated areas of squamous differentiation and smooth muscle actin showed myoepithelial cell types (Figure 4A). This histological composition is virtually identical to that of the mammary lesions observed in *Apc*1572T mice [11]. In the mammary lesions of the *Apc*1638N/*Ctnnb1*$^{-/+}$ mice, staining for Ki67 revealed moderate proliferation (Figure 4B). Expression of β-catenin was observed in epithelial cells displaying membrane-bound and nuclear β-catenin (Figure 4B). Accordingly, expression of the Wnt/β-catenin targets CD44 and Cyclin D1 was observed in the epithelial compartment of *Apc*1638N/*Ctnnb1*$^{-/+}$ mammary lesions (Figure 4B). Thus, *Apc*1638N/*Ctnnb1*$^{-/+}$ mice develop heterogeneous mammary tumors resembling those observed in *Apc*1572T mice histologically and showing active Wnt/β-catenin signaling.

Our data show that by reducing β-catenin levels, the characteristic *Apc*1638N-related intestinal tumor phenotype shifts towards mammary tissues, where tumors typically develop in *Apc*1572T mice [11]. Also, the reduced incidence of cysts and desmoids is in accordance with that observed in *Apc*1572T mice. The mammary tumors observed in *Apc*1638N/*Ctnnb1*$^{-/+}$ mice resembled those of *Apc*1572T mice histologically, although remaining smaller. We propose that in *Apc*1638N mice following loss of the wild-type *Apc* allele required for tumor initiation, we

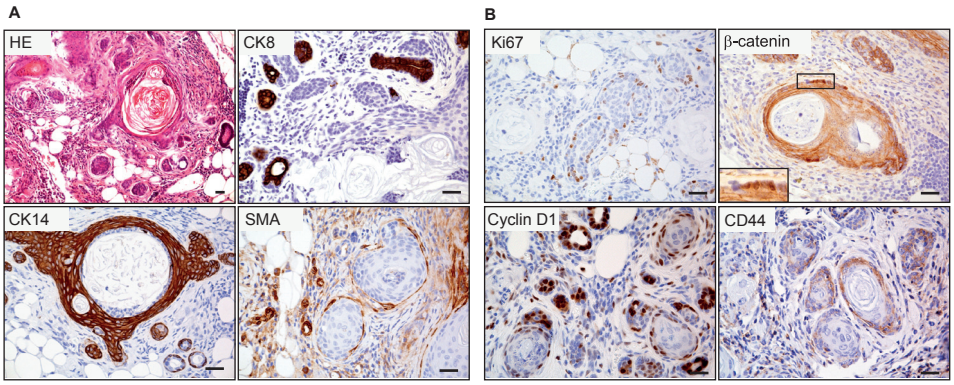

Figure 4. Characterization of *Apc*1638N/*Ctnnb1*$^{-/+}$ mammary tumors. (A) HE staining reveals heterogeneous histology. Immunohistochemical staining for cytokeratin-8 showing luminal epithelial differentiation, cytokeratin-14 indicating squamous differentiation and smooth muscle actin showing myoepithelial cells. **(B)** Ki67 indicating moderate proliferation and β-catenin staining revealing membrane-bound and nuclear localization. Active Wnt/β-catenin signaling is further indicated by the expression of Cyclin D1 and CD44.

reduced the β-catenin dosage by heterozygous β-catenin knockout to levels approaching those associated with *Apc*1572T mice, thereby enabling successful mammary tumorigenesis [10-11, 27]. However, the exact β-catenin signaling level preferred to sustain fully penetrant mammary tumor growth and metastasis may not have been reached most optimally, explaining the smaller tumors observed in *Apc*1638N/*Ctnnb1*$^{-/+}$ mice compared to *Apc*1572T mice.

Uncovering this shift in tumor phenotype from the gastrointestinal tract towards mammary tissues following β-catenin dosage reduction provides direct in vivo evidence that β-catenin dosage by itself dictates tissue-specific tumor predisposition in the setting of *Apc*-driven cancer. This is in accordance with previously described *APC* genotype–tumor phenotype correlations and associated β-catenin signaling dosages among *Apc*-mutant mouse models and sporadic and familial cancer patients [2]. Comparably, tissue-specific biological output being determined by specific dosage has been reported for the proto-oncogene *c-Myc* as well, which is one of the main target genes of β-catenin signaling [28-29]. In line with our findings, Buchert et al. presented specific β-catenin signaling thresholds being important for hepatic and intestinal tumorigenesis [20]. Hence, they showed that hepatic tumor formation as observed in a hypomorphic *Apc*-mutant model was prevented by heterozygous *Ctnnb1* knockout [20]. In addition, following heterozygous *Ctnnb1* loss, they observed reduced intestinal tumor incidence and multiplicity in an *Apc*-mutant mouse model associated with a relatively high β-catenin signaling dosage [20]. We currently show that intestinal tumor formation is even prevented completely by reducing β-catenin levels below a hypothetical threshold, using the *Apc*1638N mouse model associated with

intermediate β-catenin signaling dosage. Importantly, we hereby demonstrate that for *Apc*-driven tumor formation in the gut, enhanced β-catenin signaling is absolutely required. These results contradict suggested alternative explanations for *Apc*-driven cancer and strongly argue against the model presented by Phelps et al, who recently proposed that *APC*-driven tumor formation is independent of β-catenin, but instead requires the transcriptional corepressor CtBP [14]. CtBP has been shown to interact with APC at its 15-AARs thereby competing with β-catenin binding, and CtBP's levels appear to increase upon *Apc* loss in early adenomas [14-15]. In their paper, Phelps and coworkers suggest that in contrast to CtBP, nuclear β-catenin cannot be detected following *Apc* loss alone using immunofluorescence, and suggested the additional activation of oncogenic KRAS to impose nuclear accumulation of β-catenin [14]. As discussed by Fodde and Tomlinson, nuclear staining of β-catenin is a reliable indicator of active Wnt signaling, but its absence does not exclude the robust activation of β-catenin target genes [26]. Using immunoperoxidase-based methods most investigators detect nuclear β-catenin accumulation in early adenomas, independent of *KRAS* mutation status [25-26]. As described above, nuclear β-catenin can be detected in *Apc*1638N intestinal tumors (Figure 2D), while *K*-, *N*-, or *H-Ras* mutations are not found in these tumors [22]. In addition, whereas oncogenic *CTNNB1* mutations have been detected in a large number of tumor types and expression of oncogenic β-catenin leads to the development of numerous tumors in the mouse intestine, equivalent data indicating tumor-initiating capacity of CtBP do not exist. Furthermore, as CtBP binds the more N-terminal located 15-AARs of APC, it can not explain the selection of specific truncated APC proteins retaining between 1-3 20-AARs that is observed in tumors, whereas this is the case for β-catenin [2, 30]. The same argument holds true for the C-terminal microtubular functions of APC, which are completely lost in all APC-mutant proteins. Although loss of these C-terminal regions has been implicated in disturbed cell migration and chromosomal segregation [12-13], *Apc*-mutant mouse studies have shown that the C-terminal domains of Apc do not influence intestinal tumorigenesis. Hence, *Apc*1638T mice lacking the C-terminal regions of Apc but retaining an axin-binding repeat remain tumor-free [31], and the tumor phenotype of *Apc*1322T mice expressing a truncated Apc retaining only 1 20-AAR is not influenced by reintroduction of the C-terminal regions of Apc [32].

Although our findings provide genetic evidence for the dominant role of β-catenin signaling dosage in dictating tissue-specific predisposition for *Apc*-driven tumorigenesis, mechanisms underlying tissue preferences for specific levels of β-catenin signaling remain largely unknown. In the intestine, β-catenin signaling is one of the main regulatory pathways, however, it operates in concerted action with multiple other signaling routes. This complex interplay is

poorly understood. Other tissues including the mammary gland have unique architectural organizations and other signaling pathways are likely to play a role into different degrees. Unravelling how the complexity of all those signalling pathways influences which β-catenin signaling dosage dictates tissue-specific tumor predisposition in *Apc*-driven tumorigenesis represents a challenge for future investigation.

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CHAPTER 6

SOX2 redirects the developmental fate of the intestinal epithelium toward a premature gastric phenotype

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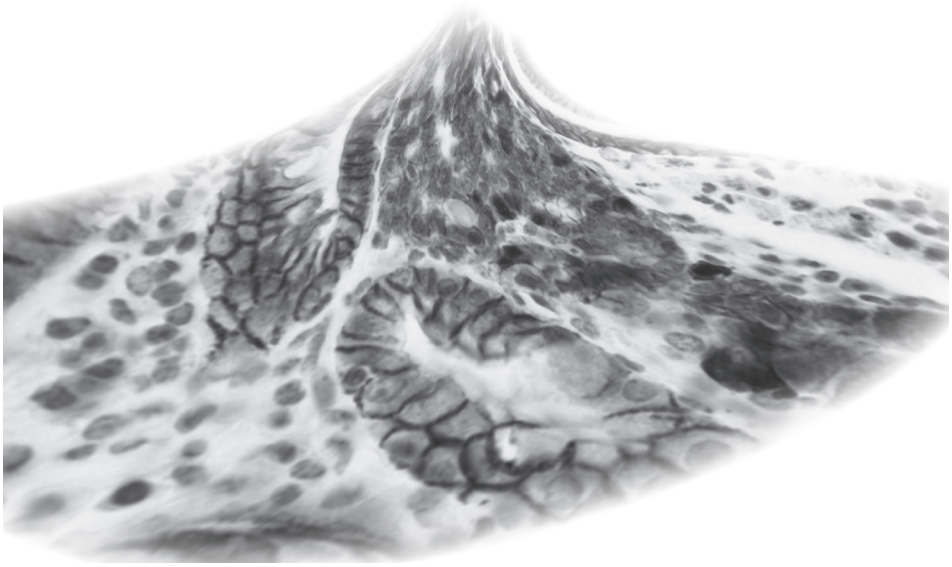

Abstract

Various factors play an essential role in patterning the digestive tract. During development, Sox2 and Cdx2 are exclusively expressed in the anterior and the posterior parts of the primitive gut, respectively. However, it is unclear whether these transcription factors influence each other in determining specification of the naïve gut endoderm. We therefore investigated whether Sox2 redirects the fate of the prospective intestinal part of the primitive gut. Ectopic expression of Sox2 in the posterior region of the primitive gut caused anteriorization of the gut toward a gastric-like phenotype. Sox2 activated the foregut transcriptional program, in spite of sustained co-expression of endogenous Cdx2. However, binding of Cdx2 to its genomic targets and thus its transcriptional activity was strongly reduced. Recent findings indicate that endodermal Cdx2 is required to initiate the intestinal program and to suppress anterior cell fate. Our findings suggest that reduced Cdx2 expression by itself is not sufficient to cause anteriorization, but that Sox2 expression is also required. Moreover, it indicates that the balance between Sox2 and Cdx2 function is essential for proper specification of the primitive gut and that Sox2 may overrule the initial patterning of the primitive gut, emphasizing the plasticity of the primitive gut.

Introduction

The vertebrate digestive tract develops through a complex mechanism of patterning, expansion and differentiation of the primitive gut, which itself is formed after gastrulation as a result of folding of the definitive endodermal layer [1-3]. One of the earliest steps in patterning of the primitive gut is the regionalization into an anterior and posterior domain, which correlates with the expression of two transcription factors, Sox2 and Cdx2 respectively [4]. During development these domains become more subdivided and by E9.5 the foregut, midgut and hindgut regions can be discriminated. The foregut eventually gives rise to the esophagus, stomach and proximal part of the duodenum; the midgut develops into the caudal part of the duodenum, jejunum, ileum, caecum and proximal part of the colon; and the hindgut forms the caudal part of the colon and rectum. Once regionalization has occurred, the gastro-intestinal tract becomes specified and cells differentiate into the characteristic types found in the various regions.

Sox2 is a member of a highly conserved family of transcription factors founded by the sex-determining gene Sry. Although individual Sox proteins share common DNA binding properties, their specificity is the result of their expression pattern and their ability to associate with different partners [5-6]. Sox proteins are recognized as key players in the regulation of embryonic development and determination of cell fate and we recently described two novel partners for Sox2 [7-8]. Sox2 plays an important role during early vertebrate embryogenesis and is required later in development in the brain, neural tube, germ cells and in the foregut and its derivatives [9-14].

Expression of Sox2 starts at the 4-8 cell stage of embryonic development and *Sox2* gene inactivation results in early peri-implantation lethality [9]. Modulating expression in mice showed the importance of Sox2 in the differentiation of the esophageal epithelium and morphogenesis of the esophagus and trachea [11, 13, 15]. Several congenital malformations in humans, for instance anophthalmia, microphthalmia and the AEG (anophthalmia-esophageal-genital) syndrome [16], have been associated with heterozygous mutations in *SOX2*. In addition, Sox2 is one of the key players in the induction of pluripotent stem cells from somatic cells [17], confirming the importance of Sox2 in development and differentiation processes.

Cdx2 is a homeobox transcription factor involved in the establishment of the anterior-posterior polarity of the gut endoderm and is expressed from E8.5 in the posterior part of the gut [18]. Later in development Cdx2 becomes restricted to the intestinal epithelium, with a sharp boundary, marking the transition from stomach to duodenum [4, 19-21].

Conditional ablation of *Cdx2* from the developing gut endoderm results in

severe malformation of the intestinal tract and anteriorization of the posterior gut, evidenced by changes in the expression pattern of various transcriptional regulators [22]. Interestingly, quantitative RNA expression analysis of *Cdx2* mutant intestinal samples demonstrated ectopic activation of Sox2 expression in the posterior region. The aberrant expression of Sox2 after *Cdx2* ablation coincided with an early anteriorization event of the gut. The apparent reciprocal expression pattern of Sox2 and Cdx2 suggests that these factors are able to influence each other. However, it remains unclear whether Sox2 or Cdx2 has a dominant role in determining the fate of the developing gut endoderm. In addition, aberrant SOX2 expression may underlie the formation of gastric metaplasia associated with intestinal inflammation [23], and the formation of various human congenital anomalies of the gut, such as Meckel's diverticulum in which ectopic gastric tissue can be present in the small intestine [24]. Furthermore, our group and others have observed aberrant expression of SOX2 in human colorectal cancers, suggesting a role for SOX2 in colorectal tumor initiation or progression (unpublished data) [25].

Therefore, we ectopically expressed Sox2 in epithelial cells of the prospective intestinal part of the primitive gut to investigate whether Sox2 is able to change the differentiation of the developing gastro-intestinal tract. Our data show that expression of Sox2 in the embryonic mouse intestinal epithelium leads to a fluid-filled, translucent swollen intestine with aberrant villi. The proliferation of epithelial cells in the Sox2 expressing intestines are no longer confined to the crypts, but rather distributed randomly throughout the intestinal epithelium. Moreover, Sox2 induced the expression of esophageal and stomach specific markers, resulting in an anteriorization of the intestinal epithelium. Although the expression level of Cdx2 is unaffected by ectopic Sox2 expression, we further show that induction of Cdx2 target gene expression is strongly reduced, because Sox2 leads to a reduced binding of Cdx2 to its target genes.

These findings provide strong evidence that expression of Sox2 in the developing gut drives the activation of the foregut transcriptional program and leads to conversion from an intestinal into a premature gastric epithelium, despite simultaneous expression of Cdx2. This indicates that the balance between Sox2 and Cdx2 function is essential for proper specification of the primitive gut and that Sox2 can exert a dominant effect over Cdx2 to alter intestinal cell fate by redirecting the intestinal transcriptional program, emphasizing the plasticity of the primitive gut.

Materials and methods

Transgenic animals
The myc-*Sox2* and Villin-*rtTA* transgenic mouse lines were previously described [11, 26]. Administration of doxycycline to dams from E8.5 or E14.5 onward in the drinking water (2mg/ml doxycycline, 5% sucrose) induced expression of myc-*Sox2* in intestinal epithelium of double transgenic embryos. Each experiment was performed on at least three independent litters containing double transgenic, single transgenic and wild type pups. All double transgenic animals receiving doxycycline expressed nuclear Sox2 in the intestinal epithelium, throughout the entire intestinal tract and showed described phenotype.

Histology
Embryos were obtained by caesarean section at E18.5. The gastro-intestinal tract was isolated and stretched on pieces of filter paper, followed by fixation in 4% PBS-buffered paraformaldehyde (PFA) overnight at 4°C. Intestines were cut into 4-5 pieces and pre-embedded in 5% bacto-agar (BD) in PBS, before being embedded in paraffin according to routine protocols.
IHC, PAS staining and electron microscopy were performed as described previously [38,39]. Antibodies are listed in Supplementary Table I. Differentiated and proliferative cells in the epithelium were quantified by counting at least 3 microscopic fields relative to the total number of epithelial cells.

Microarray analysis
The intestinal tracts of three control and two double transgenic embryos, which received doxycycline from E8.5 onwards, were isolated at E18.5 and individually used for transcriptome analysis using Affymetrix Mouse Genome 430 2.0 microarrays as previously described [11]. Subsequent analysis of the data was performed as described in detail in Supplementary Materials and methods.

Chromatin immunoprecipitation
Small intestines of, 10 double transgenic and 10 control E18.5 embryos were pooled and mechanically disrupted. Subsequently, the material was crosslinked, sonicated and used to immunoprecipitate either Cdx2 or Sox2 complexes. The precipitated material was de-crosslinked and the co-immunoprecipitated DNA fragments were used as template in qPCR reactions to amplify specific target sequences (primers are listed in Supplementary Table II). Details of the protocol are provided in Supplementary Materials and methods.

Quantitative polymerase chain reaction
RNA isolation, cDNA synthesis and subsequent qPCR analysis was performed as previously described [40]. Data were analyzed using the $2^{-\Delta\Delta Ct}$-method [41]. The gene-specific primers used are listed in Supplementary Table III.

Results

Ectopic Sox2 expression in the caudal part of the developing gut endoderm

Sox2 expression in the developing gut is restricted to the anterior part, which forms the stomach and other foregut derivatives. The posterior part is devoid of Sox2 expression, but expresses the homeobox protein Cdx2. Expression of Sox2 and Cdx2 is thought to be mutually exclusive, but it is unclear whether one has a dominant effect on the determination of the fate of the developing gut endoderm. Therefore we investigated the role of Sox2 in the patterning of the primitive gut and its influence on Cdx2 expression.

Conditional expression of Sox2 in the intestinal epithelium was achieved by crossing mice carrying a myc-tagged *Sox2* gene under the control of a tet-inducible promoter with the Villin-*rtTA* transgenic mouse line, which drives expression of the reverse tetracycline transactivator (*rtTA*) gene in all intestinal epithelial cells [11, 26]. The rtTA protein drives expression of the *Sox2* transgene in the intestinal epithelium in a doxycycline dependent manner. Induction of Sox2 in embryos was achieved by administration of doxycycline to the mother from E8.5 onwards. Double transgenic pups that received doxycycline through their mother were born at Mendelian ratio, but failed to thrive. Therefore, pups were isolated at E18.5 by caesarean section in order to investigate the intestinal abnormalities.

