

Hindgut defects and transformation of the gastro-intestinal tract in *Tcf4*^{-/-}/*Tcf1*^{-/-} embryos

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Wnt signalling plays a critical role in both initiating and patterning of the anterior–posterior axis during development. Wnts exert their biological effects, in part, by activating specific target genes through members of the TCF/LEF family of transcription factors. To gain new insight into the role of T-cell factors (or Tcf's) during development, we analysed *Tcf4* and *Tcf1* compound null embryos. These mutants showed severe caudal truncations, as well as duplications of the neural tube. Unlike other mutations affecting Wnt signalling, paraxial mesoderm formation was not impaired and early caudal markers, such as *T*, were unaffected. Analysis of endodermal markers uncovered early and specific defects in hindgut expansion, and later an anterior transformation of the gastro-intestinal tract. Our results reveal a novel role for Wnt signalling in early gut morphogenesis and suggest that specific Wnt-driven patterning events are determined by the unique tissue distribution of *Tcf/Lef* family members.

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Introduction

During mouse development, the anterior–posterior (A–P) axis becomes morphologically evident at the onset of gastrulation. At this point, the epiblast cell layer at the posterior end of the embryo forms a structure known as the primitive streak. Cells at the streak delaminate and migrate forward and laterally to form both mesodermal and endodermal layers. As gastrulation proceeds at the posterior end, body structures such as head, trunk, limbs and tail are specified along the A–P axis. This process of patterning and later organogenesis depends on a complex network of instructive signals, ultimately determining whether cells within the developing embryo proliferate, differentiate, or die.

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Among the most important regulators of cell fate decisions during both formation and patterning of the A–P axis are members of the Wnt/Wg family of secreted factors. Wnts make up a large family of cystein-rich glycoproteins that activate signalling cascades that induce cytoplasmic responses and/or transcription of target genes (for reviews, see Wodarz and Nusse, 1998; Yamaguchi, 2001; Mlodzik, 2002; He, 2003). The latter depends on cytoplasmic accumulation of β -catenin and its translocation to the nucleus, where it associates with the TCF/LEF family members (comprised of TCF1, LEF, TCF3, TCF4). Formation of this bipartite complex provides a template for the recruitment of additional transcriptional activators that finally turn on expression of specific target genes. Importantly, in the absence of Wnts, TCF/LEF proteins can actively block transcription by recruiting general repressors like Groucho and CtBP (Nusse, 1999; Hurlstone and Clevers, 2002). In this way, TCF/LEF proteins act as molecular switches that control cell fate decisions.

Perhaps the first indication that the Wnt/ β -catenin/TCF pathway was required for determining the A–P axis came from microinjection experiments conducted in *Xenopus* embryos. Ectopic expression of Wnts, for example, induces axis duplications and can rescue UV-irradiated embryos (McMahon and Moon, 1989; Sokol *et al.*, 1991). In addition, gene-targeting experiments in mice have provided a wealth of information confirming an essential role for Wnt signalling in axis formation. Mutations disrupting Wnt signalling, such as *Wnt3* and β -catenin knockouts, completely impair formation of the primitive streak and the resulting embryos remain as egg cylinders lacking posterior specification (Liu *et al.*, 1999; Huelsken *et al.*, 2000). On the other hand, mutations in *Axin* and *APC*, which lead to increased β -catenin/TCF-mediated transcription, cause axis duplications similar to those observed in *Xenopus* (Zeng *et al.*, 1997; Ishikawa *et al.*, 2003). Similarly, ablation of *Tcf3* results in duplications of the node and notochord, as well as upregulation of axial markers, such as *Foxa2*, implying that *Tcf3* acts primarily as a transcriptional repressor involved in restricting AP axis induction (Merrill *et al.*, 2004).

Deletion of other components of the Wnt cascade has shown that Wnt signals are also required for patterning and/or expansion of the mouse embryonic axis once it has formed. Mutations in *Wnt1* lead to deletion of the midbrain and cerebellar component of the hindbrain (McMahon and Bradley, 1990). *Dkk*^{-/-} embryos display a disruption of tissues forebrain and cephalic neural crest-derived tissues, as well as limb morphogenesis defects (Mukhopadhyay *et al.*, 2001). *Wnt3a*^{-/-} and *Lef*^{-/-}/*Tcf1*^{-/-} embryos show severe caudal truncations of the embryonic axis, resulting from an early loss of mesodermal structures (ie somites) (Takada *et al.*, 1994; Greco *et al.*, 1996; Galceran *et al.*, 1999). The absence of *Wnt5a* results in a general shortening of the embryonic axis, including limbs (Yamaguchi *et al.*, 1999a). Finally, loss of *LRP6*, an essential Wnt coreceptor, recapitulates many of the phenotypes described above, with

