Depletion of epithelial stem-cell compartments in the small intestine of mice lacking Tcf-4

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Mutations of the genes encoding APC or β-catenin in colon carcinoma induce the constitutive formation of nuclear β-catenin/Tcf-4 complexes, resulting in activated transcription of Tcf target genes[1,2]. To study the physiological role of Tcf-4 (which is encoded by the *Tcf7l2* gene), we disrupted *Tcf7l2* by homologous recombination. *Tcf7l2*$^{-/-}$ mice die shortly after birth. A single histopathological abnormality was observed. An apparently normal transition of intestinal endoderm into epithelium occurred at approximately embryonic day (E) 14.5. However, no proliferative compartments were maintained in the prospective crypt regions between the villi. As a consequence, the neonatal epithelium was composed entirely of differentiated, non-dividing villus cells. We conclude that the genetic program controlled by Tcf-4 maintains the crypt stem cells of the small intestine. The constitutive activity of Tcf-4 in APC-deficient human epithelial cells may contribute to their malignant transformation by maintaining stem-cell characteristics.

Members of the Tcf/Lef family of HMG box transcription factors have recently been implicated as being the downstream effectors of Wingless/Wnt signalling in *Xenopus laevis* and *Drosophila melanogaster*[3–9]. Signalling through the cascade leads to the formation of nuclear complexes between β-catenin and Tcf proteins. In these complexes, β-catenin provides an essential transactivation domain. One of the mammalian family members, *Tcf7l2*, is specifically expressed in the developing central nervous system, where it coincides with *Lef1* expression, as well as in the intestinal epithelium[10]. In the gut, expression is observed directly before the overt transition of gut endoderm into epithelium, a process initiating at E13.5 in the mouse. *Tcf7l2* remains expressed in the epithelium throughout life.

We have previously proposed that TCF-4 mediates transformation of colon epithelial cells upon loss of the tumour-suppressor protein APC. In non-stimulated cells, APC and another component of the Wnt pathway, glycogen-synthase-kinase-3β, have been shown to bind β-catenin in the cytoplasm, leading to the rapid degradation of the latter[11]. In APC-deficient colon-carcinoma cell lines, we observed the constitutive presence of transcriptionally active nuclear β-catenin/TCF-4 complexes[1]. Comparable complexes exist in APC-positive colon carcinoma[2] and melanoma[12] cells as a result of dominant mutations affecting any of four conserved Ser/Thr residues in the amino terminus of β-catenin.

To investigate the *in vivo* role of Tcf-4, we disrupted one of the exons encoding the DNA-binding HMG box in the mouse germ line by conventional gene targeting (Fig. 1). Similar targeting strategies have resulted in null mutations in the related genes *Tcf7* (ref. 13) and *Lef1* (ref. 14). Heterozygous mice were viable and fertile. Homozygous mutants were born at mendelian ratios, but died within 24 hours after birth despite the presence of milk in their stomachs. We performed immunohistochemical staining using an anti–Tcf-4 antibody on small intestine from wild-type, heterozygous and homozygous mutant animals. This analysis showed the apparent absence of Tcf-4–specific nuclear staining in the intervillus regions of the small intestinal epithelium of

