UNIVERSITY OF GOTHENBURG

FoxF Genes in Development and Disease

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To my family

FoxF genes in development and disease

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Abstract

Forkhead transcription factors of the *FoxF* group are important during embryonic development, and mutation of either of the members, *Foxf1* and *Foxf2*, has fatal consequences. In this thesis, I present our recent findings about the mechanism of action of *FoxF* genes in development and disease.

Haploinsufficiency for *FOXF1* in humans causes alveolar capillary dysplasia with misalignment of pulmonary veins (ACDMPV), a rare lethal congenital disorder with incomplete penetrance. We report a new ACDMPV case and define the genomic rearrangement which consists of a pericentric inversion on chromosome 16 (p11.2q24.1), which disrupts the *FOXF1* 5'-flanking region 134 kb upstream of the first exon. We further use this information in combination with chromatin modification data from the ENCODE data set to predict the extent of the *FOXF1* regulatory domain and the critical genomic regions for ACDMPV.

Gastrointestinal cancer, which is the result of uncontrolled proliferation of intestinal stem cells, is one of the most prevalent causes of death in the West. We show that *Foxf2* regulates the number of intestinal stem cells and the proliferation rate in adult mouse intestine, with consequences for initiation and growth of intestinal tumors. *Foxf2* limits the size of the stem cell niche by activating the expression of the extracellular Wnt inhibitor Sfrp1 in mesenchymal cells surrounding the crypts of Lieberkühn. During this work we also developed a novel method for separation of intact intestinal epithelium from mesenchyme.

Cleft palate is a common congenital malformation, associated with many genetic alterations and environmental teratogens. Loss of *Foxf2* results in cleft palate in mouse. We found that the cleft palate is the result of reduced proliferation and decreased extracellular matrix production in the neural crest-derived palatal shelf mesenchyme at a critical stage of palatal formation. The mechanistic basis appears to be a diminished Tgfβ signaling, and decreased expression of integrins required for activation of latent Tgfβ.

Papers discussed

This thesis is based on the following publications, referred to by roman numerals in the text:

I. Inversion upstream of FOXF1 in a case of lethal alveolar capillary dysplasia with misalignment of pulmonary veins.

Parris T, Nik AM, Kotecha S, Langston C, Helou K, Platt C, Carlsson P.
American Journal of Medical Genetics Part A
Volume 161, Issue 4, pages 764–770, April 2013

II. Separation of intact intestinal epithelium from mesenchyme.

Nik AM, Carlsson P .
BioTechniques, Vol. 55, No. 1, pp. 42–44, July 2013.

III. Foxf2 in intestinal fibroblasts reduces numbers of Lgr5(+) stem cells and adenoma formation by inhibiting Wnt signaling.

Nik AM, Reyahi A, Pontén F, Carlsson P
Gastroenterology. 2013 May;144(5):1001-11.

IV. *Foxf2* enhances Tgfβ signaling in secondary palate development.

Ali M.Nik, Jeanette Astorga-Johansson, Azadeh Reyahi, Mozhgan Ghiami, Fredrik Pontén and Peter Carlsson
Submitted

Table of Contents

Introduction

FoxF transcription factors

The "F" group of forkhead ("Fox") transcription factors is in mammals encoded by two genes, *FoxF1* and *FoxF2*, in humans located on chromosome 6 and 16 respectively. Evidence such as presence of a single homologue in *Drosophila*, high sequence similarity between *FoxF1* and *FoxF2*, partially overlapping expression and partially redundant function, suggest that an ancestral *FoxF* homologue was duplicated during deuterostome evolution (Carlsson and Mahlapuu, 2002). Additional support for this is derived from the observation that each *FoxF* gene is clustered with a *FoxC* gene, and the presence in *C. elegans* of what appears to be a single gene (F26B1.7) ancestral to both *FoxF* and *FoxC* groups hints at an even older duplication event.

Despite the common evolutionary origin and partial expression redundancies, *Foxf1* and *Foxf2* have important differences in their expression patterns, which make each of them indispensable for normal embryonic development and physiological homeostasis in the adult.

Foxf1

Foxf1 expression

Detailed description of *Foxf1* expression during mouse embryonic development has been described elsewhere (Kalinichenko et al., 2003; Mahlapuu et al., 2001b; Mahlapuu et al., 1998; Peterson et al., 1997). In early embryonic development, *Foxf1* is expressed in the extra-embryonic and lateral plate mesoderm. Later on, during organogenesis, the expression of *Foxf1* becomes restricted to the splanchnic mesoderm, which provides the mesenchymal cells of the intestinal tract and gut derivatives, such as lungs and liver, but is also turned on in the sclerotomes of the developing axial skeleton, and in the neural crest mesenchyme of the branchial arches and derived craniofacial structures (Jeong et al., 2004)

In the adult mouse, *Foxf1* continues to be expressed in the mesodermal tissue of the gastrointestinal tract and gut-derived organs. *Foxf1* expression in the central nervous system (CNS) has been detected in pituitary gland, outer nuclear layer of the retina and a population of cerebral astrocytes as well as meningeal cells and pericytes of the brain blood vessels (Kalinichenko et al., 2003).

Expression studies in human, suggest that, in both embryonic and adult stages, the lung expresses the highest level of *FOXF1* mRNA (Pierrou et al., 1994).

Murine *Foxf1* mutant phenotype

Inactivation of *Foxf1* in mouse is embryonically lethal. *Foxf1* null mutants die at mid-gestation due to multiple abnormalities such as defective coelom formation and amniotic expansion. The immediate reason for resorption of mutant embryos, however, is ischaemia due to lack of vasculogenesis in extra-embryonic structures and failed placentation (Astorga and Carlsson, 2007; Mahlapuu et al., 2001b).

Foxf1 heterozygous mouse pups suffer from abnormal development of foregut and mid-gut derived organs such as lungs, trachea, esophagus and gallbladder (Kalinichenko et al., 2001; Kalinichenko et al., 2002; Mahlapuu et al., 2001a). Interestingly, the penetrance and expressivity of developmental defects associated with *Foxf1* haploinsufficiency differ among mouse strains. For example, in CD1 lethality of *Foxf1* heterozygotes is over 90%, whereas a clear majority of *Foxf1$^{-/+}$* on C57Bl/6 background survives.

Human *FOXF1* mutant phenotype

Alveolar capillary dysplasia with misalignment of pulmonary veins (ACDMPV) is a life threatening congenital disorder, which often appears together with developmental anomalies in gastrointestinal tract, cardiovascular and genitourinary systems (Langston, 1991; Sen et al., 2004). Clinically, ACDMPV is characterized by severe pulmonary hypertension, which does not respond to treatment. Histopathological findings of the post mortem autopsies show failure of the alveolar capillaries to make intimate contact with the respiratory epithelium as well as thickening of intraacinar arterioles and abnormal arrangement of the pulmonary veins.

Genetic studies of affected individuals linked ACDMPV to deletions in the region between q24.1-q24.2 in chromosome 16, which contains a small cluster of genes encoding three forkhead transcription factors: *FOXF1*, *FOXC2* and *FOXL1*. Three observations made it possible to conclusively link ACDMPV to loss-of-function of *FOXF1*: the lung malformations in murine *Foxf1* mutants; the identification of ACDMPV cases with point mutations in the coding region of *FOXF1*, rather than deletions; and the absence of ACDMPV in a child that exhibited other parts of the syndrome and with a deletion that affected *FOXC2* and *FOXL1*, but not *FOXF1* (Stankiewicz et al., 2009).

Paper I

We reported a case of lethal ACDMPV with alveolar septal defect and duodenal atresia, and showed that a pericentric inversion on chromosome 16 (p11.2q24.1) disrupts the *FOXF1* 5'-flanking region 134 kb upstream of the first exon. We further used this information in combination with chromatin modification data from the ENCODE data set to predict the extent of the *FOXF1* regulatory domain and the critical genomic regions for ACDMPV. Our analysis suggests that cis-regulatory elements of *FOXF1* are distributed over more that 300 kb, and perhaps as much as 433 kb, upstream of the gene. This report, further strengthens the link between *FOXF1* and ACDMPV, and aids molecular diagnostics by defining the critical genomic region for ACDMPV.

Foxf2

Foxf2 expression

Expression of *Foxf2* during mouse embryonic development has been investigated with mRNA *in situ* hybridization (Aitola et al., 2000), which has provided a detailed view of the expressing tissues, but the lack of specific antibodies against the Foxf2 protein has hampered identification of the *Foxf2* expressing cell types.

From gastrulation stage embryos, *Foxf2* mRNA is detected in the posterior primitive streak, lateral plate mesoderm, and in mesodermal derivatives of the extra-embryonic tissues. At embryonic day E9.5, expression of *Foxf2* emerges in mesenchyme adjacent to oropharynx and stomodeum, as well as around Rathke's pouch, which will later form the pituitary gland. *Foxf2* is also expressed in the neural crest, and in neural crest-derived tissues and cells, such as palatal shelf mesenchyme and brain pericytes. In the embryonic gastrointestinal tube *Foxf2* mRNA forms an anteroposterior gradient with lowest levels in the stomach and proximal parts of the small intestine, and the highest in the colon. A radial gradient is also formed, with highest concentration closest to the epithelium (Aitola et al., 2000), which is consistent with activation of *Foxf2* expression by Hedgehog ligands secreted by the epithelium (Ormestad et al., 2006).

Foxf2 is also expressed in several other tissues, such as the sclerotomes, the leading edge of the condensed mesenchyme of the growing ribs, the pre-skeletal condensations in the limb buds, the genital tubercle, and in the periocular mesenchyme (Ormestad et al., 2004).

## Murine *Foxf2* mutant phenotype

Murine *Foxf2* null mutants are born with several severe malformations, and although most have incomplete penetrance, no *Foxf2* mutant pup survives birth with more than a few hours. Cleft palate (Wang et al., 2003); anal atresia; hyperproliferative, disintegrating intestinal epithelium; and megacolon (Ormestad et al., 2006) are examples of published *Foxf2* null phenotypes. Recently, we discovered that Foxf2 deficiency gives rise to microvessel aneurysms, and a leaky blood-brain barrier (manuscript submitted).