Externally, the littermates showed no significant morphological abnormalities and all embryos were equally well developed. Intestinal tracts isolated from pups carrying only one of the two transgenes, or non-induced myc-*Sox2*/Villin-*rtTA* double transgenic animals, appeared indistinguishable from non-transgenic control animals and showed no macroscopic or microscopic abnormalities. However, isolation of the gastro-intestinal tract of double transgenic embryos that received doxycycline immediately revealed major differences compared to single or non-transgenic animals (Figure 1A). In all double transgenic pups ectopic expression of Sox2 in the intestinal tract resulted in markedly abnormal intestines that were translucent and fluid-filled. Immunohistochemical analyses using an antibody against Sox2 (Figure 1B), or an antibody against the N-terminal myc-epitope present in the transgenic Sox2 protein (data not shown), revealed that nuclear Sox2 was expressed throughout the entire intestinal epithelium of the double transgenic animals. Assessment of Sox2 expression at specific time points of development, i.e. E.10.5, E12.5, E14.5 and E16.5, revealed that robust Sox2 expression could only be detected in the double transgenic intestinal epithelium from E14.5 onwards (Figure S1A), coinciding with the phase of cell specification in the embryonic gut. The level of ectopic Sox2 expression was compared with endogenous Sox2 levels in the stomach by qPCR analysis, showing a 6-fold increase on RNA level (Figure S1B). As Sox2 expression in the

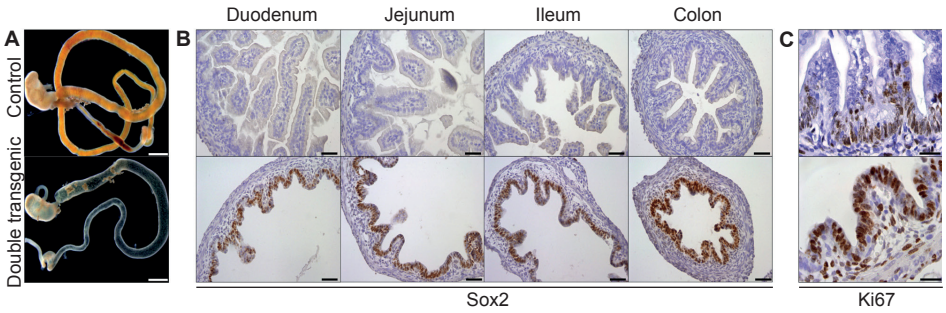

Figure 1. Ectopic expression of Sox2 severely affects the intestinal tract. (A) Macroscopic appearances of the digestive tracts from stomach until rectum isolated at E18.5 of a non-transgenic embryo (top) and double transgenic embryo treated with doxycycline (bottom), showing that Sox2 induction leads to dilated and fluid-filled intestines. Scale bar, 2 mm. **(B)** IHC using an antibody against Sox2 on cross-sections of the duodenum, jejunum, ileum, and colon reveals specific nuclear staining in the epithelium of double transgenic animals throughout the intestinal tract, whereas Sox2 is absent in the control. Scale bar, 50 μm. **(C)** IHC using an antibody against Ki67 shows an increased number of cycling cells in the double transgenic embryos, compared to control intestine. Moreover, proliferating cells were randomly distributed throughout the intestinal epithelium of the double transgenic animals, whereas proliferation is restricted to the prospective crypt compartment at the base of villi in control intestines. Scale bar, 20 μm.

E18.5 embryonic stomach is restricted to the basal cells of the forestomach, the quantitative polymerase chain reaction (qPCR) analysis will likely overestimate transgenic Sox2 expression. Therefore, we compared expression levels by immunohistochemistry (IHC) at limiting antibody concentrations, showing that on a cell-to-cell basis transgenic Sox2 is only expressed at slightly higher levels than endogenous stomach Sox2 (Figure S1C).

Ectopic Sox2 expression causes alterations in proliferation and malformation of the developing intestinal tract

Cross-sections of the intestines ectopically expressing the Sox2 transgene showed a two-fold increase in luminal space (Figure S2). In addition, the small intestines did not form true, morphological villi. Even the epithelial folds that resembled villi were markedly reduced per equivalent surface area (Figure 1B).

One possible explanation for the dilated intestinal tract could be a disturbed balance between proliferation and apoptosis. Using Ki67 staining to mark cycling cells (Figure 1C and S3A), we observed that proliferation in the cells of the double transgenic animals was no longer confined to the intervillous, primitive crypt regions as in the controls, but distributed randomly throughout the epithelium of Sox2 expressing embryos. In addition, the total number of Ki67-positive epithelial cells increased from approximately 35%-40% in controls to 85%-90% in the Sox2 expressing embryos (Figure S3A). Staining for activated caspase 3 revealed no differences in apoptosis (data not shown).

Thus, ectopic expression of Sox2 impairs villus formation and greatly alters the appearance of normal intestinal epithelium. These changes are accompanied by an increase and mislocalization of cycling cells.

Ectopic Sox2 expression in the intestine results in loss of intestinal identity

Next, we analyzed the differentiation potential of the embryonic intestinal epithelium to understand the phenotypic abnormalities observed in the Sox2 expressing intestines. Both mucin2 (Muc2) IHC and qPCR showed a dramatic loss in the number of intestinal mucin producing goblet cells in the double transgenic animals (Figure 2A,C and S3B). Similar results were obtained for enterocytes using qPCR for Lactase (*Lct*) (Figure 2C). We also observed reduced numbers of synaptophysin (Syp) positive cells and reduced expression of Chromogranin A (*ChgA*), both markers for enteroendocrine cells though not exclusive for the intestinal epithelium (Figure 2B,C and S3C). In addition, periodic acid schiff (PAS) staining demonstrated that the intestinal brush border layer was entirely absent in the double transgenic animals, while clearly present in the controls (Figure 2D). As the brush border contains several transporters for micronutrients, lack of absorption across this border may explain both the dilatation of the small intestine and the fluid retention we observed. We tested the expression level of several transporters expressed in the intestinal epithelium by qPCR. Analysis of two members of the solute carrier family, *Slc2a2* and *Slc5a1*, which are responsible for active and passive glucose transport, respectively [27], showed that these genes were downregulated in the double transgenic animals (Figure 2C). In addition, the expression levels of several members of the Aquaporin family, which are of major importance for water transport, were downregulated, as shown by qPCR (Figure S4).

Overall, ectopic expression of Sox2 in the intestinal epithelium results in loss of intestinal identity which is accompanied by reduced expression of various transporters.

Sox2 expression in the intestine results in cell fate conversion

In order to better understand the underlying changes induced by expression of Sox2 in the intestinal epithelium, we performed microarray analysis using intestines of E18.5 pups of three control and two double transgenic animals, which received doxycycline from E8.5 onwards. Hierarchical clustering of differentially expressed genes revealed large differences between controls and double transgenic intestines (Figure 3A). Differentially expressed genes in the Sox2 induced animals are involved in development and signal transduction, and are associated with a more immature and proliferative phenotype (Figure S5A-C).

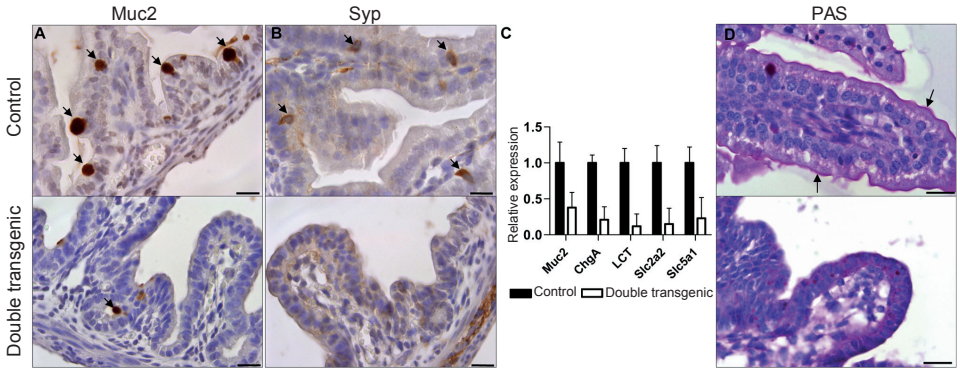

Figure 2. Sox2 affects the normal differentiation of intestinal epithelium. Cross-sections of the duodenum at E18.5 of controls and double transgenic embryos, which received doxycycline. IHC using antibodies against Mucin2 **(A)** and synaptophysin **(B)**, showed a reduced number of goblet cells and enteroendocrine cells, respectively, in the double transgenic animals (positive cells are indicated by arrows). **(C)** Analysis of the expression level of marker genes of goblet cells (*Muc2*), enteroendocrine cells (*ChgA*) and enterocytes (*Lct*) by qPCR showed a significant reduction of expression in the small intestine of double transgenic embryos at E18.5. Additionally, qPCR was used to determine the expression levels of two genes specific for the intestinal brush border, i.e. members of the solute carrier family (Slc), *Slc2a2* and *Slc5a1*, which are involved in glucose transport. Both are downregulated in double transgenic embryos. **(D)** PAS staining revealed the absence of the intestinal brush border in Sox2 induced animals compared to the controls (arrows indicate the positive lining of the brush border). Scale bar, 25 μm.

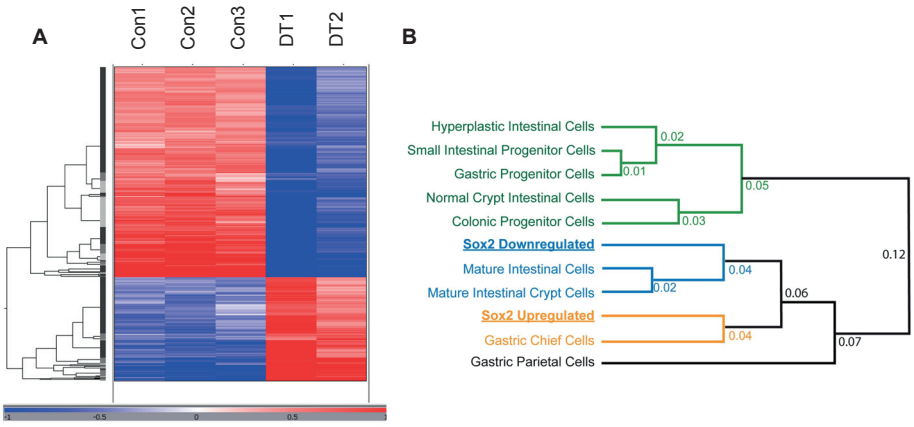

Figure 3. Transcriptome analysis reveals upregulation of gastric cell-specific transcripts by Sox2. (A) OmniViz Treescape showing the hierarchical clustering of Affymetrix probe sets that matched the selection query. Gene expression levels compared with the geometric mean are indicated in red for up-regulated genes and in blue for down-regulated genes. The color intensity correlates with the degree of change. **(B)** Sox2-upregulated and Sox2-downregulated gene lists were compared based on their associated GO term fractional representations with previously described GO term profiles of several other gastrointestinal cells and tissues [42]. "Mature intestinal crypt cells" refers to an expression profile of genes derived from β-catenin deleted mice [43], which causes crypt cells to mature. "Hyperplastic intestinal cells" refers to PTEN-deficient intestinal cells, which become hyperplastic [44].

In addition, close examination showed that the downregulated genes in the Sox2 overexpressing animals are involved in intestinal transport and lipid handling, typical processes for the absorptive function of the intestine (Figure S5D).

We next used the pattern of GO term enrichment to integrate our Sox2 profiles with a database of gastric and intestinal cells and tissues of varying degrees of differentiation (Figure 3B). That analysis showed that genes whose expression was induced by ectopic Sox2 most resembled the gene expression profile of gastric zymogenic chief cells, whereas genes whose expression was decreased by Sox2 most resembled genes expressed in mature intestinal cells. Thus, by GO term enrichment, Sox2 induced genes expressed by gastric cells and reduced expression of normal intestinal genes. Ectopic Sox2 had mixed effects on parietal cell genes, potentially due to overlap in the types of genes that parietal cells express and those that intestinal enterocytes express. For example, genes regulating lipid metabolism are characteristic of both enterocytes and parietal cells [28].

We followed up the global patterns of gene expression by confirming expression changes in individual markers with defined rostral-caudal patterns of expression in the gastro-intestinal tract. We analyzed markers identifying the presence of the esophagus and non-glandular gastric basal cells, the acid producing glandular gastric parietal cells, and gastric mucous neck cells. This showed that unlike the controls, the double transgenic animals had focal expression of p63-positive basal cells (Figure 4A and S3D), characteristic of basal cells of forestomach and esophagus. Sox2-expressing intestines also showed abundant H^+/K^+ ATPase4β positive cells of the gastric parietal lineage (Figure 4B and S3E) and GSII lectin positive mucous neck cells (Figure 4C) within the intestinal epithelium. Mist1, a marker for the zymogenic chief cell lineage in adult stomach [29], did not reveal a specific staining pattern (data not shown), which is in accordance with the lack of mature chief cells in the embryonic stomach. In addition, ectopic expression of the stomach specific *p63*, *H^+/K^+ ATPase4α*, *Mucin5ac* (Muc5ac, marks gastric surface pit cells) and *Keratin13* (Ker13, marks suprabasal cells) genes was detected using qPCR in the double transgenic intestine (Figure 4D). Ectopic expression of Sox2 in the intestinal epithelium did not clearly influence the underlying mesenchyme, as indicated by the unchanged low expression of the stromally expressed stomach-specific gene *Barx1* (Figure 4D) [30]. Checking the expression of *Gastrin* revealed a reduced expression (Figure 4D), which is in line with Gastrin producing cells being present embryonically in the intestine and pancreas, while emerging in the stomach only at birth [31]. Next, electron microscopy confirmed the lack of microvilli on the double transgenic intestinal epithelial cells (Figure 4E and S6). Moreover, it showed that ectopic Sox2 expression induced a more apical localisation of the nuclei, in contrast to the basal orientation in controls (Figure 4E).

Collectively, our data show that Sox2 expression in the intestinal epithelium causes anteriorization of the intestine with intestinal epithelial cells adopting the morphological and gene expression profile of immature gastric cells.

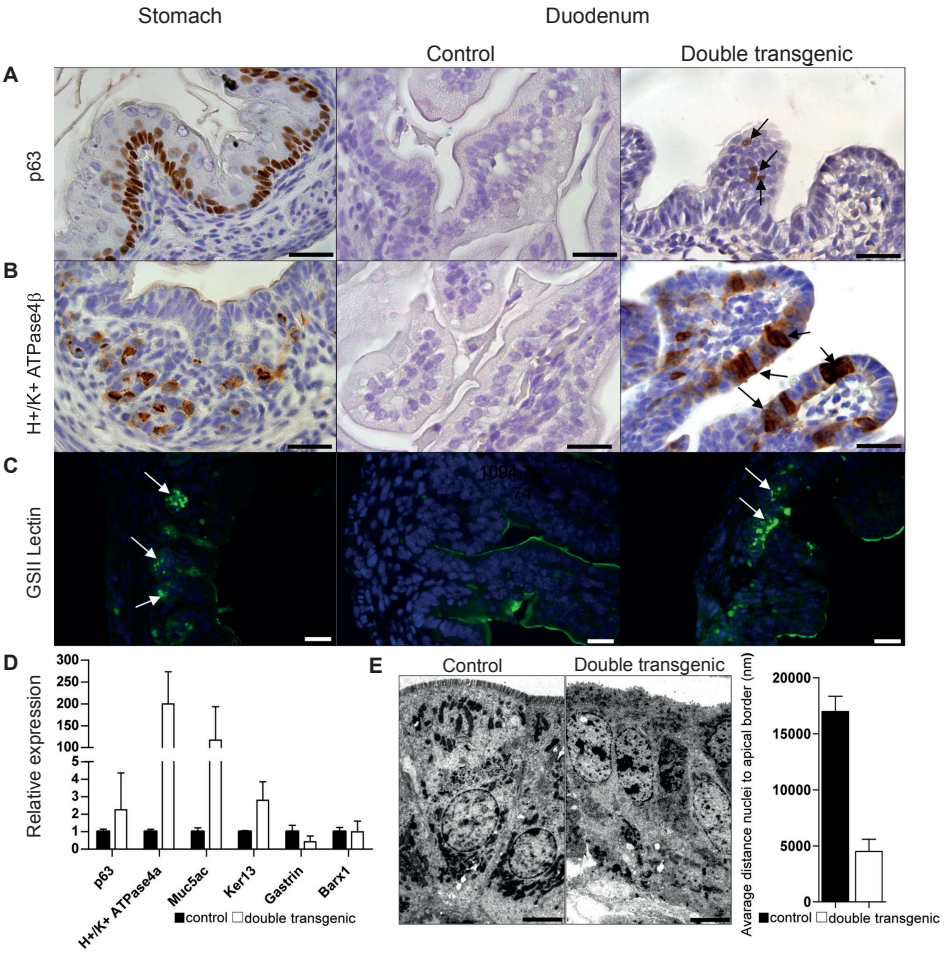

Figure 4. Sox2 induces stomach-like cells in the intestinal environment. Cross-sections of the stomach and duodenum at E18.5 of controls and double transgenic embryos, which received doxycycline. IHC using markers for basal cells (p63) **(A)** and parietal cells (H^+/K^+ATPase4β) **(B)** showed positive staining in the stomach. Ectopically expressed Sox2 induced the appearance of basal cells and parietal cells in duodenum of double transgenic animals (arrows), whereas control duodenum is devoid of staining. Immunofluorescence for GSII lectin **(C)**, a marker for the mucous neck cells in the stomach, showed positive cells in the double transgenic intestine (arrows), while no expression was found in the control. **(D)** Analysis of the expression of marker genes for basal cells (*p63*), suprabasal cells (*Ker13*), parietal cells (*H^+/K^+ATPase4α*) and gastric pit/surface-cell mucin (*Muc5ac*) by qPCR showed an increase in the small intestine of double transgenic animals. No significant change in expression level of the stomach-specific mesenchymal marker *Barx1* was detected. Expression of the Gastrin hormone was reduced in double transgenic animals. **(E)** Nuclei of normal intestinal epithelium are oriented towards the basal membrane, whereas the nuclei of the double transgenic intestinal epithelial cells are positioned more apically, shown by EM. Bar diagram represents the quantification of the distance of the nuclei to the apical border in control and double transgenic intestines. Scale bar, 6 μm.

Ectopic Sox2 expression in the intestine alters the functionality of Cdx2, but not its expression level

Considering all the changes caused by Sox2 expression, we examined whether Cdx2 expression was also decreased, thereby fitting the suggestion that Sox2 and Cdx2 are expressed in a mutually exclusive manner. qPCR showed that expression of the *Cdx2* mRNA was not altered (data not shown). Surprisingly, IHC for Sox2 (Figure 5A) and Cdx2 (Figure 5B) revealed that both transcription factors were expressed throughout the entire intestinal epithelium of the Sox2 induced animals, whereas only Cdx2 was expressed in the control. Moreover, nuclear co-localization of Cdx2 and Sox2 was shown by immunofluorescence using confocal microscopy (Figure 5C).

