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Foxf1 and *Foxf2* control murine gut development by limiting mesenchymal Wnt signaling and promoting extracellular matrix production

Mattias Ormestad[1], Jeanette Astorga[1], Henrik Landgren[1], Tao Wang[2], Bengt R. Johansson[3], Naoyuki Miura[2] and Peter Carlsson[1,*]

Development of the vertebrate gut is controlled by paracrine crosstalk between the endodermal epithelium and the associated splanchnic mesoderm. In the adult, the same types of signals control epithelial proliferation and survival, which account for the importance of the stroma in colon carcinoma progression. Here, we show that targeting murine *Foxf1* and *Foxf2*, encoding forkhead transcription factors, has pleiotropic effects on intestinal paracrine signaling. Inactivation of both *Foxf2* alleles, or one allele each of *Foxf1* and *Foxf2*, cause a range of defects, including megacolon, colorectal muscle hypoplasia and agangliosis. Foxf expression in the splanchnic mesoderm is activated by Indian and sonic hedgehog secreted by the epithelium. In Foxf mutants, mesenchymal expression of *Bmp4* is reduced, whereas *Wnt5a* expression is increased. Activation of the canonical Wnt pathway – with nuclear localization of β-catenin in epithelial cells – is associated with over-proliferation and resistance to apoptosis. Extracellular matrix, particularly collagens, is severely reduced in Foxf mutant intestine, which causes epithelial depolarization and tissue disintegration. Thus, Foxf proteins are mesenchymal factors that control epithelial proliferation and survival, and link hedgehog to Bmp and Wnt signaling.

KEY WORDS: Forkhead, Intestine, Hedgehog, Wnt, Bmp

INTRODUCTION

Gut development in higher metazoans involves a complex combination of anteroposterior and radial patterning, orchestrated by communication between the endodermal epithelium and a surrounding mesenchyme derived from the splanchnic mesoderm. In vertebrates, hedgehog ligands (Indian and sonic hedgehog; Ihh and Shh) signal from the epithelium to the mesenchyme and are important for mesenchymal proliferation and radial as well as anteroposterior patterning (Ramalho-Santos et al., 2000; Roberts et al., 1998; Sukegawa et al., 2000). The mesenchyme produces several factors that control proliferation, apoptosis and morphogenesis in the epithelium. The canonical Wnt pathway activates expression of target genes for Tcf transcription factors in epithelial cells through inhibition of β-catenin degradation, and an unequal distribution of β-catenin activity shapes the pattern of proliferation and cell migration along the crypt-villus axis (Batlle et al., 2002; van de Wetering et al., 2002). The importance of Wnt signaling for driving the intestinal developmental program in the endoderm is also illustrated by conversion of lung epithelium into an intestine-like phenotype by ectopic activation of the Wnt pathway (Okubo and Hogan, 2004). Bmps contribute to radial patterning of the gut (Sukegawa et al., 2000) and restrict epithelial (Haramis et al., 2004) as well as mesenchymal (Roberts et al., 1998) proliferation.

Forkhead transcription factors participate in specification of the different mesodermal subpopulations that arise during gastrulation. For example are Foxc genes expressed in paraxial mesoderm and required for somitogenesis (Kume et al., 2001; Wilm et al., 2004), whereas *Foxa2* is essential for formation of the axial mesoderm (notochord) (Ang and Rossant, 1994; Weinstein et al., 1994). The lateral – and later splanchnic – mesoderm expresses Foxf genes (Aitola et al., 2000; Mahlapuu et al., 2001b; Mahlapuu et al., 1998; Ormestad et al., 2004; Peterson et al., 1997), but little is known about their role in specification and differentiation of this tissue.

Foxf1 is required for completing the split of the lateral plate into a splanchnic and a somatic component (Mahlapuu et al., 2001b); expression of *Irx3* – a marker for somatic mesoderm – expands into the splanchnic layer and coelom formation is defective in *Foxf1*$^{-/-}$ embryos (Mahlapuu et al., 2001b). Embryonic lethality of null mutants precludes evaluation of the role of *Foxf1* at later stages of gut development, but in lung and foregut its expression is activated by exogenous Shh, and lung hypoplasia in heterozygotes suggests that *Foxf1* mediates the mitogenic effect of Shh on the mesenchyme (Mahlapuu et al., 2001a). Morpholino knockdown of *Xenopus Foxf1* interferes with gut coiling and inhibits development of intestinal smooth muscle cells (Tseng et al., 2004).

Drosophila has a single Foxf gene, *biniou*. Similar to murine *Foxf1*, *biniou* is required for the separation between splanchnic and somatic mesoderm, and like its *Xenopus* ortholog it is essential for differentiation of the gut musculature (Zaffran et al., 2001). Foxf genes thus appear to have been involved in coelom formation and gut morphogenesis in organisms that predate the protostome-deuterostome split, estimated to have occurred approximately one billion years ago.

The second murine Foxf gene, *Foxf2*, is also expressed in the splanchnic mesoderm, but of subordinate importance in early embryonic development (Aitola et al., 2000; Ormestad et al., 2004). In contrast to *Foxf1*, it is also expressed in the neural crest, in vasculature of the CNS and in limb mesenchyme. Null mutants of *Foxf2* die at birth (Wang et al., 2003). A cleft in the secondary palate causes air filling of the gastrointestinal tract, which is likely to

[1]Department of Cell and Molecular Biology, Göteborg University, Göteborg, Sweden. [2]Department of Biochemistry, Hamamatsu University School of Medicine, Hamamatsu, Japan. [3]The Electron Microscopy Unit, Department of Anatomy and Cell Biology, Göteborg University, Göteborg, Sweden.

*Author for correspondence (e-mail: peter.carlsson@molbio.gu.se)

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interfere with the ability of the newborn to breath and suckle, and to be the immediate cause of death. Here, we investigate the role of murine Foxf genes in gut development and its relation to paracrine signaling by analyzing the intestinal phenotype of *Foxf2*$^{-/-}$ and *Foxf1*$^{-/+}$; *Foxf2*$^{-/+}$ compound heterozygote mutants.

MATERIALS AND METHODS

Mouse strains

Targeted mutants of *Foxf1* (Mahlapuu et al., 2001a; Mahlapuu et al., 2001b), *Foxf2* (Wang et al., 2003) and the *Bmp4*^{lacZ} knock-in (Lawson et al., 1999) have been described elsewhere. All mutants were maintained on a C57Bl/6J background.

Histology, immunohistochemistry and in situ hybridization

Tissues were fixed in 4% paraformaldehyde and processed either for routine paraffin or cryosectioning. The following antibodies were used for immunohistochemistry: collagen I (Biomex), collagen IV (Biomex), laminin (Abcam), smooth muscle α-actin (SMA, Sigma, Clone 1A4, Alkaline Phosphatase conjugate), neurofilament M (Chemicon International), E-cadherin (Zymed, Clone ECCD-2), β-catenin (Transduction Laboratories), PCNA (Dako, Clone PC10), Entactin (NeoMarkers, Clone ELM1), Syndecan1 (Research Diagnostics, Clone 281-2) and Perlecan (Chemicon International, Clone A7L6). For detection, biotinylated secondary antibodies were used with HRP-streptavidine and amplification with TSA Biotin System (NEN Life Science Products). TUNEL assay was performed as previously described (Blixt et al., 2000) and X-gal staining according to Hogan et al. (Hogan et al., 1994). Automated whole-mount in situ hybridization with digoxigenin-labeled RNA probes was performed on an InsituPro instrument (Intavis AG, Germany). The following in situ probes were used: *Foxf1* (Mahlapuu et al., 2001b), *Foxf2* (Ormestad et al., 2004), *Wnt5a* (IMAGE 3487288), *Wnt4* (IMAGE 445179), *Wnt11* (IMAGE 349486), *Sfrp5* (IMAGE 1395864), *Ptch1* (Goodrich et al., 1996). Histological staining and electron microscopy followed standard procedures.

Transfection of primary intestinal fibroblasts

Primary fibroblasts were prepared by tryptic dissociation of the mesenchyme from E18.5 intestine, plated on glass slides and transfected (Lipofectamine, Invitrogen) with a plasmid encoding a dominant-negative FoxF protein (DNA-binding domain only) fused to GFP (Hellqvist et al., 1998). Cells were fixed (0.5% formalin in PBS for 5 minutes at room temperature followed by 100% methanol for 1 minute at –20°C) and stained with collagen I antiserum (visualized with Alexafluor 568, Molecular Probes) 24 hours post-transfection. Transfected cells were identified by their nuclear GFP fluorescence and nuclei by DAPI staining.