Foxf2 in intestinal homeostasis and neoplasia

Anatomy of the small intestine

Despite its name, the small intestine constitutes the largest part of the mouse gastrointestinal tract, and connects the stomach to the cecum and large intestine. The intestinal wall is made up of four layers: the mucosa, which consists of a single columnar epithelial layer and an underlying loose connective tissue called lamina propria; the submucosa, which consists of dense connective tissue; the bi-layered muscularis externa, made up by smooth muscle cells; and the outer, thin mesothelial layer called serosa (Fig 1).

The epithelium lining the lumen of the intestine accomplishes the essential task of absorbing the nutrients from the food. In order to increase the contact surface between the chyme and the intestine, the mucosa form finger-like protrusions into the lumen, called villi. Between the villi, the epithelium invaginates deep in to the lamina propria and form pear-shaped structures called crypts of Lieberkühn. A villus and its associated crypt form the functional unit of the intestine (Fig 2).
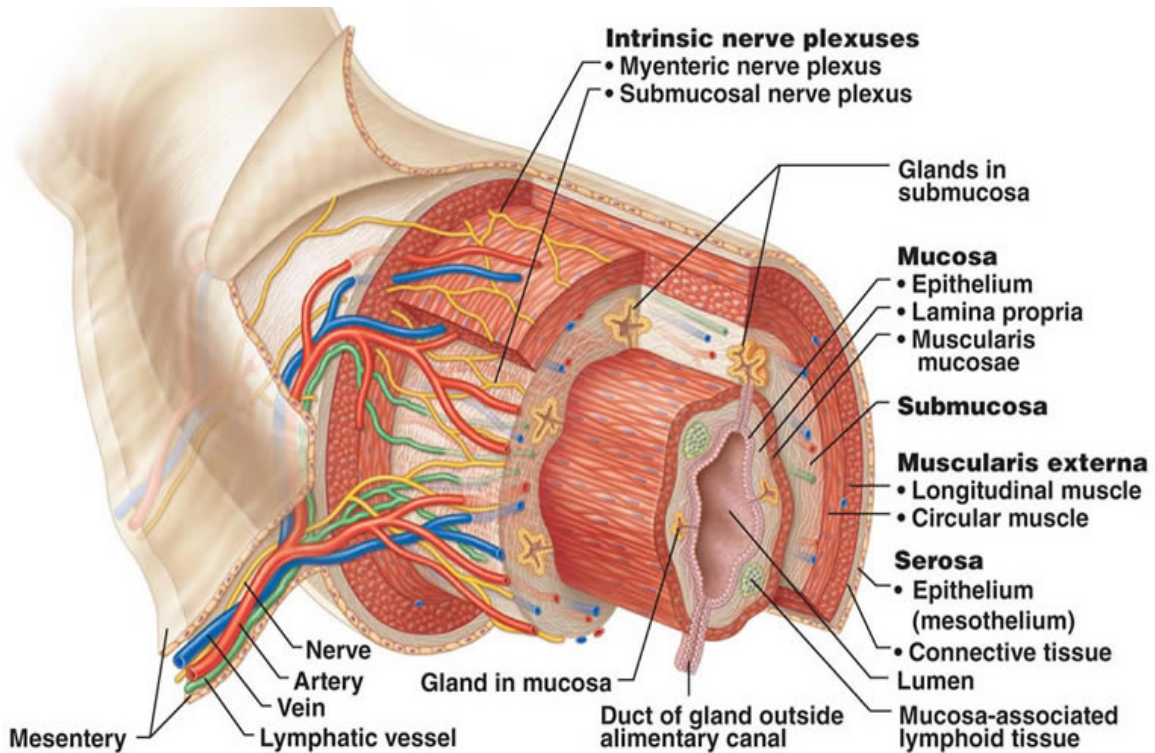
Fig. 1 Schematic view of small intestine. The small intestine consists of four distinct layers: mucosa (epithelium + lamina properia), submucosa, muscle layers (inner circular and outer longitudinal layer) and serosa.

Villus and crypt homeostasis

The mesenchymal core of the villus consists of blood capillaries, lacteals (lymph capillaries) and fibroblasts, all embedded in a complex extracellular matrix. The simple columnar epithelium, which completely covers the villus mesenchyme, consists of postmitotic and terminally differentiated cells, such as absorptive enterocytes, mucin releasing goblet cells, hormone producing entroendocrine cells, and sensory tuft cells. The crypt hosts the epithelial stem cells, undifferentiated transiently proliferative cells and Paneth cells (van der Flier et al., 2009a).

Microvilli
(brush border)

Absorptive cells

Lacteal

Goblet cell

Blood capillaries

Mucosa-associated lymphoid tissue

Intestinal crypt

Muscularis mucosae

Duodenal gland

Villus

Enteroendocrine cells

Venule

Lymphatic vessel

Submucosa
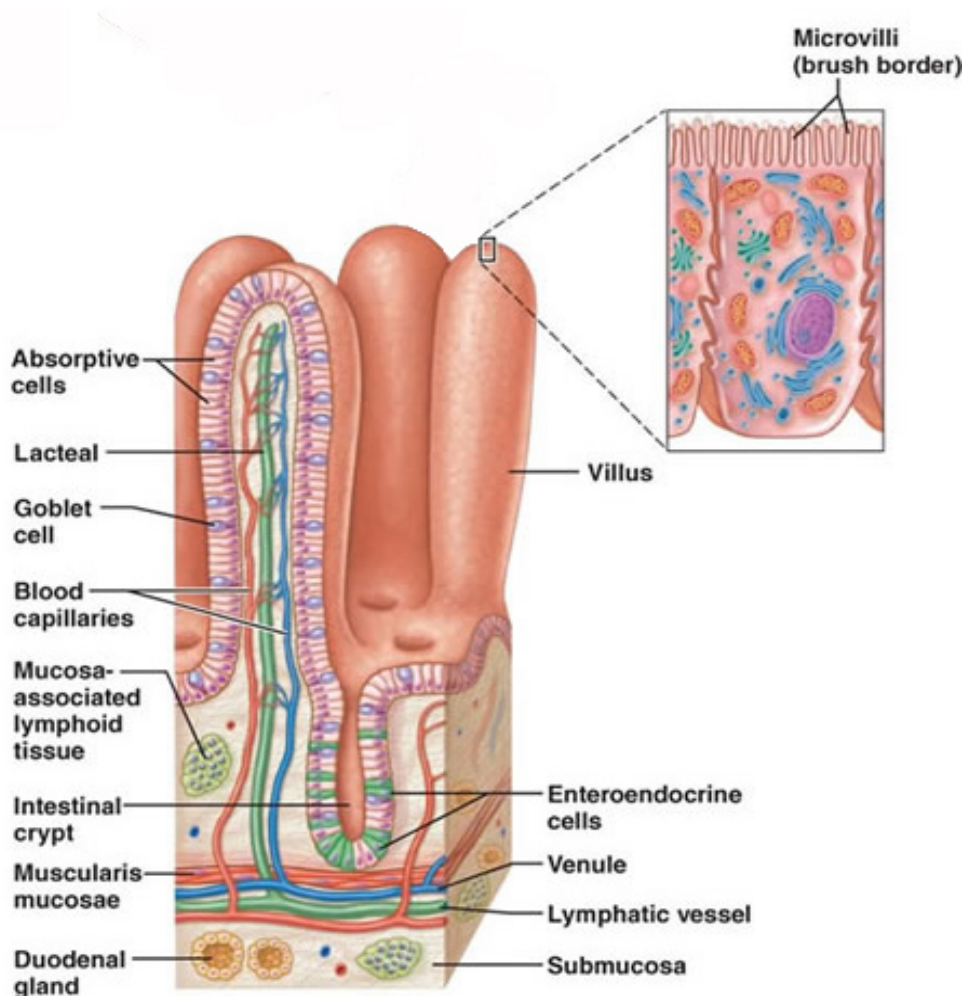
Fig. 2 Schematic illustration of an intestinal villus and its associated crypt of Lieberkühn. The mesenchymal core of the villus is covered by a single layer of epithelial cells consisting of different cell types all of which are derived from intestinal stem cells, which reside in the crypt of Lieberkühn.

The process of epithelial self-renewal was described already in the middle of the previous century (Stevens and Leblond, 1947), but the mechanistic basis began to be understood during the last decades (Morrison and Spradling, 2008; Radtke and Clevers, 2005). The epithelial cells are constantly produced in the crypts, where the epithelial stem cells reside. The stem cell niche is the restricted microenvironment that supports survival and division of a stem cell (Morrison and Spradling, 2008). Duplication of intestinal stem cells results in identical daughter cells, but the limitations of the stem cell niche will maintain a constant number of stem cells, whereas the redundant cells become transiently proliferative cells, which keep dividing as they migrate up the crypt. At the crypt–villus boundary they exit the cell cycle and initiate differentiation to one of the terminally differentiated epithelial cell types. Migration continues all the way to the tip of the villus, where the cells eventually undergo apoptosis and are shed into the intestinal lumen. The exception being the Paneth cells, which instead descend to the base of the crypt where they survive for an average of three weeks (Troughton and Trier, 1969).

Search for specific intestinal stem cell markers has led to identification of two distinct types of stem cells: long-term, normally quiescent, stem cells characterized by expression of Bmi1, which are activated only in response to tissue damage (Reinisch et al., 2006; van der Flier et al., 2009b), and the rapidly dividing Lgr5+ cells responsible for everyday epithelial renewal (Barker et al., 2007).

Regulation of the intestinal stem cells

As pluripotent and rapidly dividing cells, stem cells must be strictly regulated. Alteration or deregulation of such a control could lead to physiological changes such as senescence and pathological problems such as neoplasia and cancer.