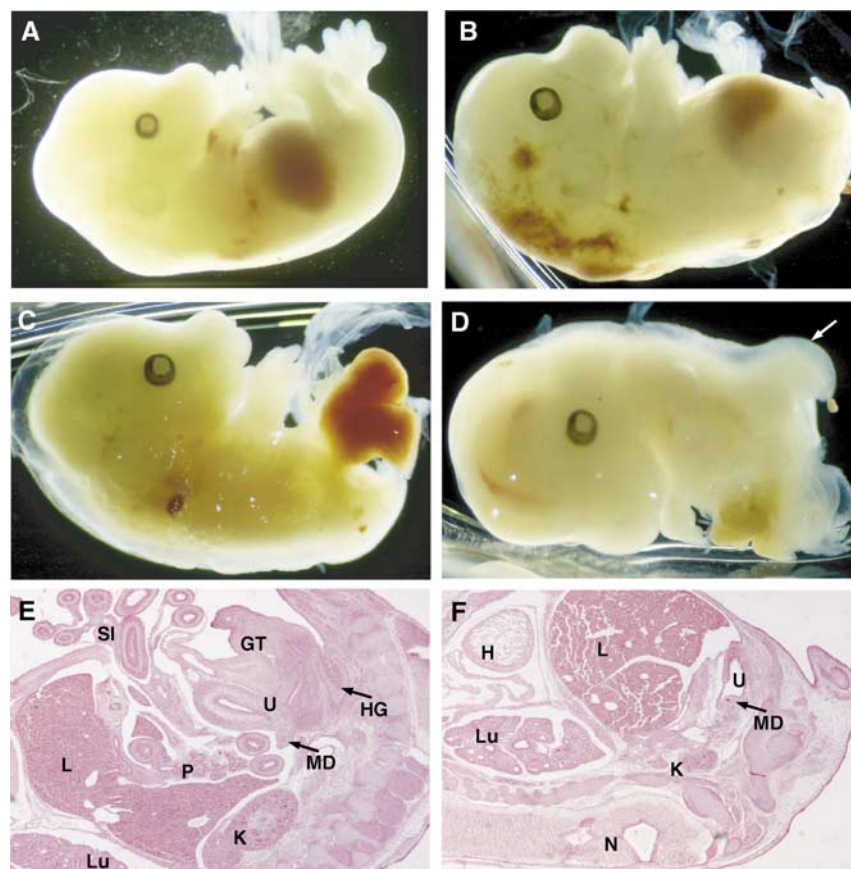


Figure 1 $Tcf4^{-/-}/Tcf1^{-/-}$ embryos show severe caudal truncations. Lateral views of E14.5 embryos. (A) $Tcf4^{+/-}/Tcf1^{-/-}$ and (B–D) $Tcf4^{-/-}/Tcf1^{-/-}$ littermates. Panel B shows vestigial hindlimbs and the absence of a tail in mutant embryos. In more severe cases as depicted in panels C and D, internal organs such as the gut and liver are exposed. In several instances, the neural tube branched out in mutant embryos as shown in (D, F) (see arrow). Panels E and F represent haematoxylin and eosin stainings of sections of embryos depicted in (A, B), respectively. Various structures are identified as follows: (H) heart, (Lu) lungs, (L) liver, (K) kidneys, (U) urogenital sinus, (MD) mesonephric duct, (GT) genital tubercle, (SI) small intestine, (HG) hindgut and (N) neural tube.

disrupted patterning in the brain, limbs, neural tube and tail (Pinson *et al*, 2000).

Besides the phenotype of $Lef^{-/-}/Tcf1^{-/-}$ and $Tcf3^{-/-}$ embryos described above, limited information is available about the function of other *Tcf/Lef* genes during early mouse development. $Lef^{-/-}$ mice die perinatally, lacking teeth, hair follicles and mammary glands (van Genderen *et al*, 1994). Further analysis has also demonstrated impaired hippocampus development and generation of dentate gyrus granule cells in *Lef*-deficient mice (Galceran *et al*, 2000). $Tcf1$ homozygous null mice are viable but demonstrate an early blockage in thymocyte differentiation, as well as increased susceptibility to form mammary gland and intestinal neoplasms (Verbeek *et al*, 1995; Roose *et al*, 1999). $Tcf4^{-/-}$ mice die at birth due to a loss of epithelial stem cells in the small intestine (Korinek *et al*, 1998a). In order to gain further insight into the function of *Tcf/Lef* genes during morphogenesis, we generated compound $Tcf4^{-/-}/Tcf1^{-/-}$ mice. These mutants display profound caudal truncations similar to $Wnt3a^{-/-}$ and $Lef^{-/-}/Tcf1^{-/-}$ embryos, although, unlike the latter, $Tcf4^{-/-}/Tcf1^{-/-}$ embryos retain paraxial mesoderm. Rather, the primary patterning defect appears to result from aberrant hindgut expansion, ultimately leading to homeotic shifts of the fetal gastro-intestinal tract.

Results

$Tcf4^{-/-}/Tcf1^{-/-}$ mutants show posterior truncations

To study the consequences of inactivating both *Tcf1* and *Tcf4* during embryonic development, we crossed $Tcf4^{+/-}$ and $Tcf1^{-/-}$ mice. Although smaller than wild-type counterparts, $Tcf4^{+/-}/Tcf1^{-/-}$ mice appeared normal. Compound homozygous null embryos, however, did not survive past gestation. Viable double mutants were obtained at the expected frequency until E14.5. Examination of $Tcf4^{-/-}/Tcf1^{-/-}$ embryos at this stage showed major abnormalities in the development of caudal structures, while anterior structures were spared. As shown in Figure 1, $Tcf4^{-/-}/Tcf1^{-/-}$ mutants lacked hindlimbs, posterior body and tail. Although the formation of hindlimbs was affected, forelimbs were normal, suggesting that the loss of hindlimbs reflects a general patterning defect, rather than a specific problem in limb development. In more severely truncated embryos, internal organs were exposed, indicating a failure of the abdominal wall to develop properly (Figure 1C and D). Haematoxylin/eosin stainings of sections of the specimen depicted in Figure 1B revealed that most internal structures, including the lungs, heart, pancreas and liver, were clearly distinguished and histologically appeared normal (Figure 1F and data not shown). Even more caudal

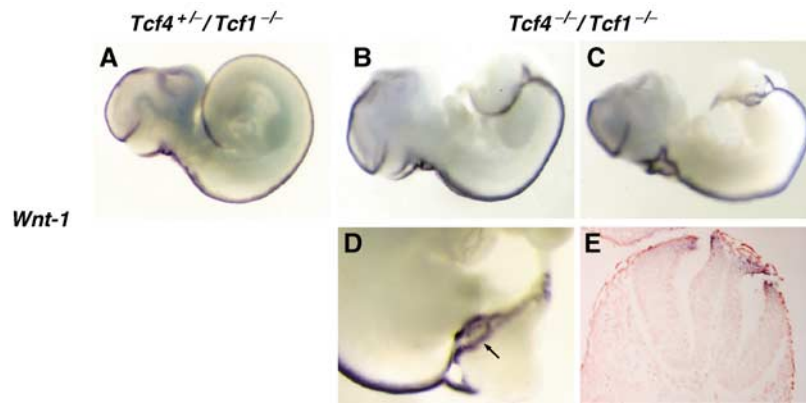


Figure 2 Duplications of the neural tube in *Tcf4*^{-/-}/*Tcf1*^{-/-} embryos. Whole-mount *in situ* hybridizations of E10.5 littermates using a *Wnt1* probe as a marker for neural tube. (A) *Tcf4*^{+/+}/*Tcf1*^{-/-} embryo; (B) right side, (C) left side and (D) close-up views of a representative *Tcf4*^{-/-}/*Tcf1*^{-/-} embryo. Lateral and close-up views reveal that the neural tube appears to split at the caudal end of mutant embryos. In some regions (see the arrow in (D) and the corresponding section in (E)), complete duplications of the neural tube are observed. Note that *Wnt1* expression appears normal in the anterior half of *Tcf4*^{-/-}/*Tcf1*^{-/-} embryos.

structures such as kidneys and mesonephric ducts had formed and differentiated. This may imply that *Tcf4* and *Tcf1* are not required for early morphogenesis of these organs. Closer examination of *Tcf4*^{-/-}/*Tcf1*^{-/-} mutants, however, showed a complete absence of the genital tubercle, hindgut and only a remnant of the urogenital sinus, as well as signs of aberrant branching of neural tube (Figure 1D and F and below).