Thus, although Sox2 induced cellular changes leading to the occurrence of anterior gastro-intestinal cell types normally devoid of Cdx2, Cdx2 remained co-expressed in the Sox2 double transgenic intestines. However, the microarray analysis revealed several known Cdx2 target genes to be clearly downregulated in these Cdx2/Sox2 positive cells, including the well-established target gene *Cdh17* [32]. These reduced expression levels were confirmed by qPCR for the target genes, *Hnf4α*, *Heph*, *Cdx1* and *Mep1α* (Figure 6A) [22, 33]. Using IHC we confirmed that the number of Hnf4α positive cells was strongly decreased in the double transgenic animals (Figure 6B).

These results suggest that ectopic expression of Sox2 does not directly affect the expression of Cdx2 itself, but interferes with the subsequent activation of Cdx2 target genes. Therefore, we analyzed the binding of Cdx2 to the promoter regions of two known target genes by chromatin immunoprecipitation (ChIP) using embryonic intestines from control and double transgenic animals (Figure 6C and S7). We show that ectopic expression of Sox2 in the intestine leads to a strong decrease in binding of Cdx2 to its targets *Cdh17* and *Hnf4α*, whereas Sox2 does not bind to these sites. Specificity of the Sox2 ChIP was confirmed by the detection of increased binding of Sox2 to its target Sox21 in the double transgenic embryos (Figure S7). Thus, ectopic Sox2 expression in the intestinal tract does not affect the expression of Cdx2, but inhibits its function as a transcriptional regulator by interfering with the binding of Cdx2 to its gene target sites, which in turn prevents the activation of the intestinal transcriptome.

Discussion

During development of the gastro-intestinal tract, Sox2 is expressed in the anterior part of the primitive gut, while the homeobox gene Cdx2 is expressed in the posterior part [4]. Various studies have suggested that the expression of

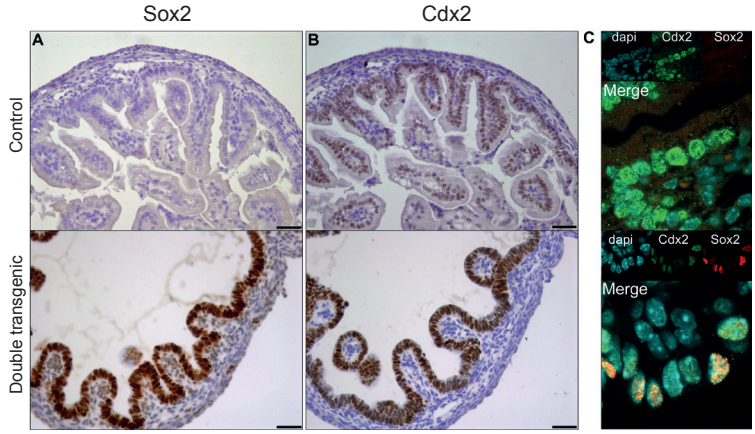

Figure 5. Sox2 and Cdx2 are co-expressed. IHC on sequential sections of E18.5 duodenum showed Sox2 expression only in double transgenic intestines **(A)**, and Cdx2 expression throughout the epithelium in both control and double transgenic embryos **(B)**. Confocal microscopy **(C)** of control and double transgenic animals showed that Cdx2 and Sox2 are co-expressed in the same intestinal epithelial cells in Sox2 expression embryos. Individual images of DAPI (blue), Cdx2 (green) and Sox2 (red) stainings are shown as inserts. Scale bar, 50 μm

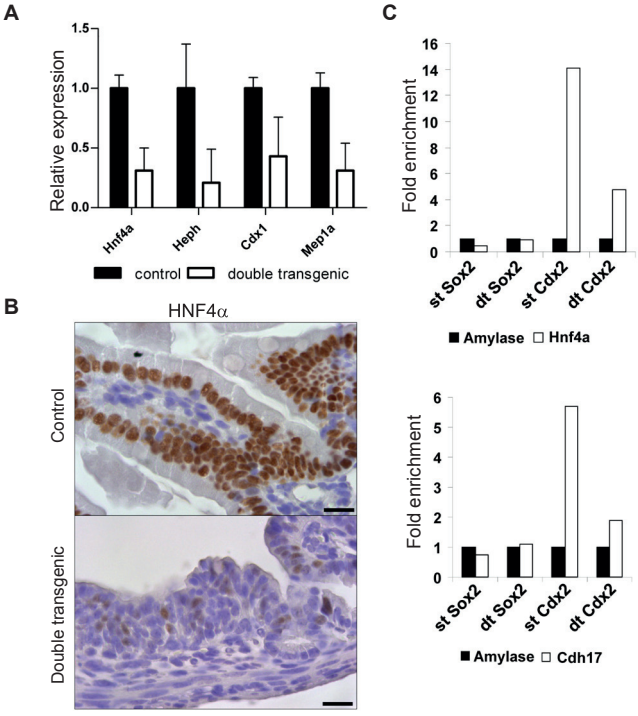

Figure 6. Sox2 affects the expression of Cdx2 target genes by inhibiting Cdx2 binding to target genes. (A) The expression levels of the Cdx2 target genes *Hnf4α*, *Heph*, *Cdx1* and *Mep1α* are significantly downregulated in the small intestine of double transgenic animals at E18.5. **(B)** IHC with an antibody against Hnf4α on cross-sections of duodenum at E18.5 of control and Sox2 overexpressing animals showed a dramatic loss of staining in the double transgenic animals. Scale bar, 20 μm. **(C)** ChIP assay showed a dramatic loss of binding of Cdx2 to *Cdh17* and *Hnf4α* in the double transgenic animals. Amylase served as a negative control.

Sox2 and Cdx2 is mutually exclusive, but it remains elusive whether one of the two is dominant over the other. Therefore, we ectopically expressed Sox2 in the prospective intestinal part of the primitive gut to investigate whether Sox2 has a dominant role over Cdx2 in the specification of the developing endoderm. We show that ectopic expression of Sox2 in the posterior region of the primitive gut caused aberrant activation of the foregut transcriptional program, leading to anteriorization of the gut with features resembling those of an immature embryonic stomach. Sox2 induction did not alter Cdx2 expression, but interfered with its function as a transcriptional regulator, providing evidence that Sox2 can exert a dominant effect over Cdx2 on cell fate.

Ectopic expression of Sox2 in the embryonic intestinal tract leads to a translucent and dilated intestine, which had a reduced number of villus-like outfoldings per surface area. These villi were poorly developed and showed increased numbers of cycling cells that were no longer confined to the intervillous regions, but were localized randomly throughout the intestinal epithelium. The differentiation towards goblet cells, enteroendocrine cells and enterocytes was dramatically affected. Instead, the Sox2 expressing epithelium differentiated into gastric-like cell types, such as parietal cells and basal cells, demonstrated by IHC, qPCR and GO term analysis. GO term comparative expression profile analysis also revealed that the genes upregulated by Sox2 were closer to cells of mature gastric zymogenic chief cell differentiation than to any of a variety of normal intestinal tissue gene expression profiles. These dramatic changes were accomplished in the short time-frame that ectopic Sox2 becomes expressed, i.e. E14.5-E18.5, demonstrating the dominant influence of Sox2 in the developing gut. Apparently, Sox2 is able to convert the cell fate of already committed embryonic intestinal cells into stomach-like cells. The prominent effect of Sox2 in determining cell fate was also highlighted by our previous study, in which we have shown that ectopic expression of Sox2 in the lung also results in dramatic changes in differentiation [11].

The fluid filled, swollen appearance of intestines of the Sox2 induced animals is most likely caused by the dramatic decreased number of enterocytes combined with the disruption of the intestinal brush border. As a consequence of the decreased absorptive area, fluid can not be absorbed by the intestine, resulting in fluid retention. Our studies with ectopic Sox2 expression in intestines of mouse embryos complement a recent study reporting the effect of *Cdx2* ablation in mouse embryos [22]. *Cdx2* ablation severely affects the normal intestinal development, since mutant embryos fail to form the colon and rectum properly. Furthermore, the duodenum of the *Cdx2* mutant animals was distended and became translucent. Also the disturbed proliferative pattern strongly resembles our observations. In our study the total number of epithelial cells was not significantly altered (data

not shown), which is in accordance with the equal number of cells in the mitotic phase of the cell cycle as revealed by phospho-Histone H3 staining (20%-25% for both genotypes, data not shown). These results indicate that although Sox2 expression strongly affects the number and position of cycling cells, it most likely also leads to an increased duration of the cell cycle, effectively resulting in an equal production of epithelial cells.

Interestingly, Gao et al. also showed ectopic activation of Sox2 in the *Cdx2* mutant intestine, which implied that Sox2 may have been responsible for inducing the phenotypes that were observed [22]. Our results strongly support this concept, as we demonstrated that Sox2 induces the differentiation toward a premature stomach phenotype, in spite of sustained Cdx2 expression. We conclude that loss of Cdx2 by itself is not sufficient to drive the activation of the foregut transcriptional program, but requires expression of Sox2 to dominantly induce anteriorization.

Inactivation of *Cdx2* in adult intestines induced the expression of gastric markers, but did not lead to major morphological changes [30]. Although these authors showed that the gut stem cells in the crypts showed some plasticity to express gastric genes, the plasticity of the cells in the developing gut is much greater since we and Gao et al. showed complete induction of gastric genes and cell types [22]. This indicates that the adult intestinal epithelial cells have partly lost their potential to change their fate upon *Cdx2* ablation. In support of this, we also observed reduced plasticity when Sox2 is ectopically induced in adult animals (unpublished data).

More recently, Cdx2 was shown to also regulate epithelial cell polarity [34]. Depletion of *Cdx2* in the developing mouse embryo from E15.5 onwards, i.e. equivalent to the time point that we induce Sox2, led to the formation of irregular packed epithelial cells containing large subapical vacuoles and a disorganized pattern of several apical and basolateral markers. In our case, staining for ZO-1 (apical), E-cadherin (basolateral), and laminin-1 (basement membrane) revealed that epithelial polarity was not clearly affected (data not shown). We also did not observe the vacuoles and irregular packing of epithelial cells. These differences in phenotype are possibly explained by the incomplete functional downregulation of Cdx2 induced by Sox2 expression.

Ectopic expression of Sox2 did not affect the expression of the intestinal specific transcription factor Cdx2. This is in apparent contrast with work published by Benahmed and co-workers, who investigated the regulation of Cdx2 expression [35]. Using cellular approaches with a 13 kb genomic fragment covering part of the *Cdx2* locus, they showed that Sox2 had a profound inhibitory effect on Cdx2 transcriptional activity. As our study shows that the endogenous locus is not affected by Sox2, this either implies that the proposed Sox2 binding site in the 13 kb fragment is not easily accessible in the endogenous setting or that for proper

Cdx2 regulation additional domains are required, e.g. the 3' located enhancer. Previous studies have provided limited data to suggest that Cdx2 regulates Sox2 expression, but no direct binding of Cdx2 to the *Sox2* promoter region was shown [4, 22, 36]. Nishiyama et al. recently described a genome wide analysis of Cdx2 target sites using ChIP technology and clearly demonstrated that Cdx2 did not bind to the *Sox2* locus [37]. Verzi et al. analyzed the Caco-2 intestinal carcinoma cell line for putative CDX2 binding sites and identified a ChIP peak in an intergenic region in proximity to the *SOX2* locus [33]. However, it remains unclear whether the binding site had functional relevance to the *SOX2* gene.

Although Cdx2 was not affected at the transcriptional level, its function is severely affected as deduced from the strong downregulation of several Cdx2 target genes. We show that Sox2 interferes with the binding of Cdx2 to its genomic target sites, thereby preventing its transcriptional activity. At present, the precise molecular mechanism to explain the inhibitory effect on Cdx2 DNA binding is unclear. Possibly Sox2 competes with co-factors that are needed by Cdx2 for its binding activity [33], or Sox2 may induce differentiation into cell types that lack the expression of factors required by Cdx2 to bind efficiently to its target sequences.

In conclusion, we show that ectopic expression of Sox2 leads to impairment of villus formation and strongly alters the appearance of the normal intestinal epithelium. We show anteriorization of the primitive gut, demonstrated by cell fate conversion from intestinal epithelial into immature stomach-like cells. This demonstrates the dominant influence of Sox2 in the developing gut, since Sox2 is able to convert the cell fate of already committed intestinal cells into stomach-like cells. Sox2 induction does not alter Cdx2 expression, but inhibits its function as transcriptional activator by interfering with its DNA binding capacity. This supports the idea that a balance between Sox2 and Cdx2 may dictate proper patterning of the foregut endoderm and that Sox2 may exert a dominant effect on intestinal cell fate by altering the differentiation program, emphasizing the plasticity of the primitive gut.

Our work improves the understanding of the mechanisms underlying the formation of the gastrointestinal tract and provides insight into the possible origin of various human congenital anomalies of the gut, such as Meckel's diverticulum, in which ectopic gastric tissue can be present in the small intestine. In addition, it may clarify the functional consequences of the ectopic SOX2 expression that we and others observed in human colorectal cancer (unpublished data) [25].

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Supplementary Figures

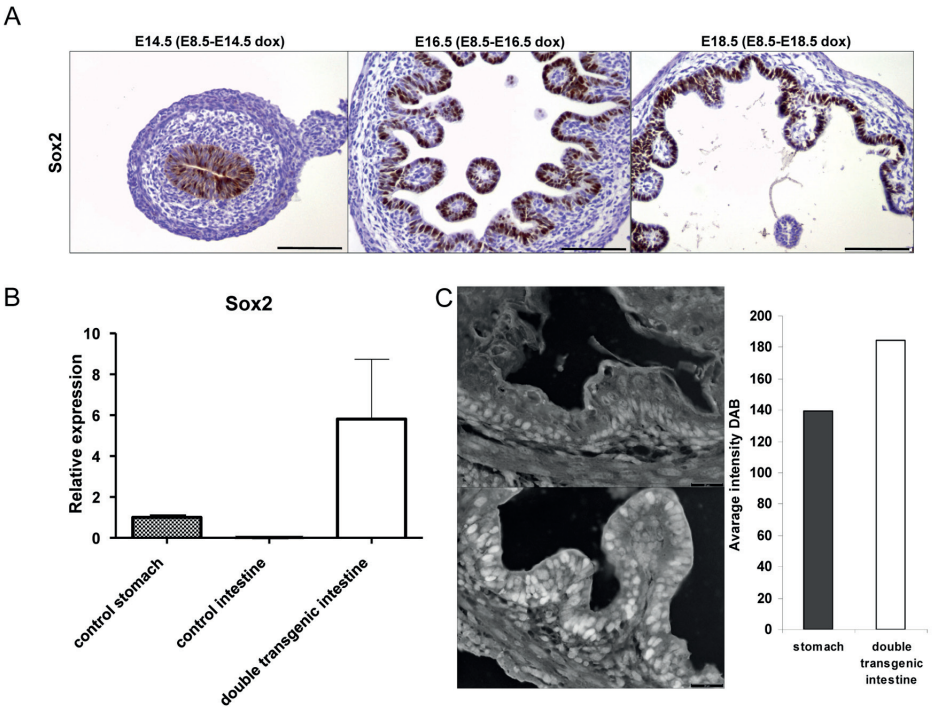


Figure S1. Robust expression of induced Sox2 at E16.5. IHC using an antibody against Sox2 on intestinal cross-sections at E14.5, E16.5 and E18.5 of double transgenic animals shows that robust Sox2 expression is observed from E14.5 onwards **(A)**. In order to evaluate the expression level of induced Sox2, we compared *Sox2* mRNA expression levels of Dox-induced double transgenic embryos with the stomach and intestine of control animals. We show an approximate 6-fold increase of expression of induced *Sox2* compared to the endogenous level in the stomach. As expected *Sox2* expression in the control intestine was negligible **(B)**. Additionally, we analysed the unaffected stomach and the intestinal tract of the induced double transgenic animals with immunohistochemistry using limiting amounts of Sox2 antibody. Because the titration of the antibody showed diminishing staining, we could perfectly compare the intensity of the staining in de stomach and intestine and quantify the expression level of the ectopically expressed Sox2 in the intestinal epithelium using ImageJ. This revealed an average increase in intensity of 32% in the intestine, compared to endogenous Sox2 expression in the stomach **(C)**. Scale bar, 50μm.

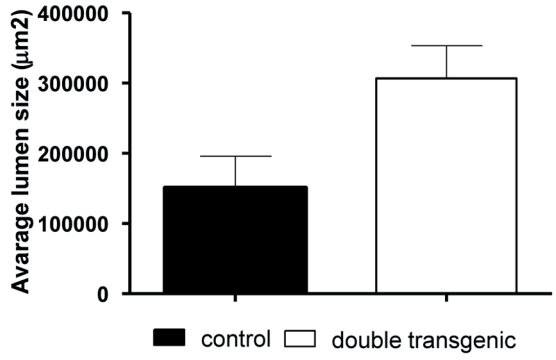


Figure S2. Ectopic Sox2 induces significant enlargement of the intestine. The average intestinal lumen size was measured in at least three controls and double transgenic animals. The double transgenic animals show a two-fold increase in lumen size.

