Tcf3: a transcriptional regulator of axis induction in the early embryo

Bradley J. Merrill¹, H. Amalia Pasolli¹, Lisa Polak¹, Michael Rendl¹, Maria J. García-García², Kathryn V. Anderson² and Elaine Fuchs¹,*

¹Howard Hughes Medical Institute, Laboratory of Mammalian Cell Biology and Development, The Rockefeller University, New York, NY 10021, USA
²Department of Developmental Biology, Sloan Kettering Memorial Institute, New York, NY 10021, USA
*Author for correspondence (e-mail: fuchs1b@rockefeller.edu)

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Summary

The roles of Lef/Tcf proteins in determining cell fate characteristics have been described in many contexts during vertebrate embryogenesis, organ and tissue homeostasis, and cancer formation. Although much of the accumulated work on these proteins involves their ability to transactivate target genes when stimulated by β-catenin, Lef/Tcf proteins can repress target genes in the absence of stabilized β-catenin. By ablating Tcf3 function, we have uncovered an important requirement for a repressor function of Lef/Tcf proteins during early mouse development. Tcf3°/° embryos proceed through gastrulation to form mesoderm, but they develop expanded and often duplicated axial mesoderm structures, including nodes and notochords. These duplications are preceded by ectopic expression of Foxa2, an axial mesoderm gene involved in node specification, with a concomitant reduction in Lefty2, a marker for lateral mesoderm. By contrast, expression of a β-catenin-dependent, Lef/Tcf reporter (TOPGal), is not ectopically activated but is faithfully maintained in the primitive streak. Taken together, these data reveal a unique requirement for Tcf3 repressor function in restricting induction of the anterior-posterior axis.

Key words: Wnt, Gastrulation, Tcf3, Node, Axis

Introduction

Wnt signaling guides cell fate decisions in many physiological contexts, and the molecular nature of its signal transduction provides the Wnt pathway with the ability to have varied effects on cells. Although the stability of β-catenin is central to the activation of Wnt signaling target genes, Lef/Tcf proteins also play pivotal roles, tailoring the transcriptional output to suit particular cellular contexts. An intensive subject of investigation has been to elucidate how Lef/Tcfs interact with stabilized β-catenin to either modify Tcf factors and/or stimulate the recruitment of core transcriptional machinery to activate target gene transcription (Bienz and Clevers, 2003; Chan and Struhl, 2002). Irrespective of mechanism, demonstrated targets of Lef/Tcf-β-catenin activation regulate diverse processes such as tumor formation (Korinek et al., 1998b; Travis et al., 1991; van de Wetering et al., 1991). Although a double knockout of Lef1 and Tcf1 indicates that some contexts allow certain Tcfs to share a degree of functional redundancy (Galceran et al., 1999), different Lef/Tcf family members do not always behave similarly when expressed in the same cell type. Indeed, in mouse skin Lef1 appears to function with β-catenin to activate genes involved in hair cell differentiation (Gat et al., 1998; van Genderen et al., 1994; Zhou et al., 1995), but when transgenically expressed in the same cells, Tcf3 appears to act as a repressor to specify an alternative cell fate (Merrill et al., 2001).

In the developing mouse embryo, anteroposterior (AP) axis formation initiates during gastrulation beginning at embryonic day 6.5 (E6.5), when ectodermal cells acquire different fates: at the posterior embryonic/extra-embryonic border (EEX), an epithelial-mesenchymal transition occurs to form the mesoderm germ layer at the primitive streak region. The primitive streak expands distally, and a special group of cells at the anterior primitive streak (APS) form the axial mesoderm, which gives rise to the embryonic organizer, i.e. node, which is both necessary and sufficient to induce the AP axis.
(Beddington, 1994). Analogous embryonic organizers in other animals are also necessary and sufficient to induce the primary embryo axis, either the AP axis (Hensen’s node in avians) or the DV axis (Spemann’s organizer in amphibians, the embryonic shield in fish) (Beddington, 1994; Harland and Gerhart, 1997; Hensen, 1876). Further patterning within the mesoderm yields other, non-axial populations (e.g. lateral and paraxial mesoderm), which produce somites and other mesodermal structures.