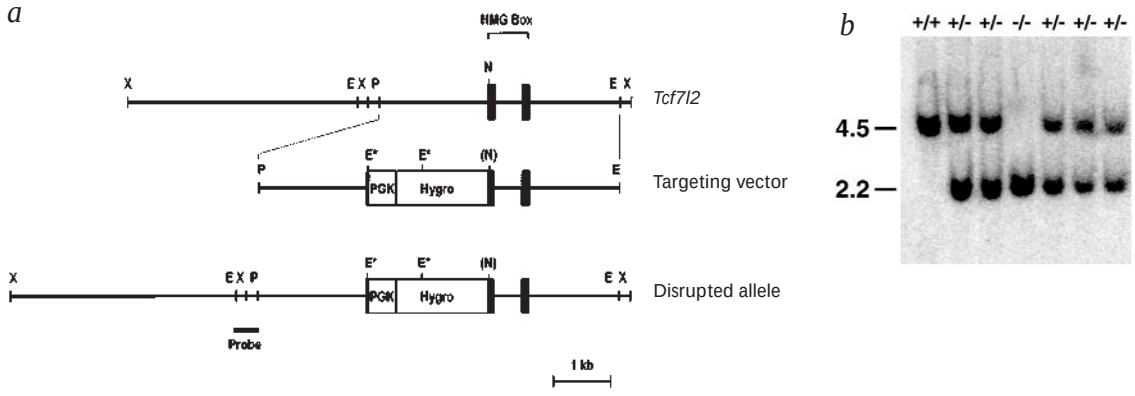

Fig. 1 Targeted disruption of *Tcf7l2*. ***a***, The targeting construct. Top, a partial restriction-endonuclease map of mouse *Tcf7l2*; middle, the structure of the targeting construct containing the hygromycin (hygro) resistance gene under the control of the phosphoglycerate kinase promoter (PGK); bottom, gene structure of the targeted allele. Homologous recombination results in the insertion of the resistance gene cassette into the exon encoding the N-terminal part of the HMG box in the same orientation. The probe used for genomic Southern-blot analysis is indicated by the thick black line. Restriction sites: E, *Eco*RI; N, *Nsi*I; P, *Pst*I; X, *Xba*I. The *Eco*RI sites E* were introduced during construction of the targeting vector. ***b***, Southern-blot analysis of the *Tcf7l2* wild-type (+/+), heterozygous (+/–) and homozygous (–/–) genomic DNA digested with *Eco*RI and hybridized with the probe indicated in (*a*). The 4.5-kb band corresponds to the wild-type allele and the 2.2-kb band corresponds to the disrupted allele.

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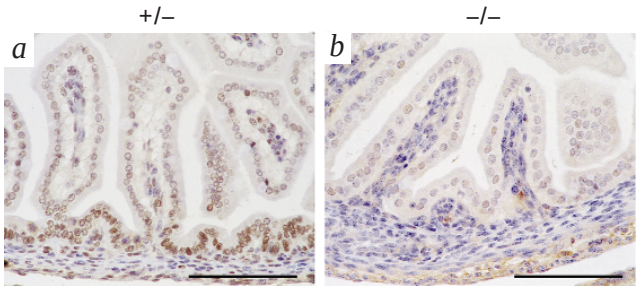

Fig. 2 Tcf-4 protein is absent in *Tcf7l2*$^{-/-}$ mice. Paraffin sections of E16.5 mouse embryos (section through the midgut) stained with anti–Tcf-4 antibody. Nuclear staining present in the intervillus epithelium of the heterozygous embryos (***a***) is not detected in the homozygous embryo (***b***). Horizontal bars indicate 0.1 mm.

homozygous mutant animals, when compared with wild-type or heterozygote littermates (Fig. 2).

Examination of body weight and anatomical appearance of the mutants did not reveal any gross developmental defects. A histological survey of sagittal sections from homozygous newborn animals showed a single abnormality; the epithelial organization of the small intestine was altered compared with wild-type or heterozygous littermates. We observed a decreased number of villi and a reduced amount of epithelial cells in the intervillus regions (Fig. 3). This resulted in extensive stretching and occasional tearing of the epithelial layer, providing a possible explanation for the neonatal lethality. Careful examination of all other organs, particularly of the central nervous system, did not reveal any differences between wild-type, heterozygous and mutant mice.

The basic tissue architecture of the mouse intestine is established during mid- to late-gestation[15–18]. From E13.5 to E18.5, the mouse gut endoderm develops through a stratified epithelium into a simple columnar epithelium overlying nascent villi. By E16.5, the lumen of the small intestine is occupied with villi, and from this day onwards a remarkable functional division of the epithelium becomes evident. Non-cycling, differentiated epithelial cells are located on the villi. The intervillus regions, which later form the crypts, are populated by actively dividing but less differentiated cells[19].