Duplications of the neural tube in *Tcf4*^{-/-}/*Tcf1*^{-/-} embryos

The posterior truncations observed in *Tcf4*^{-/-}/*Tcf1*^{-/-} embryos were reminiscent of *Wnt3a*^{-/-} mutants. Analysis of these mice, as well as *Lef*^{-/-}/*Tcf1*^{-/-} embryos, revealed an early loss of paraxial mesoderm and concomitant formation of ectopic neural tubes (Yoshikawa *et al*, 1997; Galceran *et al*, 1999). To characterize the fate of paraxial mesoderm versus neural tissue in *Tcf4*^{-/-}/*Tcf1*^{-/-} embryos, we began our analysis by examining the expression of the neural tube marker *Wnt1* in E10.5 embryos. As shown by whole-mount *in situ* hybridization, *Wnt1* expression along the anterior portion of *Tcf4*^{-/-}/*Tcf1*^{-/-} embryos was comparable to that of normal littermates (Figure 2). Towards the posterior end, ectopic *Wnt1* expression was detected, reflecting bifurcations or splitting of the neural tube (Figure 2D, see arrow). Sectioning through the mutant embryos confirmed the occurrence of duplications of the neural tube (Figure 2E).

Paraxial mesoderm formation is unaffected in *Tcf4*^{-/-}/*Tcf1*^{-/-} embryos

To follow the fate of paraxial mesoderm, we employed three somitic markers, *Paraxis*, *Pax1* and *Myf5*. Detection of *Paraxis* transcripts, normally present in sclerotome and dermamyotome compartments, clearly distinguished the presence of somitic tissue along the entire A-P axis of *Tcf4*^{-/-}/*Tcf1*^{-/-} embryos (Figure 3B). Note, however, that the somites at the caudal end did appear disorganized. Even in a specimen displaying a more severe truncation, the sclerotome marker *Pax1* revealed that, despite being smaller and less well defined, somites were present (Figure 3D). Finally, we tested

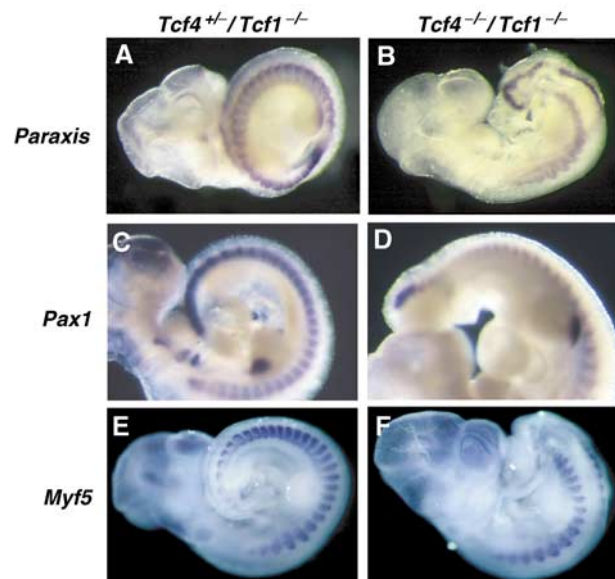


Figure 3 Paraxial mesoderm is intact in *Tcf4*^{-/-}/*Tcf1*^{-/-} embryos. Whole-mount *in situ* hybridizations of E10.5 littermates using markers for paraxial mesoderm. (A, C, E) *Tcf4*^{+/+}/*Tcf1*^{-/-} embryos and (B, D, F) *Tcf4*^{-/-}/*Tcf1*^{-/-} embryos. Panels A and B show stainings for *Paraxis*, panels C and D show *Pax1* stainings. Panels E and F show expression of *Myf5*. All three somitic markers confirm the presence of paraxial mesoderm throughout the axis of *Tcf4*^{-/-}/*Tcf1*^{-/-} mutants.

the myogenic marker *Myf5*, and found once again that caudal somites had formed (Figure 3F). The maintenance of somitic markers was in stark contrast to the previously described *Lef*^{-/-}/*Tcf1*^{-/-} phenotype, in which *Pax1* expression was completely absent below the forelimbs (Galceran *et al*, 1999). To confirm the differences between the two crosses, we generated *Lef*^{-/-}/*Tcf1*^{-/-} embryos and verified the expression of *Paraxis* in these mutants (data not shown). As expected, *Paraxis* staining was abolished in the caudal portion of these mutants, providing additional proof that, contrary to *Tcf4*^{-/-}/*Tcf1*^{-/-} embryos, *Lef*^{-/-}/*Tcf1*^{-/-} mutants lack somitic tissue. These results suggest that,

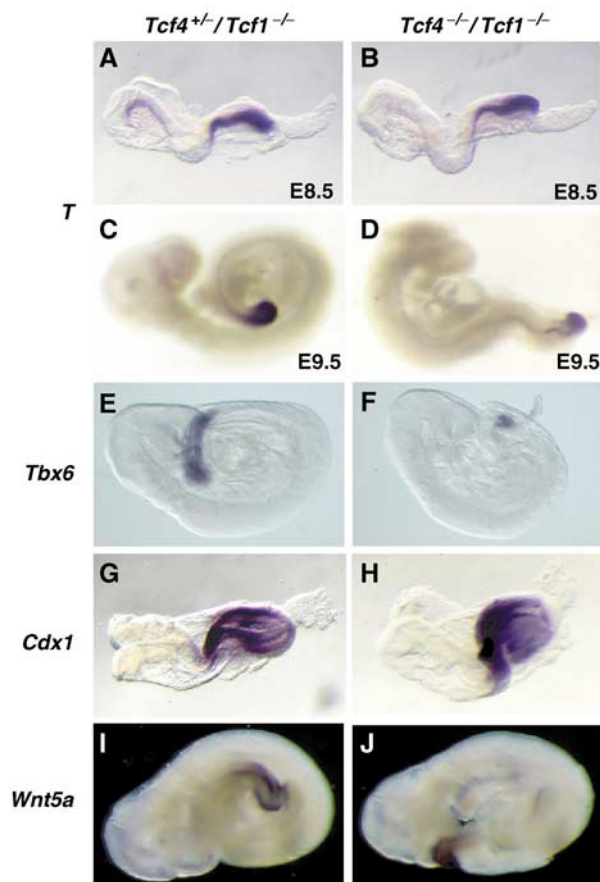


Figure 4 Expression of early posterior markers is unaffected in *Tcf4*^{-/-}/*Tcf1*^{-/-} embryos. Whole-mount *in situ* hybridizations of E8.5–9.5 littermates using various posterior markers (see above). (A, C, E, G, I) *Tcf4*^{+/-}/*Tcf1*^{-/-} embryos and (B, D, F, H, J) *Tcf4*^{-/-}/*Tcf1*^{-/-} embryos. Panels (A, B) and (C, D) represent E8.5 and E9.5 embryos, respectively, and show that initiation and maintenance of *T* expression is unchanged in *Tcf4*^{-/-}/*Tcf1*^{-/-} embryos.