In mouse, Wnt3 is expressed at the appropriate time and location to promote primitive streak induction, and Wnt3+/– embryos fail to undergo gastrulation (Liu et al., 1999). Similarly, β-catenin-null embryos also fail to specify an AP axis (Huelsken et al., 2000). Ectopic activation of Wnt signaling in early mouse embryos either by mutations affecting Axin or Apc, negative regulators of β-catenin stability, or by transgenic expression of Wnt8c all lead to ectopic AP axis specification and formation of multiple nodes (Ishikawa et al., 2003; Popperl et al., 1997; Zeng et al., 1997). Thus, Wnt signaling appears to be both necessary and, in some circumstances, sufficient for AP axis specification and formation of the node in developing mouse embryos.

Despite the proven requirement for upstream members of the Wnt signaling pathway, the role of specific Lef/Tcf proteins in mouse AP axis and node formation has remained unclear. As judged by gene targeting, ablation of Tcf1, Tcf4 or Lef1 results in either viable or neonatal lethal pups, without consequence to early embryonic development. The Tcf1+/– Lef1+/– double knockout embryos form excess neural ectoderm at the expense of paraxial mesoderm, as well as multiple neural tubes in the tail (Galceran et al., 1999). Although the Tcf3 locus has not yet been targeted for mutation in any organism, in zebrafish morpholino knockdown of both Tcf3 homologs (Hdl and Tcf3b) results in postgastrulation defects in neural patterning and anterior neural truncations (Dorsky et al., 2003; Kim et al., 2000). All of these effects in the Tcf1+/– Lef1+/– mice and the hdl– tcf3b– zebrafish, however, occur after the induction of the primary embryonic axis and after the formation of the embryonic organizer has already occurred. Interestingly, the antisense RNA-mediated knockdown of maternal and zygotic Xenopus XTCf3 leads to a markedly different phenotype characterized by the expansion of organizer cell fates and dorsoanteriorization of embryos (Houston et al., 2002). However, a similar role for mammalian Lef/Tcf proteins in either organizer formation or axis induction has yet to be identified.

Taken together, the findings to date suggest that either (1) additional Lef/Tcf functional redundancy accounts for the role of Wnt signaling in axis and node specification, (2) mouse Lef/Tcf proteins do not play a role in this process as they appear to do in Xenopus or (3) Tcf3, the lone Lef/Tcf family member left to be targeted in mice, is the crucial Lef/Tcf member in this process. Interestingly, Tcf3 is expressed throughout the mouse embryo at E6.5, prior to primitive streak formation (Korinek et al., 1998b), which makes Tcf3 a prime candidate to be either a positive or a negative regulator of Wnt-mediated AP axis specification. We have now tested this hypothesis directly by generating a null mutation in the murine Tcf3 gene, and examining the consequences to Wnt signaling and early mouse embryogenesis. Our results reveal an essential and unique role for mouse Tcf3 in restricting AP axis induction during the onset of gastrulation. Similar to its Xenopus and zebrafish homologs, mouse Tcf3 appears to function by repressing target genes in the early embryo.

**Materials and methods**

**Generation and genotyping of Tcf3–/– mice**

Standard molecular biology techniques were used to construct the targeting vector (Fig. 2A). Briefly, a BAC clone containing the 5′ region of Tcf3 was isolated from the Genome System library Down to The Well system, and a 6kb Xbal fragment was subcloned into pBSK (Stratagene). The 3′ arm of the vector [a 3 kb XhoI (blunted)–SacII fragment] was inserted into the NorI (blunted)–SacII digested pGKneobpAloxX2 PGKDTA vector kindly provided by Dr Phil Soriano (Seattle). PCR was used to insert a BglII site between exon 1 and 2, into which annealed oligos containing a loxp site were inserted. This loxp containing fragment (1.7 kb PvuII-XhoI fragment) was used as the 5′ arm and it was ligated into HincII-SacII sites to complete the construction of the targeting vector. Fragments that were amplified by PCR were sequenced prior to usage.

The targeting vector was digested with NorI and electroporated in GS-1 ES cells (Sw/129 background; Genome Systems). Approximately 300 primary clones were screened by PCR, and Southern blotting was used to determine if faithful homologous recombination occurred in PCR-positive clones (Fig. 2B). A CMV-Cre recombinase expression plasmid was then transiently transfected into two separate ‘Tcf3+/neo’ clones, and 300 additional clones were isolated and genotypes for either loss of ‘neo’ only or loss of ‘neo’ and exon 2 to produce the Tcf3−/− cells. Primers used for genotyping described in Fig. 2C are: ‘rev’, 5′-tcgcaagaggattgctctcc-3′; and ‘for’, 5′-agctgcctgctgaagatcgg. Two independent clones Tcf3−/− ES clones were injected into recipient C57Bl6 blastocysts to produce male chimeras that were selected for mating with normal C57Bl6 females to obtain Tcf3−/− mice.