To examine the developmental defect observed in the *Tcf7l2* mutants, we analysed a set of sagittal and cross-sections taken from comparable regions of the small intestines of E14.5, E16.5 and E18.5 homozygous embryos and their littermates. At E14.5, there were no observable differences in the organization of the stratified epithelium of the small intestine (Fig. 3*a*,*b*). At E16.5, a reduced amount of cells was evident in the intervillus regions. We also observed a reduction in the number of villi. However, the two predominant epithelial cell types present on villi, columnar enterocytes and goblet cells, were present in the mutant animals (Fig. 3*c*,*d*,*e*,*f* and Fig. 4*a*,*b*). In contrast, enteroendocrine cells were absent from the *Tcf7l2*$^{-/-}$ small intestine, probably as a direct result of the lack of mature stem cells from which this cell-type normally differentiates (Fig. 4*c*,*d*). The defect became increasingly more pronounced at E18.5 (data not shown), and in newborn animals (Fig. 3*g*,*h*). The mesodermal tissue underlying the epithelium showed no histological differences between mutant and control mice.

The morphological changes that accompany cellular differentiation of a stem cell to terminally differentiated villus cells can be studied in detail using transmission electron microscopy[20] (TEM). TEM analysis at E18.5 showed differences between stem-cell compartments in the intervillus regions of *Tcf7l2*$^{-/-}$ embryos and littermates (Fig. 5). In control mice, the intervillus epithelium was relatively broad, with nuclei appearing at several levels, yielding a pseudostratified appearance. A mitosis frequency of 1.5% (1500 cells counted) was observed in these compartments (Fig. 5*a*). Higher magnification revealed several distinguishing features in comparison to the epithelial cells on the villi. These included an elongated nucleus and a cytoplasm with abundant free ribosomes and polyribosomes, but few mitochondria, Golgi complex, rough-ER and lysosomes. The lateral membranes of these cells were straight, whereas the apical surface carried limited numbers of short microvilli and little endocytic activity (Fig. 5*c*). In contrast, the villus epithelium presented as a single layer of enterocytes. These cells were tall and cylindrical. The round nuclei were found basally (Fig. 5*e*). The cytoplasm contained many rod-shaped mitochondria, abundant rough-ER, but relatively few ribosomes and polyribosomes. A prominent Golgi apparatus was localized to the supranuclear cytoplasm. The lateral cell membranes of adjacent cells showed complex interdigitation. Microvilli of the brush border were well developed and closely packed. Many coated endocytic pits, endocytic vesicles and lysosomes were observed in the apical region of the cells (Fig. 5*g*).

The *Tcf7l2*$^{-/-}$ enterocytes on the villi were reduced in number and consequently broader, but displayed the same features as their wild-type or heterozygous counterparts (Fig. 5*f*,*h*). In contrast, the cells in the intervillus regions did not show any of the distinguishing characteristics of the intervillus prospective crypt cells (Fig. 5*b*,*d*). Instead, these cells resembled villus cells, as evidenced by the absence of the pseudostratified layers, and by the