Since gastrointestinal cancers are common malignancies, intestinal stem cells and the mechanisms controlling their homeostasis and physiology have been under intensive investigation. A tribute to the level of the current understanding of the molecular mechanisms underlying maintenance of the crypt stem cell niche is the recent success with *in vitro* culture of crypt organoids, and regeneration of complex organoid structures from single stem cells (Sato and Clevers, 2013). However, the analysis of the intestinal stem cells and their niche requirements have focused heavily on the epithelial cells – as stem cells and as niche factors. The crucial role of the surrounding mesenchyme/fibroblasts for shaping the paracrine gradients that define the stem cell niche is less well understood. Several signaling pathways are known to be part of the epithelial–mesenchymal cross talk along the crypt–villus axis and to influence the intestinal stem cell niche. Below, I will briefly describe four of these.

Hedgehog

For a detailed review of Hedgehog (Hh) signaling, please refer to Varjosalo and Taipale (Varjosalo and Taipale, 2008). Briefly, binding of the Hh ligand (Sonic [Shh], Indian [Ihh], or Desert [Dhh] Hh) to the membrane bound receptor, Patched (Ptch1), leads to de-repression of the signaling component of the membrane receptor, Smoothened (Smo), and stabilization of the zinc finger transcription factors Gli, which in turn activates the transcription of specific target genes. The Hh pathway is not only important in embryonic development, but also has an essential role for stem cell maintenance and associated diseases in several organs (Beachy et al., 2004). For reviews of the Hh pathway in development and disease, see (Briscoe and Therond, 2013; Jiang and Hui, 2008).

In the gut, Hh ligands (Shh and Ihh) are secreted exclusively from the epithelium and acts in a paracrine fashion on Ptch1/Smo in the mesenchyme. In the embryonic colon, Ihh is expressed equally throughout the epithelium, whereas Shh mRNA is detected only in the crypt epithelium. In the small intestine, the expression of both ligands is concentrated at the base of the villi (Ramalho-Santos et al., 2000).

Perturbation in Hh signaling in the intestine leads to abnormal development in both epithelial and mesenchymal compartments, which illustrates the entangled nature of their reciprocal cross-talk (Kolterud et al., 2009; Madison et al., 2005). The model is complicated further by the profound differences in phenotype between *Shh* and *Ihh* mutants. Murine null mutants of Shh and Ihh exhibit mirrored phenotypes in the intestinal epithelium. While *Shh$^{-/-}$* have proliferation deficiencies and depletion of the progenitor cells, *Ihh$^{-/-}$* shows overgrowth of the duodenal villi (Madison et al., 2005; Ramalho-Santos et al., 2000).

The effects on the epithelium is believed to be, at least in part, a consequence of inhibition of Wnt signaling, triggered by paracrine Hh and originating from the underlying

mesenchyme (Kolterud et al., 2009). Hh was shown to antagonize the activity of Wnt signaling in colon cancer cell lines in an autocrine manner, mediated by the Hh effector Gli1. Since Wnt is an essential stem cell factor and promotes survival and proliferation in the epithelium, the net effect of Hh signaling on intestinal homeostasis is to limit the size of the intestinal stem cell niche (van den Brink et al., 2004). *Foxf2* is a mesenchymal target of epithelial Hh (Ormestad et al., 2006). How activation of *Foxf2* leads to paracrine inhibition of Wnt signaling is the focus of Paper III (see below).

Notch

Ligands of Notch (Delta-like and Jagged) are membrane bond and require cell-to-cell contact to bind their receptors. Ligand binding induces proteolytic release of the intracellular domain of Notch, which translocates to the nucleus and activates transcription of target genes. Mechanistic details of Notch signaling pathway and its regulation are discussed in recent reviews (Andersson et al., 2011; Fortini, 2009; Guruharsha et al., 2012).

Notch signaling is highly conserved among metazoans (Artavanis-Tsakonas et al., 1999), but the role in intestinal development appears to be different in mammals and insects. Abrogation of Notch signaling in the mouse intestine depletes the stem cell population, whereas in the fruit fly it increases the number of stem cell-like cells (Fre et al., 2011). A likely explanation for this discrepancy is the fundamental structural differences between the vertebrate and arthropod intestine (Fre et al., 2011).

In the intestinal crypt, Notch is an essential stem cell niche factor. In its absence, Lgr5+ cells cease to proliferate and undergo apoptosis (VanDussen et al., 2012). The stimulatory effect of Notch on proliferation of crypt cells is Wnt dependent (Fre et al., 2009). Notch also has a role in the cell fate determination of epithelial cells, and promotes differentiation into the absorptive, rather than secretory, cell lineage (Fre et al., 2005; Gerbe et al., 2011; Jensen et al., 2000; Pellegrinet et al., 2011) .

Notch signaling components are expressed in the crypt proliferative region. Lineage tracing and enrichment of Notch1 in the crypt base columnar (Lgr5+) cells argues for active Notch signaling in the stem cells (Pellegrinet et al., 2011; van der Flier et al., 2009b). Paneth cells, which surround the Lgr5+ cells, seem to be the major source of Notch ligands in the crypt (Sato et al., 2011). Successful *in vitro* culture of crypt organoids, using the Lgr5+ cells mixed with Paneth cells, suggests that Notch ligand-expressing Paneth cells are essential constituents of the stem cell niche (Sato et al., 2011). It has been shown that the expression of the stem cell marker, Olmf4 is directly dependent on Notch signaling (VanDussen et al., 2012).

Bmp

Paracrine signaling by Bmp:s rely on binding of the ligand to the membrane-bound Type-2 receptor, which in turn recruits the Type-1 receptor. The receptor complex phosphorylates the cytoplasmic signaling molecules Smad1, Smad5, or Smad8, which heterodimerize with the common Tgfb/Bmp signal transducer Smad4. The heteromeric complex translocates into the nucleus, and with the help of other transcription factors initiate transcription of target genes (Derynck and Zhang, 2003; Massague, 2000; Miyazono et al., 2000; Shi and Massague, 2003).

Expression of Bmp signaling components in the intestine has been studied in detail (Batts et al., 2006; Haramis et al., 2004; He et al., 2004). Briefly, Bmp2 is present in the villus tip epithelium, whereas Bmp4 is expressed in the mesenchymal compartment of

the intestine. In the developing embryonic intestine, *Bmp4* is expressed in the mesen-chyme of the villus tip (Karlsson et al., 2000). The Bmp receptor Bmpra1 is found, at a very low level, in the intestinal stroma and at high level in the epithelium. Bmpra1 has the highest expression in the *Bmi1-* expressing quiescent stem cells and also in the villus tip epithelium. Unlike the Bmp ligands and receptors, which are expressed both in the epithelium and mesenchyme, Bmp antagonists mainly originate from the mesenchyme (Hardwick et al., 2008; Kosinski et al., 2007; Li et al., 2007).

Unlike Notch and Wnt signaling, Bmp signaling generally restricts the stem cell niche and prevents the stem cell self-renewal. Inhibition of Bmp signaling in the intestine by transgenic expression of the Bmp antagonist noggin, leads to *de novo* ectopic crypt for-mation in the flank of the villus (Haramis et al., 2004). The inhibitory effect of Bmp sig-naling on the stem cell self-renewal is exerted through inhibition of the Wnt pathway (He et al., 2004). A reflection of this is the requirement for addition of high concentra-tions of noggin to the growth media in order to grow crypt organoids *in vitro*.

Wnt

The Wnt signaling pathway is the master regulator of intestinal epithelial homeosta-sis (Korinek et al., 1998; Pinto and Clevers, 2005; Scoville et al., 2008). Activation of the Wnt pathway in the intestinal crypt is the main driving force for stem cell renewal, as well as for proliferation of the transiently proliferative cells of the crypt. Blocking the Wnt pathway in the embryonic and adult intestine through overexpression of the Wnt antagonist, Dkk1, or removal of the Wnt transducers Tcf4 or β-catenin, leads to a dra-matic loss of proliferative cells (Fevr et al., 2007; Ireland et al., 2004; Korinek et al., 1998; Kuhnert et al., 2004; Pinto et al., 2003). In contrast, over-activation of the Wnt pathway leads to expansion of stem cells (Gat et al., 1998; Kim et al., 2005). Many of the regulatory effects of other signaling pathways on stem cell regulation are exerted trough modulation of Wnt signaling (Fre et al., 2009; He et al., 2004). The fact that intestinal stem cell markers such as Lgr5, Msi1 and CD44 are direct Wnt targets suggests that the crypt stem cell fate is directly dependent on Wnt signaling (Barker et al., 2007; Hou et al., 2011; Rezza et al., 2010).

Below, I will generally describe the canonical Wnt pathway and discuss its im-portance for adenoma/cancer formation. For detailed accounts of these subjects, refer to the following reviews: (Clevers and Nusse, 2012; Valkenburg et al., 2011).

The canonical Wnt pathway leads to activation of transcription of Wnt target genes by a protein complex, which forms after binding of β-catenin to transcription factors of the Tcf/Lef family in the cell nucleus. Upon activation of Wnt signaling, β-catenin enters the nucleus and replaces the transcriptional inhibitor Groucho, which is associated with Tcf/Lef in the absence of β-catenin. The Tcf/β-catenin complex recruits additional pro-teins, such as transcriptional co-activators and histone modifiers (Cavallo et al., 1998; Roose et al., 1998). Thus the amount of β-catenin in the nucleus is a limiting factor for Wnt signaling.