**Embryo in situ hybridization, immunofluorescence and histology**

The age of embryos was determined based on the time of day harvested assuming noon on the day of plug discovery corresponds to day 0.5. For E6.5–E7.5 embryos staging criteria described (Downs and Davies, 1993) were used to determine the stage of gastrulation of embryos.

In situ hybridization was performed essentially as described (Wilkinson and Nieto, 1993). Briefly, embryos were fixed overnight in 4% PFA at 4°C, dehydrated in a graded methanol series and stored at −20°C in 100% methanol until in situ hybridization was performed. Embryos were then rehydrated, bleached in methanol/H2O2 (4:1) for 1 hour, washed in PBT, treated with proteinase K (2-3 minutes E7.5, 4-5 minutes E8.5), post-fixed in 4% PFA/0.2% glutaraldehyde, and hybridized with digoxigenin-labeled cRNA probes. Hybridized cRNA probes were detected with sheep anti-DIG AP FAB antibody (Roche). After BCP/NBT (Roche) reaction to detect signal, embryos were dehydrated through a graded methanol series to develop the purple colored precipitate, rehydrated and cleared in 50% glycerol prior to imaging. Embryos were identified as Tcf3+/− or Tcf3−/− after imaging by digesting embryos in proteinase K and PCR as described above. Probes used in this study were specific for Hexx1 (P. Thomas), Enl (A. Joyner), Krox20 (Egr2 – Mouse Genome Informatics) (D. Wilkinson), Six3 (G. Oliver), Foxa2 (E. DeRobertis) and brachury (D. Wilkinson).

Immunofluorescent detection of proteins was performed on PFA-fixed, frozen sections as described previously (Merrill et al., 2001) with the following antibodies: guinea pig anti-Tcf3 (Merrill et al., 2001), mouse anti-β-catenin (Sigma) and rat anti-E-cadherin (M. Takeichi). Secondary antibodies, FITC or Texas Red-conjugated donkey antibodies (Jackson Labs) were used at 1:200 dilution.
Scanning electron microscopy (SEM)

SEM procedures were performed essentially as described (Sulik et al., 1994). Embryos were fixed overnight at 4°C in 2.5% glutaraldehyde and post-fixed in 2% osmium tetroxide for 2 hours. They were dehydrated in a graded series of ethanol. Crucial point drying and sputter coating with palladium/gold was used to preserve structures and highlight surface features. Embryos were mounted on metal stubs exposing the ventral/distal surface for optimal viewing of the node. Imaging was performed with a JEOL microscope.

Results

Activation of Wnt signaling and Tcf3 protein expression during gastrulation

Knockout mice lacking the gene(s) for Tcf4, Tcf1, Lef1 or Lef1 and Tcf1, all maintain the formation of the primitive streak and node, whereas ablation of either Wnt3 or β-catenin prevents the formation of the primitive streak (Galceran et al., 1999; Huelsken et al., 2000; Korinek et al., 1998a; Liu et al., 1999). The lack of defects in Lef/Tcf mutant gastrulae raises the possibility that Lef/Tcf-β-catenin complexes may not be active at these early stages of gestation and suggest the possibility that stabilized β-catenin may be exerting its effects independently of Lef/Tcfs. If Lef/Tcf proteins do play a role in mouse AP axis formation, then the activation or derepression of Lef/Tcf target genes would be expected to accompany Wnt expression.

To address this issue, we first assessed whether Tcf/Lef/β-catenin regulated target genes are activated in the early mouse embryo with the TOPGal transgenic reporter, which is responsive specifically to Tcf/Lef/β-catenin complexes (DasGupta and Fuchs, 1999). The first signs of TOPGal activity were seen at E6.5-E7.0, where X-Gal staining was detected beginning at the early streak (ES) stages of gastrulation [Fig. 1; for details of staging criteria for gastrulating embryos, see Downs and Davies (Downs and Davies, 1993)]. β-Galactosidase activity concentrated along the forming primitive streak (ps) region extending along both sides of the posterior EEX border (Fig. 1A) and was subsequently expanded distally in late streak (LS; Fig. 1B) and neural fold stages (Fig. 1C,D). These results were consistent with those previously reported for the BAT-Gal Wnt reporter transgene (Maretto et al., 2003) Sagittal sections of TOPGal stained neural fold stage embryos revealed activity in ectodermal and mesodermal cell types in the primitive streak region (Fig. 1E) and activity in the posterior half of the ventral node (Fig. 1E’).