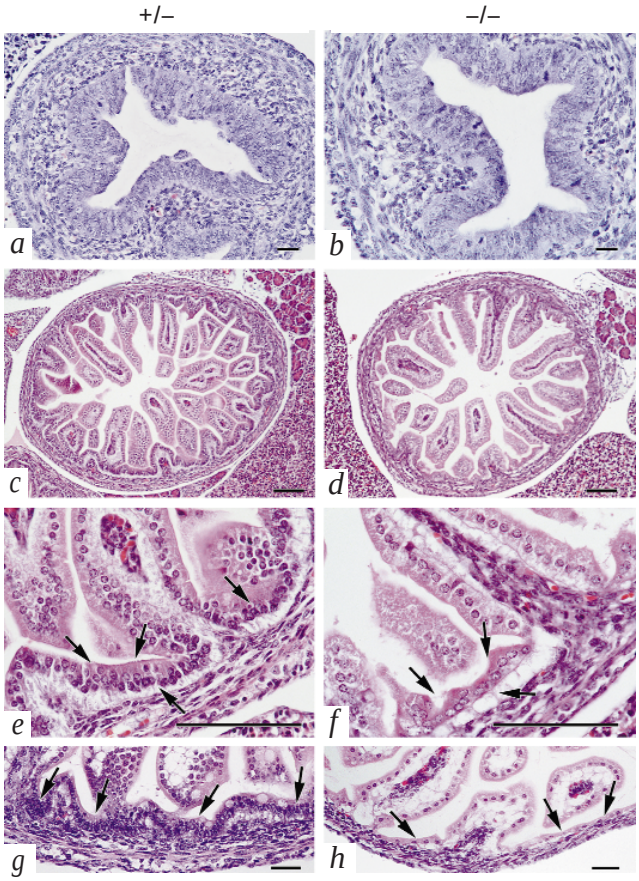

Fig. 3 Embryonic development of the small intestine in *Tcf7l2*$^{-/-}$ mice. Haematoxylin & eosin-stained paraffin sections of the proximal midgut from heterozygous (left) or homozygous mutant (right) mice. ***a***,***b***, At E14.5, the invagination of the mesenchyme to form epithelial ridges occurs normally in heterozygous (*a*) and homozygous mutant (*b*) gut; no differences are observed. ***c***,***d***,***e***,***f***, At E16.5, fewer villi are apparent in the homozygotes (*d*,*f*), and the intervillus regions (arrows) contain a reduced amount of cells. ***g***,***h***, The same structural defect is more pronounced in newborn animals.

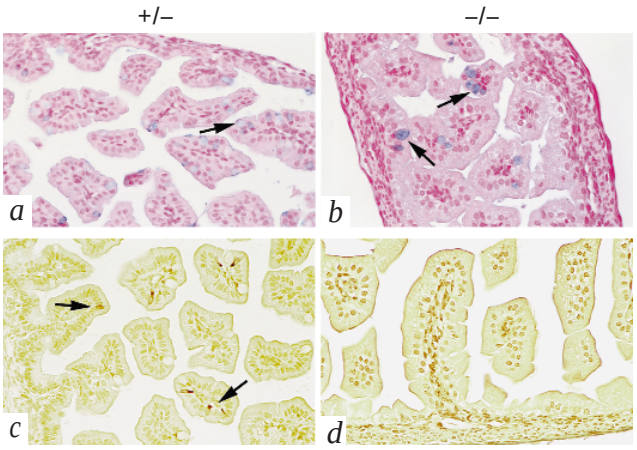

Fig. 4 Cytodifferentiation in the small intestine of *Tcf7l2*$^{-/-}$ mice. Paraffin sections of small intestine from E18 heterozygous (left) or homozygous mutant (right) stained with Alcian Blue (goblet cell-specific, ***a***,***b***) or Grimelius (enteroendocrine cell-specific, ***c***,***d***). Goblet cells are present in the small intestine of both *Tcf7l2*$^{+/-}$ (*a*) and *Tcf7l2*$^{-/-}$ (*b*) mice (arrows), but no enteroendocrine cells are evident in the *Tcf7l2*$^{-/-}$ mice (*d*).

presence of closely packed long microvilli, round nuclei, a supranuclear Golgi, many rod-shaped mitochondria, an extensive endocytic compartment including coated-pit formation on the apical membranes, interdigitating lateral membranes and the absence of mitotic figures. We never observed any morphological sign of apoptosis in either mutant or control epithelium, nor did we see any differences in the formation of junction complexes between lateral membranes.

To confirm the absence of cell cycling, we performed immunohistochemistry using the nuclear proliferation marker Ki-67 (ref. 21). At E14.5, numerous proliferating cells were randomly distributed throughout the epithelium; no difference was observed between mutant and control mice (Fig. 6*a*,*b*). At E16.5 and later stages, Ki-67 staining revealed a marked difference between control and *Tcf7l2*$^{-/-}$ mice. In the control epithelium, proliferating cells were abundant and compartmentalized in the intervillus regions. In contrast, the epithelium of the small intestine of the mutant embryos did not show any Ki-67 staining at this stage (Fig. 6*c*,*d*). Analysis of 5-bromodeoxyuridine (BrdU) incorporation in E17 mutant embryos confirmed the lack of proliferative activity in the intervillus regions of the developing small intestine (Fig. 6*e*,*f*). This correlated with the absence of mitoses, as observed by TEM. Ki-67 staining in the tissue layers directly underneath the epithelium as well as in other organs (data not shown) was not affected in the mutants.