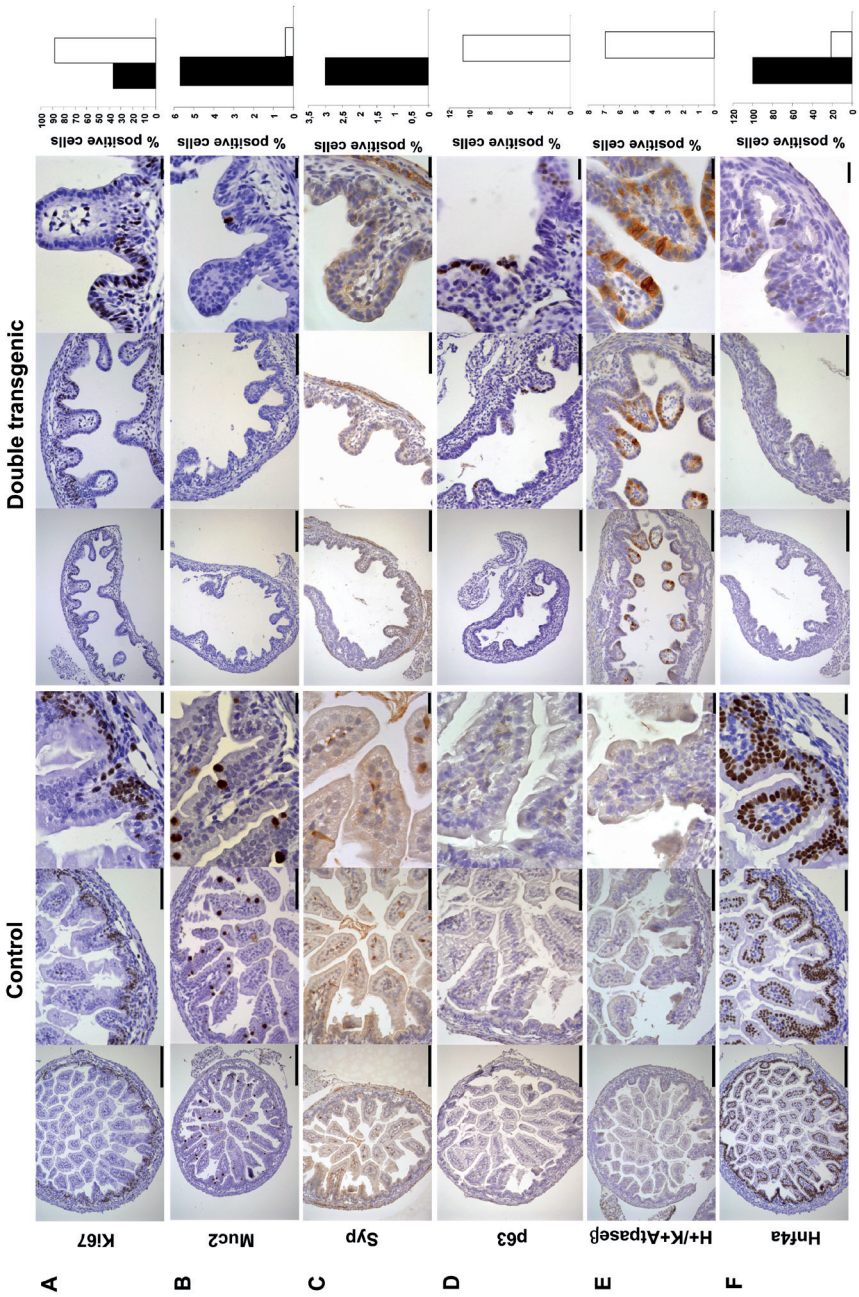


Figure S3. Overview of IHC on control and double transgenic intestines. Overview of IHC results with antibodies against Ki67 **(A)**, Muc2 **(B)**, Syp2 **(C)**, p63 **(D)**, H^+/K^+ Atpase4β **(E)** and Hnf4α **(F)** on cross-sections of duodenum of control (left panels) and double transgenic (center panels) animals at E18.5. Each panel consists of three images, representing 100, 200 and 400 times magnification (scale bars: 200, 100 and 20 μm, respectively). Quantification is represented as the number of positive cells per total number of epithelial cells in at least 3 microscopic fields (right panels). Black bars represent the control and white bars the double transgenic animals.

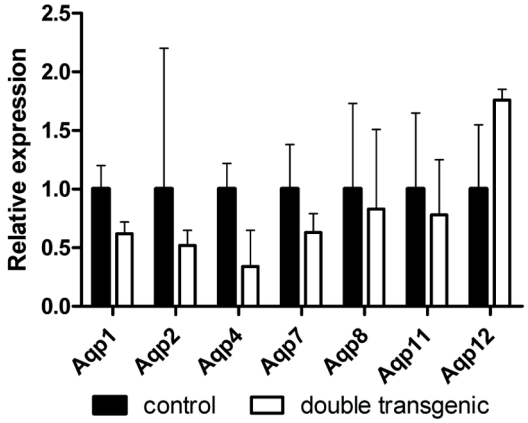


Figure S4. Quantification of the expression of aquaporin family members. Analysis of the expression of Aquaporin family members *Aqp1*, *Aqp2*, *Aqp4*, *Aqp7*, *Aqp8*, *Aqp11* showed a downregulation in the double transgenic animals, whereas *Aqp 12* was upregulated compared to the controls.

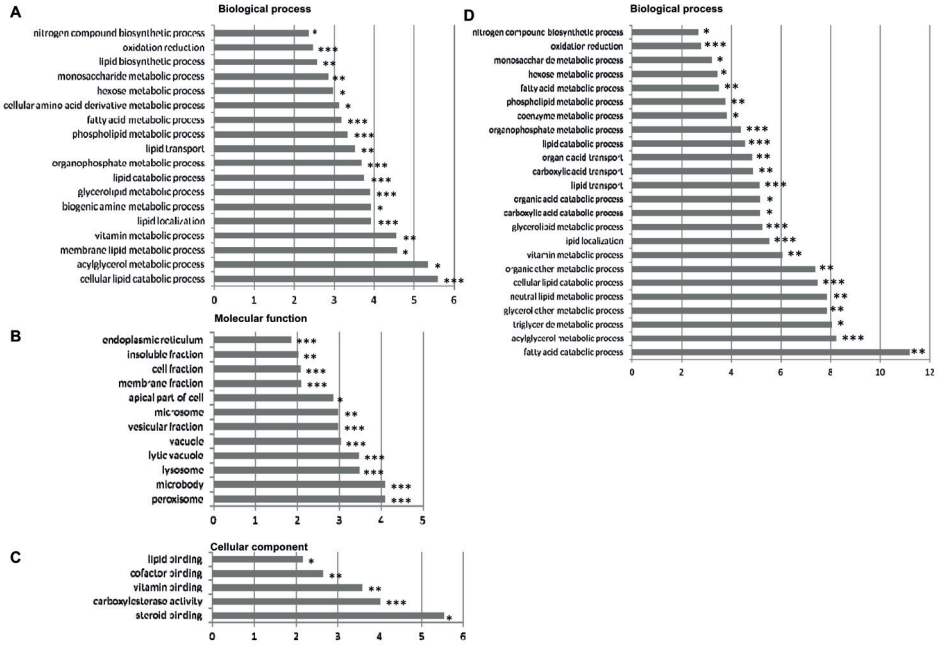


Figure S5. GO enrichment analysis of control and double transgenic intestines. Enriched GO terms within the set of 1,354-regulated probesets (both up- and downregulated genes) for GO biological processes **(A)**, GO molecular function **(B)** and GO cellular component **(C)**. Enriched categories are those identified as significantly enriched (P<0.05) after multiple testing. *P < 0.05; **P < 0.01; ***P < 0.001. **(C)** Enriched GO terms within the set of 906-downregulated probesets for GO biological processes **(D)**. Enriched categories are those identified as significantly enriched (P<0.05) after multiple testing. *P < 0.05; **P < 0.01; ***P < 0.001.

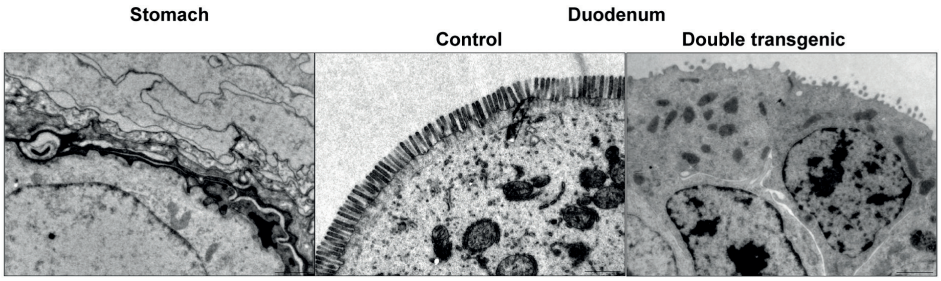


Figure S6. Sox2 affects the intestinal brush border. Close up electron microscopy images of the intestinal brush border of control and double transgenic E18.5 pups, and of the embryonic stomach, which does not have a brush border. Scale bar, 1.4μm.

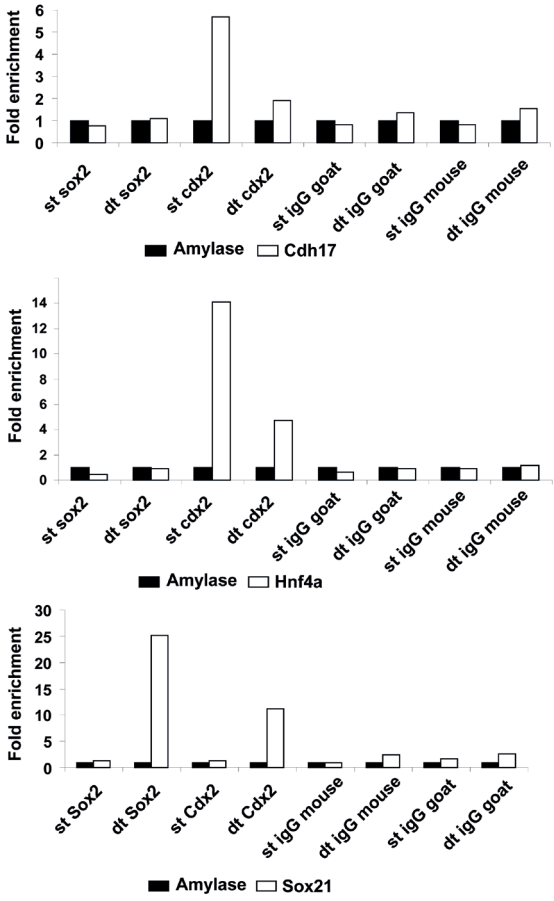


Figure S7. Sox2 interferes with Cdx2 binding to target genes. ChIP assay for Cdx2 shows loss of binding to *Cdh17* (top panel) and *Hnf4*α (middle panel) in the double transgenic animals, compared to the control. Sox2 does not bind to *Cdh17* or *Hnf4*α, but does bind to its downstream target *Sox21* (bottom). Mouse IgG or goat IgG serve as negative antibody controls Cdx2 and Sox2, respectively. Amylase served as negative control for the qPCR.

Supplementary Materials and methods

Microarray analysis

The intestinal tract of three control and three double transgenic embryos, which received doxycycline from E8.5 onwards, were isolated at E18.5 and individually used for total RNA isolation with Trizol reagent (Invitrogen life technologies, Carlsbad, CA, USA). RNA was purified using RNeasy MinElute Cleanup kit (Qiagen, Valencia, CA, USA) and cDNA was synthesized using the GeneChip Expression 3'-Amplification Reagents One-Cycle cDNA Synthesis kit (Affymetrix, Santa Clara, CA, USA). Biotin-labelled cRNA synthesis, purification and fragmentation were performed according to standard conditions. Fragmented biotinylated cRNA was subsequently hybridised onto Affymetrix Mouse Genome 430 2.0 microarray chips.

To examine the quality of the various arrays, several R packages (including affyQCreport) were run starting from the CEL files. All created plots, including the percentage of present calls, noise, background, and ratio of GAPDH 39 to 59 (1.4) indicated a high quality of all samples and an overall comparability, except for one sample, which was excluded from further analysis. Raw intensity values of all samples were normalized by RMA normalization (Robust Multichip Analysis), background correction and quantile normalization using Partek version 6.4 (Partek Inc., St. Louis, MO).

The normalized datafile was transposed and imported into OmniViz version 6.0.1 (BioWisdom Ltd., Cambridge, UK) for further analysis. For each probe set, the geometric mean of the hybridization intensity of all samples was calculated. The level of expression of each probe set was determined relative to this geometric mean and 2log-transformed. The geometric mean of the hybridization signal of all samples was used to ascribe equal weight to gene expression levels with similar relative distances to the geometric mean. Differentially expressed genes were identified using statistical analysis of microarrays (SAM). Cutoff values for significantly expressed genes were the number of falsely called less than 1 (FDR of 0.0006 or less) and a fold change of 2. Functional annotation of the statistical analysis of microarrays results was done using Ingenuity Pathway Analysis (Ingenuity, Mountain View, CA) and DAVID (http://david.abcc.ncifcrf.gov). DAVID calculates significant overrepresentation of gene ontology (GO)-classified biological processes. The results are shown for biological processes, which are significantly ($P < 0.05$) enriched after multiple testing.

To analyze higher-order differentiation patterns defined by the 449 genes induced and 906 downregulated significantly by Sox2, each gene list was re-expressed in terms of the fractional representation of GO terms associated with its member genes using the GOurmet software suite [1]. The distribution of GO term fractional representations were then used as a metric to classify the Sox2 expression profiles to a database of expression profiles of mature, proliferating, and hyperplastic gastrointestinal tissues, as described before [2].

Chromatin immunoprecipitation

The small intestines of, respectively, 10 double transgenic and 10 control E18.5 embryos were pooled and mechanically disrupted, after incubating for 20 minutes in ColagenaseII at 37°C. Next we filtered the cell suspension using a 70μm cell strainer. Formaldehyde was added to a final concentration of 1% for 10 min on RT to crosslink the samples and this process was stopped by adding 0.15 M glycine. Cells were washed with PBS and lysed for 10 minutes on ice in cell lysis buffer (10mM Tris pH 8.0, 10 mM Nacl, 0.2% NP-40 and complete protease inhibitors). After centrifugation the nuclei were resuspended in nuclei lysis buffer (50mM Tris pH 8.0, 10mM EDTA, 1% SDS and complete protease inhibitors) and the chromosomal DNA was fragmented by sonication (20 cycles of 15 seconds, 45 seconds in between cycles) to yield DNA fragments with an average size of 500bp.

Equal amounts of sample were diluted 1:10 with ChIP dilution Buffer (0.01% SDS, 1.1% Tx-100, 1.2 mM EDTA, 16.7mM Tris-HCL pH 8.1 and 167 mM Nacl) and from each sample 50μl was removed to serve as input control. The samples were pre-cleared with 80μl Prot A/G agarose beads for 1 hour. Next, these samples were incubated O/N with pre-formed complexes of 10 μg antibody specific for Sox2 or Cdx2, or control IgG (goat and mouse) with 150 μl Prot A/G agarose beads.
Beads were washed with Low salt immune complex buffer (0.1% SDS, 1% Tx-100, 2mM EDTA, 20mM Tris-HCL pH 8.0 and 50 mM Nacl), High salt immune complex buffer (0.1% SDS, 1% Tx-100, 2mM EDTA, 20mM Tris-HCL pH 8.0 and 150 mM Nacl), LiCl immune complex buffer (0.25 M LiCl, 1% NP-40, 1% deoxycholate, 1mM EDTA and 10mM Tris-HCL pH 8.0) and twice with TE (10mM Tris-HCL pH 8.0, 1mM EDTA pH 8.0). The DNA was eluted by adding twice 250 μl elution buffer (1% SDS and 0.1m NaHCO3). Next the samples and the input were incubated at 65°C O/N using 20 μl 5M NaCl to de-crosslink the DNA and proteins. The eluted material was phenol-extracted and ethanol-precipitated. The DNA was resuspended in 26μl of water and qPCRs were performed to analyze the enrichment of Cdx2 for binding to *Hnf4α* and *Cdh17*, and Sox2 for binding to *Sox21*, using Amylase as control.

1. Doherty JM, Carmichael LK, Mills JC. Gourmet: a tool for quantitative comparison and visualization of gene expression profiles based on gene ontology (GO) distributions. BMC Bioinformatics 2006;7:151.
2. Doherty JM, Geske MJ, Stappenbeck TS, Mills JC. Diverse adult stem cells share specific higher-order patterns of gene expression. Stem Cells 2008;26:2124-30.

Supplementary Tables

Supplementary Table I

Antigen	Clone	Target	Source	Concentration	Dilution	Kit
Myc-epitope	9E10	Myc-epitope	Roche	5mg/ml	1:800	None
Sox2	401196	Sox2	Immune systems	1mg/ml	1:500	ABC-kit
Cdx2	Cdx2-88	Cdx2	Biogenex	10-15mg/ml	1:20	ABC-kit
P-histoneH3	32219	mitosis	Upstate	1mg/ml	1:800	ABC-kit
Ki67	TEC-3	proliferation	Dakocytomation		1:50	ABC-kit
Cl.Casp. 3	5A1E	apoptosis	Cell Signaling	100µg/ml	1:100	ABC-kit
Mucin 2	H-300 Sc-15334	goblet cells	Santa Cruz	1mg/ml	1:400	ABC-kit
Synaptophysin	A0010	enteroendocrine cells	DakoCytomation	300µg/ml	1:250	Envision
p63	4A4 Sc-8431	basal cells	Santa Cruz	200µg/ml	1:200	ABC-kit
H^+/K^+ ATPase 4β	2G11	parietal cells	Thermo scientific	10µg/ml	1:2000	ABC-kit
HNF4	K9218	HNF4	Abcam	1mg/ml	1:100	ABC-kit
GSII Lectin	L21415	mucous neck cells	Molecular probes	1mg/ml	1:2000	None
Mist1		zymogenic chief cells	Gift of Jason Mills		1:500	None
ZO-1	61-7300	Apical membranes	Invitrogen	250µg /ml	1:50	None
Laminin	L9393	Basement membranes	Sigma		1:400	None
E-cadherin	24E10	Lateral membranes	Cell Signaling		1:20	ABC-kit

ABC-kit: StreptABCcomplex/HRP (Dako)
Envision: Envision+systems (Dako)

Supplementary Table II

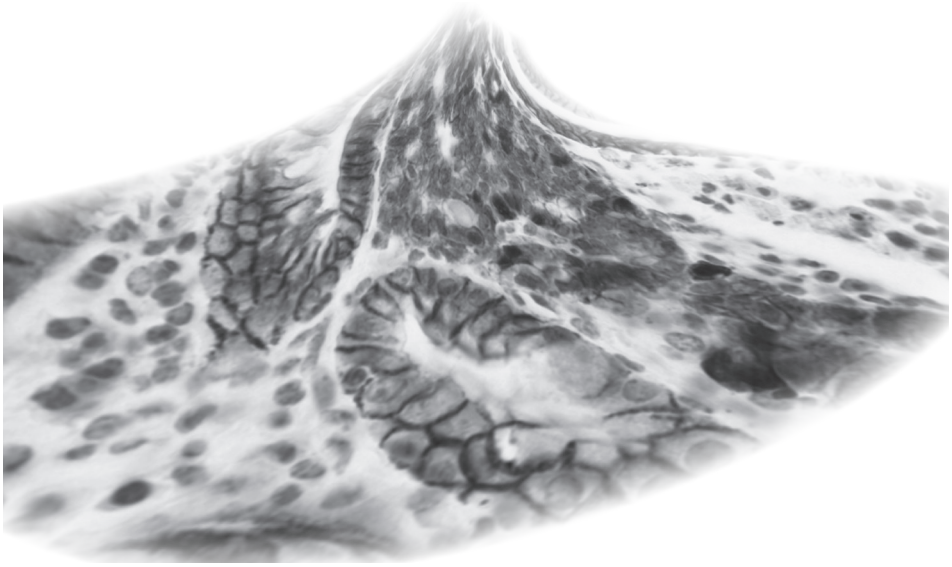
Genomic region	Sequence (forward)	Sequence (reverse)
Sox21	GCAGGCGCATAAATAAATAA	ATATCCATTCAAAGGGCATT
Cdh17	TTAAAACAACACCACCACCAC	CCCCAGTCAAACATTAACCAC
Hnf4α	AGGCTGAGGCTATGAGAAC	AACTCTCCCCTGACTCCTTGC
Amylase	CTCCTTGTACGGGTTGGT	AATGATGTGCACAGCTGAA

Supplementary Table III

Gene	Sequence (forward)	Sequence (reverse)
Aqp4	CTGTGATTCCAAACGAACTG	GGCTCCAGTATAATTGATTGCA
Aqp8	CTACTGGGACTTCCATTGGA	CCGATGAGGAGCCTAATGAG
H^+/K^+Atpase4α	GACCACTGATGATAATCTGTACCT	GATATTTGTGCTCTTGAACTCCTG
Cdx2	GTATGTCTGTGTTGTAAATGCC	AAACAATTCCGGTCTTCTTCAG
ChgA	CAGAAGTGTTTGAGAACCAGAG	TTCTCTTCTCCATAGTGTCCC
Krt13	CTGACTCTGGCTAAGACTGAC	AATTCCTTCATCTCCTCTTCGT
Muc2	TGCAACAACTTAACTGCTCTG	TCAGTATGGTAATAGCCAGCC
Muc5ac	CATGACCTGTTATAGCTCCGA	CTCAGTAACAACACAGCCTC
p63	CATTGTCAGTTTCTTAGCAAGG	CTCAATCTGATAGATGGTGGT
Slc2a2	CAGAAGACAAGATCACCGGA	GCATTGATCACACCGATGTC
Slc5a1	ATTGAAATAGACACAGAAGCCC	GTCATCTTTGGTCCTTTATCCT
Lct	GCTTCCTATCAGGTTGAAGGT	GTCGTCATTCCCAATCTTCAG
Hnf4α	CTTTGATCCAGATGCCAAGG	GGTCGTTGATGTAATCCTCCA
Mep1a	CATCTTCAGCTATAAATGGCTC	CTTCTGAAACAATCACAGTCCT
Heph	GCAGAAGAGATAGAGTGGGA	ATAGCTGTCTTTCTCAGATGTG
Cdx1	AAAGGAGTTTCACTACAGCC	GAACCAGATCTTTACCTGCC
Aqp1	CTCCCTAGTCGACAATTCAC	CCAATGATCTCAATGCCAG
Aqp2	ACCTCCTTGGGATCTATTTCAC	ATCATCAAACTTGCCAGTGAC
Aqp7	GAGCTACAGTTCAGTTGCAG	ATGAAGTAGGTTCTCTGAAGTG
Aqp11	TCACAGGAGCATTGTTTAACC	ATCAGCACACCTACAGAAGG
Aqp12	CACAGCCTTCTTGTCTACAG	GGATTGAAGAAGGCAGATGTG

CHAPTER 7

General Discussion



Wnt signaling is a main regulatory signaling network that is crucially involved in embryonic development, maintenance of tissue homeostasis and often deregulated in cancer. Although considerable knowledge on the role of Wnt signaling pathways in the intestine has been gained over the past decades, several aspects remain incompletely understood. Among these, the dynamics and activities of non-canonical Wnt5a signaling in the intestine during life are unclear. Furthermore, the impact of β-catenin signaling dosage on tumor formation in the intestinal tract and other organs remained to be solidly proven and elucidated further. In this chapter, the novel insights that we gained by investigating these topics will be discussed, finalizing by prospecting on possible therapeutic opportunities.