Next, we assessed whether Tcf3, the only Tcf/Lef member not previously targeted in knockout mice, could be a candidate to affect primitive streak or node formation. Previous studies indicated that Tcf3 mRNA is expressed at E6.5 and then begins to decline in following days of gestation (Korinek et al., 1998b). To detect expression of Tcf3 protein at these stages, we conducted immunofluorescence microscopy on sectioned E6.5 to E7.5 embryos encapsulated by their decidua. As judged by a monospecific Tcf3 antibody (Merrill et al., 2001), Tcf3 expression preceded gastrulation (Fig. 1F). At this early stage, Tcf3 was restricted to the nuclei (purple) of the ectodermal cells of the developing embryo. Analyses of sagittal and serial transverse sections showed that Tcf3 was present in the embryonic ectoderm throughout the proximodistal length (data not shown).

As mesoderm formed at the primitive streak region, the pattern of Tcf3 expression changed dramatically (Fig. 1G-I). Tcf3 immunoreactivity was weak or absent in the primitive streak, while it became intense in the ectoderm and mesoderm anterior to the node. (Fig. 1G). By E7.5, Tcf3 was also detected in a portion of the anterior endoderm. Thus, whereas the E6.5 embryo displayed relatively uniform anti-Tcf3 staining, the E7.5 embryo displayed an anterior gradient of Tcf3 immunoreactivity, with the transition at or near the node (Fig. 1I). Overall, reduction of Tcf3 protein in the posterior of the E7.5 embryos preceded the activation of TOPGal. Intriguingly, the earliest activation of TOPGal at E6.5 preceded Tcf3 downregulation.

Targeted ablation of Tcf3 in ES cells and in mice

If Tcf3 functions prior to gastrulation, then it might be expected to act in concert with early Wnt signaling to affect the formation and patterning of the primitive streak. If, however, the function of Tcf3 does not occur until after the formation of the AP axis, then based on the retention of Tcf3 in the anterior of the gastrula, Tcf3 might be expected to act in patterning the anterior of the embryo, similar to the functions previously revealed for Tcf3b and Hdi (a truncated Tcf3 gene) in zebrafish development.

To distinguish between these possibilities, we created a null mutation in the Tcf3 gene in mice. The 64 bp exon 2 of the Tcf3 gene was targeted for excision, so that if the resulting Tcf3 mRNA lacking exon 2 were stable, it would contain a frameshift and an early termination codon. Such nonsense-mutation containing mRNAs are typically unstable and rarely translated into protein (Wilkinson and Shyu, 2002). Moreover, previous mice containing potential truncation mutations residing much further downstream in the Tcf1, Tcf4 and Lef1 genes have not caused dominant negative effects (Korinek et al., 1998a; van Genderen et al., 1994; Verbeek et al., 1995).

The targeting vector was designed to provide flexibility in engineering either embryonic stem (ES) cells lacking exon 2, or ES cells harboring a floxed exon 2 for conditional knockouts (Fig. 2A). For the present study, we focused on the straight knockout. Shown in Fig. 2B are representative examples of the Southern blot analyses of ES cell genomic DNA, revealing a 5.9 kb HinCII fragment diagnostic for the floxed PGK-Neo cassette between exons 2 and 3 of the Tcf3 gene, and a 3.3 kb BgIII fragment revealing the presence of the loxP site and new BgIII site inserted between exons 1 and 2 of the Tcf3 gene (+/neo). After transfection with CMV-Cre plasmid, the loss of the PGK-Neo cassette was confirmed by Southern blot (+/– in Fig. 2B), and PCR verified the loss of the 170 bp exon 2 fragment (Fig. 2C).

From 26 litters involving two Tcf3+/– heterozygote parents, 60 mice (36%) were Tcf3 +/– and 104 mice (64%) were Tcf3+/–, as judged by PCR analyses (data not shown). These animals appeared normal, indicating no dominant-negative or haploinsufficiency defects from the single Tcf3 allele alteration. However, between newborn and E11.5, no intact Tcf3+/– embryos were found, suggesting that the loss of Tcf3 resulted in early embryonic lethality.

The first intact Tcf3+/– embryos were recovered from three E9.5 litters (8+/–, 20+/– and 6+/–), although they were only 20–40% the size of wild-type littermates. Although a beating heart and somites were present in some E9.5 Tcf3+/– embryos,
anterior neural structures were conspicuously absent (not shown). At E8.5 and earlier, Tcf3–/– embryos were recovered at Mendelian ratios and no signs of excessive resorption were noted. PCR analyses confirmed the existence of the Tcf3 mutation and loss of the Tcf3 wild-type allele (Fig. 2C), and anti-Tcf3 immunoblot analysis confirmed the loss of Tcf3 protein in E8.5 Tcf3–/– embryos (Fig. 2D).