Explant cultures

Intestine and stomach were dissected from E12.5 and E13.5 embryos, and cultured on filters as previously described (Mahlapuu et al., 2001a). Beads (Affi-gel blue, BioRad) soaked in Bmp4 (10 ng/μl), Noggin (100 ng/μl) (both from R&D Systems) or BSA (as control) were grafted into the mesenchyme of the explants. For inhibition of hedgehog signaling, cyclopamine was used in the culture medium at a concentration of 20 μM. After in vitro culture for 24 hours, explants were fixed briefly in 4% paraformaldehyde and analyzed by whole-mount in situ hybridization with probes for *Wnt5a, Ptch1*, *Foxf1* and *Foxf2*.

RESULTS

Foxf1 and *Foxff2* have overlapping functions in gut development

To investigate the role of Foxf genes in gut development we examined the intestines of embryos with various combinations of mutations in *Foxf1* and *Foxf2*. Neither heterozygote (*Foxf1*$^{-/+}$ and *Foxf2*$^{-/+}$) had any obvious intestinal defects and *Foxf1*$^{-/-}$ embryos were resorbed by E10, before gut morphogenesis had begun (Mahlapuu et al., 2001b). All *Foxf2*$^{-/-}$ and an estimated 94% of *Foxf1*$^{-/+}$; *Foxf2*$^{-/+}$ compound heterozygotes died shortly after birth (three out of 154 surviving offspring from *Foxf1*$^{-/+}$× *Foxf2*$^{-/+}$ crosses were compound heterozygotes and survived for up to a few weeks). *Foxf1*$^{-/+}$ heterozygotes have increased perinatal mortality – on some genetic backgrounds exceeding 90% – owing to lung and foregut malformations (Kalinichenko et al., 2001; Mahlapuu et al., 2001a). However, the C57Bl/6J strain used here is particularly resistant to *Foxf1* haploinsufficiency and heterozygotes have approximately the same survival rate and life expectancy as wild type (Mahlapuu et al., 2001a). The inviability of compound heterozygotes therefore represents non-allelic non-complementation and indicates a functional overlap between *Foxf1* and *Foxf2*. Examination of E18.5 fetuses revealed defects and malformations in the intestine of *Foxf2*$^{-/-}$ and *Foxf1*$^{-/+}$; *Foxf2*$^{-/+}$, whereas cleft palate was only observed in *Foxf2*$^{-/-}$ (Wang et al., 2003). The two mutant genotypes had similar intestinal abnormalities and will be collectively referred to as Foxf mutants. In cases where consistent phenotypic differences between them were observed, this will be specifically commented on.

Reduced Foxf gene dosage causes aganglionic megacolon

At E18.5 the mesodermal component of the murine intestine has given rise to fibroblasts in a subepithelial mesenchyme, longitudinal and circular muscular layers, and a mesothelium that delimits the gut from the coelom (Fig. 1J). In most Foxf mutant fetuses, the distal colon was thinwalled and dilated (megacolon; Fig. 1B,E,F,H,I), and in some *Foxf2*$^{-/-}$ ended in a blind sac (intestinal atresia or imperforate anus; Fig. 1F). Congenital megacolon in human infants is caused by a defective innervation of the colon (aganglionic colon or Hirschsprung's disease) (Carrasquillo et al., 2002) and we therefore examined the distribution of enteric neurons. Immunostaining for the neuronal markers neurofilament (Fig. 1L,M) and PGP9.5 (not shown) showed that the innervation of distended parts of the distal colon in Foxf mutants was weak and patchy. The reduction in enteric neurons correlated with the degree of colon dilation; in the worst affected regions, neurons were virtually absent.

The distended distal colon of mutants typically had a flat epithelium and lacked well-developed fibroblasts or mesothelium (Fig. 1K). The mesodermal component instead consisted of a lax disorganized assembly of cells with the appearance of incompletely differentiated smooth muscle cells (SMCs, Fig. 1K). The poor development of the musculature in colon and rectum was confirmed by a faint and incoherent immunostaining for smooth muscle α-actin (SMA; Fig. 1N,O). Proximal, non-dilated, parts of the colon maintained a more normal histology, but generally had a smaller diameter than wild type.

Foxf genes are important for extracellular matrix production by intestinal fibroblasts

Throughout the intestine, Foxf mutants showed signs of poor adhesion between cells, a defect that was more pronounced in *Foxf2*$^{-/-}$ than in *Foxf1*$^{-/+}$; *Foxf2*$^{-/+}$. The epithelium, the mesenchyme and the two muscular layers all separated from each other, and in the worst affected parts each layer dissolved into individual cells. Spontaneous disintegration and increased susceptibility to mechanical stress suggested a deficiency in the extracellular matrix (ECM). When viewed by electron microscopy (TEM), cells of the colon from *Foxf1*$^{-/+}$; *Foxf2*$^{-/+}$ mutants appeared loosely assembled (Fig. 2). The basal laminae surrounding SMCs as well as the basement membrane underneath the epithelium were indistinct and frequently replaced by gaps of extracellular space (Fig. 2D). Immunohistochemistry revealed a striking deficiency of fibrillar (type I) as well as sheet-forming (type IV) collagens throughout the