In the absence of Wnt ligand, cytoplasmic β-catenin is phosphorylated by Gsk3 in the cytoplasmic destruction complex. The phosphorylated β-catenin becomes ubiquitinated by βTrCP and degraded inside proteasomes (Fig 3A). The Wnt destruction complex con-sists of proteins such as Adenoma polyposis coli (Apc), Gsk3, Ck1, Axin, Dvl. Upon inter-action of Wnt ligands with the heterodimeric Wnt receptor complex (Frizzled & LRP5/6), and phosphorylation of LRP5/6, the β-catenin destruction complex binds to the LRP and Gsk3 is released from the destruction complex. Inactivation of the destruc-

8

tion complex leads to accumulation of β-catenin in the cytoplasm and its translocation to the nucleus where β-catenin associates with Tcf and drives the transcription of Wnt target genes.
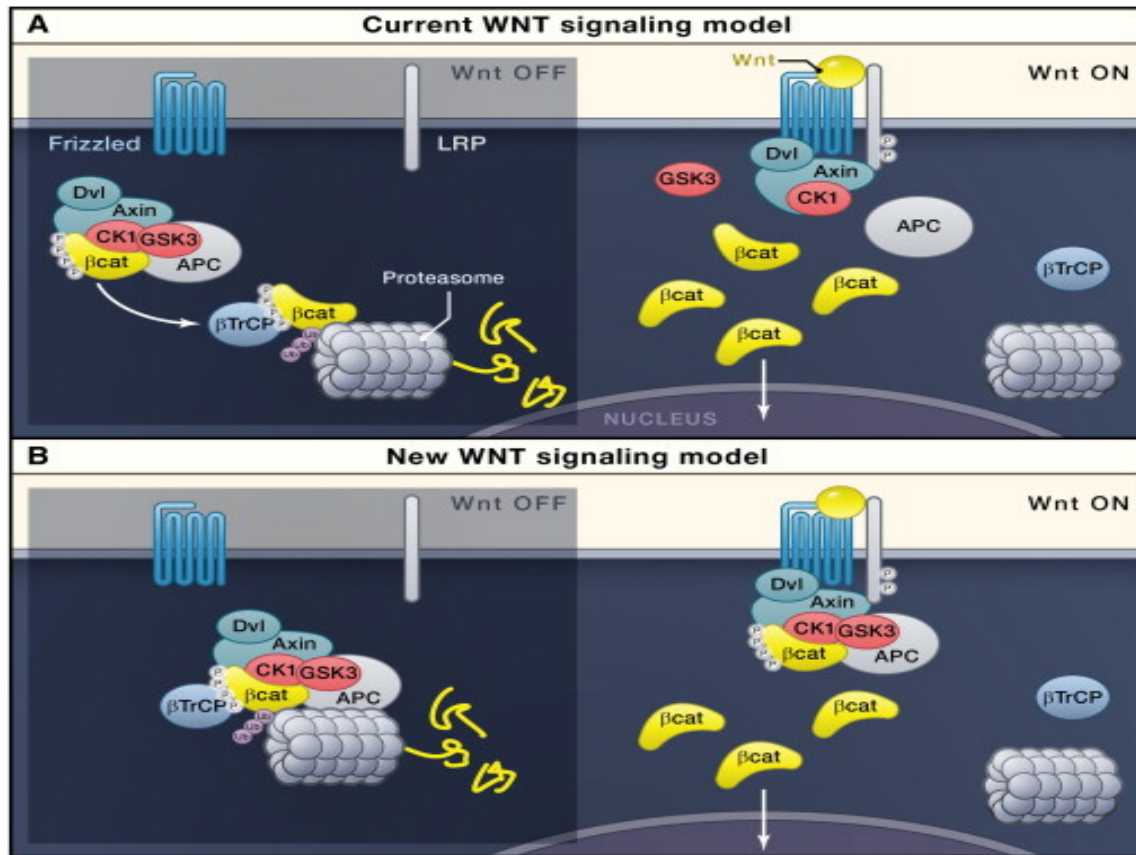

Fig. 3 β-catenin kinetics in the presence and absence of active Wnt ligand: A) The current model of Wnt signaling. In the absence of Wnt ligands the β-catenin is phosphorylated by the destruction complex and subsequently ubiquitinated by β-TrCP in the cytoplasm and degraded by the proteasome. Upon Wnt ligand binding to the receptor, the destruction complex disassembles and β-catenin is stabilized. B) The new model of Wnt signaling suggested by Li et al., 2012. Both phosphorylation and the ubiquitination of the β-catenin is accomplished by the destruction complex in the absence of Wnt ligands. Upon Wnt ligand binding, β-TrCP is excluded from the complex. The destruction complex can still capture and phosphorylate β-catenin, but ubiquitination is impaired. The newly synthetized β-catenin accumulates. The figure is adopted from (Clevers and Nusse, 2012).

Based on new studies, Li et al proposed a different model of action for the β-catenin destruction complex in witch the β-TrCP, which ubiquitinates the phosphorylated β-catenin, is a part of the destruction complex. Upon binding to LRP, the destruction complex can still phosphorylate the β-catenin, but ubiquitination by β-TrCP is blocked. The phosphorylated β-catenin then accumulates and translocates to the nucleus (Clevers and Nusse, 2012; Li et al., 2012). Fig 3 summarizes the traditional and the newly proposed Wnt activation mechanisms.

Canonical Wnt signaling is regulated at various levels and through different mechanisms, many of which involve Wnt inhibitors. At the ligand-receptor level, Wnt signaling is inhibited by small, secreted inhibitory proteins such as Dickkopf (Dkk1), WISE/SOST, Frizzled related proteins (Sfrps), Kremen, and Wnt inhibitory protein (Wif). Another type of Wnt inhibitor is the membrane bound glycoprotein APCDD1, which interacts both with Wnt ligands and the LRP co-receptor (Shimomura et al., 2010).

WISE/SOST and Dkk1 antagonize Wnt through binding Wnt co-receptors, LRP5/6. Dkk1 disrupts the formation of the heterodimeric receptor complex, Fz-LRP6. The antagonistic effect of Wif and Sfrp is through binding the Wnt ligands and making them unavailable for the receptors (Bovolenta et al., 2008; Ellwanger et al., 2008; Glinka et al., 1998; Semenov et al., 2005; Semenov et al., 2008). Trafficking of β-catenin to the nucleus is another event that is actively regulated by diverse mechanisms. For instance, the PI3K pathway is necessary for nuclear entry of β-catenin (Wu et al., 2008).

Most of the Wnt signaling pathway components are expressed in the epithelium (Gregorieff et al., 2005), but although the mechanisms are less well-understood, indirect evidence indicate an important role for the surrounding mesenchyme in modulating the Wnt pathway. One example of a mesenchymal transcription factor that influences epithelial Wnt signaling is Foxl1. *Foxl1* null mutants have increased formation of intestinal adenomas and exhibit an elevated activity of the Wnt pathway. Although the mechanism is not fully understood, control of deposition of heparan sulfate proteoglycans in the extracellular matrix has been proposed to be controlled by Foxl1 and to affect the efficiency of Wnt signaling (Perreault et al., 2001; Perreault et al., 2005).

Wnt ligands can trigger several alternative signaling events in the target cells that do not depend on β-catenin as transcriptional activator. These are collectively referred to as non-canonical pathways. Indirectly, the canonical pathway interacts with other signaling pathways. For example, Wnt shares Gsk3 with the mTOR pathway. Upon the activation of Wnt and disassembly of the β-catenin destruction complex, released Gsk3 phosphorylates the mTOR inhibitor Tsc2, which in turn leads to activation of mTOR and promotes proliferation.

Stem cells and colorectal neoplasia.

Tight regulation of Wnt signaling is absolutely essential in the intestinal stem cell niche, and deregulation of Wnt signaling is the most frequent cause of gastrointestinal tract neoplasia.

In which cells do mutations affecting Wnt signaling lead to adenocarcinoma development? Under normal conditions, the canonical Wnt pathway is active in the crypt stem cells and the transiently proliferative cells of the crypt. Barker et al showed that inactivation of *Apc*, encoding an essential component of the destruction complex and the most commonly mutated gene in human colorectal cancer, in Lgr5+ cells immediately caused widespread transformation, whereas the same mutation in transiently proliferative cells, or in the differentiated cells, did not lead to transformation, even after one month (Barker et al., 2009). Additional support for the notion that stem cells are the direct targets of Wnt mutations in intestinal cancer comes from a study in which deletion of a stem cell marker, Cd44 in *Apc*^*Min/+* mice (*i e Apc* heterozygotes with high spontaneous rate of adenoma formation due to loss of heterozygosity) resulted in a dramatic reduction of adenoma formation (Zeilstra et al., 2008). A prediction from this model is that the probability of initiation of an adenocarcinoma is proportional to the number of stem cells and the frequency of their division. From this follows that genetic perturbations

that modulates the size of the stem cell niche, and/or stem cell proliferation, are likely to affect the risk of developing intestinal neoplasias. This prediction is addressed in Paper III.

Paper II

In order to answer questions about the origin of expression of different genes in the intestine, I developed a method for separation of intact epithelium from the mesodermally derived (non-epithelial) tissues of mouse small intestine and colon. Contrary to previously available protocols, our method uses neither enzymes, nor harsh physical treatments, or chelating agents. Other advantages are purity, speed (the whole procedure takes half an hour), and that the tissue is kept on ice during the whole procedure. Taken together, this translates into pure preparations with very little cross contamination, and excellent viability.

Paper III

In this paper, we investigated the effect of modest alterations in expression level of the mesenchymal transcription factor Foxf2 on adenoma formation in the $Apc^{Min/+}$ strain, and on the intestinal stem cell niche.

We showed that Foxf2 is localized to nuclei of a subset of sub-epithelial myofibroblasts in the mesenchymal compartment of the intestine. *Foxf2* expression is higher in the villus than around the crypt base, consistent with activation of mesenchymal *Foxf2* by epithelial Hh signaling.