Morphological defects in Tcf3–/– embryos

Just after gastrulation at e8.5, Tcf3+/+ and +/- embryos (WT) had well-established AP axial structures, distinct neural-folds, somites and a heart (Fig. 3A,A’). The rostral ends of the neural-folds extend anteriorly past the developing heart, and forebrain, midbrain and hindbrain regions are identifiable (Fig. 3A’). All E8.5 Tcf3–/– embryos were unmistakably aberrant, although morphological abnormalities exhibited variable penetrance in a range from mild (48%; Fig. 3B-D) to severe (52%; Fig. 3E-F’). Most striking in the mildly affected Tcf3–/– embryos was the duplication of developing neural-folds (Fig. 3B,B’). Although the appearance of supernumerary neural-folds was most common in the anterior of Tcf3–/– embryos, their pattern and structure were variable. Histological staining of semithin transverse sections through the neural folds revealed that instead of a single neural groove as in wild-type embryos (Fig. 3G), multiple neural grooves and abundant neurectodermal cells were present in Tcf3–/– embryos (Fig. 3H). In mildly affected E8.5-E9.5 Tcf3–/– embryos, somites were often visible along the AP midline, and occasionally an extra row was observed (asterisks in Fig. 3C,C’). These data showed that Tcf3 is not required for the generation of paraxial mesoderm or its condensation into somites. Mildly affected embryos also frequently displayed an enlarged heart (Fig. 3B’), and all embryos displayed anterior truncations (Fig. 3B’,D compare with 3A’).

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Although supernumerary neural-folds were a hallmark of mildly affected E8.5 Tcf3+/– embryos, these structures were grossly runted or absent in severely affected embryos, which also failed to produce somites and heart (Fig. 3E,F). However, consistent with the presence of multiple neural folds in mildly affected mutants, multiple grooves (ng) along the ventral surface were evident in the severely affected mutants (Fig. 3E). These grooves extended along the anterior half of the embryo, which was frequently bent laterally. Distally, the severely affected embryos frequently exhibited atypically large areas
The earliest recognizable defects were detected in E7.5 Tcf3−/− embryos, where a frequent bulging and less frequent duplication of the primitive streak were detected (Fig. 3I). Histological examination of these morphological perturbations revealed an aberrant accumulation of mesoderm (Fig. 3I). Overall, these data were consistent with defects involving partial and sometimes complete duplications of AP axis structures, including neural grooves and primitive streaks. Other abnormalities, including enlarged cardiac sacs, multiple large blood vessels (v) and foregut defects (fg) seemed to be secondary consequences of partial AP axis duplication. We pursue this avenue in greater detail below.

Defective neural patterning in Tcf3−/− embryos

The forebrain defects in postgastrulation E8.5 Tcf3−/− embryos bore a strong resemblance to those seen upon ectopic activation of Wnt signaling (Mukhopadhyay et al., 2001; Popperl et al., 1997), and in hdl, tcf3b knock-down zebrafish embryos (Dorsky et al., 2003; Kim et al., 2000). To explore the extent to which these later stage defects might resemble artificial Wnt activation, we examined the expression of neural-specific genes in E8.5 embryos. Fig. 4 illustrates representative whole-mount in situ hybridization performed on groups of ~10 E8.5 embryos (6–9 Tcf3+/+ or +/− and 3–4 Tcf3−/−). Data for +/+ or +/− embryos were indistinguishable and are referred to as ‘wild-type’.

As expected, well-known forebrain-specific markers, such as Hesx1, Six3 and Bf1 mRNAs, were expressed in the anterior neurectoderm of WT E8.5 embryos (data shown for Hesx1 in Fig. 4A,A′). In mutant embryos, expression of these mRNAs was markedly reduced. Of the three markers examined, the only definitive expression of forebrain markers was for Hesx1, and this was weak and only detected in the rostral neurectoderm of the most mildly affected Tcf3−/− animals (Fig. 4B,B′).