despite forming ectopic neural tissue, paraxial mesoderm fate is unaffected in *Tcf4*^{-/-}/*Tcf1*^{-/-} embryos.

Next, we assessed whether expression of early caudal markers was impaired. Several genes expressed in the primitive streak and presomitic mesoderm have been reported to play an essential role during patterning of the caudal end of the mouse embryo. For example, mutations in *T* (*Brachyury*) cause a kinked tail phenotype and maintenance of *T* expression was shown to depend on intact TCF/LEF-binding sites in its promoter (Yamaguchi *et al*, 1999b; Galceran *et al*, 2001). However, *in situ* hybridization on both E8.5 and E9.5 embryos demonstrates that *T* expression is unaltered in *Tcf4*^{-/-}/*Tcf1*^{-/-} embryos (Figure 4A–D). These results contrast the analysis of *Lef*^{-/-}/*Tcf1*^{-/-} embryos, where maintenance of *T* expression was abolished in the tail bud of E9.5 embryos. We also tested *Tbx6*, another T box-containing family member implicated in posterior patterning (Chapman and Papaioannou, 1998). As shown in Figure 4E and F, *Tbx6* transcripts were still detected, although the levels and range of expression were reduced compared to normal littermates. *Cdx1* expression was shown to be downregulated as early as E8.5 in *Wnt3a*-deficient embryos, and in the fetal gut of *Tcf4*^{-/-} mice (Lickert *et al*, 2000; Ikeya and Takada, 2001; Prinos *et al*, 2001). In spite of these observations, Figure 4G

and H shows that *Cdx1* is not dependant on *Tcf1*/*Tcf4*, at least during gastrulation. Finally, we tested *Wnt5a* because of its essential role in expansion of the tailbud, but as with other markers tested (including *Cdx2*, *Lef*, *Evx1* and *Wnt3a*, data not shown) no changes in expression were observed. Altogether, these results provide further evidence that the patterning defects in *Tcf4*^{-/-}/*Tcf1*^{-/-} embryos cannot be directly attributed to loss of mesodermal precursors.

Expression of *Tcf4*, *Tcf1* and *Lef* in the primitive gut

To gain further insight into the underlying causes of the *Tcf4*^{-/-}/*Tcf1*^{-/-} phenotype, we re-examined the expression of *Tcf*/*Lef* family members. Previous analysis has shown overlapping expression of *Tcf1* and *Lef* in the forelimbs and primitive streak region of gastrulating embryos (Oosterwegel *et al*, 1993; Galceran *et al*, 1999). Somewhat surprisingly, in the light of the caudal truncations observed in *Tcf4*^{-/-}/*Tcf1*^{-/-} embryos, *Tcf4* transcripts were mainly detected in rostral structures, such as di- and mesencephalon and pharyngeal arches (Korinek *et al*, 1998b; Galceran *et al*, 1999). To address this discrepancy, we performed *in situ* hybridization on sections of E8.5, and E10.5 wild-type embryos, focusing our attention on posterior structures. Sections through the primitive streak showed that the overall expression pattern of *Tcf*/*Lef* genes was only partially overlapping. At E8.5 *Tcf4* was specifically detected in the hindgut. *Tcf1* was expressed in the primitive ectoderm, presomitic mesoderm and hindgut. *Lef* was restricted to the presomitic mesoderm (Figure 5A–C). At later stages, we could distinguish *Tcf4* expression in the neural tube and hindgut. Comparable sections showed that *Tcf1* expression was maintained in all three germ layers, including the neural tube, presomitic mesoderm and hindgut. Importantly, *Lef* was excluded from the primitive gut and was expressed in mesodermal structures (Figure 5D–F). The tissue specificities of *Tcf*/*Lef* genes provided a rationale to explain the disparities between the phenotypes of the compound null mutants. The overlapping expression of *Tcf1* and *Lef* in the presomitic mesoderm, coupled with the exclusion of *Tcf4* in this compartment, may explain why *Lef*^{-/-}/*Tcf1*^{-/-} embryos, but not *Tcf4*^{-/-}/*Tcf1*^{-/-} embryos, lose caudal somites. By analogy, we reasoned that the primary anomaly in *Tcf4*^{-/-}/*Tcf1*^{-/-} embryos may be impaired hindgut development. Based on these observations, we decided to analyse endodermal fate in *Tcf4*^{-/-}/*Tcf1*^{-/-} embryos.

Caudal endoderm is impaired in *Tcf4*^{-/-}/*Tcf1*^{-/-} but not *Lef*^{-/-}/*Tcf1*^{-/-} embryos

To follow gut development, we analysed the expression of three endodermal markers *Sox17*, *Foxa1* and *Shh* in *Tcf4*^{-/-}/*Tcf1*^{-/-} and *Lef*^{-/-}/*Tcf1*^{-/-} embryos. At E8.5, endodermal tube formation is initiated by folding of the endodermal lining at the anterior and posterior ends, creating anterior and caudal intestinal portals (AIP and CIP) (for reviews, see Wells and Melton, 1999; Roberts, 2000). At this stage, *Sox17* and *Foxa1* are normally expressed along the entire endodermal lining in wild-type embryos (Figure 6A and D). In *Tcf4*^{-/-}/*Tcf1*^{-/-} embryos, however, the caudal endoderm showed an absence of both *Sox17* and *Foxa1* staining (Figure 6B and E, see arrows). Conversely, in *Lef*^{-/-}/*Tcf1*^{-/-} embryos, expression of both markers is uninterrupted (Figure 6C and F). At E9.5, the AIP and CIP join to close the endodermal tube, resulting in the formation of a proper foregut, midgut