We conclude that Tcf-4 plays a unique role in the maintenance of the epithelial stem-cell compartment of the small intestine. In contrast, Tcf-4 is not essential for the primary induction of epithelial cells from endoderm, as shown by the presence of differentiated villus enterocytes and goblet cells in the mutants. The observed phenotype is unique to the small intestine. We currently do not know why stem-cell compartments in other parts of the intestinal tract are not affected. One possible explanation is functional redundancy with *Tcf7l1*, a member of the Tcf family that is expressed in gut[10]. The phenotype of the *Apc*^{Min} mice and of *Apc*-deficient mice is largely restricted to the small intestine; however, polyps are occasionally found in the colon[22–25].

These observations may provide insight into the molecular mechanism of APC's tumour-suppressor function. Wild-type APC functions as a negative regulator of β-catenin/TCF-4 signalling. In its absence, complexes are formed between β-catenin and Tcf-4. Such complexes are normally formed only in response to Wnt signalling and activate transcription of reporter genes, and, presumably, of endogenous target genes. We have proposed that in APC-deficient colon cancer, ectopic activation of TCF-4 target genes represents one of the early steps in cellular transformation. The current study provides some insight into the mechanism, as signals transduced *via* Tcf-4 seem to help establish the stem-cell phenotype in (nascent) crypts of the small intestine. An immediate prediction based on the current observation would be that constitutive activation of TCF-4 in APC-deficient cells leads to maintenance of stem cell characteristics (cycling and longevity, for example) in cells that should normally undergo terminal dif-