Perturbations were also detected in midbrain and hindbrain gene expression. En1 is typically expressed in neurectoderm at the caudal end of the midbrain (Fig. 4C,C′). In mutant embryos, En1 was expanded rostrally, and its distance from the rostral tip of the embryo was shortened, consistent with the severe reduction of the forebrain (compare bar in Fig. 4C to bar in Fig. 4D). Frontal views revealed that neurectoderm retained En1 expression but did not develop a neural groove (Fig. 4D′) like wild-type embryos did (Fig. 4C′). For the hindbrain marker Krox20, wild-type embryos exhibited robust expression in rhombomeres 3 and 5 (Fig. 4E,E′), whereas Krox20 expression in knockout embryos was restricted to a single band on each neural fold, and was markedly reduced in intensity (Fig. 4F,F′). Additionally, rather than being confined to the neural groove, Krox20 was detected on the lateral surfaces of Tcf3 mutant neural-folds (Fig. 4F′). Thus, loss of Tcf3 not only resulted in a failure to specify the most rostral neurectoderm, but also more broadly caused abnormalities in caudal brain regions.

Multiple nodes and notochords in Tcf3−/− embryos

Defects in early patterning events that produced duplications and expansions of axial structures in Tcf3-null mouse embryos had not been observed in the morpholino knockdowns of Tcf3 gene expression in zebrafish. By contrast, gross expansion of Spemann’s organizer was observed in knockdown of XTcf3
from *Xenopus* embryos, although distinct duplications of either the organizer or the primary embryo axis in X*Tcf3*−/− embryos were not reported (Houston et al., 2002). Given the importance of the node and notochord in postgastrulation patterning of axial structures, the appearance of expanded or duplicated nodes and notochords in *Tcf3*−/− embryos could explain many of the observed morphological defects. Therefore, we next addressed whether the node and notochord were duplicated and/or expanded in *Tcf3*−/− embryos.

Towards the end of gastrulation (E7.5), node and notochord are readily visible by scanning electron microscopy (SEM) as an indented club-like structure on the embryo surface (Fig. 5A). In the normal embryo, the ventrally located node is most obvious, and is composed of small, rounded, monociliated progenitor cells (Fig. 5A,A′; green arrow). Arising anterior of the node, the derivative cells of the notochord are similar in appearance, forming a structure that is two to three cells wide (double blue arrows in Fig. 5A). Both the node and the notochord are readily distinguished from the surrounding, more superficial endodermal cells, which are flatter and larger in appearance.

The organization of node and notochord cells within severely affected E8.0 *Tcf3*−/− embryos was strikingly abnormal. Two distinct nodes (green arrows) were separated by a thin strip of endodermal cells (red arrow) at the posterior end of the structure (Fig. 5B–B″). These seemingly duplicated nodes merged rostrally, forming an expanded notochord-like structure along the ventral surface of the embryo (blue arrows in Fig. 5B). As the notochord-like structures extended rostrally (below the blue arrows in the example shown), they branched dorsolaterally into numerous streaks of what appeared to be notochord strands along the ventral surface of the mutant (boxed region in Fig. 5B, at higher magnification in B′). In some areas, the width of notochord-like structures was larger than that of the node of wild-type embryos.
To assess whether these node-like and notochord-like structures exhibited molecular characteristics of organizer cell populations, we examined a pair of marker genes, brachyury and Foxa2, which are important for organizer function at this stage. In wild-type embryos, brachyury is expressed in the primitive streak and then concentrates in the node and notochord (ncd) (Fig. 5C,C'). In Tcf3−/− embryos, brachyury was often grossly expanded (Fig. 5D,D'). These patterns varied, but were a direct reflection of the degree of expanded/duplicated node and notochord cells in a given Tcf3−/− embryo. In the most severely affected embryos, brachyury expression nearly covered the entire ventral surface. Similar findings were obtained with Foxa2. In wild-type embryos, Foxa2 is expressed in the node and notochord as well as in the neural floor plate when dorsoventral polarity is established in the neural tube (Fig. 5E,E'). In mutant embryos, Foxa2 expression was considerably broader and variable, but always reflective of the morphological expansions and duplications/multiplications of node and notochord (Fig. 5F,F').

The use of Foxa2 and brachyury as markers in E8.5 embryos provided graphic illustrations of the various different axis defects observed in our Tcf3−/− embryos. The most striking were cases where the axis duplications appeared to occur on opposite sides of the embryo (Fig. 5G,G').