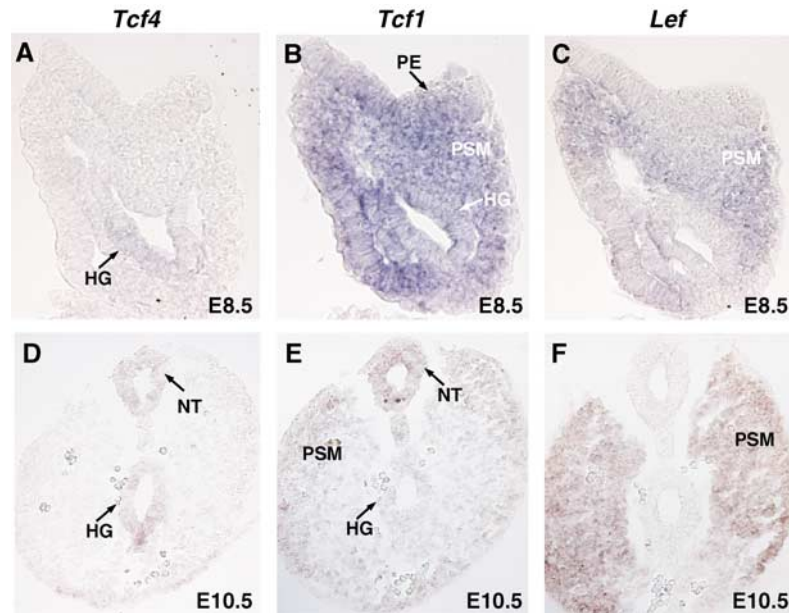


Figure 5 Comparison of *Tcf4*, *Tcf1* and *Lef* expression. *In situ* hybridizations on sections of E8.5 and E10.5 wild-type embryos were performed using specific probes recognizing *Tcf4* (A, D), *Tcf1* (B, E) and *Lef* (C, F). Top panels A–C depict sections through the primitive streak region of E8.5 embryos, while bottom panels D–F show sections through the tail bud of E10.5 embryos. Various structures are identified as follows: (PE) primitive ectoderm, (NT) neural tube, (PSM) presomitic mesoderm and (HG) hindgut. *Tcf4* was specifically expressed in the hindgut at E8.5 and E10.5 in the neural tube and hindgut. *Tcf1* was found in all three germ layers at both E8.5 and E10.5. *Lef* was detected in the presomitic mesoderm in both stages examined and was absent from the primitive gut.

and hindgut. During this stage, *Shh* expression is detected throughout the newly formed endodermal tube and clearly distinguishes the hindgut from the notochord at the posterior end (Figure 6G–I). In *Tcf4*^{-/-}/*Tcf1*^{-/-} embryos, only notochord staining is observed, indicating a complete lack of the hindgut. As a result of the loss of underlying gut, the notochord appears exposed at the tail end of these embryos (Figure 6H, see arrowhead). *Shh* expression in *Lef*^{-/-}/*Tcf1*^{-/-} embryos was maintained in the hindgut (Figure 6I, see arrow), implying once again that the observed effects in *Tcf4*^{-/-}/*Tcf1*^{-/-} are specific. To confirm these data, we prepared haematoxylin/eosin sections of wild-type, *Tcf4*^{-/-}/*Tcf1*^{-/-}, as well as *Lef*^{-/-}/*Tcf1*^{-/-} embryos. Sections through the midgut region (just posterior to the stomach) of *Tcf4*^{-/-}/*Tcf1*^{-/-} embryos revealed that the endodermal tube had not closed (Figure 6K). More caudal sections showed ectopic neural tissue and the absence of any hindgut (Figure 6N). Equivalent regions in *Lef*^{-/-}/*Tcf1*^{-/-} embryos demonstrated that the gut tube had formed despite the loss of somites and formation of multiple neural tubes (Figure 6L and O). Altogether, these results implied that specification and expansion of the hindgut is aberrant in *Tcf4*^{-/-}/*Tcf1*^{-/-} embryos.

Anterior transformation in the gastro-intestinal tract of *Tcf4*^{-/-}/*Tcf1*^{-/-} embryos

Given the specific defects in the formation of caudal endoderm in *Tcf4*^{-/-}/*Tcf1*^{-/-} embryos, we were also interested in examining the later patterning events of the gastro-intestinal tract. As expected, serial transverse sections of E14.5 embryos showed that the intestine of *Tcf4*^{-/-}/*Tcf1*^{-/-} mutants was severely truncated and ended blindly (Supplementary Figure 1, bottom panels m–o). A closer

inspection of mutant embryos revealed profound patterning defects in the stomach/duodenum region. Normally, the stomach is restricted by the oesophageal junction at the proximal end and by the duodenal junction at the distal end (Supplementary Figure 1, top panels a–e). While the boundary between the oesophagus and stomach was normal in *Tcf4*^{-/-}/*Tcf1*^{-/-} embryos, the duodenal opening was not clearly defined. In fact, serial sections suggested that the duodenum was dilated, giving the impression that the stomach had duplicated at the distal end (Supplementary Figure 1, bottom panels). To better visualize this, we dissected out the stomach and intestine from E14.5 embryos in order to verify the expression of the epithelial stomach marker *Sox2* (Ishii *et al*, 1998). In *Tcf4*^{+/-}/*Tcf1*^{-/-} littermates, *Sox2* expression was restricted to the stomach, with no transcripts detected beyond the duodenal boundary. In *Tcf4*^{-/-}/*Tcf1*^{-/-} embryos, *Sox2* expression was present throughout the stomach and truncated intestinal tract. These results implied that the *Tcf4*^{-/-}/*Tcf1*^{-/-} gastro-intestinal tract was indeed anteriorized. To confirm this, we performed *in situ* hybridizations on consecutive sections of normal and mutant stomach/duodenum preparations in order to verify the expression of the intestinal marker *Cdx2*, alongside *Sox2*. As depicted in Figure 7, *Sox2* and *Cdx2* are confined to the stomach and duodenum, respectively, in normal littermates (see figure legends for details). In mutant embryos, comparable sections showed that the duodenum only partially expressed *Cdx2*. Moreover, regions of the duodenum devoid of *Cdx2* (see black arrowheads, comparing top and bottom sets of panels i–vi) abundantly expressed *Sox2*. Thus, the duodenum was apparently transformed into the stomach, effectively leaving *Tcf4*^{-/-}/*Tcf1*^{-/-} embryos with little or no intestine.