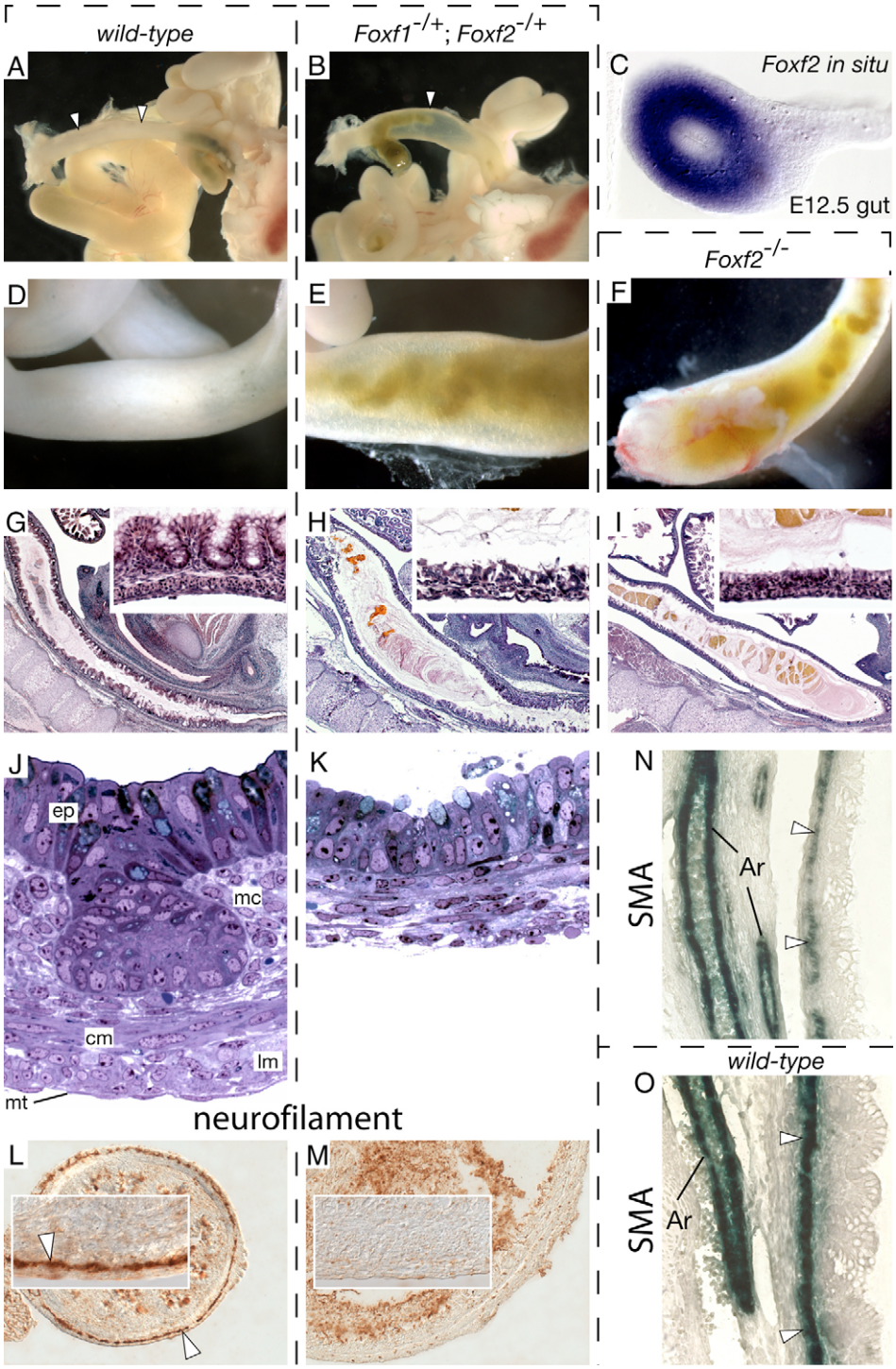

Fig. 1. Foxf mutants have aganglionic megacolon with smooth muscle hypoplasia and occasional anal atresia. (**A**,**B**) Lower gastrointestinal tract of E18.5 wild-type (A) fetus and *Foxf1*$^{-/+}$; *Foxf2*$^{-/+}$ litter mate (B). Arrowheads indicate the colon, which is thin-walled and distended in the mutant. (**C**) Vibratome section of the intestine of a wild-type E12.5 embryo, whole-mount in situ hybridized with a *Foxf2* probe, showing *Foxf2* expression in the mesenchyme next to the endodermal epithelium. (**D**,**E**) Higher magnification of the colon in A and B. (**F**) Anal atresia and megacolon in E18.5 *Foxf2*$^{-/-}$ mutant. (**G-I**) Hematoxylin and Eosin stained sagittal sections through the rectum and lower colon of E18.5 wild-type (G), *Foxf1*$^{-/+}$; *Foxf2*$^{-/+}$ (H) and *Foxf2*$^{-/-}$ (I); insets show higher magnifications of the distal colon wall, which is thin and flat in the mutants. (**J**,**K**) Thin (1 μm) section of wild-type (J) E18.5 distal colon showing the stratification with epithelium (ep), mesenchyme (mc), circular (cm) and longitudinal (lm) musculature, and mesothelium (mt). The mesodermal layer in the *Foxf1*$^{-/+}$; *Foxf2*$^{-/+}$ (K) is hypoplastic and consists of loosely associated, poorly differentiated SMCs. (**L**,**M**) Immunostaining with antiserum against the neuronal marker neurofilament shows the plexus of enteric nerves in the mesodermal layer of E18.5 wild-type distal colon (L), whereas no neurons are detected in the mutant (M; *Foxf1*$^{-/+}$; *Foxf2*$^{-/+}$). Insets show the distal colon wall at higher magnification and arrowheads indicate the enteric plexus. Staining in the gut lumen is due to the non-specific stickiness of the meconium and goblet cell mucins. (**N**,**O**) Immunostaining with anti-SMA reveals SMC hypoplasia in *Foxf2*$^{-/-}$ distal colon (arrowheads), but normal SMC investment of arteries (Ar).

intestines of E18.5 *Foxf2*$^{-/-}$ mutants (Fig. 3). Laminin staining was also weaker, although the reduction was not as dramatic (not shown). In *Foxf1*$^{-/+}$; *Foxf2*$^{-/+}$ mutants the ECM staining was reduced (Fig. 3D), but to a lesser degree than in *Foxf2*$^{-/-}$. The compound heterozygote also had ectopic expression of smooth muscle α-actin in intravillus mesenchyme (Fig. 3M).

Mesodermal cells of *Foxf1*$^{-/+}$; *Foxf2*$^{-/+}$ colon had poorly developed endoplasmic reticulum (ER; Fig. 2D,F), suggestive of a lower production of secreted proteins. To investigate if inhibition of Foxf activity in fibroblasts would reduce secretion of ECM proteins, we transfected primary fibroblasts from wild-type E18.5 small intestine with a plasmid expressing a dominant-negative Foxf protein fused to GFP (Hellqvist et al., 1998) (Fig. 3P,Q). Immunostaining showed abundant collagen I in ER vesicles of untransfected cells, as well as in fibers on the growth surface underneath. Transfected cells – identified by their GFP positive nuclei – contained almost no collagen I and similar results were obtained for collagen IV (not shown). Expression of GFP had no effect on collagen production (Fig. 3N,O). These results suggest that inhibiting transcriptional regulation of Foxf target genes leads to loss of collagen synthesis and indicates a cell-autonomous requirement for Foxf proteins in ECM production by fibroblasts.

ECM deficiency leads to epithelial depolarization and inter-villus adhesion, but surprisingly little apoptosis

Polarization of epithelial cells is induced by interaction with the basement membrane through integrin receptors (Kedinger et al., 2000) and an expected consequence of the ECM deficiency in Foxf mutants was therefore loss of polarity. Indeed, epithelial cells in Foxf mutants showed typical signs of depolarization: rounded in shape with central, rather than basal, nuclei. This anomaly was apparent also in areas where the physical integrity was not yet affected. The subcellular distribution of E-cadherin, a component of adherence junctions normally confined to lateral membranes of epithelial cells,

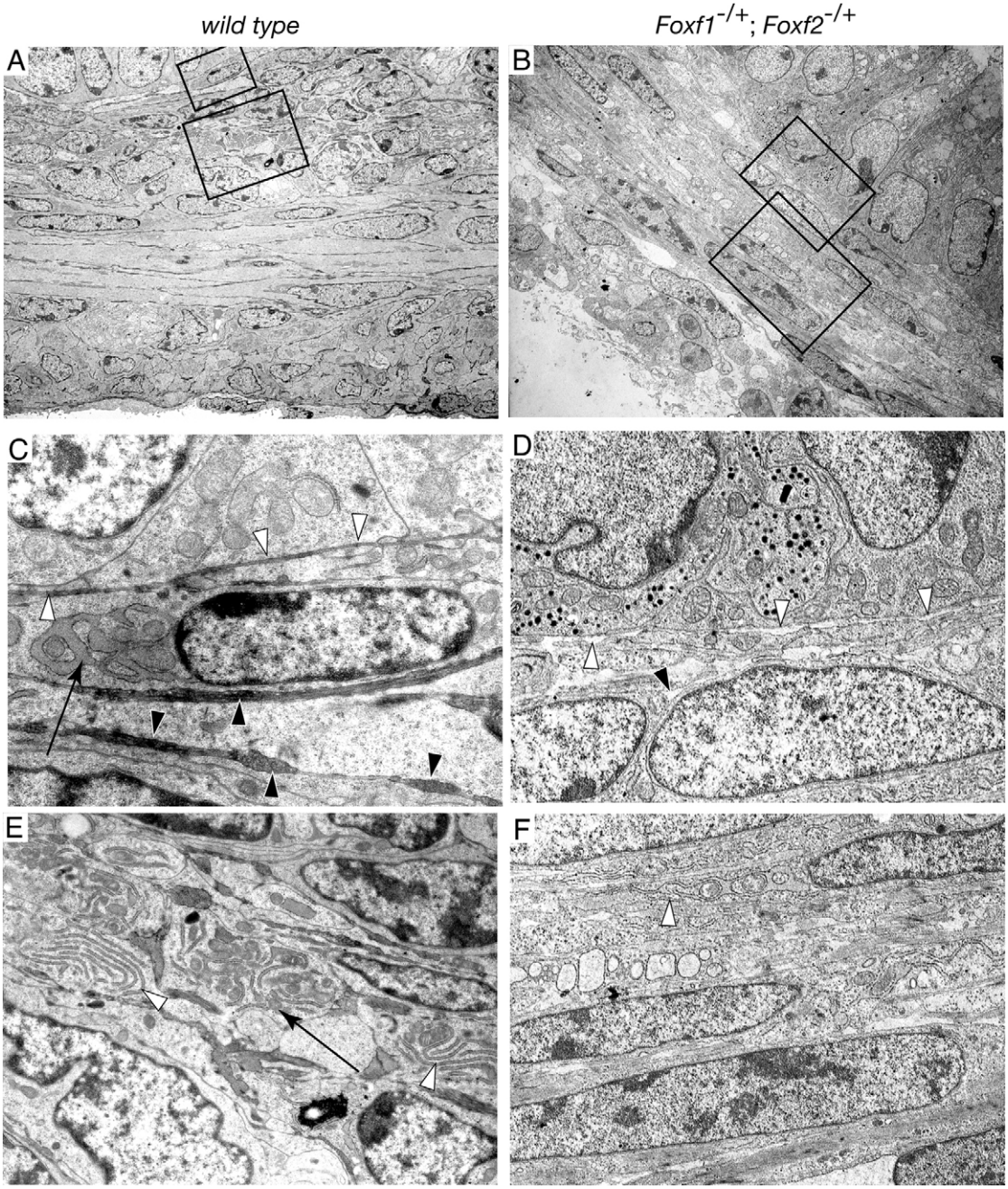

Fig. 2. Lack of extracellular matrix, weak cell adhesion and poorly developed endoplasmic reticulum in fibroblasts of Foxf mutant gut. TEM images of E18.5 wild-type (A,C,E) and *Foxf1*$^{-/+}$; *Foxf2*$^{-/+}$ (B,D,F) distal colon. (**A**) Normal stratified colon with (from top) epithelium, mesenchyme, the two muscle layers and mesothelium (compare Fig. 1J). (**B**) *Foxf1*$^{-/+}$; *Foxf2*$^{-/+}$ colon with flat epithelium (top right) and poorly differentiated, dissociating SMCs (compare with Fig. 1K). (**C**) A basement membrane (white arrowheads) delimits the epithelium (top) from the mesenchymal layer and bundles of fibrillar collagens (black arrowheads) are embedded in the basal laminae surrounding individual fibroblasts and smooth muscle cells. Endoplasmic reticulum in fibroblasts is well developed and filled with electron-dense proteins for secretion (arrow). (**D**) Hardly any ECM or collagen fibers can be seen in the mutant colon. The basement membrane is indistinct and instead gaps of extracellular space (white arrowheads) separate epithelium (top) from mesenchyme. Gaps are also frequent between mesodermal cells (black arrowhead). (**E**) Wild-type fibroblasts have well-developed endoplasmic reticulum (white arrowheads), often filled with proteins (arrow). (**F**) Foxf mutant colon mesodermal cells have a poorly differentiated SMC phenotype with little endoplasmic reticulum (white arrowhead).

expanded into the basal and apical membranes, and in the most depolarized cells was circumferentially distributed (Fig. 4D-F). A similar shift from lateral to more or less ubiquitous membrane staining was also observed for β-catenin, another component of adherence junctions. One consequence of cell adhesion proteins being exposed on the apical surface is adherence between epithelial cells from separate villi. In areas with overt depolarization the result was extensive inter-villus cross-linking, which lead to complete luminal obstruction (Fig. 4C).