**Mesodermal patterning defects in Tcf3−/− gastrulae**

To begin to elucidate the causes of the AP axis duplications and expansions of axial mesendoderm in Tcf3−/− embryos, we examined molecular events known to be important for proper AP axis induction and subsequent patterning of the primitive streak mesoderm. The extra-embryonic organizer, anterior visceral endoderm (AVE), is required early to pattern the gastrulating epiblast for AP axis formation (for a review, see Lu et al., 2001). Expressed in the AVE, the Nodal inhibitors Cer1 and Lefty1 (Lefta – Mouse Genome Informatics) are crucial to this process, as judged by the AP axis duplications caused by expanded Nodal signaling in Cer1−/− Lefty1−/− embryos (Perea-Gomez et al., 2002).

To determine whether loss of Tcf3 interferes with the ability of the AVE to restrict Nodal activity, we examined the expression of endodermal markers in Tcf3−/− gastrulae. Cer1 expression was detected in the AVE of all wild-type and Tcf3−/− embryos examined, although it was expanded in the posterior endoderm of the mutant embryos (Fig. 6A–B'). An additional AVE marker, the homeobox gene Hex, was also largely unaffected in its expression (Fig. 6C,D). The intact expression of AVE markers indicated that the axis duplications are not caused by loss of Nodal antagonists in the AVE.

As the epiblast cells flow into the primitive streak region and form nascent mesoderm, the mesoderm is patterned to follow specific developmental fates. Axial mesoderm, which develops into node and notochord organizers, is specified early compared with the paraxial and lateral mesoderm (Kinder et al., 1999; Lawson et al., 1991). To assess whether the loss of Tcf3 affects axial mesodermal patterning in early gastrulation, we examined the expression of a series of marker genes that specify early mesodermal populations. Brachyury is one of the first mesodermal markers that is normally expressed along the midline of the primitive streak region (Fig. 6E). In Tcf3−/− embryos, brachyury was maintained in the primitive streak region. Brachyury staining either revealed a crooked or bent path in Tcf3−/− embryos (Fig. 6F,F') (57% of mutants) or staining intensity was slightly diminished (28% of mutants; not shown). Normal Cripto (Cfc1 – Mouse Genome Informatics) expression was detected in all wild-type and knockout embryos examined.
Overall, the pattern of these markers indicated that the loss of Tcf3 did not result in gross defects in general mesoderm formation.

In E7.0 embryos, Foxa2 is normally expressed in the axial mesoderm that forms the APS, where it is required for node formation (Ang and Rossant, 1994; Weinstein et al., 1994) (Fig. 6I). In 75% of the Tcf3−/− embryos examined, Foxa2 expression was altered in variable patterns (Fig. 6J,J¢). In half of the Tcf3−/− embryos, Foxa2 expression was clearly expanded but still localized at its normal position at the distal tip of the primitive streak (Fig. 6J). In 25% of Tcf3−/− embryos, signs of ectopic expression were visible such as shown in Fig. 6J¢, where strong hybridization was seen along the perimeter of the extra-embryonic/embryonic border.

Complementary to the axial mesoderm of the anterior primitive streak, the lateral mesoderm is marked by expression of Lefty2 (Leftfb – Mouse Genome Informatics) (Meno et al., 1999) (Fig. 6K). Expression of Lefty2 was diminished in most (78%) Tcf3−/− embryos (Fig. 6L,L¢). Concomitant with the reduction in Lefty2 was a corresponding expansion of the region where axial mesoderm normally develops (area below the red line in Fig. 6K-L¢). Together, these findings suggested that the loss of Tcf3 results

Fig. 5. Duplications and expansions of node and notochord in Tcf3−/− embryos. (A–B”) Scanning electron microscopy (SEM) images of the ventral surface of wild-type (WT; A,A¢) and Tcf3−/− (KO; B–B”) embryos (anterior towards the lower left-hand corner). Arrows: Green, nodes; blue, notochords; red, endodermal strip. White line indicates notochord-endoderm boundary in B and B’. White boxes in A and B outline areas magnified in A’ and B’, respectively. Note the club-like structure of the single node and notochord in the wild-type embryo and the duplicated node and expanded notochord in mutant embryo.

(C–F’) Whole-mount in situ hybridizations of E8.5 wild-type and knockout embryos, probed with digoxigenin-labeled cRNAs for brachyury or Foxa2 as indicated. Anterior is towards the top of each image. The three embryos in each panel in D and F show different representative aberrant patterns, reflective of the extent of node/notochord multiplication. Opposing arrowheads indicate thickness of notochords (ncd); arrows indicate splitting of the notochord, often seen in mutant embryos. (G,G’) Tcf3−/− embryo with a rostral extension probed for brachyury expression. Anterior is leftwards for both images. The primary primitive streak (1°PS) and a secondary primitive streak (2°PS) are positive for brachyury expression. Emerging from the secondary primitive streak are structures similar to a node (arrows) and notochord (arrowheads).
Tcf3 and axis induction in expansion of axial mesoderm at the expense of other mesodermal cell types.