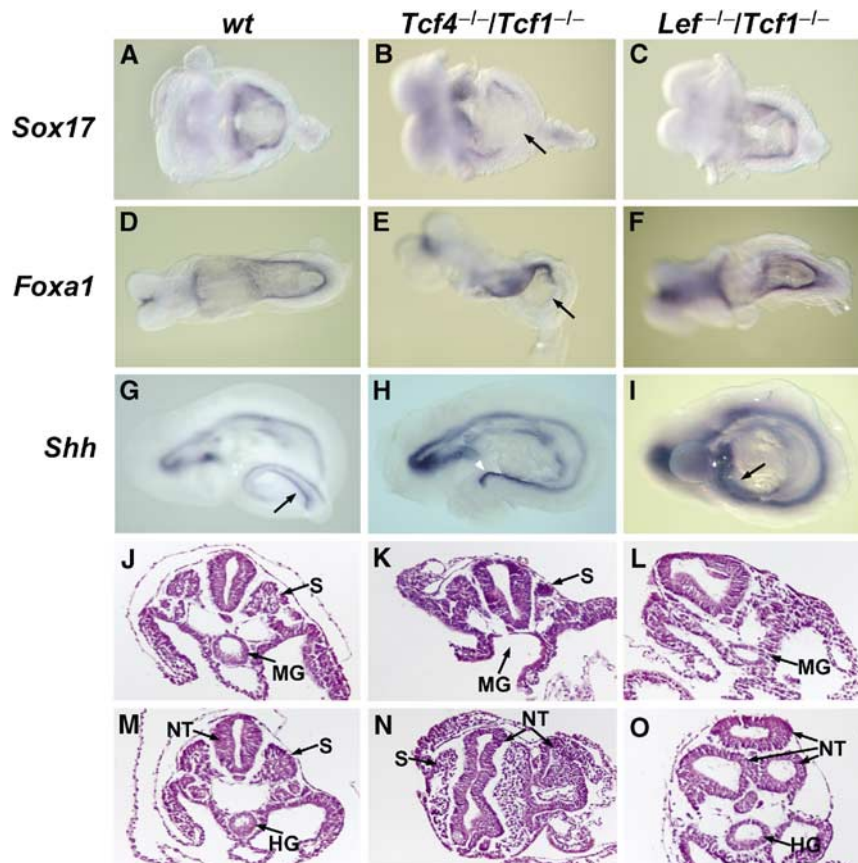


Figure 6 Impaired expansion of caudal endoderm in *Tcf4*^{-/-}/*Tcf1*^{-/-} embryos. Whole-mount *in situ* hybridizations of E8.5–9.5 littermates using endodermal markers and histology of E9.5 normal and mutant embryos. (A, D, G, J, M) Wild-type embryos, (B, E, H, K, N) *Tcf4*^{-/-}/*Tcf1*^{-/-} embryos and (C, F, I, L, O) *Lef*^{-/-}/*Tcf1*^{-/-} embryos. The arrows in (B) and (E) point to the loss of *Sox17* and *Foxa1* staining in the caudal endoderm of *Tcf4*^{-/-}/*Tcf1*^{-/-}, while staining is normal in *Lef*^{-/-}/*Tcf1*^{-/-} embryos. Arrows in (G) and (I) point to *Shh* expression in the hindgut of wild-type and *Lef*^{-/-}/*Tcf1*^{-/-} embryos. The notochord in *Tcf4*^{-/-}/*Tcf1*^{-/-} embryos appears exposed due to the absence of an underlying gut tube (arrowhead in (H)). Panels J–L and M–O represent haematoxylin and eosin stainings of sections through the midgut region and hindgut, respectively. Various structures are identified as follows: (S) somites, (NT) neural tube, (MG) midgut and (HG) hindgut. Note that in panel K *Tcf4*^{-/-}/*Tcf1*^{-/-} embryos maintain somites but the primitive gut tube has not closed. Similar sections in *Lef*^{-/-}/*Tcf1*^{-/-} embryos show a properly formed gut tube and the absence of somites (panel L). The caudal sections (M–O) also reveal the presence of ectopic neural tissue in both *Tcf4*^{-/-}/*Tcf1*^{-/-} and *Lef*^{-/-}/*Tcf1*^{-/-} embryos.

Discussion

In this report, we show that deletion of *Tcf1* and *Tcf4* leads to an absence of caudal structures in the mouse embryo. These embryos displayed duplications of the neural tube and, unlike other null mutants affecting Wnt signalling, *Tcf4*^{-/-}/*Tcf1*^{-/-} embryos retain paraxial mesoderm. By examining the expression of endodermal markers, we found an early defect in the development of the hindgut and later patterning anomalies, consistent with homeotic transformations of the gastro-intestinal tract. The specific defects in caudal endoderm and later axial truncations are reminiscent of recent experiments with chick embryo explants, where surgical removal of the caudal endoderm leads to blunted tail development (de Santa and Roberts, 2002). Likewise, the impaired gut development in *Sox17*^{-/-} embryos is accompanied by disorganized posterior trunk development, which can partially be rescued in chimaera (Kanai-Azuma *et al*, 2002; Tam *et al*, 2003). Together, these findings suggest that posterior endoderm may produce signals required for proper development not only of the hindgut but also adjacent structures. Therefore, in *Tcf4*^{-/-}/*Tcf1*^{-/-} embryos, the basis for the

severe posterior truncations may be the result of arrested gut development. In the light of this, the disorganized aspect of the paraxial mesoderm in some embryos may be attributed to the hindgut defects. Further analysis will be required to test this hypothesis.