The integrin-mediated attachment to the basement membrane, and the resulting polarization, are also essential for epithelial cell survival (Frisch and Ruoslahti, 1997). This is illustrated by the presence of apoptotic cells in sites of poor epithelio-mesenchymal contact of wild-type intestine (Fig. 4K). Surprisingly, TUNEL assay did not reveal the expected massive apoptosis in *Foxf2*$^{-/-}$ mutants, in spite of the severe deficiency of several ECM components (Fig. 4L). Loosely attached, completely depolarized cells failed to

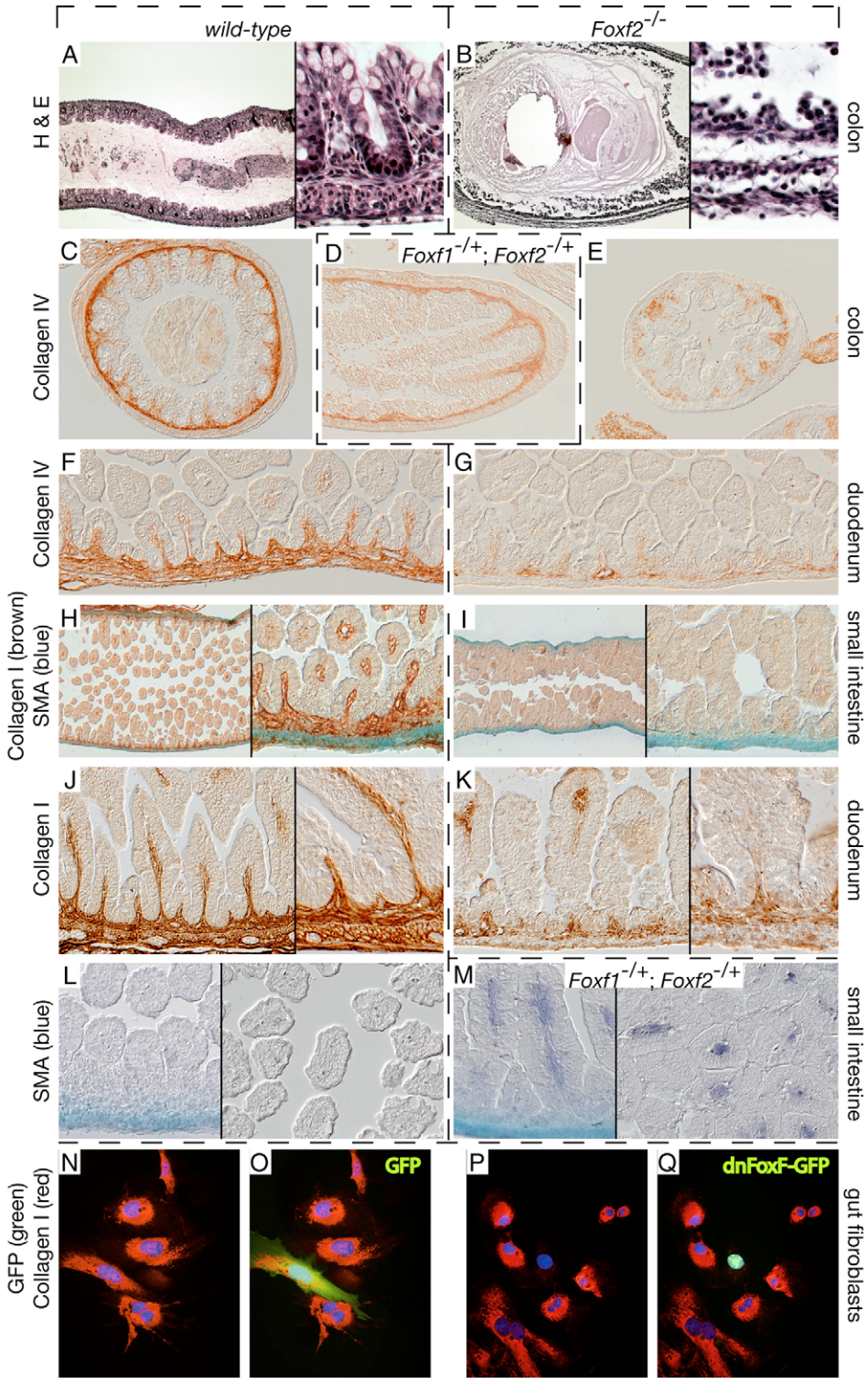

Fig. 3. Tissue disintegration due to ECM deficiency in E18.5 *Foxf2*$^{-/-}$ intestine. (**A**,**B**) Hematoxylin and Eosin stained sections of colon from wild type (A) and *Foxf2*$^{-/-}$ (B). The mutant has a distended distal colon and the mesodermal layers separate from each other and from the epithelium (top in close-up) because of poor cell adhesion. (**C**-**K**) Immunostaining with antisera against a sheet forming collagen (type IV), a fibrillar collagen (type I) and SMA. Both collagens are reduced throughout the length of the intestine (from duodenum to rectum) in *Foxf2*$^{-/-}$. The mutant intestinal wall (I) is flimsy in appearance compared with the wild type (H), owing to lack of collagen fibers. Both the small intestine (I) and colon (E; here from a proximal, non-distended part) has a smaller diameter in the mutant than in wild-type littermates (C,H). *Foxf1*$^{-/+}$; *Foxf2*$^{-/+}$ (D; proximal colon) also has reduced amounts of ECM components, but less extreme than in *Foxf2*$^{-/-}$. (**L**,**M**) Immunostaining with anti-SMA in wild type (L) and *Foxf1*$^{-/+}$; *Foxf2*$^{-/+}$ (M) E18.5 small intestine shows ectopic expression of SMA in intravillus mesenchyme of the mutant. (**N-Q**) Cell-autonomous requirement for Foxf proteins for collagen expression in intestinal fibroblasts. Primary fibroblasts were prepared from E18.5 wild-type intestine and transfected with a plasmid expressing GFP (N,O) or a dominant-negative Foxf protein fused to GFP (P,Q). After 24 hours, cells were fixed and stained with an antiserum against collagen I (red). Collagen staining is seen in cytoplasmic vesicles and fibers between the cell and the glass substrate. In cells expressing the dominant-negative Foxf protein, identified by their green nuclear fluorescence, collagen staining is reduced dramatically. Blue, DAPI nuclear staining.

undergo apoptosis, and only in areas that had reached the final stages of tissue disintegration did the frequency of TUNEL-positive cells increase significantly (Fig. 4M).

Epithelial overgrowth in small intestine of *Foxf1*$^{-/+}$; *Foxf2*$^{-/+}$ compound heterozygotes

Cell adhesion and ECM production was less affected in the compound heterozygotes than in *Foxf2* knockouts, and tissue layers of the small intestine did not normally disintegrate. Instead, the villi were large and club-shaped with multilayered epithelia (Fig. 4B). Normal villi are covered by a monolayer of epithelial cells (Fig. 4A) and the continuous addition from the basal, proliferative compartment is balanced by apoptosis and desquamation at the tip of villi (Stappenbeck et al., 1998). The large clusters of epithelial cells in *Foxf1*$^{-/+}$; *Foxf2*$^{-/+}$ small intestine, particularly in the distal parts of villi, therefore suggested overproliferation or loss of normal control of cell survival. Epithelial proliferation in E18.5 small intestine is confined to the intervillus pockets, predecessors of crypts