Non-canonical Wnt5a in intestinal development

Wnt5a has already been demonstrated to be indispensable for proper embryonic development of multiple structures, including the gastrointestinal tract [1-2]. The most solid evidence was presented by Cervantes et al, showing that *Wnt5a* knockout mice display a shortened gastrointestinal tract with an imperforate anus [1]. However, the dynamics and mechanisms of Wnt5a functioning during gut development remained unclear. We generated an inducible transgenic Wnt5a mouse model and induced Wnt5a expression during different timeframes of embryonic gut development. We learned that Wnt5a overexpression from E10.5 but not from E13.5 onwards also results in a shortened gastrointestinal tract with an imperforate anus, without affecting intestinal cytodifferentiation.

As mentioned, the activities of Wnt5a during gut development are not completely understood. The gut elongation defect as observed in *Wnt5a* knockout mice was explained by the authors to result partly from reduced epithelial cell proliferation [1]. However, despite resulting in a comparably shortened gastrointestinal tract, we did not observe gross changes in intestinal cell proliferation following Wnt5a overexpression from E10.5 and also *Ror2* knockout embryos show no altered intestinal cell proliferation (**Chapter 2**) [3]. The other part of the explanation for the intestinal elongation defect in Wnt5a knockout mice proposed by Cervantes et al. was that post-mitotic cells show defective intercalation into the growing gut epithelium [1]. Hence, they describe that at E11.5, the gut epithelium is thickened in *Wnt5a* knockout mice, resulting from accumulation of improperly intercalated cells, forming 3-4 cell layers rather than the single layer in control mice and thereby resulting in reduced gut elongation. Recently, a study by Grosse et al. has indicated that although during normal development, the gut epithelium is thickened and appears multi-layered at E12.5 and E14.5, it still constitutes of a

single cell layer of which the cells are more elongated in an apicobasal direction [4]. A process called interkinetic nuclear migration (INM) is suggested to be involved, wherein nuclei shuttle between the apical and basal sites in concert with the cell cycle. A similar process has been suggested in *Ror2* knockout mice [3]. With this knowledge, the multi-layered gut epithelium at E11.5 shown by Cervantes et al. might well represent a single cell layer in which different intracellular locations of nuclei were wrongly interpreted as multiple cell layers. However, whether Wnt5a indeed plays a role in nuclear movements during the cell cycle remains to be confirmed.

In abovementioned explanations it is assumed that the epithelium is the main determinant of intestinal elongation. However, tissue layers other than the epithelium, e.g. the underlying stromal or muscle layers, might as well provide the driving force for intestinal elongation. Supportively, studies in Xenopus have indicated that explants of a single layer of mesodermal cells (made from mid gastrula stage) converge and extend by mediolateral intercalation, leading to tissue elongation, independent of the endodermal epithelium [5-7]. In addition, in the embryonic mouse gut, Wnt5a is produced by mesenchymal cells, and Ror2 is expressed in both the epithelium and the mesenchyme [1, 3]. Thus, the mesenchymal layer might drive intestinal elongation, whereby loss of *Wnt5a* would perturb elongation of the mesenchymal layer. According to such a hypothesis, the epithelium would be a passive follower of the space created by the expansion of the mesenchymal tissue, rather than dictating elongation actively by itself. This could also explain the reduced epithelial proliferation as described by Cervantes et al. in the *Wnt5a* knockout mice, since limited space would consequently reduce the need for proliferation of the epithelial cells. Altogether, although some explanations for the defective gut elongation in *Wnt5a* knockout mice have been suggested, the mechanisms through which Wnt5a manipulation results in defective elongation of the gastrointestinal tract remain to be solidly demonstrated.

Our results show that Wnt5a gain of function during embryonic development results in a phenotype resembling a Wnt5a loss of function situation, a phenomenon reported for other genes as well [8-9]. Moreover, it was shown in Xenopus explants that both *XWnt5a* knockdown and overexpression resulted in a comparable phenotype, showing decreased explant constriction [10]. Additional studies in non-mammalian species including Drosophila, Xenopus and Zebrafish have indicated that loss and gain of Wnt5a function deregulate convergent extension (CE) and planar cell polarity (PCP) movements during embryonic morphogenesis [11-16]. When we induced transgenic Wnt5a expression in mice from E10.5 onwards, intestinal length was severely reduced, suggestive of deregulated morphogenic movements that probably involve PCP and CE. Given that the mechanisms

underlying the involvement of Wnt5a in mammalian gut elongation are poorly characterized to date, we can only speculate on how both gain and loss of Wnt5a function might result in defective gut elongation in mice. During normal embryogenesis, intestinal Wnt5a expression is restricted to the mesenchyme of the caudal midgut and rostral hindgut, thereby providing strict positional information directing intestinal elongation (Figure 1A) [1, 3, 17]. Both in the Wnt5a overexpression and *Wnt5a* knockout situation, a regionalized endogenous Wnt5a gradient is lost, resulting in lack of positional cues required for directional cell migration and gut elongation (Figure 1A-C). The strictly regionalized Wnt5a expression might cooperate with the complex expression pattern of Ror2, providing morphogenic Wnt5a gradients for Ror2 expressing cells in the developing gut. Supportively, it has been suggested that Ror2 mediates Wnt5a signaling in regulating polarized cell migration at locations other than the gut, such as in the developing palate and in various cell lines [3, 11, 18-20]. However, such a direct cooperation of Wnt5a and Ror2 expression patterns in the developing gut remains to be shown. Irrespective of whether indeed Ror2 is involved, we propose that the disturbance of strictly organized endogenous Wnt5a expression gradients results in loss of directional information during gut elongation, likely explaining the comparable gut elongation defects in both loss and gain of Wnt5a function scenarios. In this respect, it would be very interesting to know where new cells are generated along the developing and elongating gut, and how Wnt5a directs these cells to their proper position. Unfortunately, this is not known to date.

In support of our postulated explanation for the elongation defect following gain of Wnt5a function, the time dependency of our induced Wnt5a-mediated gut phenotype coincided with the period during which endogenous Wnt5a is normally expressed most prominently, i.e. between E10.5 and E13.5 [1, 3, 17]. Induction of Wnt5a expression after this period, starting from E13.5 did not affect gut elongation. Together, this indicates the requirement for properly regionalized Wnt5a expression in this crucial phase of gut elongation during embryonic development.

Other cellular components of non-canonical Wnt signaling have also been implicated in PCP and CE. These include Frizzled and Dishevelled, of which asymmetric localization is important as well, although at the cellular level [7]. In these cases, the source of polarizing information is described to be unclear, however, Wnt5a presents a logic candidate. Supportively, Wnt5a has been described to regulate the intracellular localization of Dishevelled and of focal adhesion and migration machineries [21-22]. On the cellular level, Wallingford et al. propose that in the absence of Dishevelled, there will be no positional cue and polarity in these cells [7]. Likewise, if there is too much Dishevelled protein, it can overload the localization machinery and consequently become uniformly

distributed. In both scenarios, polarity and thereby a chance for polarized cell migration would be lost. Although this was suggested for cellular Dishevelled expression, it supports and illustrates our proposal with regard to the possible consequences of either gain or loss of protein function. Altogether, we propose that both in the Wnt5a overexpression and *Wnt5a* knockout situation in mice, a regionalized morphogenic Wnt5a gradient is lost, resulting in lack of positional cues required for directional cell migration during gut elongation.

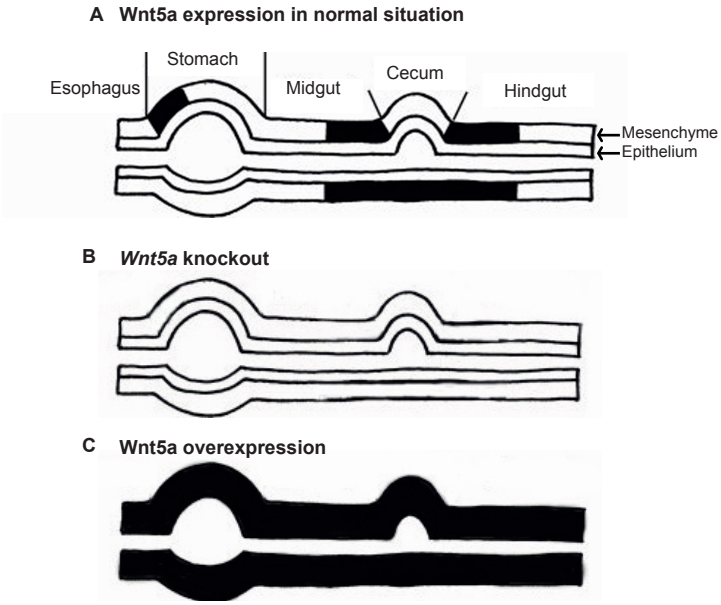


Figure 1. Hypothetical model of loss and gain of morphogens both resulting in loss of regionalized gradients and positional information. Schematic representation of the developing mouse gastrointestinal tract. (A) During normal development, Wnt5a expression is restricted to the mesenchyme of the caudal midgut and rostral hindgut in the intestine, providing positional cues. **(B)** The *Wnt5a* knockout situation and **(C)** Wnt5a overexpression scenario both lacking regionalized Wnt5a gradients, resulting in lack of positional cues required for directional cell migration and gut elongation.

An alternative candidate explanation for the observed intestinal shortening possibly involves the strongly downregulated Ror2 protein levels in our Wnt5a^{ind} embryos, thereby potentially phenocopying a *Ror2* knockout situation [3]. However, we do not consider this a solid explanation, given that residual Ror2 protein expression is retained and levels of the downstream signaling components pDvl2 and pJNK in Wnt5a^{ind} embryonic intestines are increased, indicating a net gain of Wnt5a signaling. Although we cannot formally exclude that the enhanced signaling is mediated via alternative Wnt5a receptors, such as the Frizzleds, it suggests that Ror2 reduction has not hampered Wnt5a signaling. We believe

that the reduced Ror2 expression is the result of high ligand exposure, followed by high ligand-receptor endocytosis and enhanced directing of receptors for degradation. Ror2 has been shown to undergo internalization upon Wnt5a stimulation and to present in early endosomes, but its lysosomal degradation remains to be proven [23-24]. Other Wnt5a receptors including Fz2, Fz4 and Fz5 have been suggested to undergo lysosomal degradation following internalization [25-27]. Such a mechanism where elevated ligand exposure directs more receptors towards lysosomal degradation has been described for the EGF receptor, in order to restrict signaling [28].

Taken together, Wnt5a gain of function before E13.5 in mice mimics the scenario of Wnt5a loss of function, both resulting in shortened gastrointestinal structures, anal malformation but unaffected cellular differentiation. This provides evidence that controlled spatial Wnt5a expression is required for proper elongation of the gastrointestinal tract and that Wnt5a is especially involved in this process before E13.5. Summarizing the timeframes of overall Wnt signaling activity during embryonic gut development, it is interesting to note that canonical and non-canonical Wnt5a signaling seem to alternate. Initially, transient canonical Wnt/β-catenin signaling is active from E7.5-E8.5, ceasing thereafter [29]. This is followed by the appearance of non-canonical Wnt5a activity, most prominently between E10.5-E13.5, which is necessary to direct the process of intestinal elongation. Finally, approaching birth, canonical Wnt/β-catenin signaling reactivates, contributing to the generation of proliferative intervillus regions and preparing for crypt development following birth. Thus, oversimplified, it appears that canonical Wnt/β-catenin signaling is involved in tissue specification during the earliest and latest stages of fetal intestinal development, whereas non-canonical Wnt5a mainly drives tissue elongation between these stages.

Non-canonical Wnt5a in intestinal homeostasis

In addition to a role for Wnt5a during embryonic development as indicated by in vivo studies, Wnt5a has also been implicated in postnatal processes. These include cell growth, differentiation, migration and invasion and are mostly indicated by in vitro studies [30-33]. Also, enhanced Wnt5a expression has been observed in several human diseases, including colon cancer [34-35]. Together this suggests a relevant postnatal role for Wnt5a, but, the perinatal lethality of *Wnt5a* knockout mice has hampered in vivo investigation of Wnt5a in the context of an adult organism. We induced Wnt5a expression in adult mice, using our newly generated inducible Wnt5a mouse model as described in **Chapter 2**. We strikingly found that induced Wnt5a expression was tolerated well by the mice. Thorough

examination of the intestinal tract revealed no abnormalities in length, morphology, cellular differentiation or proliferation. Also, intestinal Wnt/β-catenin signaling was unaltered following Wnt5a induction. Thus, in contrast to the possible involvement of Wnt5a in intestinal homeostasis as suggested in the General Introduction (**Chapter 1**), our results indicate that induced Wnt5a expression has no influence on proliferation, differentiation and oriented cell migration of epithelial cells along the crypt-villus axis during homeostasis. During the completion of this thesis, a study was published by Miyoshi et al. that supported the lack of phenotype that we observed following Wnt5a overexpression during intestinal homeostasis. Hence, their work indicated no consequences of conditional *Wnt5a* knockout under homeostatic circumstances in the adult mouse intestine, whereas crypt regeneration following intestinal wounding was disturbed in these *Wnt5a* deficient mice [98].

Initially, the absence of a phenotype following Wnt5a manipulation in the homeostatic adult mouse intestine might appear surprising, given the multiple signaling routes and cellular processes in which Wnt5a has been implicated [30-33]. On the other hand, our results and those of Miyoshi et al. [98] suggest a functional role of Wnt5a in morphogenic outgrowth processes and tissue reorganization, which occur during embryonic development and tissue repair, but not during intestinal homeostasis. Also, our findings that induced Wnt5a does not affect adult intestinal cell fate is in line with the absence of a cellular differentiation phenotype in the embryonic gut with either knockout or overexpression of Wnt5a (**Chapter 2**) [1]. Moreover, even the embryonic involvement of Wnt5a seems to be restricted to the time frame before E13.5, when rapid gut elongation occurs. Supportively, endogenous Wnt5a expression levels are relatively low in the adult intestine compared to the embryonic intestine, also applying to its receptor Ror2 (**Chapter 2**) [1, 3, 17, 34]. In this regard, it can be tempting to speculate that sufficient Ror2 expression is needed to acquire an effect following high Wnt5a ligand exposure. However, Wnt5a induction from E13.5 onwards did not cause a phenotype while Ror2 is clearly present in this stage, showing that the phenotypic consequences are not entirely dependent on Ror2 presence. After all, our data indicate no significant impact of induced Wnt5a expression during postnatal intestinal homeostasis.

Cancer of the intestinal tract

Canonical Wnt/β-catenin signaling in intestinal cancer

The vast majority of colorectal cancer cases results from aberrant activation of the Wnt/β-catenin signaling pathway. Specific *APC* genotype-tumor phenotype

correlations have been described and indicated the requirement of specific dosages of enhanced Wnt/β-catenin signaling to achieve successful tumor formation at different locations. According to the just-right signaling model, APC function must be impaired sufficiently to allow a specific degree of nuclear β-catenin signaling most optimal for successful tumor formation. Although the *APC* genotype-tumor phenotype correlations can be explained by the associated dosages of β-catenin signaling, several aspects remain unclear. Among these, tissue-specific tumor predisposition has not been formally proven to be the direct consequence of β-catenin signaling dosage and alternative models of colorectal cancer development have been proposed. Furthermore, the location preference of tumors within the colorectal tract depending on their type of genetic instability remained unexplained. We addressed these aspects in **Chapter 5** and **4** respectively.

Regarding the location preference of tumors within the colorectal tract, tumors harboring a deficient MMR system most often develop in the proximal colorectal tract (also referred to as right-sided colon), whereas most others occur in the distal (left-sided) colon [36-40]. The phenotype of MMR deficient tumors is generally distinct, being characterized by lymphocytic infiltration, mucinous histology, lack of chromosomal instability and LOH, and associated with a better prognosis than MMR proficient ones [41-43]. The mechanism underlying the side preference of MMR deficient tumors for the proximal colon has not properly been explained to date. Both distal and proximal sides of the colon differ in various aspects. For example, the proximal colon originates from the embryonic midgut, while the distal colon develops from the hindgut, concomitantly with their different blood supply. Moreover, features including bile acid metabolism, water resorption, luminal content, bacterial colonization, short-chain fatty acid production and various others are different in the proximal versus the distal colon [41, 44]. However, none of these aspects can solidly explain the side preferences of colorectal tumor formation.