Tcf/Lef genes regulate distinct patterning events

The comparison between *Tcf4*^{-/-}/*Tcf1*^{-/-} and *Lef*^{-/-}/*Tcf1*^{-/-} embryos has allowed us to uncover distinct roles for *Tcf/Lef* family members during A–P patterning. Previous work and our own analysis indicate that *Lef* and *Tcf1* are necessary for early specification of paraxial mesodermal *Lef*^{-/-}/*Tcf1*^{-/-} embryos phenocopy null mutations in *Wnt3a* and also the putative target gene *Tbx6* (Chapman and Papaioannou, 1998). The primary defect in these mutants appears to result from the formation of ectopic neural tissue at the expense of paraxial mesoderm. One model that has emerged to explain this phenomenon suggests that Wnt signals (namely Wnt3a-LEF/TCF1) instruct epiblast cells to adopt a mesodermal fate. When this signal is disrupted, involuting cells from the primitive streak fail to differentiate into the mesoderm and, possibly by default, adopt a neuronal differentiation pro-

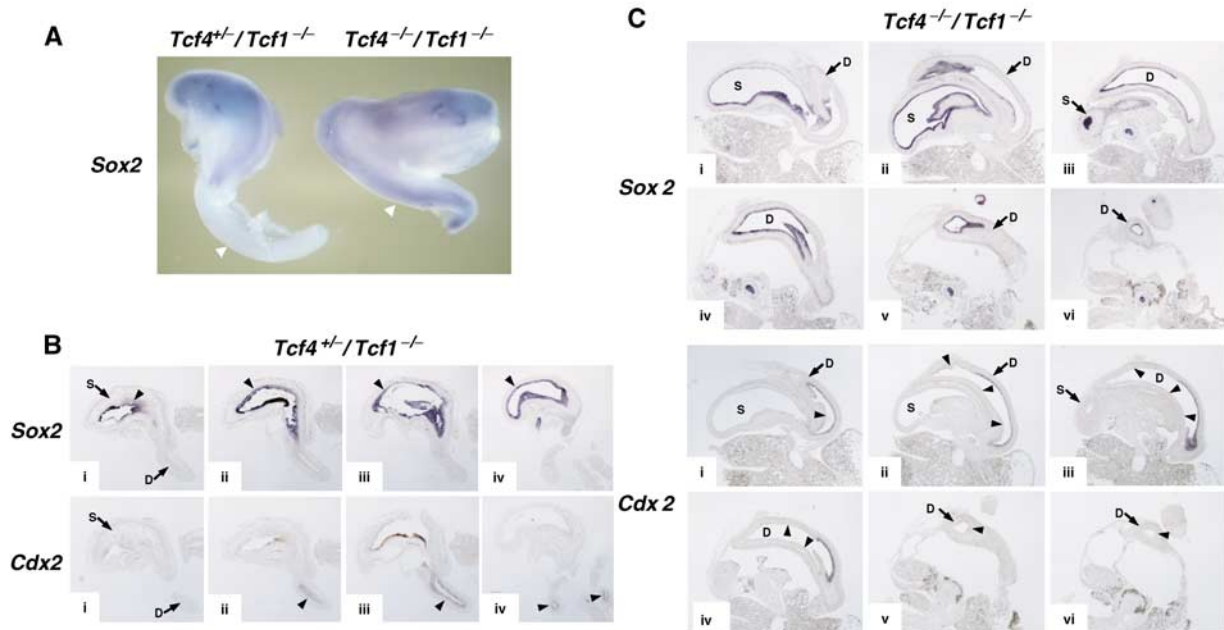


Figure 7 Anteriorization of the gastro-intestinal tract in *Tcf4*^{-/-}/*Tcf1*^{-/-} embryos. **(A)** Whole-mount *in situ* hybridizations of dissected anterior gastro-intestinal tracts of E14.5 *Tcf4*^{+/+}/*Tcf1*^{-/-} (left) and *Tcf4*^{-/-}/*Tcf1*^{-/-} (right) embryos using a stomach marker, *Sox2*. The gastro-intestinal tract of the normal embryo was severed to remove the intestinal tube. The truncated gastro-intestinal tract of *Tcf4*^{-/-}/*Tcf1*^{-/-} embryos is shown in its entirety. As shown by the arrowheads, *Sox2* expression is confined to the stomach in normal littermates, while ectopic expression in the duodenum is apparent in mutants. **(B)** *In situ* hybridizations on consecutive sections (i–iv) of a single E13.5 *Tcf4*^{+/+}/*Tcf1*^{-/-} gastro-duodenal preparation. The top and bottom rows of panels were stained for *Sox2* (stomach) and *Cdx2* (intestine), respectively. The top and bottom sections i–iv represent equivalent regions and should be compared with each other. The arrowheads indicate regions of positive staining for both probes. **(C)** *In situ* hybridizations on consecutive sections (i–vi) of a single E13.5 *Tcf4*^{-/-}/*Tcf1*^{-/-} gastro-duodenal preparation. The top and bottom sets of panels were stained for *Sox2* and *Cdx2*, respectively. Sections i–vi represent equivalent regions and should be compared with each other. Various structures are identified as follows: (S) stomach, (D) duodenum. The arrowheads in panels stained for *Cdx2* represent regions of the duodenum that are devoid of *Cdx2* expression but show high expression of *Sox2*. Note how the duodenum in mutants appears dilated compared to normal littermates (B). In addition, *Cdx2* transcripts are confined to a small portion of the duodenum, and the remaining tissue expresses *Sox2*. Altogether, these data provide evidence for the occurrence of an anterior transformation in the gastro-intestinal tract of *Tcf4*^{-/-}/*Tcf1*^{-/-} embryos.

gramme, ultimately contributing to the formation of ectopic neural tissue where normally somites are formed.

In *Tcf4*^{-/-}/*Tcf1*^{-/-} embryos, expression of posterior paraxial mesoderm markers is maintained, suggesting that *Tcf4* is not required for specifying this tissue type. Rather, *Tcf4* in concert with *Tcf1* is necessary for the development of caudal endoderm. By analogy to the above model, our results could also imply that during germ layer formation *Tcf4* and *Tcf1* regulate the formation and/or expansion of caudal endoderm at the expense of neural tissue. Thus, the neural tube duplications in these embryos may result from unspecified epiblast cells forming ectopic neural tubes, as described in *Wnt3a*^{-/-} and *Lef*^{-/-}/*Tcf1*^{-/-} mutants. Alternatively, the duplications in *Tcf4*^{-/-}/*Tcf1*^{-/-} embryos may reflect later intrinsic patterning defects caused by loss of *Tcf4* and *Tcf1* expression in the neural tube.

Wnt signals, therefore, pattern specific caudal structures through the differential usage or tissue distribution of *Tcf4*, *Tcf1* and *Lef*. The absence of any defect in either paraxial mesoderm or caudal endoderm development in the single knockouts, however, argues that TCF/LEF factors are functionally redundant at the biochemical level. In other words, LEF/TCF1 in the paraxial mesoderm and TCF4/TCF1 in the caudal endoderm most likely regulate overlapping sets of target genes. However, these results do not exclude the possibility that within other cell types, coexpressed TCF/LEF factors control unique genetic programmes. In favour

of this notion, promoter studies have demonstrated that unique domains present within individual TCF/LEF proteins allow for the regulation of specific target genes (Atcha *et al*, 2003; Hecht and Stemmler, 2003). Moreover, forced expression of mutant version of *Tcf3* and *Lef* in different model systems, such as *Xenopus* embryos and skin stem cells, results in distinct developmental outcomes (Merrill *et al*, 2001; Roel *et al*, 2002). Thus, it appears that the exact contribution of individual TCF/LEF factors in response to Wnt signals depends on the cellular and developmental context.