Heterozygosity for *Foxf2* resulted in enhanced activity of the canonical Wnt pathway in the epithelium, an increase in the number of Lgr5+ cells in the crypts, elevated proliferation of crypt cells, and to formation of more and larger adenomas on $Apc^{Min/+}$ background. Interestingly, a moderate overexpression of *FoxF2*, by means of an extra copy of human *FOXF2* driven by its endogenous promoter, produced a phenotype that was a mirror image of the heterozygosity; smaller and fewer adenomas, fewer stem cells, less proliferation and less expression of a key Wnt target gene, c-Myc. Thus, a negative correlation exists between *Foxf2* gene dosage/expression level and the size of the crypt stem cell niche. Based on expression analysis of candidate genes, we conclude that a major part of the paracrine mechanism through which Foxf2 in mesenchymal cells inhibits Wnt signaling in epithelial consists of increased expression of *Sfrp1*, encoding an extracellular Wnt inhibitor.

Palatogenesis

The secondary palate forms the roof of the oral cavity, and separates it from the nasal cavity. Palatogenesis is a complex developmental process, which in mouse occurs between embryonic day 11.5 and 15.5. The palatal shelf mesenchyme, which differentiates into cartilage and connective tissue, originates from cranial neural crest, whereas the epithelia that cover the upper and lower palatal surfaces are continuous with those of the nasal and oral cavities, respectively (Ito et al., 2003).

The process and the molecular mechanism of murine palatogenesis have been reviewed previously (Bush and Jiang, 2012). At E11.5, neural crest derived cells from the maxillary processes begin to form ridges along the anteroposterior axis. These ridges continue to grow vertically, down the sides of the tongue, forming the palatal shelves. The expansion is driven in roughly equal parts by proliferation, and by accumulation of extracellular matrix (ECM) produced by the mesenchyme. The ECM mainly consists of glycosaminoglycans (GAG), collagens and fibronectin. GAGs in the ECM are hygroscopic, which contributes to the increase in volume. Since GAG deposition is slightly asymmetric, the swelling elevates the palatal shelves to a horizontal position, above the tongue. Continued horizontal growth, brings the two palatal shelves together at the midline, where they fuse. In C57Bl/6 palatal fusion is completed by E15.5 (Fig. 4).
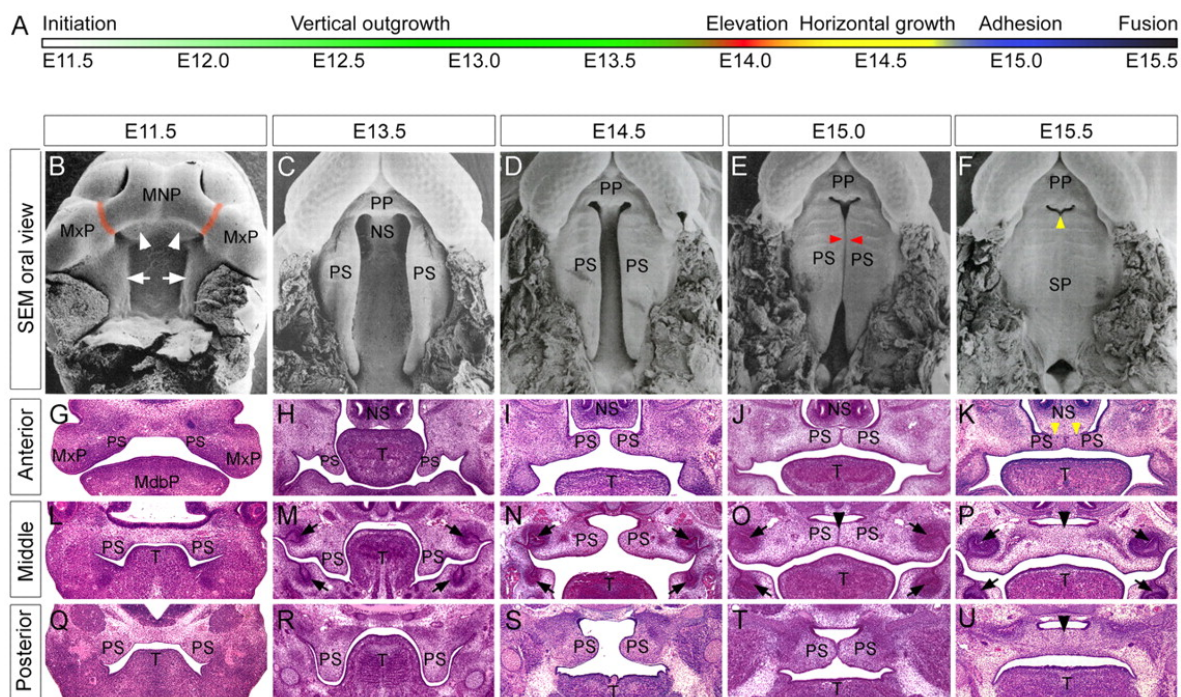

Fig. 4 Palatogenesis in the mouse embryo. (A) Time course of secondary palate development. (B-F) Scanning electron micrographs showing ventral views of the secondary palate at representative developmental stages. (G-U) Histological frontal sections from anterior, middle and posterior regions of the developing palate at indicated stages. Image adopted from (Bush and Jiang, 2012).

Cleft palate

Palatogenesis is thus a multistep developmental process, which involves proliferation, migration, ECM deposition and differentiation. Cleft palate (CP) results when one of these events fails. The sensitivity of palatogenesis to genetic and teratogenic disturbances is illustrated by the high incidence of CP; in humans around one in a thousand newborns. CP is listed as part of about 300 syndromes, but it also occurs isolated.

In humans, CP can usually be corrected surgically, but in rodents, which are obligatory nose breathers, it is fatal at birth due to its interference with breathing and suckling. Reverse genetics in mouse has identified a large number of proteins, from transcription factors to signaling molecules and ECM components, as essential for normal palatogenesis. In addition to genetic defects, environmental factors such as smoking, dioxin, and viruses have been shown to cause CP in humans. Among the many signaling pathways involved in palatogenesis, I will focus on Tgfβ and integrin signaling, which are relevant to Paper IV.

Tgfβ signaling in palatogenesis

Secreted Tgfβs are held in a latent complex, which consist of the mature Tgfβ ligand, latent Tgfβ binding protein (LTBP) and processed Tgfβ propeptide (latency associated protein, LAP)(Saharinen et al., 1996). Covalent bonds between the LTBP and components of the ECM incorporate the Tgfβ large latent complex (LLC) into the ECM (Taipale et al., 1996). An important level of control of Tgfβ signaling is therefore mobilization of the active form of the Tgfβ ligand in the extracellular space. Releasing of the active Tgfβ ligand, involves detachment of the LLC from the ECM and also removal of the latency associated peptide from the 25kD active ligand dimer.

Liberating the LLC from the ECM could be done either through degradation of the ECM microfibrils or by enzymatic cleavage of the LTBP. These processes are mediated by a wide range of proteases including mast cell chymase, plasmin and thrombin and matrix metaloproteinases (Annes et al., 2003; ten Dijke and Arthur, 2007).

Another essential step in liberating the mature Tgfβ ligand is to eliminate the LAP. The ECM protein thrombospondin-1 (THBS1) is a major activator of Tgfβ1 (Crawford et al., 1998). Conformational changes in the LAP upon binding to the THBS1, prevents the LAP from conferring latency on Tgfβ (Murphy-Ullrich and Poczatek, 2000).

Integrins are another important mediator of Tgfβ activation and fibronectin plays an important role in the activation process as ligand for the relevant integrins. The LAPs of Tgfβ1 and Tgfβ3 contain an integrin binding RGD sequence, whereas that of Tgfβ2 does not. Experimental data indicate that in the absence of integrin αvβ1, activation of latent Tgfβ is inefficient (Fontana et al., 2005). Integrins αvβ6 and αvβ8 directly interact with LAP's RGD sequences and activate the respective Tgfβ (Munger et al., 1999; Sheppard, 2005). Integrins are so important for activation of Tgfβ1 that transgenic mice harboring a non-functional RGD in its Tgfβ1 phenocopy the Tgfβ1 null mutant (Yang et al., 2007). Furthermore, mice with mutated αv or β8 integrins, exhibit similar phenotypes as Tgfβ1 and Tgfβ3 mutants (Bader et al., 1998; Zhu et al., 2002). The exact mechanism of activation is yet unknown, but conformational changes in the LAP and liberation or exposure of the Tgfβ after interaction with integrins have been suggested (ten Dijke and Arthur, 2007).

After being released from the ECM and elimination of the LAP, the active form of the Tgfβ ligand binds to the trans-membrane threonine/serine kinase receptor heteromeric complexes of Tgfβ receptors I and II (Cheifetz et al., 1987; Wrana et al., 1992). Tgfβ receptors III, also known as betaglycan, plays an important role in this process as co-receptor. It is not a signaling protein, but a large, membrane anchored proteoglycan that efficiently binds extracellular Tgfβ. Its role in the receptor activation process is not fully understood, but it is believed to act as reservoir of ligand and to facilitate binding by TgfβrII. Upon binding of ligand to the receptor complex, TgfβrI is phosphorylated by TgfβrII. This phosphorylation attracts the Smad2/3 signal transducer to the complex.

Smad2/3 is phosphorylated by the receptor, binds Smad4, translocates to the nucleus and activates the transcription of Tgfβ target genes (Shi and Massague, 2003).

Tgfβ signaling is essential for several steps in secondary palate development. Mutation of the genes encoding the ligands: Tgfβ1, Tgfβ2, Tgfβ3; receptors: TgfβrI (Alk5), TgfβrII, TgfβrIII; and signal transducer, Smad2, leads to defects in palatal formation, or fusion, in mice (Dudas et al., 2006; Ito et al., 2003; Kaartinen et al., 1995; Sanford et al., 1997; Shiomi et al., 2006). Tgfβ can also act through alternative, non-canonical pathways, which do not depend on TgfβrII /Smad2/3. Iwata *et al* showed that canonical and non-canonical Tgfβ signaling are antagonistic in palatogenesis, and that abrogation of the non-canonical pathway can rescue cleft palate in *TgfβrII* mutant animals (Iwata et al., 2012).