As described in **Chapter 4**, the mutation spectra that are observed in tumors found on both sides of the colon are different. Focusing on *APC* mutations in tumors with a functional MMR system, distal colonic tumors preferentially acquire mutations retaining one 20-AAR, while APC truncations retaining two or three 20-AARs are predominantly found in proximal tumors [45-52]. These findings indicate that a preferred gradient of β-catenin signaling dosage for tumor initiation exists along the colorectal tract, with moderate levels being preferred proximally and higher levels preferred distally. A comparable phenomenon is also observed in different *Apc*-mutant mouse models, where in both the colon and the small intestine, the proximal parts prefer lower levels of β-catenin signaling to achieve tumor formation than the distal counterparts.

Then, how can we explain that different genetic defects lead to tumor development at different colorectal locations? Tumors with a functional MMR mostly harbor *APC* mutations with one 20-AAR remaining, a genetic signature preferred by the distal colon. Explanations for the high frequency of these *APC* mutations in case of MMR proficient tumors includes that the sequence length between the first and second 20-AARs is relatively large (**Chapter 4**, Figure 8). Logically, this region will thereby have an enhanced chance to acquire mutations compared to the shorter sequences in between the other 20-AARs. Moreover, the region between the first and second 20-AAR contains a AAAAG(2) repeat, prone to a deletion of 5 base pairs and representing one of the most common somatic *APC* mutations [53]. Thus, colorectal cells with a functional MMR will have a larger chance to acquire *APC* mutations with one 20-AAR remaining, thereby providing the optimal β-catenin signaling dosage for tumor development in the distal colon. On the other hand, MMR deficiency causes other types of mutations, associated with a 10-30 fold higher induction of single base pair substitutions [54-56]. In this case, sequences containing mono- or dinucleotide repeats are of special sensitivity; their mutation frequency can be increased up to 100-fold. The *APC* gene contains such repeats between the second and third 20-AARs and between the third 20-AAR and the first Axin binding domain (**Chapter 4**, Figure 8). Consequently, mutation at these repeats will give rise to truncated APC proteins with either two or three 20-AARs, associated with a relatively low gain in β-catenin signaling and thus being most ideal for tumor development in the proximal colon. Also the β-catenin gene will be subjected to enhanced induction of point mutations in case of MMR deficiency. Since β-catenin can become oncogenic following a single base pair substitution in one of the Ser/Thr amino acids in the N-terminus involved in its breakdown, one would expect to find relatively more *CTNNB1* mutations in MMR deficient than proficient tumors. This is indeed the case; whereas *CTNNB1* mutations are rarely found in MMR proficient tumors (<1%), 10% of MMR deficient colon tumors harbor oncogenic β-catenin mutations. *CTNNB1* mutations are associated with a comparable gain in β-catenin signaling as is the case following *APC* mutation retaining 2-3 repeats. Therefore, *CTNNB1* mutations are expected to be selected in the proximal colon, which is in accordance with over 80% of *CTNNB1* mutation carrying tumors indeed being found on this side of the colon. In summary, we propose that the side preferences of colorectal tumors can be explained by their particular mutational mechanism, resulting in *APC* or *CTNNB1* mutations associated with a specific level of β-catenin signaling that is optimal for either side of the colorectal tract.

Although the concept of β-catenin signaling dosage determining the success of tumor formation among tissues is gaining acceptance, tissue-specific tumor

predisposition was not formally proven to date to be the direct consequence of β-catenin signaling dosage rather than other APC-related functions. Furthermore, alternative models of colorectal cancer development have been proposed. Hence, APC is a multifunctional, large protein implicated in various cellular processes other than β-catenin regulation. At its C-terminus, APC can bind cytoskeletal components and loss of these regions has been implicated in disturbed cell migration and chromosomal segregation [57-58]. However, *Apc*-mutant mouse studies have shown that the C-terminal domains of Apc do not greatly influence intestinal tumorigenesis. Hence, *Apc*1638T mice lacking the C-terminal regions of Apc but retaining an axin-binding repeat remain tumor-free [59], and the tumor phenotype of *Apc*1322T mice expressing a truncated Apc retaining only 1 20-AAR is not influenced by reintroduction of the C-terminal regions of Apc [60]. Another protein which can bind APC is the transcriptional corepressor C-terminal binding protein (CtBP) [61-62]. Phelps et al. challenged the relevance of β-catenin signaling for colorectal tumor formation, suggesting that adenoma formation is triggered by enhanced levels of CtBP rather than β-catenin [63]. According to their observations nuclear β-catenin is only present following the acquisition of oncogenic *KRAS* mutations [63]. They based their argument against a role of β-catenin in tumor formation on the lack of its nuclear detection in early adenomas using immunofluorescence. However, as already put forward by Fodde and Tomlinson, although nuclear β-catenin expression is a reliable indicator of Wnt activation, its absence does certainly not exclude the robust activation of β-catenin target genes [64]. Moreover, the detection of nuclear β-catenin is generally recognized as a technical challenge, not achieved on frozen sections or using fluorescence. However, using immunoperoxidase methods, nuclear β-catenin is detected in human early adenoma, independent of *KRAS* mutation [64-65]. Further supporting these findings, we routinely detect nuclear β-catenin staining in the epithelial tumor cells of *Apc*1638N intestinal tumors (**Chapter 5**). Importantly, *Apc*1638N intestinal tumors and those of other *Apc*-mutant mouse models do not spontaneously acquire *Ras* mutations [66-68], indicating that oncogenic Ras is not required for nuclear accumulation of β-catenin in mouse intestinal tumors. This provides evidence that for both human and mouse intestinal cancer, oncogenic *KRAS* mutations are not absolutely required to impose nuclear β-catenin accumulation.

Subsequently we aimed to provide direct evidence for the importance of β-catenin signaling in intestinal tumor development. To this end, we used *Apc*1638N mice that normally develop approximately 1-7 gastrointestinal tumors and are highly susceptible for extra-intestinal manifestations, consisting of desmoids and cysts [69-70]. Reducing the pool of β-catenin in these mice by crossing them with heterozygous β-catenin knockout mice revealed interesting

results. Hence, by reducing the β-catenin levels in the *Apc*1638N setting, these mice were completely protected against gastrointestinal tumor formation, whereas all control *Apc*1638N mice with unaltered β-catenin alleles developed gastrointestinal tumors. In addition, the incidence of desmoids and cysts was strongly reduced following heterozygous β-catenin knockout. Strikingly, we demonstrated that the incidence of mammary tumor development was enhanced in the *Apc*1638N/*Ctnnb1*$^{-/+}$ mice. Normally, mammary tumors are rarely observed in *Apc*1638N mice but are characteristic for *Apc*1572T mice, which is a mouse model associated with a relatively lower gain in β-catenin signaling compared to *Apc*1638N mice. Also, characterization of the mammary tumors revealed a heterogeneous histology that was similar to that observed in *Apc*1572T mammary tumors [71]. Summarizing our results, we demonstrated that by simply reducing β-catenin levels in *Apc*1638N mice, the tumor phenotype shifted towards an *Apc*1572T-related phenotype, showing no gastrointestinal tumors, low numbers of desmoids and cysts but enhanced mammary tumor formation. We believe that in *Apc*1638N mice following loss of the wild type *Apc* allele, which is required for tumor initiation, we reduced the β-catenin dosage by heterozygous β-catenin knockout to levels approaching those associated with *Apc*1572T mice, thereby enabling successful mammary tumorigenesis.

Uncovering this shift in tumor phenotype from the gastrointestinal tract towards mammary tissues following β-catenin dosage reduction provides direct in vivo evidence that β-catenin dosage by itself dictates tissue-specific tumor predisposition in the setting of *Apc*-driven cancer. This is in accordance with previously described *APC* genotype–tumor phenotype correlations and associated β-catenin signaling dosages among *Apc*-mutant mouse models and sporadic and familial cancer patients as described in **Chapter 4**. In line with our findings, a previous study by Buchert et al. presented specific β-catenin signaling thresholds being important for hepatic and intestinal tumorigenesis. They observed a reduced intestinal tumor incidence and multiplicity in an *Apc*-mutant mouse model associated with a relatively high β-catenin signaling, following heterozygous β-catenin knockout [72]. Our findings are the first to demonstrate complete prevention of gastrointestinal tumor formation by reducing β-catenin levels below a hypothetical threshold, using the *Apc*1638N mouse model associated with intermediate β-catenin signaling. Importantly, we hereby demonstrate the absolute requirement for sufficiently enhanced β-catenin signaling to enable *Apc*-driven tumor formation in the gastrointestinal tract.

Our findings strongly argue against the model proposed by Phelps et al, stating that intestinal tumor formation is independent of β-catenin but instead requires CtBP [63]. Although we proved the relevance of β-catenin for intestinal tumor development, the specific aspect considering the impact of CtBP on

intestinal tumor formation has not been the focus during our studies. Thus, it is possible that CtBP does contribute to *Apc*-driven intestinal tumor formation, however, it does not exclude the role for β-catenin that is now solidly proven. CtBP has been shown to interact with APC at its 15-AARs thereby potentially competing with β-catenin binding, and CtBP's levels appear to increase upon *Apc* loss in early adenomas [61-63]. However, data showing oncogenic *CtBP* mutations or indicating tumor-initiating capacity of CtBP have not been reported to date, while these data are available for β-catenin (**Chapter 4**) [73]. Furthermore, as CtBP binds the more N-terminal located 15-AARs of APC, it cannot explain the selection of specific truncated APC proteins retaining between 1-3 20- AARs that is observed in intestinal tumors, whereas this is the case for β-catenin (**Chapter 4**) [73].

Our findings provide genetic evidence for the dominant role of β-catenin signaling dosage in dictating tissue-specific predisposition for *Apc*-driven tumorigenesis. However, mechanisms underlying tissue preferences for specific levels of β-catenin signaling remain largely obscure. Considering the different β-catenin signaling windows preferred for tumor formation along the intestinal tract, Leedham et al. have shown that gradients in physiological Wnt activity exist in the mouse and human intestine [48]. Examining Wnt target gene expression along the gastrointestinal tract indicated that in the human colorectal tract, physiological Wnt/β-catenin signaling is higher in the proximal part than distally. This implicates that to reach an identical hypothetical threshold of Wnt/β-catenin signaling to allow intestinal tumorigenesis, the gain in Wnt/β-catenin signaling should be higher in the distal than in the proximal colorectal tract. As such, the physiological Wnt gradients along the colorectal tract might contribute to the different mutation spectra and associated favored increase in Wnt/β-catenin signaling observed in tumors along the colorectal tract. Although β-catenin signaling is one of the main regulatory pathways in the intestine, it operates in concerted action with multiple other signaling routes and the concomitant complex interplay is poorly understood. Each tissue has its own unique architectural organization and multiple signaling pathways are likely to play a role into different degrees. As such, various β-catenin dosages might initiate different gene expression profiles in a tissue-specific manner [74]. A low β-catenin signaling level mainly modulates the expression of the most accessible and responsive genes, whereas high doses will also affect the expression of less responsive ones [75]. Unraveling how the complexity of all those signaling pathways influences which β-catenin signaling dosage dictates tissue-specific tumor predisposition in *Apc*-driven tumorigenesis represents a challenge for future investigation.

Non-canonical Wnt5a in intestinal cancer

In contrast to the well-established importance of canonical Wnt signaling in intestinal cancer development, a possible role of non-canonical Wnt5a signaling remains elusive. Although the majority of the currently available data indicate that Wnt5a is upregulated in intestinal tumors, controversial results have been published. Moreover, the functional consequences of Wnt5a expression during intestinal carcinogenesis are unclear. As described in **Chapter 3**, we investigated the contribution of Wnt5a to intestinal tumorigenesis using various approaches.
With regard to the controversy that exists considering the expression of Wnt5a in intestinal cancer, most reports described an increase in *WNT5A* RNA expression produced by the stromal cells of the tumor, following an augmenting trend during progression from normal intestine through adenoma to carcinoma [34-35, 76-78]. Our findings indicating that enhanced *WNT5A* expression is associated with poor prognosis in colon cancer patients is in line with this. In contrast, a single report by Dejmek et al. described reduced expression of WNT5A protein in the epithelial compartment of a subset of colorectal tumors and suggested that reduced epithelial WNT5A expression is associated with tumor progression [79]. In this report, epithelial expression was detected using a home-made polyclonal antibody proposed to detect WNT5A. However, their observation of epithelial WNT5A expression in colorectal tumors has not been validated by an independent report using alternative antibodies. Ourselves, we did not succeed in detecting WNT5A expression in human colorectal tumors using a generally used commercial Wnt5a antibody. On the other hand, we were able to verify enhanced endogenous Wnt5a expression in the stroma of *Apc*1638N mouse intestinal tumors on protein level, supporting the reports describing enhanced stromal WNT5A expression during intestinal tumorigenesis. In line with the presumed expression of Wnt5a to be stromal, we showed that the majority of the human colorectal cancer cell lines, derived from the epithelial compartment, do not express WNT5A. Also, during mouse gut development Wnt5a expression is typically restricted to the mesenchyme [3]. Altogether, most solid indications exist for the upregulation of Wnt5a expression in the stromal compartment of intestinal tumors. Irrespective of the exact source, Wnt5a proteins are secreted from their producing cells and subsequently act on cells present in the short range neighborhood. This implicates that it will not be of great importance whether Wnt5a is produced by either stromal or epithelial cells in intestinal tumors, since these cells are in close proximity. In line with this, we showed in **Chapter 2** that expression of Wnt5a causes downregulation of its main receptor Ror2 in a paracrine fashion. Hence, Ror2 expression was reduced not only in cells overexpressing Wnt5a themselves but also in the surrounding cells.

We investigated the actions of Wnt5a in intestinal cancer by examining the

consequences following *WNT5A* knockdown in SW480 human colorectal cancer cells. Moreover, we used our newly generated inducible Wnt5a transgenic mouse model to induce enhanced Wnt5a expression during the development of intestinal tumors in *Apc*1638N mice. First of all, as Wnt5a can modulate canonical Wnt/β-catenin signaling, of which aberrant activation underlies most colorectal cancer cases, Wnt5a manipulation might have consequences for intestinal tumorigenesis via this action [80-82]. Although the mechanisms by which Wnt5a modulates canonical Wnt/β–catenin are incompletely understood, Topol et al. have indicated that the inhibitory action by WNT5A is dependent on intact APC [83]. As most colorectal cancers acquire loss of function mutations in both copies of the *APC* gene, WNT5A is not expected to grossly affect intestinal tumor growth through modulation of β–catenin signaling within colorectal tumor cells. Accordingly, we observed that *WNT5A* knockdown in SW480 cells does not affect intrinsic Wnt/β-catenin signaling and we observed no changes in staining of β-catenin and its target Cyclin D1 in *Apc*1638N intestinal tumors following transgenic Wnt5a expression. In line with our findings, ectopic WNT5A expression in SW480 cells has been shown previously not to affect intrinsic Wnt/β-catenin signaling [83]. Few exceptions have been reported demonstrating that WNT5A inhibits intrinsic β-catenin signaling in colorectal cancer cell lines. However, these cell lines express either full length APC (HCT116 and SW48) or a long truncated APC protein with a considerable level of residual activity (HT29) [83-85]. In addition to the condition of APC, the cellular context, especially defined by the availability of WNT5A receptors such as Frizzleds and Ror2, is also important in dictating the activities of WNT5A [81, 86-87]. Although we showed that Ror2 receptors are present in the intestinal tumors of *Apc*1638N mice, we did not establish the expression of the different Frizzled receptors in current study, precluding us to state whether the receptor context played a role in the observed unaffected Wnt/β-catenin signaling. Corresponding with the unaltered Wnt/β-catenin signaling in intestinal cancer cells, we observed no discernible alterations in intestinal tumor cell proliferation following *WNT5A* knockdown in SW480 cells or Wnt5a induction in intestinal tumors in vivo. Accordingly, tumor initiation in the gastrointestinal tract and extra-intestinal were not affected by induced Wnt5a expression.

As our data indicated no influence of Wnt5a on intestinal tumor initiation, which is the phase most critically involving canonical Wnt/β-catenin signaling, we subsequently determined possible roles of Wnt5a during tumor progression. WNT5A has been implicated in regulating cellular migration and invasion in various cell types [19, 31-33, 88-91], however, not much comprehensive data have been reported to date considering WNT5A in colon cancer cells. Our findings show that when SW480 colon cells are deprived from their endogenous *WNT5A*, especially their efficient, directional cell migration is reduced. Moreover, also their

three-dimensional dispersion throughout collagen is decreased. Together this provides evidence that WNT5A promotes migration and invasion of colon cancer cells, corresponding with the tumor promoting activities of WNT5A reported for gastric cancer cells and melanoma cells [88-91]. Tumorigenesis is often considered to involve reactivation of embryonic programs. In this regard, the observed directional migration promoting effect exhibited by Wnt5a on colon cancer cells appears in line with the embryonic function of Wnt5a, which predominantly involves regulation of oriented cell migration. In an attempt to elucidate how WNT5A regulates the directional migration of colon cancer cells, we investigated adhesion characteristics. We showed that WNT5A promotes the formation of cellular focal adhesion sites to occur in a focal fashion. Hence, in *WNT5A* knockdown SW480 colon cancer cells, actin, p-FAK and p-Paxillin present in less focal, more diffusely distributed cellular adhesion sites, instead of focal adhesion sites. As indicated in other cell types, enlargement of cellular adhesion sites is associated with their reduced turnover and concomitantly, with reduced cellular migration [21, 88, 92]. As such, the promotion of colon cancer cell adhesion in a focal manner by WNT5A likely explains the reduced directional migration of *WNT5A* knockdown colon cancer cells.

Although these in vitro findings indicate a pro-tumorigenic role for Wnt5a in intestinal cancer by promoting tumor cell migration and invasion, we observed no gross differences in tumor growth, composition or malignancy when Wnt5a expression was induced in *Apc*1638N mice. Local invasion was not clearly altered upon Wnt5a induction and metastasis to distant organs was not observed. This provides evidence that by itself, enhanced Wnt5a expression is not sufficient to augment intestinal tumor malignancy or induce metastasis in *Apc*1638N mice. Despite the relevance of *Apc*-mutant mouse models to study intestinal tumorigenesis in a representative in vivo context, no genetically modified mouse models for intestinal cancer have been reported to date that show metastasis to distant organs. A possible explanation for this lack of metastasis is represented by the sequential accumulating mutations that are normally acquired in genes additional to *APC* during the adenoma to carcinoma sequence but that are absent in *Apc*1638N tumors. Among these, *KRAS* and *p53* mutations contribute to the progression of human colon cancer, whereas mutations in *K*-, *N*-, *H-RAS* and *p53* are not found in *Apc*1638N intestinal tumors [68]. Considering this, a potential role for Wnt5a during intestinal cancer progression remains likely, however, Wnt5a is not a sufficiently strong inducer on its own to overcome the hurdles of lack of sequential mutations necessary for colon cancer progression and lack of metastasis in this mouse model. Taken together, our results further support previous reports indicating enhanced Wnt5a expression in the stromal compartment of intestinal tumors. We demonstrated that WNT5A promotes adhesion sites to form focally,

and stimulates the directional migration and invasion of colorectal cancer cells. These properties can contribute to local invasion and metastasis and thereby to colon cancer progression. Moreover, these WNT5A activities can explain the association between enhanced *WNT5A* expression and poor prognosis that we observed.