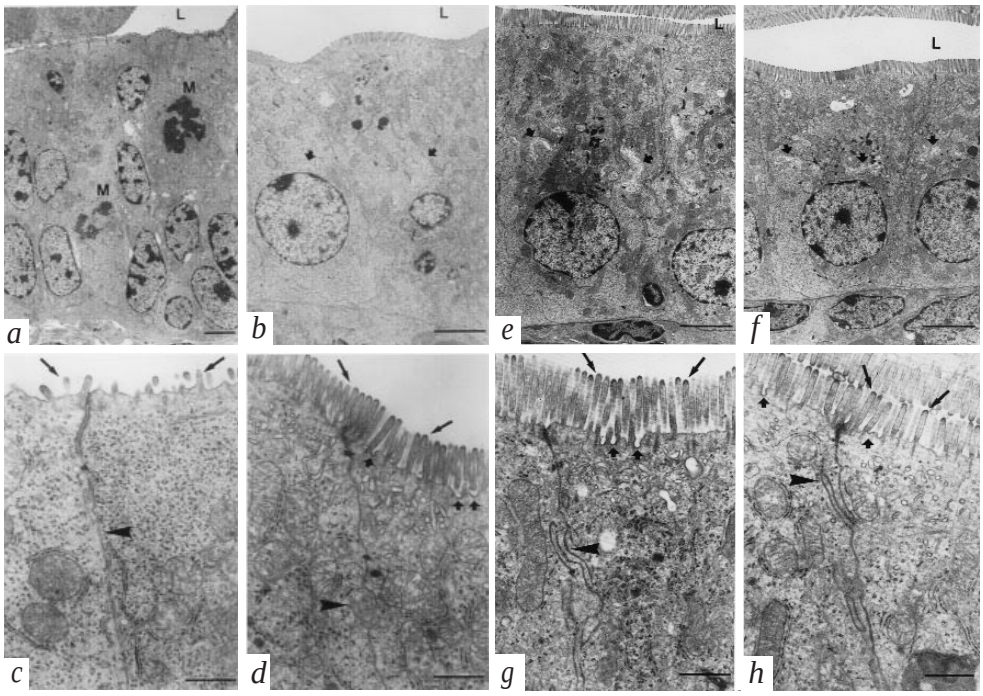

Fig. 5 Ultrastructural investigations show a defect in the intervillus region. *a*–*d*, TEM (ref. 20) of the intervillus region. *e*–*h*, Villus zone. *a*,*c*,*e*,*g*, Control embryo. *b*,*d*,*f*,*h*, *Tcf7l2*$^{-/-}$ embryo. ***a***, Two mitotic cells (M) and the pseudostratified character of the epithelial layer. ***b***, The epithelial intervillus cells of the *Tcf7l2*$^{-/-}$ embryo have morphological characteristics similar to the villus cells of the control (***e***) or the *Tcf7l2*$^{+/-}$ (***f***). Thick arrows indicate the Golgi complex; L, gut lumen. At high magnification (***c***,***d***,***g***,***h***) we observed many endocytic vesicles and coated pits on the apical membrane (thick arrows) of the intervillus cell in the *Tcf7l2*$^{-/-}$ (*d*) comparable to villus cells (*g*,*h*). In addition, interdigitations were observed in the homozygote (arrowhead in *d*) that were not seen in the control (*e*) and resembled the interdigitation as seen in the villus cells (*g*,*h*). Horizontal bars indicate 2 μm in *a*,*b*,*e*,*f*; 0.5 μm in *c*,*d*,*g*,*h*.

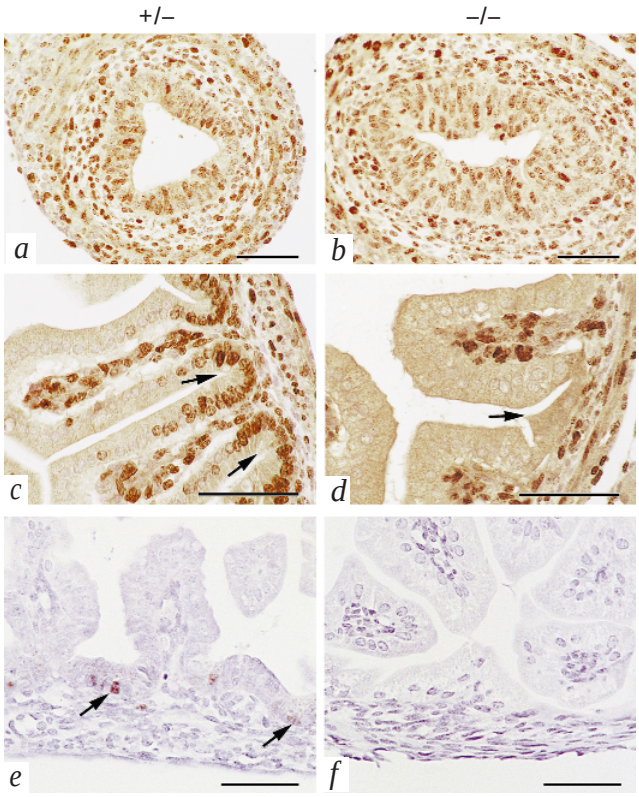

Fig. 6 Absence of cycling cells in *Tcf7l2*$^{-/-}$ small intestinal epithelium. Paraffin sections of the proximal small intestine from E14.5 heterozygous (***a***) or homozygous (***b***), and E16.5 heterozygous (***c***) or homozygous mutant (***d***) embryos stained with an antibody against proliferating cell antigen Ki-67, and E17 heterozygous (***e***) or homozygous (***f***) mutant embryos labelled with BrdU and stained with an anti-BrdU monoclonal antibody. At E14.5, numerous proliferating cells are present throughout the epithelium of both heterozygous and homozygous mutant embryos (*a,b*). At E16.5, proliferating cells are restricted to the intervillus regions in the epithelium of the *Tcf7l2*$^{+/-}$ embryos (arrows), but are absent from the *Tcf7l2*$^{-/-}$ embryos (arrows in *c,d*). There is no apparent difference between the staining of underlying mesenchymal tissue in the heterozygous and the homozygous mutant intestine. BrdU-labelled cells are present in the intervillus regions of the *Tcf7l2*$^{+/-}$ embryos (arrows), but are absent from the *Tcf7l2*$^{-/-}$ embryos (*e,f*). Horizontal bars indicate 0.1mm.