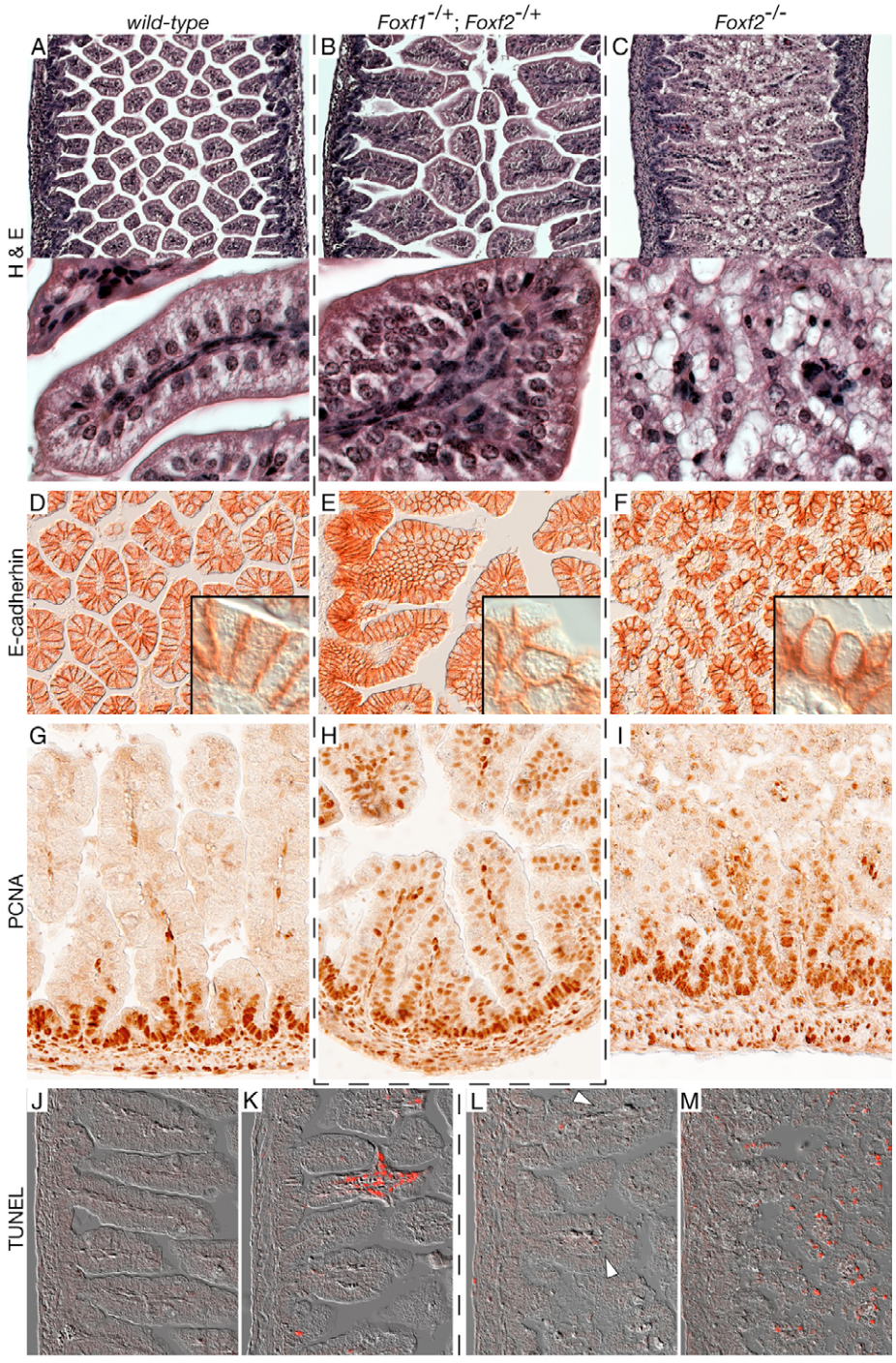

Fig. 4. Overproliferation and failure to induce apoptosis generate excessive epithelial cells in Foxf mutants. (**A-C**) Low (top) and high (bottom) magnifications. Hematoxylin and Eosin staining of E18.5 small intestine sections from wild-type (A), *Foxf1*$^{-/+}$; *Foxf2*$^{-/+}$ (B) and *Foxf2*$^{-/-}$ (C). Normal villi (A) are covered by a smooth monolayer of highly polarized epithelial cells with basal nuclei. Villi in the compound heterozygote (B) are large, club-shaped with multilayered epithelia, whereas in *Foxf2* null mutants (C), the epithelial cells detach from the mesenchyme and adhesion between epithelial cells from adjacent villi create inter-villus cross linking and luminal obstruction. (**D-F**) Altered distribution of E-cadherin immunostaining reveals loss of epithelial polarity. In normal, polarized epithelial cells (D), E-cadherin is confined to the lateral membrane, where adherence junctions connect adjacent cells, and basal and apical membranes lack this cell-adhesion molecule. In the compound Foxf heterozygote (E), internal layers of epithelial cells have ubiquitous membrane-associated staining, but the outer layer has apical membranes free of E-cadherin. In *Foxf2*$^{-/-}$ small intestine (F) the epithelial cells show many signs of lost polarity, including E-cadherin staining along the entire circumference. (**G-I**) Dissolution of the proliferation boundary in Foxf mutants. Immunostaining for PCNA – a proliferation marker – identifies actively cycling cells, which in wild-type E18.5 small intestine epithelium (G) are confined to the intervillus pockets (predecessors of crypts of Lieberkühn). In Foxf mutants (H,I), the boundary between the basal proliferative and the distal post-mitotic compartments is missing, and PCNA-positive epithelial cells are found throughout the villi. (**J-M**) Partial resistance to apoptosis in *Foxf2*$^{-/-}$ intestinal epithelium. TUNEL assay (red nuclei, apoptotic cells) of E18.5 wild-type (J,K) and *Foxf2*$^{-/-}$ (L,M) small intestine. (J) Most parts of the normal intestine show no or few apoptotic cells at this stage. (K) Where local slippage between mesenchyme and epithelium causes poor contact between epithelial cells and basement membrane, apoptosis is induced in the affected cells. (L) In the light of the severe ECM deficiency and the beginning separation between epithelium and mesenchyme (arrowheads), the *Foxf2*$^{-/-}$ intestinal epithelium contains surprisingly few apoptotic cells. (M) Not until the final stages of tissue disintegration in the worst affected areas, where the epithelial cells detach completely from the villus core, does the frequency of apoptotic cells increase significantly.

of Lieberkühn which do not form until postnatally. Transition from the basal, proliferative to the distal, postmitotic, compartment is indicated by loss of proliferation markers such as PCNA (Fig. 4G). In Foxf mutants, the sharp boundary between proliferating and non-proliferating cells was dissolved and PCNA-positive cells were found throughout the villi (Fig. 4H,I). Persistent cell division therefore appears to account for the observed surplus of epithelial cells.

Nuclear β-catenin along the entire villus axis indicates ectopic Wnt signaling

Persistent proliferation and partial resistance to apoptosis in the intestinal epithelium suggested abnormal activation of the Wnt-β-catenin-Tcf/Lef pathway. On sections of E18.5 wild-type small intestine, β-catenin staining was intense in basal epithelium and faded along the villus axis (Fig. 5A). Higher magnification showed nuclear β-catenin at the base of villi, but only membrane associated staining distally (Fig. 5F,H,J), consistent with Wnt signaling emanating from mesenchyme underlying the intervillus pits and stabilizing β-catenin in basal epithelial cells. Sections of intestine from Foxf mutants showed β-catenin staining remaining strong along the villus axis (Fig. 5B) and nuclear localization also at the apices of villi (Fig. 5G,I,K). The multilayered epithelia of *Foxf1*$^{-/+}$; *Foxf2*$^{-/+}$ mutants had a mosaic pattern with clusters of epithelial cells whose β-catenin staining was significantly weaker (Fig. 5B-D). TUNEL assay produced a similar mosaic pattern, which suggests that apoptosis is prevalent within low-β-catenin areas (Fig. 5E).

How does a decreased Foxf expression in the mesenchyme lead to β-catenin stabilization in the epithelium? In mice lacking *Foxl1*, another mesenchymal forkhead gene, accumulation of proteoglycans has been suggested to facilitate Wnt signaling and thereby promote epithelial overgrowth (Kaestner et al., 1997; Katz