Although our findings provide insight into the role of Wnt5a in intestinal cancer, these data should ideally be further expanded to provide more definitive evidence for the contribution of Wnt5a to intestinal cancer. Using our inducible Wnt5a transgenic mouse model in combination with *Apc*1638N mice, no impact of Wnt5a on intestinal tumorigenesis was observed. It would be very helpful to use a genetically modified mouse model for intestinal cancer that exhibits metastasis to distant organs, so that the influence of Wnt5a on metastasis of intestinal tumors can be elucidated. Furthermore, the use of a conditional *Wnt5a* knockout mouse model to investigate the consequences of Wnt5a loss on intestinal cancer would be a valuable addition to our Wnt5a overexpression findings.

Therapeutic implications for colorectal cancer

Canonical Wnt/β-catenin signaling as a candidate therapeutic target

Our research indicated that the dosage of β-catenin signaling can explain the side preferences of colorectal tumors and that sufficiently enhanced β-catenin signaling dosage is required for intestinal tumorigenesis. This adds reason to consider modulation of β-catenin signaling strength as a therapeutic option to combat colorectal cancer. On the one hand, inhibition of canonical Wnt/β-catenin signaling below a hypothetical lower threshold will remove the driving force required by colorectal tumor cells to sustain tumor growth. On the other hand, activation of canonical Wnt/β-catenin signaling above a hypothetical threshold will cause cell death and as such suppress tumor growth. These opportunities have been investigated using a diverse array of molecular approaches, including inhibition of β-catenin expression over induction of β-catenin degradation, relocalization to the plasma membrane, and disruption of β-catenin interactions with other proteins [93]. Especially this latter approach seems effective when the interaction between β-catenin and TCF is targeted, as this is critical for transcriptional activity of β-catenin. Furthermore, this is downstream in the signaling sequence, thereby representing an effective candidate target in tumors harboring *APC* or *CTNNB1* mutations. Several compounds have been suggested to inhibit β-catenin signaling at this level, however, the specificity and efficacy remain to be established [94]. Accordingly, clinical trials of these β-catenin signaling reducing compounds have not been initiated to date [95]. Other studies have been

focusing on interfering with β-catenin signaling on different cellular components, including Axin, GSK3 and Porcupine. The latter protein is required for Wnt protein acetylation and secretion, and as such it might inhibit the paracrine action of Wnt signals to the growth of colorectal cancer cells. However, such an approach will be useful predominantly in tumors with intact APC and an intact β-catenin regulation complex, or at least with some residual β-catenin downregulating capacity remaining in case of *APC* mutant tumors. Since the majority of colorectal tumors harbor *APC* mutations, interfering with Porcupine and extracellular Wnt signals is not expected to be the most efficient treatment strategy. Also the opportunities to activate β-catenin signaling are being explored, the most widely used approach being GSK3 inactivation. However, GSK3 is a kinase involved in multiple cellular signaling routes, and its inactivation is not desirable. Altogether, the lack of specificity and knowledge about targets, working mechanisms or adverse side effects have precluded clinical implication of the β-catenin modulating compounds. Given the crucial function of Wnt/β-catenin signaling in the intestine and beyond, interference is expected to be associated with serious side effects. Therefore, it is of major importance to determine targets, working mechanisms and safety of β-catenin modulating compounds.

It will be challenging to establish the exact β-catenin thresholds that limit the tumor-favoring β-catenin windows and also to modulate this to the desired level subsequently. Importantly, we demonstrated in **Chapter 5** that by reducing β-catenin levels in an *Apc*-mutant situation, the tumor predisposition shifts from the gastrointestinal tract towards the mammary tissues in mice. This implicates that when modulation of β-catenin signaling would be used as an approach to reduce colorectal cancer development in patients with an *APC* germline mutation, it is important to realize that this might influence the tumor predisposition in tissues other than the colorectal tract. Hence, when one is successful in circumventing a tumor-associated β-catenin window in the colorectal tract, one might support the outgrowth of cells elsewhere that carry suboptimal mutations in the Wnt/β-catenin signaling pathway. On the other hand, most colorectal cancer patients do not carry *APC* germline mutations, and patients will not often coincidentally harbor initiated tumor cells outside the colorectal tract. Moreover, patients are expected to be willing to take such a minor risk when suffering from colorectal cancer. However, this phenomenon should be kept in mind as a possible risk associated with Wnt/β-catenin signaling modulation when performed in a systemic fashion.

Another drawback of using the approach of β-catenin modulation as a treatment for colorectal cancer is reflected by the time that has already elapsed from tumor initiation to the moment of colorectal cancer diagnosis. Colorectal cancer is a multistep process, generally taking several decades to fully develop into cancer with symptomatic manifestations. As such, at the moment that patients

are diagnosed with colorectal cancer, most of them will carry tumors that harbor mutations not only in *APC* or β-catenin, but also multiple subsequent mutations in additional oncogenes and tumor suppressor genes (e.g. *KRAS*, *p53*) will have accumulated. As a consequence, the preferred β-catenin signaling windows and associated thresholds will be altered upon mutations in other pathways. For example, p53 is crucially involved in apoptosis, and its mutation will most likely alter the β-catenin signaling threshold that is normally associated with apoptosis.

In summary, since aberrant Wnt/β-catenin signaling is the main Achilles heel of colorectal cancer cells, interfering with canonical Wnt/β-catenin is a candidate mechanism to approach the treatment of colorectal cancer and research hereon is ongoing. However, this approach requires fine-tuning of β-catenin signaling levels, elucidation of working mechanisms and possible side effects of the used agents, and most optimally, it requires the colorectal tumors to be diagnosed in an early stage.

Non-canonical Wnt5a as a candidate therapeutic target

In contrast to canonical Wnt/β-catenin signaling, non-canonical Wnt5a signaling has not been considered as a therapeutic target for colon cancer to date yet. Hence, the contribution of Wnt5a to colorectal cancer has been unclear. Our results indicated that Wnt5a has no major role during intestinal tumor initiation, but rather promotes intestinal tumor progression by stimulating cellular migration and invasion. Supportively, we showed that enhanced *WNT5A* expression is associated with early recurrence and metastasis in colon cancer patients. This presents WNT5A as a potential target for therapy in colon cancer patients, allowing interference with tumor cell motility, invasion and metastasis. Based upon our studies, WNT5A seems a promising candidate target. However, before being considered for patient care, the activities of WNT5A should be explored in more detail and the contribution to colon cancer progression remains to be confirmed in vivo. For cancer types where the tumor-promoting role of Wnt5a has been clearly established, i.e. gastric cancer and melanoma, inhibition of Wnt5a has been tested as a mechanism to reduce tumorigenic properties. A polyclonal anti-Wnt5a antibody was shown to successfully inhibit migration and invasion activities of gastric cancer cells in vitro and inhibited metastasis to the liver following splenic inoculation [96]. A comparable approach with a different Wnt5a inhibiting compound, Box5, has shown that antagonizing Wnt5a causes reduced migration and invasion of melanoma cells [97]. These results indicate that antagonizing Wnt5a can be promising as a therapeutic approach to reduce cancer cell migration, invasion and metastasis. However, therapeutic opportunities remain to be further investigated in vivo.

Such Wnt5a antagonizing approaches can be considered as a therapeutic option for colorectal cancer as well, if the suggested tumor-promoting role of Wnt5a in colorectal cancer is indeed confirmed in vivo in the future. Advantageously, modulation of non-canonical Wnt5a signaling is expected to be associated with less severe side effects than modulation of canonical Wnt/β-catenin signaling, since our data indicated that non-canonical Wnt5a signaling appears not as crucially involved in homeostasis of the intestine and other tissues as is canonical Wnt/β-catenin signaling. In line with the role of canonical Wnt/β-catenin signaling in tumor initiation, modulation of the canonical Wnt/β-catenin signaling is expected to be useful predominantly during early tumorigenesis, when additional mutations have not yet accumulated. On the other hand, our data present non-canonical Wnt5a as a tumor promoter during the progression of intestinal cancer. Accordingly, Wnt5a is expected to be most useful as a therapeutic target during later phases of tumor development. Since diagnosis often occurs relatively late in the adenoma to carcinoma sequence, when mutations in different genes have already accumulated, targeting non-canonical Wnt5a instead of canonical Wnt/β-catenin signaling might even represent a more effective approach in practice.

Taken together, targeting non-canonical Wnt5a signaling in colorectal cancer seems promising and has several important practical benefits compared to canonical Wnt/β-catenin modulation. However, the pro-tumorigenic role of Wnt5a in colorectal cancer needs to be investigated more thoroughly before being exploited in patient care.

Concluding remarks

In this thesis we described the novel aspects of Wnt signaling in the intestine that we identified during our research. We identified non-canonical Wnt5a as an important regulator of early embryonic gut elongation and found that Wnt5a promotes migration and invasion of colon cancer cells. On the other hand, we solidly established the absolute requirement of a sufficiently high gain in canonical Wnt/β-catenin signaling for intestinal tumorigenesis and provided evidence that β-catenin signaling dosage dictates tumor predisposition among tissues. Moreover, we proposed a mechanism explaining the side preferences of different colorectal tumor types, based on selection of specific β-catenin signaling dosages along the colorectal tract.

We evaluated the possible implications of our findings in the clinical setting. Aberrant Wnt/β-catenin signaling is recognized as the Achilles heel in the vast majority of colorectal cancers, thereby giving reason to investigate the opportunities of targeting this pathway in colorectal cancer patients. Due to

the lack of knowledge on molecular activities and efficiency, and expected side effects, no Wnt/β–catenin signaling modulating agent is used in a clinical setting to date. However, research hereon is ongoing and might turn out useful in the future. We present non-canonical Wnt5a as a novel, alternative candidate target for colorectal cancer. Hence, enhanced WNT5A expression is associated with poor prognosis in colorectal cancer patients and we showed that WNT5A promotes colon cancer cell migration and invasion. Interference with non-canonical WNT5A is expected to be predominantly useful during colorectal cancer progression and to be associated with less severe side effects then when interfering with Wnt/β-catenin signaling. However, the pro-tumorigenic activities of Wnt5a in colon cancer should first be investigated more thoroughly before considering its use in patient care.

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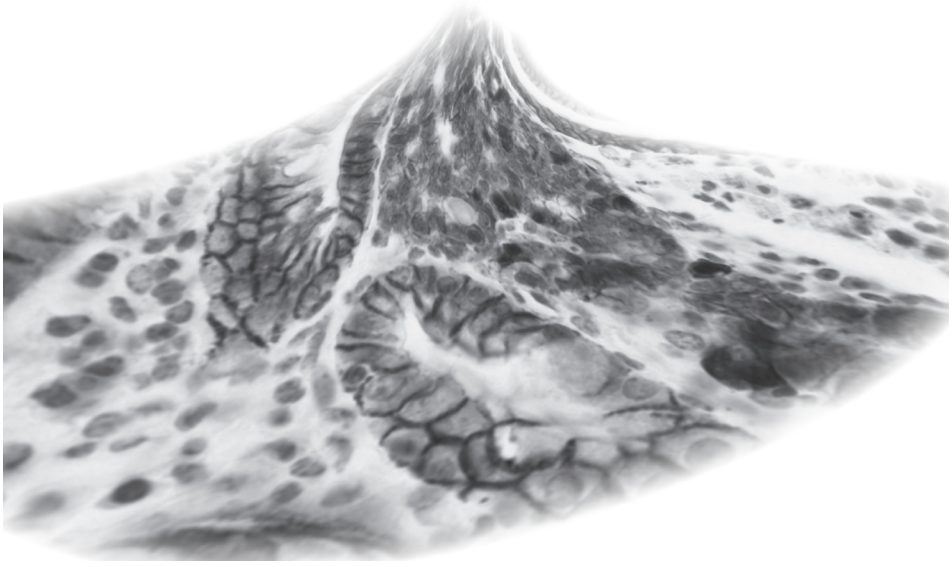
Summary

Samenvatting



Summary

Wnt signaling is a main regulatory signaling network crucially involved in embryonic development, maintenance of tissue homeostasis and often deregulated in cancer. In general, Wnt signaling is subdivided into canonical ('classical') and non-canonical ('alternative') signaling. Canonical Wnt signaling is the best-known pathway and is mediated via the key player in this route: β-catenin, which dictates canonical Wnt signaling output. Non-canonical Wnt signaling is a collective name for the Wnt signaling pathways alternative to the canonical Wnt/β-catenin pathway. Several Wnt ligands can activate non-canonical Wnt signaling, among which Wnt5a is the most extensively investigated ligand. **Chapter 1** introduces Wnt signaling and describes what is known or remains to be clarified about the involvement of its different pathways in the intestine. Our research was aimed to gain more insight into specific aspects of Wnt signaling in the intestine that were poorly understood. Among these, we investigated the dynamics and activities of Wnt5a during embryonic gut development, adult intestinal homeostasis and intestinal carcinogenesis. Furthermore, we verified the impact of β-catenin signaling dosage on tumor formation in the intestinal tract and in other tissues. Our findings are presented in this thesis.

Wnt5a-mediated non-canonical Wnt signaling is known to be involved in the outgrowth of structures during embryonic development, including the limbs, tail, facial structures and the gastrointestinal tract. Not much knowledge exist regarding the dynamics of the involvement of non-canonical Wnt5a signaling in these embryonic processes. Also, a significant impact of non-canonical Wnt5a signaling during postnatal life has not been solidly confirmed to date. In **Chapter 2**, we present our newly generated inducible Wnt5a transgenic mouse model, as a tool to investigate Wnt5a-mediated non-canonical Wnt signaling in vivo during specific time-frames. We show that Wnt5a is involved in embryonic outgrowth and intestinal elongation especially before embryonic day (E) 13.5. More specifically, enhanced Wnt5a expression caused shortening of the gastrointestinal tract, limbs, tail and facial structures, when induced from E10.5 but not from E13.5 onwards. Differentiation of the intestinal cell types was unaffected by Wnt5a when induced during embryogenesis and also when induced in adult mice. Moreover, we demonstrate that induced Wnt5a expression has no major impact on mouse intestinal homeostasis postnatally. Furthermore, we uncover a regulatory process where induction of Wnt5a causes downregulation of its receptor Ror2 at protein level and in a paracrine fashion. Altogether, these data indicated that Wnt5a directs intestinal elongation during early (<E13.5) embryonic gut development, but has no impact on cellular differentiation or adult intestinal homeostasis.

Another important player during embryonic gut development is the transcription factor Sox2, which is involved in determining organ identity. Normally, Sox2 expression is restricted to the anterior part of the developing gut, which forms the stomach and other foregut derivatives. In **Chapter 6**, we show that ectopic Sox2 expression in the posterior parts of the developing gut leads to conversion of an intestinal to a premature gastric phenotype, indicating the dominant effect that Sox2 can potentially exert on the direction of gastrointestinal tract-related transcriptional programs.

Non-canonical Wnt5a signaling has been implicated in tumorigenesis of the intestine as well, although its contribution is unclear. In **Chapter 3** we show that increased *WNT5A* expression is associated with poor prognosis in colon cancer patients. We demonstrate that WNT5A promotes directional migration and invasion of human colon cancer cells, possibly mediated through its involvement in the formation of cellular focal adhesion sites. Despite the pro-tumorigenic activities of Wnt5a exerted in vitro, in vivo induction of Wnt5a during *Apc*-driven intestinal tumorigenesis in mice was shown to be insufficient by itself to either affect initial tumor formation, augment tumor malignancy or cause metastasis. The lack of additional mutations required for malignant progression (e.g. in *Kras* and *Trp53*) in these mouse intestinal tumors possibly explains this. Although Wnt5a cannot by itself augment malignant progression or impose metastasis in the used *Apc*-mutant mouse model, we believe that the tumor promoting activities of Wnt5a by stimulating colon cancer cell migration and invasion likely explain the poor prognosis associated with increased *WNT5A* expression in colon cancer patients.

In the highly proliferative intestinal epithelium, classical Wnt signaling through β-catenin is especially important in maintaining homeostasis by balancing proliferation, differentiation and apoptosis. In this pathway, the level of β-catenin is controlled by adenomatous polyposis coli (APC), which is the gatekeeper tumor suppressor in the intestine responsible for the degradation of β-catenin. Aberrant activation of the Wnt/β-catenin pathway is well known to underlie the majority of colorectal cancer cases, mostly resulting from *APC* mutations. However, the requirement of enhanced β-catenin signaling for tumor initiation in the intestine has been debated. Furthermore, an important concept concerns the impact of the dosage of β-catenin signaling in dictating tumor predisposition among tissues, which remained to be solidly proven. In **Chapter 5**, we show that *Apc*1638N mice that normally develop intestinal tumors rather develop mammary tumors and are protected from intestinal tumors when their β-catenin levels were reduced by heterozygous β-catenin knockout. This importantly proves that in *Apc*-mutant mice, a sufficiently high gain in β-catenin signaling dosage is absolutely required to enable intestinal tumorigenesis. In addition, these findings provided formal

in vivo evidence for the dominant role of β-catenin dosage in dictating tissue-specific tumor predisposition in *Apc*-driven cancer.

The distribution preferences of different colorectal tumor types are poorly explained to date. Whereas mismatch repair (MMR) deficient tumors mostly develop in the proximal colon, the majority of MMR proficient tumors develop in the distal colorectal tract. **Chapter 4** reviews the involvement of canonical Wnt/β-catenin signaling and in particular the concept of dosing this pathway in colorectal cancer. Moreover, we present an explanation for the striking side preferences of MMR proficient versus deficient colorectal tumors. Previously, we and others have shown that not all *APC* mutations result in the same β-catenin signaling strength to the nucleus. Interestingly, we observed that proximal colon tumors prefer *APC* mutations resulting in a moderate gain in β-catenin signaling, whereas distal tumors harbor *APC* mutations leading to a higher gain in β-catenin signaling levels. Due to the preferential mutational mechanism in MMR deficient tumors, these tumors acquire *APC* mutations that result in a moderate gain in β-catenin signaling which is most optimal for tumor formation in the proximal colon. Altogether, we propose that the colorectal tumor side preferences can be explained by their particular mutational mechanisms, resulting in specific *APC* or β-catenin mutations associated with a specific level of β-catenin signaling that is selected for by either side of the colorectal tract.

In conclusion, the studies in this thesis provide novel insights into multiple aspects of Wnt signaling involvement in development, homeostasis and cancer of the intestine. Wnt5a-mediated non-canonical Wnt signaling appears especially involved in directional outgrowth and migration events during embryonic development and cancer of the intestine. Furthermore, we have formally proven that for intestinal tumor formation in *Apc*-mutant mice, a sufficiently high gain in β-catenin signaling dosage is absolutely required. Moreover, β-catenin signaling dosage was demonstrated to importantly dictate tumor predisposition among tissues in *Apc*-driven cancer. Side preferences of tumors within the colorectal tract are likely explained by different mutational mechanisms leading to different β-catenin dosages that are most optimal for tumor formation on either side of the colon. Finally, the novel aspects that we identified on Wnt signaling in the intestine and prospective therapeutic implications for colorectal cancer are evaluated in **Chapter 7**.