ferentiation into one of the epithelial cell types. Indeed, it has been proposed that tumorigenesis in *Apc*^{Min} mice affects the multipotent stem cell in the intestinal crypt[26]. The resulting expansion of these crypt cells leads to the formation of an intestinal micro-adenoma, which then expands into a neighbouring villus[27]. This notion readily explains the formation and characteristics of polyps in the *Apc*^{Min} mice and the almost obligatory activation of the transcriptional activity of TCF-4 through mutations in *APC* or *CTNNB1* (encoding β-catenin), as found in human colon cancer. The phenotype of the *Tcf7l2*$^{-/-}$ mouse helps explain how Apc functions to inhibit tumorigenesis in the small intestine. A similar mechanism might be active in melanoma. We currently do not know the nature of the physiological signal that is transduced by Tcf-4 in crypt epithelial cells. Based on existing data on epithelial/mesenchymal interactions in the gut[28–30], we assume that the mesenchymal-derived cells underlying the crypts produce soluble factors essential for the establishment and maintenance of the stem-cell compartment. Given the evidence for the involvement of other Tcf family members in Wingless/Wnt signalling, Wnt factors secreted by the gut mesenchyme are the prime candidates to deliver the signal relayed by Tcf-4.

Methods

Generation of the *Tcf7l2* targeting construct. Lambda phage clones containing the mouse *Tcf7l2* gene were isolated from 129SVJ mouse genomic library in Lambda FIX II vector (Stratagene). A 4.2-kb *Pst*I–*Eco*RI fragment containing two HMG box-encoding exons was subcloned into pBluescriptSK. The targeting construct was generated by insertion of the PKG-Hygro cassette into the *Nsi*I site located in the proximal part of the 5´ end exon. Prior to ligation, the *Nsi*I cut was blunted with T4 DNA polymerase, resulting in a 4-bp deletion in the *Tcf7l2* coding region. The construct was linearized by *Sma*I digestion and electroporated into E14 ES cells. Approximately 300 ES cell clones selected by Hygromycin B (250 μg ml^{-1}) were screened by Southern-blot analysis for the presence of a recombinant 2.2-kb *Eco*RI band, in addition to the endogenous 4.5-kb fragment, using a 300-bp *Eco*RI/*Pst*I fragment from *Tcf7l2*. Three clones had undergone correct integrations at both ends of the construct. Chimaeric mice were derived from all three clones; one of these transmitted the mutation through the germ line.

Immunohistochemical staining of embryos. Approximately 50 *Tcf7l2*$^{-/-}$ and an equivalent number of *Tcf7l2*$^{+/-}$ embryos were fixed in 4% formaldehyde-PBS, embedded in paraffin and sectioned (6 μm). For immunohistochemical analyses, sections were treated with 1.5% H_2O_2 in methanol for 20 min, the slides were then immersed in citrate buffer (0.01 M; pH 6.0) and incubated for 25 min at 90 ºC in a steam bath. Slides were washed in PBS and incubated with 2% goat non-immune serum-2% BSA for 20 min at RT to block non-specific binding. The primary antibodies were rabbit anti Ki-67 (1:500, Novacastra) and mouse anti–Tcf-4 antibody. The anti Ki-67 antibody were visualized with goat anti-rabbit biotinylated antiserum (Vector Laboratories), and streptavidin/biotin/HRP detection system (DAKO); the anti-Tcf-4 antibody was detected with rabbit anti-mouse/HRP antibody and swine anti-rabbit/HRP antibody (DAKO). The tissue was counterstained lightly with haematoxylin.

BrdU cell-proliferation assay. Mice pregnant with E17 embryos were injected IP with the BrdU-labelling reagent from an Amersham cell proliferation kit (RPN 20) according to the manufacturer's instructions. Mothers were killed 2 h later, embryos removed and fetal intestinal tissue samples prepared as described above. BrdU incorporation was measured using an anti-BrdU primary monoclonal antibody (Amersham) and a rabbit anti-mouse/HRP secondary antibody (DAKO).

Preparation of intestinal tissue samples for TEM. Tissue samples from the proximal part of the small intestine of E18.5 *Tcf7l2*$^{+/-}$ and *Tcf7l2*$^{-/-}$ embryos were fixed for at least 18 h at 4 ºC in a mixture of cold paraformaldehyde (2%, w/v) and glutaraldehyde (2.5%, v/v) in sodium cacodylate buffer (0.1 M, pH 7.4). The tissues were then cut into 2-mm^2 fragments, post-fixed for 2 h in osmium tetroxide (1%), dehydrated in graded alcohols and embedded in Epon 812. Sections (50nm) were contrasted with uranyl magnesium acetate and lead citrate staining and viewed with model 1200 electron microscope.

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