Both early and late stages of gastro-intestinal development are controlled by Wnt signalling

The specific defects in the fetal gut development of *Tcf4*^{-/-}/*Tcf1*^{-/-} embryos are consistent with our previously described single *Tcf4* knockout (Korinek *et al*, 1998a). The phenotype of these mice first becomes evident during villus formation in the small intestine at around E16.5. At this time point, proliferating epithelial cells normally detected in the intervillus regions are lost and as a result *Tcf4*^{-/-} mice die perinatally. Given the importance of *Tcf4* in maintaining epithelial stem cells in the small intestine, it is intriguing to speculate that *Tcf4* may play an equivalent role in the primitive gut. A recent study in the chick provides additional evidence that *Tcf/Lef* factors are essential for gut development (Theodosiou and Tabin, 2003). However, in this model,

expression profile analysis and loss-of-function studies predict a restricted role for *Tcf4* and *Lef* in the development of the gizzard, the duodenum and ceca.

The transformations of the gastro-intestinal tract highlight an unexpected role for *Tcf4* and *Tcf1* in later patterning of the gut tube. From E9.5 to E14.5, once the endodermal tube has formed, specialized structures such as lungs, stomach, small intestine, colon, etc., develop along the A–P axis. During this patterning phase, both *Tcf4* and *Tcf1* are expressed in foregut derivatives such as the stomach (data not shown). *Tcf4* is strongly expressed in the stomach epithelium. *Tcf1* expression appears much less abundant and transiently, with early expression in the stomach mesenchyme and later in the epithelium. As removal of *Tcf4* and *Tcf1* leads to ectopic stomach tissue, this may reflect a role for both factors in restricting growth of the stomach, while at the same time promoting the growth of more caudal endodermal structures (i.e. the midgut and hindgut).

Besides regulating the expansion and patterning of the gastro-intestinal tract, a number of other functional studies implicate canonical Wnt signalling in other aspects of gut development. For example, the initial formation of definitive endoderm is severely impaired in conditional β -catenin mutant embryos, and as a result these embryos develop ectopic cardiac tissue (Lickert *et al*, 2002). As a self-renewing tissue, the adult intestine also requires active Wnt signalling to maintain proliferation of epithelial progenitors. This was confirmed recently by two independent studies, which showed that forced expression of the Wnt antagonist *Dkk1* blocks proliferation in the intestinal mucosa (Pinto *et al*, 2003; Kuhnert *et al*, 2004). On the other hand, several lines of evidence have shown that overstimulation of the Wnt pathway, through activating mutations in components such as β -catenin and *APC*, promotes tumorigenesis (Bienz and Clevers, 2000; Polakis, 2000). Altogether, therefore, these data imply that Wnt signalling is absolutely required for the formation, growth, patterning and homeostasis of the gut endoderm.

In conclusion, the results presented here establish a novel role for Wnt signalling in early gut morphogenesis and underscore the importance of the *Tcf/Lef* genes in driving early patterning events during mouse development. Given the abundance of Wnts (19 described genes) and the probability that many have overlapping functions, future analysis of various compound *Tcf/Lef* null mutants should provide valuable models to dissect the role of canonical Wnt signalling in many other developmental processes.

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Materials and methods

Mice

Tcf4^{-/-}, *Tcf1*^{-/-} and *Lef*^{-/-} mice were described elsewhere (van Genderen *et al*, 1994; Verbeek *et al*, 1995; Korinek *et al*, 1998a). Genotyping of all embryos was performed by PCR of genomic DNA isolated from extraembryonic tissue. Detailed protocols (including primer sequences) for genotyping will be provided upon request.

Histology and in situ hybridization

Embryos were dissected in PBS, fixed overnight in 4% paraformaldehyde and kept for long-term storage in 100% methanol. For whole-mount *in situ* hybridization, embryos were rehydrated, digested in proteinase K, post-fixed and hybridized overnight at 70°C with various probes in 5 × SSC (pH 4.5), 50% formamide, 2% blocking powder (Roche), 5 mM EDTA, 50 µg/ml yeast tRNA, 0.1% Tween 20, 0.5% CHAPS and 50 µg/ml heparin. Embryos were washed as follows: 2 × SSC for 4 × 10 min at 70°C, 2 × SSC/0.1% CHAPS for 2 × 30 min at 70°C, 100 µg/ml RNase A/2 × SSC/0.1% CHAPS for 30 min at 37°C and 100 mM maleic acid/150 mM NaCl for 2 × 30 min at 70°C. Embryos were washed in TBST, and blocked for 2 h in TBST containing 0.5% blocking powder and 1% sheep serum. Next, embryos were incubated in blocking solution overnight at 4°C with preabsorbed alkaline phosphatase-conjugated anti-digoxigenin (1/2000 dilution) (Roche). Embryos were washed several times in TBST and colour reaction was performed with BM Purple AP substrate (Roche). For *Wnt1* stainings, embryos were dehydrated, embedded in paraffin, sectioned and counterstained with 1.0% Neutral Red. For *in situ* hybridization on sections, embryos were embedded in paraffin, sectioned at 10 µm and processed for hybridization as above, with the following modifications. Both CHAPS and Tween 20 were omitted from the hybridization buffer and post-hybridization washes were performed as follows: 2 × SSC for 5 min, 2 × SSC/50% formamide for 3 × 30 min at 65°C. Sections for histology studies were stained with haematoxylin and eosin.

Probes

The following probes were used for *in situ* hybridization studies: *Pax1* (IMAGE clone #1327502), *T* (Stott *et al*, 1993), *Wnt5a* (Gavin *et al*, 1990), *Cdx1* (Meyer and Gruss, 1993), *Lef* (Oosterwegel *et al*, 1993), *Foxa1* (Kaestner *et al*, 1994), *Sox17* (Kanai *et al*, 1996), *Sox2* (IMAGE clone #5707193), *Tcf1* (IMAGE clone #4016305) and *Tcf4* (IMAGE clone #4952976).

Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

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