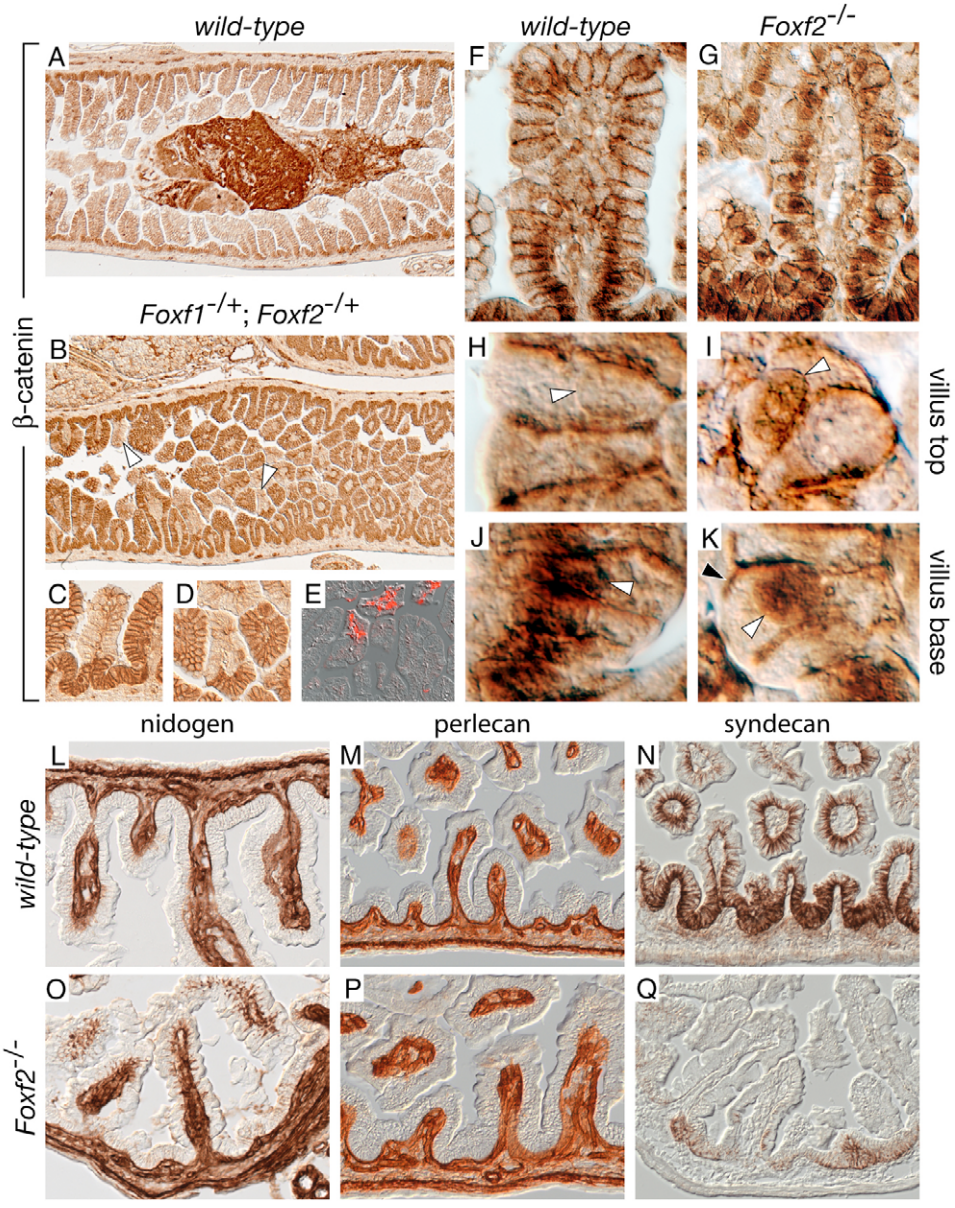

Fig. 5. Accumulation of β-catenin in Foxf mutant intestinal epithelium. β-Catenin immunostaining (A-D,F-K) and TUNEL assay (E; red nuclei indicate apoptotic cells) of E18.5 small intestine of wild type (A,F,H,J), *Foxf1*$^{-/+}$; *Foxf2*$^{-/+}$ (B-E) and *Foxf2*$^{-/-}$ (G,I,K). (**A**) In normal E18.5 small intestine, β-catenin is most abundant in the basal epithelial cells of the intervillus pits and decreases along the villus axis (non-specific staining in the gut lumen is due to the stickiness of the meconium). (**B-D**) In Foxf mutants, β-catenin concentration stays high all the way to the villus tip. *Foxf1*$^{-/+}$; *Foxf2*$^{-/+}$ differs from *Foxf2*$^{-/-}$ in having a mosaic pattern with sharp boundaries between regions of the epithelium with high and low β-catenin (C,D; arrowheads in B). (**E**) Patches of TUNEL-positive (apoptotic) cells match the low β-catenin mosaic pattern. (**F**,**G**) Higher magnifications show nuclear localization of β-catenin at base of villi in wild type (F), but ubiquitous nuclear β-catenin in Foxf mutants (G). (**H**) Only membrane-associated β-catenin is found only in cells from upper half of wild-type villus; nuclei (white arrowhead) are negative. (**I-K**) Both nuclear (white arrowheads) and membrane-associated β-catenin are found in cells from the upper half of *Foxf2*$^{-/-}$ villus (I; circular organelle in adjacent cell is a goblet cell mucus vesicle) and from the base of wild-type (J) and *Foxf2*$^{-/-}$ villus (K). Epithelial depolarization is beginning in K, in spite of the seemingly intact contact with the mesenchyme: the membrane associated β-catenin has begun to expand from the lateral and into the apical surface (black arrowhead) and nuclei are centrally located in the cells. (**L-Q**). Normal or reduced amounts of the major glycosaminoglycan proteoglycans in Foxf mutant intestine. Immunostaining with antisera against nidogen (Entactin; L,O), perlecan (M,P) and syndecan 1 (N,Q) on cryosections of E18.5 small intestine from wild type (L-N) and *Foxf2*$^{-/-}$ (O-Q). Nidogen and perlecan are constituents of the mesenchymal ECM and present in normal amounts in Foxf mutants. Syndecan 1 is membrane anchored, present on the basolateral face of epithelial cells and reduced in Foxf mutant intestine.

et al., 2004; Perreault et al., 2001). However, in Foxf mutants, the major intestinal proteoglycans were present in normal (perlecan and nidogen/entactin) or reduced (syndecan 1) amounts (Fig. 5L-Q). We therefore investigated if a reduced Foxf gene dose increases the expression of Wnt genes in the mesenchyme. To exclude secondary effects due to interrupted epithelio-mesenchymal signaling, E14.5 embryos were chosen – a stage at which the association between epithelium and mesenchyme is still intact in the mutants. Whole-mount in situ hybridization with candidate Wnt genes (*Wnt4*, *Wnt5a* and *Wnt11*) showed *Wnt5a* to be upregulated in *Foxf2*$^{-/-}$ embryos (Fig. 6A). *Wnt5a* and *Foxf2* are co-expressed in several parts of the embryo, such as limbs and genital tubercle, and an increase in *Wnt5a* mRNA was seen in all these organs of *Foxf2*-null embryos, with intermediate levels in *Foxf2*$^{-/+}$ heterozygotes (not shown). In fact, the difference in *Wnt5a* mRNA content between *Foxf2*$^{-/-}$ and wild type was greater in limbs, where *Foxf2* is the only expressed Foxf gene, than in the intestine where *Foxf1* and *Foxf2* are co-expressed.

Reduced *Bmp4* expression contributes to *Wnt5a* upregulation

Wnt5a is co-expressed with Foxf genes in the mesenchymal cells of the developing intestine and might therefore be a direct transcriptional target. However, in limbs, *Wnt5a* expression is increased in *Foxf2*$^{-/-}$ embryos, in spite of the two genes being expressed in adjacent, non-overlapping cell populations; we therefore suspected that the link between Foxf genes and *Wnt5a* may be indirect also in the gut. A candidate extracellular mediator was Bmp4; it inhibits *Wnt5a* expression in the genital tubercle (Suzuki et al., 2003) and its expression in the early splanchnic mesoderm requires Foxf1 (Mahlapuu et al., 2001b). We first compared *Bmp4* expression in *Foxf2*$^{-/-}$ and wild-type using a *Bmp4*^{lacZ} knock in. β-galactosidase activity in the intestine of *Foxf2*$^{-/-}$; *Bmp4*$^{lacZ/+}$ embryos (E15.5) was reduced (Fig. 6B,C) in the gut, whereas tissues without Foxf expression, such as eye lids and hair follicles, retained normal *Bmp4*-driven β-galactosidase