Tgfb mutant palate phenotype

Of the Tgfβ ligands, Tgfβ1 and Tgfβ3 are expressed only in the palatal shelf epithelium, whereas Tgfβ2 is produced in the mesenchyme (Fitzpatrick et al., 1990; Pelton et al., 1990). The receptor TgfβrII is expressed in both epithelium and mesenchyme, and plays important, but distinct, roles in both tissues. Targeting *TgfβrII* in the mesenchyme of the palatal shelves leads to cleft palate due to reduction in cell proliferation (Ito et al., 2003). Disabling *TgfβrII* specifically in the epithelium also leads to cleft palate, but in this case it is the fusion of the shelves at the midline that fails (Xu et al., 2006).

Paper IV

Wang *et al* (Wang et al., 2003) first described the cleft palate phenotype in the murine *Foxf2* null mutant. Spatial interference with elevation of the palatal shelves, as a result of an abnormal tongue morphology, was proposed as the mechanism responsible. We failed to observe any consistent morphological defects in the tongues of *Foxf2^-/-* embryos. Instead, we generated a conditional (floxed) *Foxf2* allele and showed that *Foxf2* is essential in neural crest, which contributes to palatal shelf mesenchyme, but not to the tongue. We also used *in vitro* culture of maxillary explants to show that the failure to expand and fuse is intrinsic to the palatal tissue and distinguishes *Foxf2* mutant from wild-type explants, even in the absence of tongue and mandible. The failed expansion of the palatal shelves was associated with a reduction in both mesenchymal proliferation, and collagen accumulation. Consistent with the importance of Tgfβ signaling for both of these processes, the phosphorylation of Smad2/3 was diminished, whereas a readout for the non-canonical signaling – phosphorylation of p38 – was increased. Tgfβ2 protein level was decreased, but the mRNA level was not. The mechanism behind the reduction in Tgfβ2 protein is not fully understood, but expression of genes encoding proteins involved in latency formation (fibronectin), activation (integrins αV and β1), and receptor binding (TgfβrIII) of Tgfβ were significantly reduced.

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References

Aitola, M., Carlsson, P., Mahlapuu, M., Enerback, S., and Pelto-Huikko, M. (2000). Forkhead transcription factor FoxF2 is expressed in mesodermal tissues involved in epithelio-mesenchymal interactions. Developmental dynamics : an official publication of the American Association of Anatomists *218*, 136-149.

Andersson, E.R., Sandberg, R., and Lendahl, U. (2011). Notch signaling: simplicity in design, versatility in function. Development *138*, 3593-3612.

Annes, J.P., Munger, J.S., and Rifkin, D.B. (2003). Making sense of latent TGFbeta activation. Journal of cell science *116*, 217-224.

Artavanis-Tsakonas, S., Rand, M.D., and Lake, R.J. (1999). Notch signaling: cell fate control and signal integration in development. Science *284*, 770-776.

Astorga, J., and Carlsson, P. (2007). Hedgehog induction of murine vasculogenesis is mediated by Foxf1 and Bmp4. Development *134*, 3753-3761.

Bader, B.L., Rayburn, H., Crowley, D., and Hynes, R.O. (1998). Extensive vasculogenesis, angiogenesis, and organogenesis precede lethality in mice lacking all alpha v integrins. Cell *95*, 507-519.

Barker, N., Ridgway, R.A., van Es, J.H., van de Wetering, M., Begthel, H., van den Born, M., Danenberg, E., Clarke, A.R., Sansom, O.J., and Clevers, H. (2009). Crypt stem cells as the cells-of-origin of intestinal cancer. Nature *457*, 608-611.

Barker, N., van Es, J.H., Kuipers, J., Kujala, P., van den Born, M., Cozijnsen, M., Haegebarth, A., Korving, J., Begthel, H., Peters, P.J.*, et al.* (2007). Identification of stem cells in small intestine and colon by marker gene Lgr5. Nature *449*, 1003-1007.

Batts, L.E., Polk, D.B., Dubois, R.N., and Kulessa, H. (2006). Bmp signaling is required for intestinal growth and morphogenesis. Developmental dynamics : an official publication of the American Association of Anatomists *235*, 1563-1570.

Beachy, P.A., Karhadkar, S.S., and Berman, D.M. (2004). Tissue repair and stem cell renewal in carcinogenesis. Nature *432*, 324-331.

Bovolenta, P., Esteve, P., Ruiz, J.M., Cisneros, E., and Lopez-Rios, J. (2008). Beyond Wnt inhibition: new functions of secreted Frizzled-related proteins in development and disease. Journal of cell science *121*, 737-746.

Briscoe, J., and Therond, P.P. (2013). The mechanisms of Hedgehog signalling and its roles in development and disease. Nature reviews Molecular cell biology *14*, 416-429.

Bush, J.O., and Jiang, R. (2012). Palatogenesis: morphogenetic and molecular mechanisms of secondary palate development. Development *139*, 231-243.

Carlsson, P., and Mahlapuu, M. (2002). Forkhead transcription factors: key players in development and metabolism. Developmental biology *250*, 1-23.

Cavallo, R.A., Cox, R.T., Moline, M.M., Roose, J., Polevoy, G.A., Clevers, H., Peifer, M., and Bejsovec, A. (1998). Drosophila Tcf and Groucho interact to repress Wingless signalling activity. Nature *395*, 604-608.

Cheifetz, S., Weatherbee, J.A., Tsang, M.L., Anderson, J.K., Mole, J.E., Lucas, R., and Massague, J. (1987). The transforming growth factor-beta system, a complex pattern of cross-reactive ligands and receptors. Cell *48*, 409-415.

Clevers, H., and Nusse, R. (2012). Wnt/beta-catenin signaling and disease. Cell *149*, 1192-1205.

Crawford, S.E., Stellmach, V., Murphy-Ullrich, J.E., Ribeiro, S.M., Lawler, J., Hynes, R.O., Boivin, G.P., and Bouck, N. (1998). Thrombospondin-1 is a major activator of TGF-beta1 in vivo. Cell *93*, 1159-1170.

Derynck, R., and Zhang, Y.E. (2003). Smad-dependent and Smad-independent pathways in TGF-beta family signalling. Nature *425*, 577-584.

Dudas, M., Kim, J., Li, W.Y., Nagy, A., Larsson, J., Karlsson, S., Chai, Y., and Kaartinen, V. (2006). Epithelial and ectomesenchymal role of the type I TGF-beta receptor ALK5 during facial morphogenesis and palatal fusion. Developmental biology *296*, 298-314.

Ellwanger, K., Saito, H., Clement-Lacroix, P., Maltry, N., Niedermeyer, J., Lee, W.K., Baron, R., Rawadi, G., Westphal, H., and Niehrs, C. (2008). Targeted disruption of the Wnt regulator Kremen induces limb defects and high bone density. Molecular and cellular biology *28*, 4875-4882.

Fevr, T., Robine, S., Louvard, D., and Huelsken, J. (2007). Wnt/beta-catenin is essential for intestinal homeostasis and maintenance of intestinal stem cells. Molecular and cellular biology *27*, 7551-7559.

Fitzpatrick, D.R., Denhez, F., Kondaiah, P., and Akhurst, R.J. (1990). Differential expression of TGF beta isoforms in murine palatogenesis. Development *109*, 585-595.

Fontana, L., Chen, Y., Prijatelj, P., Sakai, T., Fassler, R., Sakai, L.Y., and Rifkin, D.B. (2005). Fibronectin is required for integrin alphavbeta6-mediated activation of latent TGF-beta complexes containing LTBP-1. FASEB J *19*, 1798-1808.

Fortini, M.E. (2009). Notch signaling: the core pathway and its posttranslational regulation. Developmental cell *16*, 633-647.

Fre, S., Bardin, A., Robine, S., and Louvard, D. (2011). Notch signaling in intestinal homeostasis across species: the cases of Drosophila, Zebrafish and the mouse. Experimental cell research *317*, 2740-2747.

Fre, S., Huyghe, M., Mourikis, P., Robine, S., Louvard, D., and Artavanis-Tsakonas, S. (2005). Notch signals control the fate of immature progenitor cells in the intestine. Nature *435*, 964-968.

Fre, S., Pallavi, S.K., Huyghe, M., Lae, M., Janssen, K.P., Robine, S., Artavanis-Tsakonas, S., and Louvard, D. (2009). Notch and Wnt signals cooperatively control cell proliferation and tumorigenesis in the intestine. Proceedings of the National Academy of Sciences of the United States of America *106*, 6309-6314.

Gat, U., DasGupta, R., Degenstein, L., and Fuchs, E. (1998). De Novo hair follicle morphogenesis and hair tumors in mice expressing a truncated beta-catenin in skin. Cell *95*, 605-614.

Gerbe, F., van Es, J.H., Makrini, L., Brulin, B., Mellitzer, G., Robine, S., Romagnolo, B., Shroyer, N.F., Bourgaux, J.F., Pignodel, C.*, et al.* (2011). Distinct ATOH1 and Neurog3 requirements define tuft cells as a new secretory cell type in the intestinal epithelium. The Journal of cell biology *192*, 767-780.

Glinka, A., Wu, W., Delius, H., Monaghan, A.P., Blumenstock, C., and Niehrs, C. (1998). Dickkopf-1 is a member of a new family of secreted proteins and functions in head induction. Nature *391*, 357-362.

Gregorieff, A., Pinto, D., Begthel, H., Destree, O., Kielman, M., and Clevers, H. (2005). Expression pattern of Wnt signaling components in the adult intestine. Gastroenterology *129*, 626-638.

Guruharsha, K.G., Kankel, M.W., and Artavanis-Tsakonas, S. (2012). The Notch signalling system: recent insights into the complexity of a conserved pathway. Nature reviews Genetics *13*, 654-666.

Haramis, A.P., Begthel, H., van den Born, M., van Es, J., Jonkheer, S., Offerhaus, G.J., and Clevers, H. (2004). De novo crypt formation and juvenile polyposis on BMP inhibition in mouse intestine. Science *303*, 1684-1686.