Samenvatting

Wnt signalering is een belangrijk signaleringsnetwerk welke cruciaal betrokken is bij embryonale ontwikkeling, behoud van weefselhomeostase en welke vaak gedereguleerd is in kanker. Over het algemeen wordt Wnt signalering onderverdeeld in zogenaamde canonical ('klassieke') en non-canonical ('alternatieve') signalering. Canonical Wnt signalering is de meest bekende route en wordt gemedieerd via de belangrijke speler in deze route: β-catenine, welke de output van de canonical Wnt signalering bepaalt. Non-canonical Wnt signalering is een verzamelnaam voor de Wnt signaleringsroutes anders dan de canonical Wnt/β-catenine route. Verscheidene Wnt liganden kunnen non-canonical Wnt signalering activeren. Hiervan is Wnt5a het best onderzochte ligand. **Hoofdstuk 1** introduceert Wnt signalering en beschrijft wat er reeds bekend is en wat er nog opgehelderd dient te worden omtrent de betrokkenheid van de verschillende Wnt routes in de darm. Ons onderzoek was bedoeld om meer inzicht te krijgen in specifieke aspecten van Wnt signalering in de darm die nog slecht begrepen waren. Hiertoe hebben we de dynamiek en activiteiten van Wnt5a tijdens embryonale darmontwikkeling, volwassen darmhomeostase en darmkanker onderzocht. Tevens hebben we het belang van β-catenine signaleringsdosis bij tumorvorming in het darmkanaal en andere weefsels geverifieerd. Onze bevindingen zijn beschreven in dit proefschrift.

Van Wnt5a-gemedieerde non-canonical Wnt signalering is bekend dat het betrokken is bij uitgroeiprocessen van bepaalde structuren tijdens de embryonale ontwikkeling, waaronder dat van de ledematen, de staart (in het geval van de muis), het gezicht en het maag-darmkanaal. Er is vandaag de dag niet veel kennis wat betreft de dynamiek van Wnt5a betrokkenheid tijdens deze processen. Ook is een significante invloed van non-canonical Wnt5a signalering tijdens postnatale fasen momenteel niet solide bevestigd. In **Hoofdstuk 2** presenteren we ons nieuw gegenereerde induceerbare Wnt5a transgene muismodel, als een tool waarmee Wnt5a-gemedieerde non-canonical Wnt signalering in vivo onderzocht kan worden gedurende gewenste tijdspannen. We laten zien dat Wnt5a vooral betrokken is bij embryonale uitgroei en darmverlenging voor embryonale dag (E) 13.5. Verhoogde Wnt5a expressie veroorzaakte verkorting van het maag-darmkanaal, ledematen, staart en gezichtsstructuren wanneer het geïnduceerd werd vanaf E10.5, maar niet wanneer het geïnduceerd werd vanaf E13.5. Differentiatie van de verschillende intestinale celtypes werd niet beïnvloed door Wnt5a wanneer dit geïnduceerd werd tijdens embryonale ontwikkeling of in volwassen muizen. We laten bovendien zien dat geïnduceerde Wnt5a expressie geen grote invloed heeft op darmhomeostase in de volwassen muis. Tevens ontdekten we een regulatoir proces waarbij inductie van Wnt5a een sterke

afname van zijn receptor Ror2 veroorzaakt, op eiwitniveau en op een paracriene manier. Samengevat wijzen deze data erop dat Wnt5a darmverlenging aanstuurt tijdens vroege (<E13.5) embryonale darmontwikkeling, maar geen grote invloed heeft op intestinale celdifferentiatie of volwassen darmhomeostase.

Een andere belangrijke speler tijdens embryonale darmontwikkeling is de transcriptie factor Sox2, welke betrokken is bij het bepalen van orgaanidentiteit. Normaal gesproken is Sox2 expressie beperkt tot het voorste gedeelte van het zich ontwikkelende maag-darmkanaal, van waaruit de maag en afgeleiden van de voordarm gevormd worden. In **Hoofdstuk 6** laten we zien dat gedwongen Sox2 expressie in de achterste delen van het zich ontwikkelende maag-darmkanaal leidt tot conversie van een darm- naar een prematuur maagfenotype. Dit wijst op het dominante effect dat Sox2 potentieel kan hebben op de sturing van maag-darmkanaal gerelateerde transcriptieprogramma's.

Non-canonical Wnt5a signalering is ook geïmpliceerd in tumorontwikkeling in de darm, maar de bijdrage hiervan is onduidelijk. In **Hoofdstuk 3** laten we zien dat verhoogde *WNT5A* expressie geassocieerd is met een slechte prognose voor darmkankerpatiënten. We tonen tevens aan dat WNT5A de gerichte migratie en invasie van darmkankercellen stimuleert, mogelijk gemedieerd via betrokkenheid bij de formatie van focale aanhechtingspunten van de cellen. Ondanks deze tumorbevorderende activiteiten van Wnt5a in kweek blijkt in vivo inductie van Wnt5a tijdens *Apc*-gedreven darmtumorontwikkeling in muizen op zichzelf onvoldoende om initiële tumorvorming te beïnvloeden, tumor maligniteit te verhogen of uitzaaiing te veroorzaken. Het gebrek aan additionele mutaties die nodig zijn voor maligne progressie (bijv. in *Kras* en *Trp53*) in deze muizendarmtumoren kan dit mogelijk verklaren. Ondanks dat Wnt5a op zichzelf geen maligne progressie stimuleert of uitzaaiing induceert in het gebruikte *Apc*-mutante muismodel, denken wij dat de tumorbevorderende activiteiten van Wnt5a door het stimuleren van migratie en invasie van darmkankercellen vermoedelijk wel de slechte prognose kunnen verklaren die gezien wordt in darmkankerpatiënten met hoge *WNT5A* expressie.

In de zich snel vernieuwende darmbekleding is canonical Wnt signalering via β-catenine vooral belangrijk voor het behoud van homeostase door het balanceren van proliferatie, differentiatie en apoptose. In deze route wordt het niveau van β-catenine gecontroleerd door adenomatous polyposis coli (APC), wat een essentiële tumor onderdrukker is in de darm, verantwoordelijk voor de afbraak van β-catenine. Afwijkende activatie van deze route ligt ten grondslag aan de meerderheid van de darmkankergevallen en is meestal het resultaat van *APC* mutaties. Desalniettemin wordt er door sommigen getwijfeld of verhoogde β-catenine signalering vereist is voor tumor initiatie in de darm. Bovendien is nog niet definitief bewezen dat een specifieke signaleringsdosis van β-catenine ten grondslag ligt aan weefselspecifieke tumorpredispositie. In

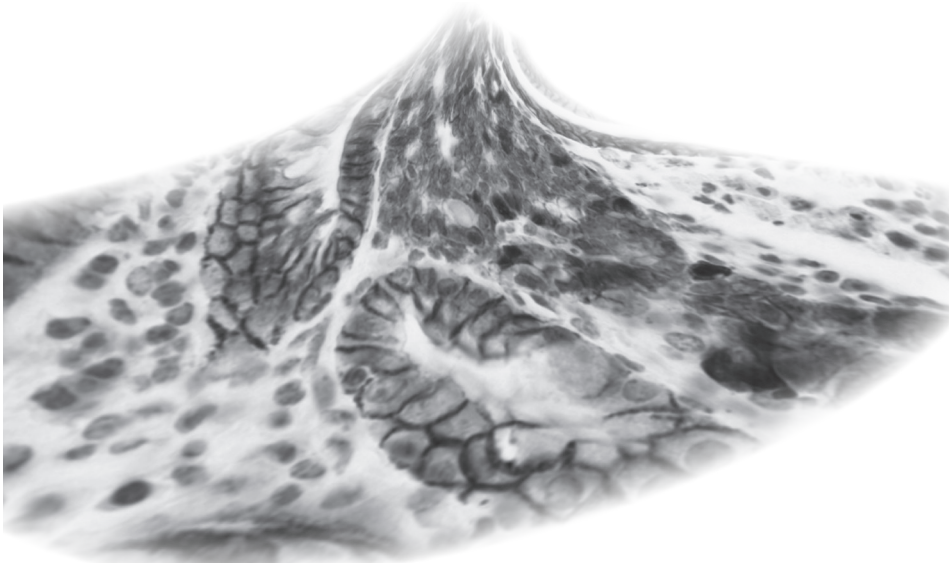
Hoofdstuk 5 laten we zien dat *Apc*1638N muizen die normaal gesproken darmtumoren ontwikkelen, juist borsttumoren ontwikkelen en volledig beschermd zijn tegen de vorming darmtumoren wanneer hun β-catenine niveaus gereduceerd worden door heterozygote β-catenine knockout. Dit bewijst dat in *Apc*-mutante muizen, een voldoende verhoogde β-catenine signaleringsdosis absoluut vereist is om tumorvorming in de darm mogelijk te maken. Daarnaast leveren we formeel in vivo bewijs voor de dominante rol van β-catenine dosering in het bepalen van weefselspecifieke tumorgevoeligheid in *Apc*-mutante kanker.

Voor de locatievoorkeuren in de dikke darm van verschillende types tumoren is momenteel geen goede verklaring beschikbaar. Terwijl mismatch repair (MMR) deficiënte tumoren zich vooral ontwikkelen in de proximale colon, ontwikkelen tumoren met een intact MMR zich juist vooral in de distale dikke darm. **Hoofdstuk 4** beschrijft de betrokkenheid van canonical Wnt/β-catenine signalering, en het concept van dosering van deze route, in dikkedarmkanker. Bovendien presenteren we een verklaring voor de opvallende locatie voorkeuren van MMR intacte versus defecte darmtumoren. Voorheen hebben wij en anderen laten zien dat niet alle *APC* mutaties resulteren in dezelfde β-catenine signaleringssterkte naar de celkern. We zagen dat proximale colon tumoren *APC* mutaties prefereren die resulteren in een matige verhoging van β-catenine signalering, terwijl distale tumoren *APC* mutaties bevatten die leiden tot een sterke verhoging in β-catenine signalering. Als gevolg van het voorkeursmechanisme waarmee mutaties gegenereerd worden in MMR defecte tumoren verkrijgen deze tumoren *APC* mutaties die resulteren in een matige β-catenine signaleringsverhoging, wat het meest optimaal is voor tumorvorming in de proximale dikke darm. Alles samengenomen stellen wij dat de locatie voorkeuren van dikke darm tumoren verklaard kunnen worden aan de hand van hun specifieke mutatiemechanismen, resulterend in specifieke *APC* of β-catenine mutaties die geassocieerd zijn met een bepaald niveau van β-catenine signalering, en waarvoor geselecteerd wordt aan een van beider kanten van de dikke darm.

Concluderend leveren de studies in dit proefschrift nieuwe inzichten op inzake velerlei aspecten van de betrokkenheid van Wnt signalering tijdens ontwikkeling, homeostase en kanker van de darm. Non-canonical Wnt5a signalering blijkt vooral betrokken bij gerichte uitgroei en migratieprocessen tijdens de embryonale ontwikkeling en carcinogenese van de darm. Verder hebben we formeel bewezen dat een voldoende sterke verhoging van β-catenine signalering absoluut vereist is voor darmtumorvorming in *Apc*-mutante muizen. Bovendien demonstreren we dat β-catenine signaleringsdosis de weefselspecifieke tumorpredispositie bepaalt. Voorkeuren voor locatie van tumoren in de dikke darm kunnen vermoedelijk uitgelegd worden aan de hand van hun verschillende mutatiemechanismen, leidend tot verschillende β-catenine signaleringsdoseringen die het meest

optimaal zijn voor tumorvorming op een bepaalde plaats in de dikke darm. Tenslotte evalueren we de nieuwe aspecten die we geïdentificeerd hebben omtrent Wnt signalering in de darm en voorziene therapeutische implicaties hiervan voor darmkanker in **Hoofdstuk 7**.

Dankwoord



Dankwoord

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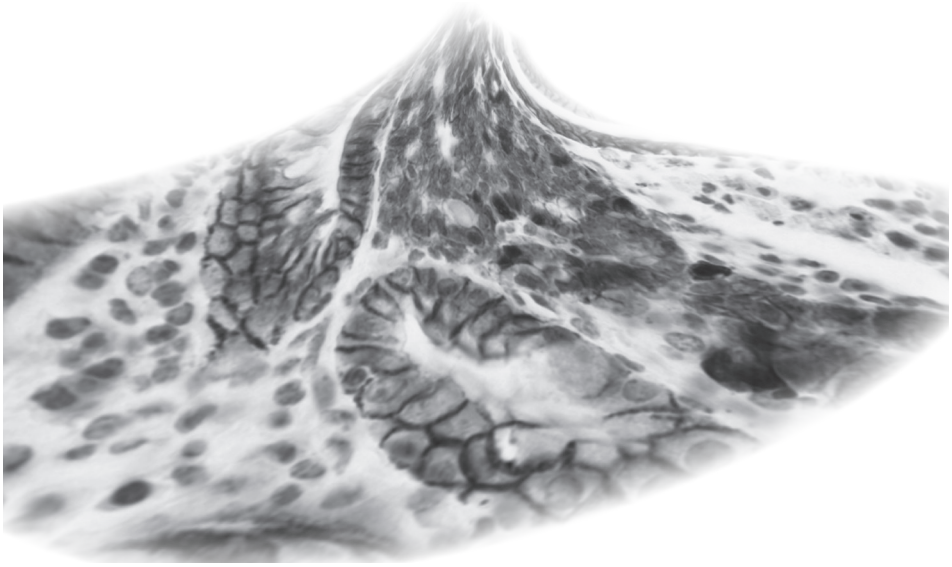
About the author



PhD portfolio

Name PhD student	Elvira R.M. Bakker
Erasmus MC Department	Gastroenterology and Hepatology
PhD period	August 2008- August 2012
Promotor	Prof. dr. E.J. Kuipers
Co-promotor	Dr. R. Smits

General academic and research skills: courses

2010 Introduction to Data Analysis, Netherlands Institute for Health Sciences

2010 Biological Interpretation of Gene Expression Data with ExPlainTM Analysis System, Molecular Medicine Postgraduate School, ErasmusMC

2008 Laboratory Animal Science (Art.9), Erasmus MC

2008 Basic and Translational Oncology, Molecular Medicine Postgraduate School, Erasmus MC

Conferences: Oral presentations

2012 Digestive Disease Week 2012, San Diego, USA
'Heterozygous knockout of β-catenin in *Apc*1638N mice prevents gastrointestinal tumor formation but predisposes for mammary lesions'

2012 Spring Meeting of Dutch Society for Gastroenterology, Veldhoven, The Netherlands
'Heterozygous knockout of β-catenin in *Apc*1638N mice prevents gastrointestinal tumor formation but predisposes for mammary lesions'

2012 Annual Day of the Molecular Medicine Postgraduate School, Rotterdam, The Netherlands
'Dosaging of the β-catenin proto-oncogene dictates tissue-specific tumor predisposition'

2011 Digestive Disease Week 2011, Chicago, USA
'Generation of an inducible Wnt5a transgenic mouse model to study the contribution of increased Wnt5a expression to intestinal tumor growth'

2010 WNT Meeting, Stockholm, Sweden
'Generation of an inducible Wnt5a transgenic mouse model to study the contribution of increased Wnt5a expression to intestinal tumor growth'

Conferences: Poster presentations

2012 Digestive Disease Week 2012, San Diego, USA
'Induced Wnt5a expression is well-tolerated in adult mice, but perturbs outgrowth and intestinal elongation in embryos'

2011 American Association for Cancer Research, Annual Meeting 2011,

Orlando, USA
'Generation of an inducible Wnt5a transgenic mouse model to study the contribution of increased Wnt5a expression to intestinal tumor growth'

2010 WNT Meeting, Stockholm, Sweden
'Generation of an inducible Wnt5a transgenic mouse model to study the contribution of increased Wnt5a expression to intestinal tumor growth'

2010 Dutch Society for Gastroenterology, Veldhoven, The Netherlands
'Generation of an inducible Wnt5a transgenic mouse model to study the contribution of increased Wnt5a expression to intestinal tumor growth'

2009 Digestive Disease Week 2009, Chicago, USA
'Establishing models for determining the role of increased Wnt5a expression in intestinal tumor growth'

Conferences: Attendance

2010 Dutch Tumor Immunology Meeting, Breukelen, The Netherlands
2009 WNT Meeting, Washington DC, USA
2008 Tumor Cell Biology Meeting, Dutch Cancer Society, Lunteren, The Netherlands

Scientific awards and grants

2012 Best Abstract Prize Dutch Experimental Gastroenterology and Hepatology Meeting, Spring Meeting of Dutch Society for Gastroenterology, Veldhoven, The Netherlands
2012 Erasmus Trustfonds Travel Grant
2011 Erasmus Trustfonds Travel Grant
2011 Dutch Society for Gastroenterology Travel Grant
2010 WNT2010 Travel Grant, Stockholm, Sweden
2010 Dutch Society for Gastroenterology Travel Grant
2010 Erasmus Trustfonds Travel Grant
2009 Erasmus Trustfonds Travel Grant

Teaching activities: supervision internships BSc students

2012 J.M. Overbeeke
2010 L. Jongeneel

Other activity:

2012 Rocket Launch Esrange Space Center, Kiruna, Sweden
'Microgravity and the activation of immune cells'

Curriculum vitae

Elvira Renate Maria Bakker was born in Rotterdam, The Netherlands, on January 13th, 1982. In 2000, she obtained her VWO-Athenaeum degree at 'Willem van Oranje', Oud-Beijerland and started her study Biology at the University of Utrecht, The Netherlands. After completing the first three years with a Bachelor degree in Biology, she switched to the master program 'Biology of Disease' at the Faculty of Biomedical Sciences, University of Utrecht. During the master program, she performed her first internship at the Department of Pathology, University Medical Center Utrecht followed by her second internship at the Unit Research & Development, Netherlands Vaccine Institute, Bilthoven, The Netherlands. She wrote her master thesis at the Department of Medical Oncology, University Medical Center Utrecht, and received her Master of Science degree in December 2005 at the University of Utrecht. From January 2006 to August 2008 she proceeded working at the Unit Research & Development at the Netherlands Vaccine Institute as a research associate. During this period, she fulfilled her wish to experience life beyond borders by traveling the world for 7 months. In August 2008 she started her PhD training on Wnt signaling in intestinal development and cancer at the Department of Gastroenterology and Hepatology, Erasmus Medical Center, Rotterdam, The Netherlands under the supervision of Prof. Dr. E.J. Kuipers and Dr. R. Smits. The results of her PhD training are described in this thesis.

List of publications

Nils O. Lindström, Elvira R.M. Bakker, Sally F. Burn, Ron Smits, Jamie A. Davies and P. Hohenstein. A β-catenin activity gradient drives cellular identity and patterning of the developing nephron. *Submitted*

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