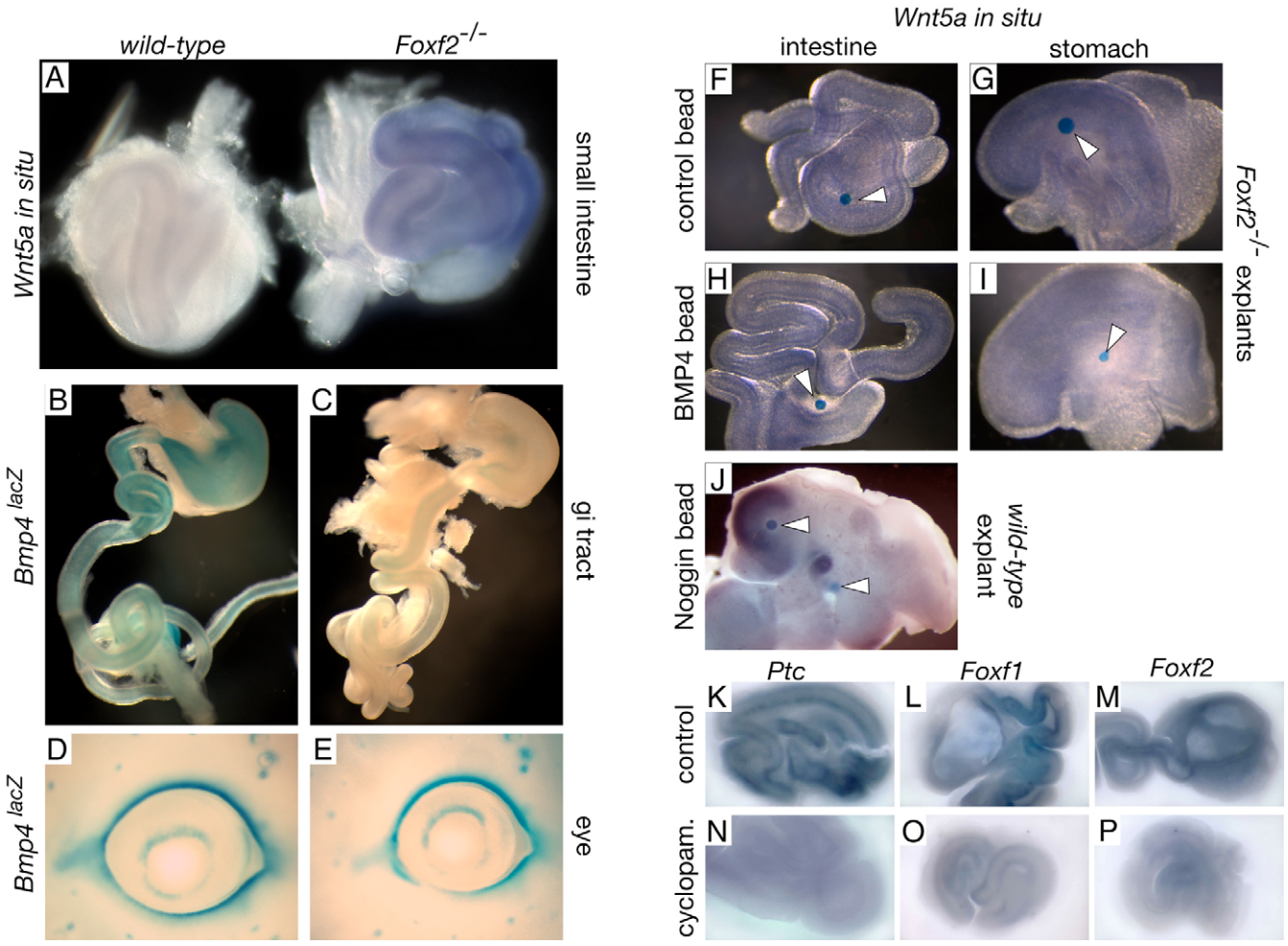

Fig. 6. Reduced Foxf gene dosage has pleiotropic effects on paracrine signaling in the intestinal mesenchyme: more Wnt and less Bmp. (**A**) Whole-mount in situ hybridization of E14.5 embryos with a *Wnt5a* probe. Expression is significantly higher in *Foxf2*$^{-/-}$ mutant (right) than in wild-type (left) small intestine. (**B-E**) X-gal staining of E15.5 *Bmp4*$^{lacZ/+}$ embryos. *Bmp4*-driven β-galactosidase activity is significantly reduced in the gastrointestinal tract of *Foxf2*$^{-/-}$ (C), compared with wild type (B). The same embryos have comparable levels of staining in organs where *Foxf2* is not expressed, such as eyelids and hair follicles (D,E). (**F-J**) Bmp signaling inhibits *Wnt5a* expression in gastrointestinal mesenchyme. *Wnt5a* whole-mount in situ hybridization of gastric (G,I) and intestinal (F,H,J) explants from *Foxf2*$^{-/-}$ (F-I) or wild-type (J) E12.5 embryos cultured for 24 hours with beads (arrowheads) implanted into the mesenchyme. Beads contained BSA (control; F,G), Bmp4 (H,I) or noggin (J). The uneven white outline of the explant in J is from remnants of the filter on which the explant was cultured. (**K-P**) Inhibition of hedgehog signaling by cyclopamine (20 μM; n-p) reduces the expression of Foxf genes (L,M,O,P) to the same extent as of a known hedgehog target, *Ptch1* (K,N). E12.5 intestinal explants were cultured for 24 hours in the presence or absence of cyclopamine and analyzed by whole-mount in situ hybridization.

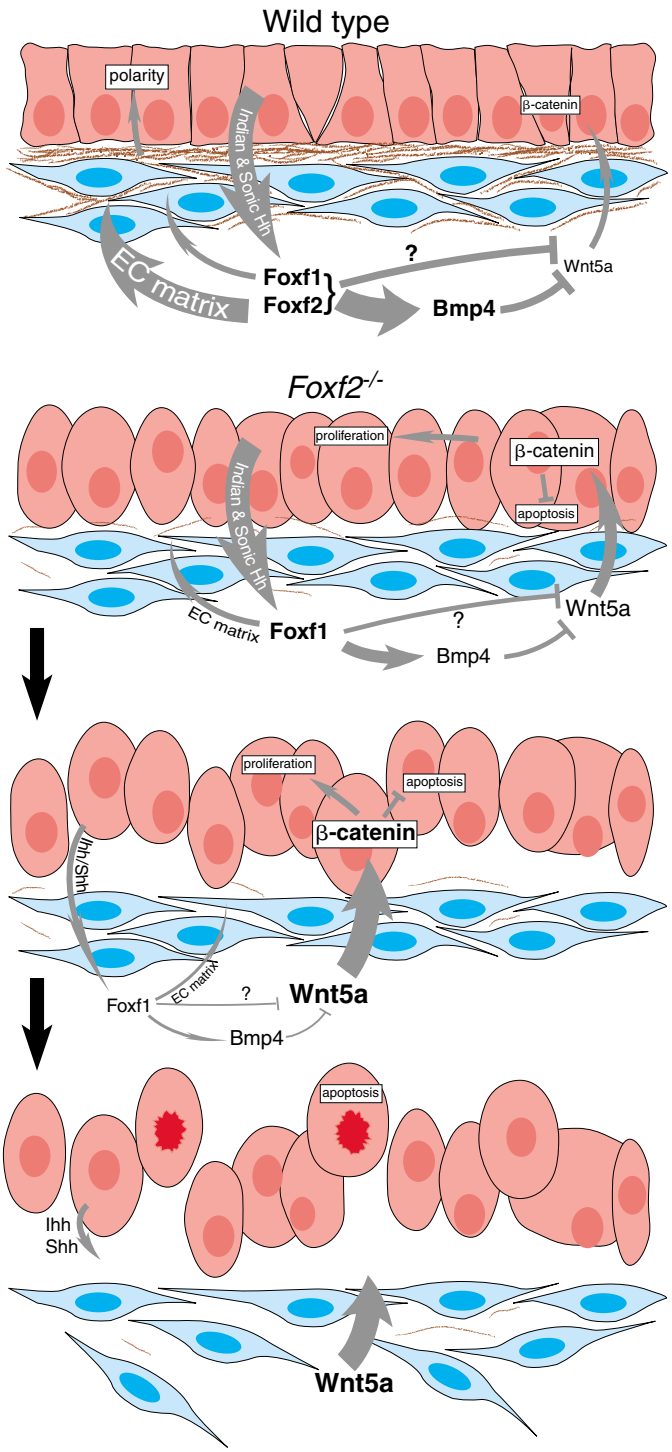

Fig. 7. Summary of paracrine interactions and a model for a degenerative cycle that could explain the local variation of phenotype in Foxf mutant intestine. In wild type, both Foxf genes are activated in mesenchymal cells by hedgehog produced by the epithelium. Cell-autonomous stimulation of ECM production is more dependent on Foxf2 than on Foxf1, whereas both proteins activate *Bmp4*. Bmp4 inhibits *Wnt5a* expression in the mesenchyme and there may also be other mechanisms ('?') through which Foxf proteins restrict *Wnt5a*. The ECM provides tight contact between epithelium and mesenchyme, induces epithelial polarity and ensures efficient signaling of both Hh and Wnt ligands. The loss of both *Foxf2* alleles will result in reduced *Bmp4* and increased *Wnt5a* expression, but foremost in a radical decrease of several ECM components. The ECM deficiency will lead to loss of epithelial polarity, but not to apoptosis at this stage because of the stabilized β-catenin that results from increased Wnt signaling. However, the poor adhesion, particularly between epithelium and the weakened basement membrane, creates an unstable situation where even moderate physical strain (e.g. from peristalsis) will separate the epithelium from the mesenchyme. Once parted, mesenchymal cells will experience a reduced Hh signaling, expression from the remaining Foxf alleles (in this example the two *Foxf1* alleles) will drop and the phenotype deteriorates further. When ECM production falls below a certain level, the tissue disintegrates and epithelial cells end up too far from the source of Wnt5a to be rescued from apoptosis. $Foxf1^{-/+}$; $Foxf2^{-/+}$ compound heterozygotes initially have a less dramatic reduction in ECM, which sustains tissue integrity longer and supports more efficient epithelio-mesenchymal signaling, allowing the epithelium to overgrow. Given the reduced Foxf gene dose and weakened basement membranes, the tissue is, however, vulnerable to the same degenerative cycle, which may be the cause of the observed low-β-catenin/apoptosis mosaic pattern.

activity (Fig. 6D,E). To investigate if Bmp signaling influences *Wnt5a* expression in the intestinal mesenchyme, we applied beads soaked in Bmp4 or the Bmp antagonist Noggin to the mesenchyme of gastric or intestinal explants (E12.5 or 13.5), cultured in vitro and analyzed *Wnt5a* expression by whole-mount in situ hybridization (Fig. 6F-J). Bmp4 inhibited *Wnt5a* expression in $Foxf2^{-/-}$ explants (Fig. 6H,I), and Noggin induced high levels of *Wnt5a* mRNA in wild-type explants, from a low basal expression (Fig. 6J). By contrast, Foxf gene expression was not influenced by Bmp4 or Noggin (not shown).