Hardwick, J.C., Kodach, L.L., Offerhaus, G.J., and van den Brink, G.R. (2008). Bone morphogenetic protein signalling in colorectal cancer. Nature reviews Cancer *8*, 806-812.

He, X.C., Zhang, J., Tong, W.G., Tawfik, O., Ross, J., Scoville, D.H., Tian, Q., Zeng, X., He, X., Wiedemann, L.M.*, et al.* (2004). BMP signaling inhibits intestinal stem cell self-renewal through suppression of Wnt-beta-catenin signaling. Nature genetics *36*, 1117-1121.

Hou, N.Y., Yang, K., Chen, T., Chen, X.Z., Zhang, B., Mo, X.M., and Hu, J.K. (2011). CD133+ CD44+ subgroups may be human small intestinal stem cells. Molecular biology reports *38*, 997-1004.

Ireland, H., Kemp, R., Houghton, C., Howard, L., Clarke, A.R., Sansom, O.J., and Winton, D.J. (2004). Inducible Cre-mediated control of gene expression in the murine gastrointestinal tract: effect of loss of beta-catenin. Gastroenterology *126*, 1236-1246.

Ito, Y., Yeo, J.Y., Chytil, A., Han, J., Bringas, P., Jr., Nakajima, A., Shuler, C.F., Moses, H.L., and Chai, Y. (2003). Conditional inactivation of Tgfbr2 in cranial neural crest causes cleft palate and calvaria defects. Development *130*, 5269-5280.

Iwata, J., Hacia, J.G., Suzuki, A., Sanchez-Lara, P.A., Urata, M., and Chai, Y. (2012). Modulation of noncanonical TGF-beta signaling prevents cleft palate in Tgfbr2 mutant mice. The Journal of clinical investigation *122*, 873-885.

Jensen, J., Pedersen, E.E., Galante, P., Hald, J., Heller, R.S., Ishibashi, M., Kageyama, R., Guillemot, F., Serup, P., and Madsen, O.D. (2000). Control of endodermal endocrine development by Hes-1. Nature genetics *24*, 36-44.

Jeong, J., Mao, J., Tenzen, T., Kottmann, A.H., and McMahon, A.P. (2004). Hedgehog signaling in the neural crest cells regulates the patterning and growth of facial primordia. Genes & development *18*, 937-951.

Jiang, J., and Hui, C.C. (2008). Hedgehog signaling in development and cancer. Developmental cell *15*, 801-812.

Kaartinen, V., Voncken, J.W., Shuler, C., Warburton, D., Bu, D., Heisterkamp, N., and Groffen, J. (1995). Abnormal lung development and cleft palate in mice lacking TGF-beta 3 indicates defects of epithelial-mesenchymal interaction. Nat Genet *11*, 415-421.

Kalinichenko, V.V., Gusarova, G.A., Shin, B., and Costa, R.H. (2003). The forkhead box F1 transcription factor is expressed in brain and head mesenchyme during mouse embryonic development. Gene expression patterns : GEP *3*, 153-158.

Kalinichenko, V.V., Lim, L., Stolz, D.B., Shin, B., Rausa, F.M., Clark, J., Whitsett, J.A., Watkins, S.C., and Costa, R.H. (2001). Defects in pulmonary vasculature and perinatal lung hemorrhage in mice heterozygous null for the Forkhead Box f1 transcription factor. Dev Biol *235*, 489-506.

Kalinichenko, V.V., Zhou, Y., Bhattacharyya, D., Kim, W., Shin, B., Bambal, K., and Costa, R.H. (2002). Haploinsufficiency of the mouse Forkhead Box f1 gene causes defects in gall bladder development. The Journal of biological chemistry *277*, 12369-12374.

Karlsson, L., Lindahl, P., Heath, J.K., and Betsholtz, C. (2000). Abnormal gastrointestinal development in PDGF-A and PDGFR-(alpha) deficient mice implicates a novel mesenchymal structure with putative instructive properties in villus morphogenesis. Development *127*, 3457-3466.

Kim, K.A., Kakitani, M., Zhao, J., Oshima, T., Tang, T., Binnerts, M., Liu, Y., Boyle, B., Park, E., Emtage, P.*, et al.* (2005). Mitogenic influence of human R-spondin1 on the intestinal epithelium. Science *309*, 1256-1259.

Kolterud, A., Grosse, A.S., Zacharias, W.J., Walton, K.D., Kretovich, K.E., Madison, B.B., Waghray, M., Ferris, J.E., Hu, C., Merchant, J.L.*, et al.* (2009). Paracrine Hedgehog signaling

in stomach and intestine: new roles for hedgehog in gastrointestinal patterning. Gastroenterology *137*, 618-628.

Korinek, V., Barker, N., Moerer, P., van Donselaar, E., Huls, G., Peters, P.J., and Clevers, H. (1998). Depletion of epithelial stem-cell compartments in the small intestine of mice lacking Tcf-4. Nature genetics *19*, 379-383.

Kosinski, C., Li, V.S., Chan, A.S., Zhang, J., Ho, C., Tsui, W.Y., Chan, T.L., Mifflin, R.C., Powell, D.W., Yuen, S.T.*, et al.* (2007). Gene expression patterns of human colon tops and basal crypts and BMP antagonists as intestinal stem cell niche factors. Proceedings of the National Academy of Sciences of the United States of America *104*, 15418-15423.

Kuhnert, F., Davis, C.R., Wang, H.T., Chu, P., Lee, M., Yuan, J., Nusse, R., and Kuo, C.J. (2004). Essential requirement for Wnt signaling in proliferation of adult small intestine and colon revealed by adenoviral expression of Dickkopf-1. Proceedings of the National Academy of Sciences of the United States of America *101*, 266-271.

Langston, C. (1991). Misalignment of pulmonary veins and alveolar capillary dysplasia. Pediatric pathology / affiliated with the International Paediatric Pathology Association *11*, 163-170.

Li, V.S., Ng, S.S., Boersema, P.J., Low, T.Y., Karthaus, W.R., Gerlach, J.P., Mohammed, S., Heck, A.J., Maurice, M.M., Mahmoudi, T.*, et al.* (2012). Wnt signaling through inhibition of beta-catenin degradation in an intact Axin1 complex. Cell *149*, 1245-1256.

Li, X., Madison, B.B., Zacharias, W., Kolterud, A., States, D., and Gumucio, D.L. (2007). Deconvoluting the intestine: molecular evidence for a major role of the mesenchyme in the modulation of signaling cross talk. Physiological genomics *29*, 290-301.

Madison, B.B., Braunstein, K., Kuizon, E., Portman, K., Qiao, X.T., and Gumucio, D.L. (2005). Epithelial hedgehog signals pattern the intestinal crypt-villus axis. Development *132*, 279-289.

Mahlapuu, M., Enerback, S., and Carlsson, P. (2001a). Haploinsufficiency of the forkhead gene Foxf1, a target for sonic hedgehog signaling, causes lung and foregut malformations. Development *128*, 2397-2406.

Mahlapuu, M., Ormestad, M., Enerback, S., and Carlsson, P. (2001b). The forkhead transcription factor Foxf1 is required for differentiation of extra-embryonic and lateral plate mesoderm. Development *128*, 155-166.

Mahlapuu, M., Pelto-Huikko, M., Aitola, M., Enerback, S., and Carlsson, P. (1998). FREAC-1 contains a cell-type-specific transcriptional activation domain and is expressed in epithelial-mesenchymal interfaces. Developmental biology *202*, 183-195.

Massague, J. (2000). How cells read TGF-beta signals. Nature reviews Molecular cell biology *1*, 169-178.

Miyazono, K., ten Dijke, P., and Heldin, C.H. (2000). TGF-beta signaling by Smad proteins. Advances in immunology *75*, 115-157.

Morrison, S.J., and Spradling, A.C. (2008). Stem cells and niches: mechanisms that promote stem cell maintenance throughout life. Cell *132*, 598-611.

Munger, J.S., Huang, X., Kawakatsu, H., Griffiths, M.J., Dalton, S.L., Wu, J., Pittet, J.F., Kaminski, N., Garat, C., Matthay, M.A.*, et al.* (1999). The integrin alpha v beta 6 binds and activates latent TGF beta 1: a mechanism for regulating pulmonary inflammation and fibrosis. Cell *96*, 319-328.

Murphy-Ullrich, J.E., and Poczatek, M. (2000). Activation of latent TGF-beta by thrombospondin-1: mechanisms and physiology. Cytokine & growth factor reviews *11*, 59-69.

Ormestad, M., Astorga, J., and Carlsson, P. (2004). Differences in the embryonic expression patterns of mouse Foxf1 and -2 match their distinct mutant phenotypes. Developmental dynamics : an official publication of the American Association of Anatomists *229*, 328-333.

Ormestad, M., Astorga, J., Landgren, H., Wang, T., Johansson, B.R., Miura, N., and Carlsson, P. (2006). Foxf1 and Foxf2 control murine gut development by limiting mesenchymal Wnt signaling and promoting extracellular matrix production. Development *133*, 833-843.

Pellegrinet, L., Rodilla, V., Liu, Z., Chen, S., Koch, U., Espinosa, L., Kaestner, K.H., Kopan, R., Lewis, J., and Radtke, F. (2011). Dll1- and dll4-mediated notch signaling are required for homeostasis of intestinal stem cells. Gastroenterology *140*, 1230-1240 e1231-1237.

Pelton, R.W., Hogan, B.L., Miller, D.A., and Moses, H.L. (1990). Differential expression of genes encoding TGFs beta 1, beta 2, and beta 3 during murine palate formation. Dev Biol *141*, 456-460.

Perreault, N., Katz, J.P., Sackett, S.D., and Kaestner, K.H. (2001). Foxl1 controls the Wnt/beta-catenin pathway by modulating the expression of proteoglycans in the gut. The Journal of biological chemistry *276*, 43328-43333.

Perreault, N., Sackett, S.D., Katz, J.P., Furth, E.E., and Kaestner, K.H. (2005). Foxl1 is a mesenchymal Modifier of Min in carcinogenesis of stomach and colon. Genes & development *19*, 311-315.

Peterson, R.S., Lim, L., Ye, H., Zhou, H., Overdier, D.G., and Costa, R.H. (1997). The winged helix transcriptional activator HFH-8 is expressed in the mesoderm of the primitive streak stage of mouse embryos and its cellular derivatives. Mechanisms of development *69*, 53-69.

Pierrou, S., Hellqvist, M., Samuelsson, L., Enerback, S., and Carlsson, P. (1994). Cloning and characterization of seven human forkhead proteins: binding site specificity and DNA bending. The EMBO journal *13*, 5002-5012.

Pinto, D., and Clevers, H. (2005). Wnt, stem cells and cancer in the intestine. Biology of the cell / under the auspices of the European Cell Biology Organization *97*, 185-196.

Pinto, D., Gregorieff, A., Begthel, H., and Clevers, H. (2003). Canonical Wnt signals are essential for homeostasis of the intestinal epithelium. Genes & development *17*, 1709-1713.

Radtke, F., and Clevers, H. (2005). Self-renewal and cancer of the gut: two sides of a coin. Science *307*, 1904-1909.

Ramalho-Santos, M., Melton, D.A., and McMahon, A.P. (2000). Hedgehog signals regulate multiple aspects of gastrointestinal development. Development *127*, 2763-2772.

Reinisch, C., Kandutsch, S., Uthman, A., and Pammer, J. (2006). BMI-1: a protein expressed in stem cells, specialized cells and tumors of the gastrointestinal tract. Histology and histopathology *21*, 1143-1149.

Rezza, A., Skah, S., Roche, C., Nadjar, J., Samarut, J., and Plateroti, M. (2010). The overexpression of the putative gut stem cell marker Musashi-1 induces tumorigenesis through Wnt and Notch activation. Journal of cell science *123*, 3256-3265.

Roose, J., Molenaar, M., Peterson, J., Hurenkamp, J., Brantjes, H., Moerer, P., van de Wetering, M., Destree, O., and Clevers, H. (1998). The Xenopus Wnt effector XTcf-3 interacts with Groucho-related transcriptional repressors. Nature *395*, 608-612.

Saharinen, J., Taipale, J., and Keski-Oja, J. (1996). Association of the small latent transforming growth factor-beta with an eight cysteine repeat of its binding protein LTBP-1. The EMBO journal *15*, 245-253.

Sanford, L.P., Ormsby, I., Gittenberger-de Groot, A.C., Sariola, H., Friedman, R., Boivin, G.P., Cardell, E.L., and Doetschman, T. (1997). TGFbeta2 knockout mice have multiple developmental defects that are non-overlapping with other TGFbeta knockout phenotypes. Development *124*, 2659-2670.

Sato, T., and Clevers, H. (2013). Growing self-organizing mini-guts from a single intestinal stem cell: mechanism and applications. Science *340*, 1190-1194.

Sato, T., van Es, J.H., Snippert, H.J., Stange, D.E., Vries, R.G., van den Born, M., Barker, N., Shroyer, N.F., van de Wetering, M., and Clevers, H. (2011). Paneth cells constitute the niche for Lgr5 stem cells in intestinal crypts. Nature *469*, 415-418.

Scoville, D.H., Sato, T., He, X.C., and Li, L. (2008). Current view: intestinal stem cells and signaling. Gastroenterology *134*, 849-864.

Semenov, M., Tamai, K., and He, X. (2005). SOST is a ligand for LRP5/LRP6 and a Wnt signaling inhibitor. The Journal of biological chemistry *280*, 26770-26775.

Semenov, M.V., Zhang, X., and He, X. (2008). DKK1 antagonizes Wnt signaling without promotion of LRP6 internalization and degradation. The Journal of biological chemistry *283*, 21427-21432.

Sen, P., Thakur, N., Stockton, D.W., Langston, C., and Bejjani, B.A. (2004). Expanding the phenotype of alveolar capillary dysplasia (ACD). The Journal of pediatrics *145*, 646-651.

Sheppard, D. (2005). Integrin-mediated activation of latent transforming growth factor beta. Cancer metastasis reviews *24*, 395-402.

Shi, Y., and Massague, J. (2003). Mechanisms of TGF-beta signaling from cell membrane to the nucleus. Cell *113*, 685-700.

Shimomura, Y., Agalliu, D., Vonica, A., Luria, V., Wajid, M., Baumer, A., Belli, S., Petukhova, L., Schinzel, A., Brivanlou, A.H., *et al.* (2010). APCDD1 is a novel Wnt inhibitor mutated in hereditary hypotrichosis simplex. Nature *464*, 1043-1047.

Shiomi, N., Cui, X.M., Yamamoto, T., Saito, T., and Shuler, C.F. (2006). Inhibition of SMAD2 expression prevents murine palatal fusion. Developmental dynamics : an official publication of the American Association of Anatomists *235*, 1785-1793.

Stankiewicz, P., Sen, P., Bhatt, S.S., Storer, M., Xia, Z., Bejjani, B.A., Ou, Z., Wiszniewska, J., Driscoll, D.J., Maisenbacher, M.K., *et al.* (2009). Genomic and genic deletions of the FOX gene cluster on 16q24.1 and inactivating mutations of FOXF1 cause alveolar capillary dysplasia and other malformations. American journal of human genetics *84*, 780-791.

Stevens, C.E., and Leblond, C.P. (1947). Rate of renewal of the cells of the intestinal epithelium in the rat. The Anatomical record *97*, 373.

Taipale, J., Saharinen, J., Hedman, K., and Keski-Oja, J. (1996). Latent transforming growth factor-beta 1 and its binding protein are components of extracellular matrix microfibrils. The journal of histochemistry and cytochemistry : official journal of the Histochemistry Society *44*, 875-889.

ten Dijke, P., and Arthur, H.M. (2007). Extracellular control of TGFbeta signalling in vascular development and disease. Nature reviews Molecular cell biology *8*, 857-869.

Troughton, W.D., and Trier, J.S. (1969). Paneth and goblet cell renewal in mouse duodenal crypts. The Journal of cell biology *41*, 251-268.

Valkenburg, K.C., Graveel, C.R., Zylstra-Diegel, C.R., Zhong, Z., and Williams, B.O. (2011). Wnt/beta-catenin Signaling in Normal and Cancer Stem Cells. Cancers *3*, 2050-2079.

van den Brink, G.R., Bleuming, S.A., Hardwick, J.C., Schepman, B.L., Offerhaus, G.J., Keller, J.J., Nielsen, C., Gaffield, W., van Deventer, S.J., Roberts, D.J., *et al.* (2004). Indian Hedgehog is an antagonist of Wnt signaling in colonic epithelial cell differentiation. Nature genetics *36*, 277-282.

van der Flier, L.G., Haegebarth, A., Stange, D.E., van de Wetering, M., and Clevers, H. (2009a). OLFM4 is a robust marker for stem cells in human intestine and marks a subset of colorectal cancer cells. Gastroenterology *137*, 15-17.

van der Flier, L.G., van Gijn, M.E., Hatzis, P., Kujala, P., Haegebarth, A., Stange, D.E., Begthel, H., van den Born, M., Guryev, V., Oving, I., *et al.* (2009b). Transcription factor achaete scute-like 2 controls intestinal stem cell fate. Cell *136*, 903-912.

VanDussen, K.L., Carulli, A.J., Keeley, T.M., Patel, S.R., Puthoff, B.J., Magness, S.T., Tran, I.T., Maillard, I., Siebel, C., Kolterud, A., *et al.* (2012). Notch signaling modulates

proliferation and differentiation of intestinal crypt base columnar stem cells. Development *139*, 488-497.

Varjosalo, M., and Taipale, J. (2008). Hedgehog: functions and mechanisms. Genes & development *22*, 2454-2472.

Wang, T., Tamakoshi, T., Uezato, T., Shu, F., Kanzaki-Kato, N., Fu, Y., Koseki, H., Yoshida, N., Sugiyama, T., and Miura, N. (2003). Forkhead transcription factor Foxf2 (LUN)-deficient mice exhibit abnormal development of secondary palate. Dev Biol *259*, 83-94.

Wrana, J.L., Attisano, L., Carcamo, J., Zentella, A., Doody, J., Laiho, M., Wang, X.F., and Massague, J. (1992). TGF beta signals through a heteromeric protein kinase receptor complex. Cell *71*, 1003-1014.

Wu, X., Tu, X., Joeng, K.S., Hilton, M.J., Williams, D.A., and Long, F. (2008). Rac1 activation controls nuclear localization of beta-catenin during canonical Wnt signaling. Cell *133*, 340-353.

Xu, X., Han, J., Ito, Y., Bringas, P., Jr., Urata, M.M., and Chai, Y. (2006). Cell autonomous requirement for Tgfbr2 in the disappearance of medial edge epithelium during palatal fusion. Dev Biol *297*, 238-248.

Yang, Z., Mu, Z., Dabovic, B., Jurukovski, V., Yu, D., Sung, J., Xiong, X., and Munger, J.S. (2007). Absence of integrin-mediated TGFbeta1 activation in vivo recapitulates the phenotype of TGFbeta1-null mice. J Cell Biol *176*, 787-793.

Zeilstra, J., Joosten, S.P., Dokter, M., Verwiel, E., Spaargaren, M., and Pals, S.T. (2008). Deletion of the WNT target and cancer stem cell marker CD44 in Apc(Min/+) mice attenuates intestinal tumorigenesis. Cancer research *68*, 3655-3661.

Zhu, J., Motejlek, K., Wang, D., Zang, K., Schmidt, A., and Reichardt, L.F. (2002). beta8 integrins are required for vascular morphogenesis in mouse embryos. Development *129*, 2891-2903.