Intestinal Foxf expression is activated by hedgehog signaling

In the early splanchnic mesoderm *Foxf1* and *Foxf2* are activated by sonic and Indian hedgehog (J.A. and P.C., unpublished), and *Foxf1* expression in foregut and lung requires Shh (Mahlapuu et al., 2001a). The overlapping expression of *Shh* and *Ihh*, together with the early lethality of the double null mutant, limit the use of genetic methods to evaluate the role of hedgehogs in activation of Foxf genes in the intestine. Instead, we used cyclopamine to inhibit hedgehog signaling in explant cultures. Cyclopamine was found to reduce the expression of both *Foxf1* and *Foxf2* to the same extent as of a known hedgehog target, *Ptch1* (Fig. 6K-P). We therefore conclude that Shh and Ihh from the epithelium activates mesenchymal transcription of Foxf genes also in the intestine.

DISCUSSION

The data presented here place Foxf proteins at the crossroad of several of the major signaling pathways in gut development. Activated by hedgehog proteins from the epithelium they control expression of Wnt and Bmp in the mesenchyme. The conservation between mammals and *Drosophila* (Zaffran et al., 2001) of a role in gut development implies that this represents an ancient and presumably primeval role of the FoxF class of forkhead transcription factors.

Phenotypic similarity between $Foxf2^{-/-}$ and the *Foxf1*; *Foxf2* compound heterozygotes indicates a functional overlap between the two proteins. Rather than redundancy, the relation between *Foxf1* and *Foxf2* represents non-allelic non-complementation, as at least three functional alleles are required for normal development.

However, although functionally overlapping, the presence of phenotypic differences between the mutants also indicates non-equivalence, in agreement with the distinct properties of the activation domains shown previously (Hellqvist et al., 1998; Hellqvist et al., 1996; Mahlapuu et al., 1998). The more severe ECM deficiency in *Foxf2*$^{-/-}$ may explain the strikingly different small intestine histology of the two mutants. Both share an activation of the canonical Wnt pathway, presence of cycling cells in the distal parts of villi and a partial resistance to apoptosis in cells with poor anchorage. In the presence of an intact, although weakened, basement membrane this will allow accumulation of excessive epithelial cells – as seen in the compound heterozygote – whereas loss of contact between epithelium and mesenchyme will impede Wnt signaling, lead to less proliferation and the more severe disintegration typical of *Foxf2*$^{-/-}$.

Regions with disintegrated tissues and crosslinked villi can be found next to areas with a grossly normal histology. This rules out difference in genetic background as the sole source of phenotypic variation and instead suggests that reduction of Foxf gene dose creates an unstable situation that will deteriorate rapidly once the defects reach a certain level. Hedgehog signaling from epithelium to mesenchyme requires an intimate contact between cells and will be hampered by loss of ECM. With Foxf genes activated by hedgehog, a degenerative cycle may be initiated once the epithelio-mesenchymal contact is disturbed: lower expression from the remaining Foxf alleles would produce even less ECM and gradually aggravate the tissue disintegration (Fig. 7). Initial small differences in the contact between cells – enough to trigger a degenerative cycle in some areas, but not in others – may be introduced when cell adhesion is challenged by the forces of the commencing peristalsis.

Similarities between the *hedgehog* and Foxf mutant phenotypes suggest that reduced Foxf expression is responsible for many of the observed defects in *hedgehog* mutants, such as megacolon (in *Ihh*$^{-/-}$), anal atresia (in *Shh*$^{-/-}$ and *Gli2/3* mutants) and smooth muscle hypoplasia (in *Ihh*$^{-/-}$) (Mo et al., 2001; Ramalho-Santos et al., 2000). Inhibition of hedgehog signaling by transgenic expression of Hhip mimics the phenotype described here, including activation of the Wnt/β-catenin pathway, epithelial overproliferation and SMA-positive cells in the villus core (Madison et al., 2005). A conserved role for Foxf proteins in intestinal smooth muscle development is further supported by the loss of gut SMCs in response to *Foxf1* morpholino knockdown in *Xenopus* embryos (Tseng et al., 2004) and the defective visceral musculature in *Drosophila biniou* mutants (Zaffran et al., 2001).

The posterior gut agangliosis, which Foxf mutants share with *Ihh*$^{-/-}$ (Ramalho-Santos et al., 2000), resembles Hirschsprung's disease in humans (Carrasquillo et al., 2002). Bmp4 inhibits enteric nerve differentiation (Sukegawa et al., 2000), and premature differentiation of migrating, neural crest-derived neuronal precursors – as a result of decreased *Bmp4* expression – could lead to their depletion preferentially in the posterior gut. SMCs produce a neurotrophic factor, neurturin, that stimulates growth of enteric nerves (Rossi et al., 1999); therefore smooth muscle hypoplasia may also contribute to the reduction of neurons.

Wnt5a can activate both the canonical and non-canonical pathways (Civenni et al., 2003; Topol et al., 2003), and the specificity appears to reside in the receptor. When acting through the Fzd5 receptor, Wnt5a is a potent activator of the canonical pathway and induces axis duplication in *Xenopus* embryos (He et al., 1997; Ishikawa et al., 2001). *Fzd5* is expressed in epithelial cells throughout the murine intestine, with highest expression in the crypts (Ishikawa et al., 2001). Activation of the canonical pathway with stabilized β-catenin in the epithelium is therefore an expected result of increased expression of *Wnt5a* in the mesenchyme.

That Bmps in the intestine are involved in limiting epithelial proliferation is illustrated by formation of ectopic crypts and adenomatous foci in response to transgenic overexpression of noggin (*Nog*) (Haramis et al., 2004). However, in contrast to Foxf mutants, *Nog*-transgenic animals develop normally up until 3 weeks postnatally and the abnormal proliferation takes place in crypt-like structures, rather than throughout the villi. Hence, lowering Foxf gene dose appears to have a pleiotropic effect on cell signaling, and mechanisms other than those caused by a reduction of *Bmp4* expression are likely to contribute to the mutant phenotype.

Deregulation of epithelial proliferation, contact inhibition and survival are hallmarks of intestinal carcinoma and the genetic lesions in epithelial cells that underlie cancerous transformation have been studied extensively (Reya and Clevers, 2005). However, recent results show that the environment provided by the tumor stroma (i.e. non-epithelial cells) influences not only tumor progression but also initiation, and that alterations in both growth factor signaling and ECM composition are important (Bhowmick et al., 2004; Kuperwasser et al., 2004). The tumor-associated, growth-promoting fibroblasts are referred to as 'activated' and, although the exact nature of the underlying changes are not known, activated fibroblasts normally express myofibroblastic markers, such as SMA. The expression of SMA in the subepithelial and intra-villus fibroblast layer of Foxf mutants suggests that there may be a common mechanistic basis for certain changes in epithelial growth and survival seen in Foxf mutants and in intestinal carcinoma. The expansion of SMA-positive myofibroblasts into the villus core in response to transgenic inhibition of hedgehog signaling (Madison et al., 2005) is consistent with this model.

Foxl1 is expressed in intestinal fibroblasts and – like Foxf genes – controls β-catenin accumulation in epithelial cells, although by a different mechanism (Kaestner et al., 1997; Perreault et al., 2001). Loss of both *Foxl1* alleles on an *Apc*$^{-/+}$ (*Min*) genetic background leads to a marked increase in tumor multiplicity in the colon, and *Apc*$^{Min/+}$; *Foxl1*$^{-/-}$ mice also develop gastric tumors not observed in *Apc*$^{Min/+}$ mice (Perreault et al., 2005). The fact that Foxf mutants have an increased intestinal Wnt signaling and that fibroblasts express a marker characteristic of activated tumor stroma raise the possibility that loss of Foxf alleles could contribute to tumor susceptibility.

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