TOPGal activity retains its expression pattern during gastrulation in Tcf3−/− embryos

Based primarily on the VegT-dependent upregulation of organizer genes in XTCf3-depleted embryos, a function of XTcf3 in Xenopus gastrulae is to repress target genes activated by non-β-catenin dependent mechanisms (Houston et al., 2002). Consistent with this model in Xenopus, the removal of Tcf-binding sites from the Siamois promoter elevates its activity in ventral blastomeres of embryos (Brannon et al., 1997). These data from Xenopus combined with similarities between Tcf3−/−, Axin−/−, ApcNeoNeo and Wnt8c transgenic phenotypes together suggest that murine Tcf3 functions to repress target genes during induction of the AP axis (Ishikawa et al., 2003; Popperl et al., 1997; Zeng et al., 1997).

To evaluate whether Tcf3 mediates its non-redundant effects on early gastrulation through repression or activation of Wnt target genes, we mated the TOPGal transgenic mice on the Tcf3−/− background, and examined TOPGAL expression in gastrulating embryos. If Tcf3 functions as a non-redundant activator of Wnt target genes, TOPGal activity should be absent from the Tcf3−/− gastrula; if Tcf3 functions as a non-redundant repressor that normally counteracts other Lef/Tcf factors in Wnt-receiving cells, then this might be reflected in ectopic TOPGal expression. If the non-redundant role of Tcf3 is to suppress genes that require other factors for their transactivation, then TOPGal should be expressed only in the axial mesodermal cells and their progeny, which are normally TOPGal positive (see Fig. 1).

The results of this experiment are compiled in Fig. 7. TOPGal expression was clearly maintained in the Tcf3−/− gastrula even at the early stages of primitive streak formation.
Fig. 7. TOPGal expression patterns maintained in Tcf3\(^{-/-}\) embryos. TOPGal transgenic, wild-type (WT) or Tcf3\(^{-/-}\) (KO) embryos of the indicated stages of development were stained with X-gal to identify the location of activation of Lef/Tcf-\(\beta\)-catenin target genes. (A) Lateral view of a wild-type late streak embryo is weakly positive for TOPGal activity in the primitive streak region. (B,C) Tcf3\(^{-/-}\) embryos display TOPGal positive staining in the primitive streak region. The intensity of staining is indistinguishable from wild type. (D,D\') Headfold staged Tcf3\(^{-/-}\) embryos display a TOPGal expression pattern indistinguishable from wild-type embryos (see Fig. 1C,D) and include positive TOPGal expression in the node. (E) Tail of a wild-type E8.5 embryo. TOPGal is present in the posterior node and the mesenchymal cells near the node. (F) Tcf3\(^{-/-}\) E8.5 embryo. TOPGal expression is found in the tail of the embryo. (F\') Dorsal view shows a duplication of the node in this embryo. TOPGal is maintained in the same pattern as wild type in the node and the surrounding mesenchyme, despite the duplication.

(KO; Fig. 7B,C). Analogous to the pattern seen in wild-type embryos, X-gal staining was in the posterior of the embryo, at the EEX border. TOPGal expression was also detected in the primitive streak and node of embryos that had formed neural-folds (Fig. 7D,D\'). Interestingly, even in embryos with a clearly duplicated node, TOPGal expression was still faithfully maintained in the correct, but now expanded, cell population (Fig. 7E-F\'). As we failed to detect suppression, elevation and ectopic activation of TOPGal, these data best fit a model whereby the non-redundant role of Tcf3 is as a repressor of genes that are not activated by Wnt signaling alone.

Discussion

Despite the conclusion that both murine and zebrafish Tcf3 act as transcriptional repressors, loss of murine Tcf3 results in early gastrulation defects that are different from the postgastrulation headless phenotype caused by knockdown of zebrafish hdl and tcf3b. The mildly affected mouse mutants displayed anterior neural truncations and other neural patterning defects similar to the zebrafish knockdown experiments; however, the zebrafish experiments did not reveal a function for hdl or tcf3b in the formation of the AP axis or patterning of primitive streak mesoderm. Possible explanations for the differences include: (1) a maternal store of hdl or tcf3b which was not affected by morpholino-mediated knockdown; (2) the presence of an additional Tcf, such as maternally expressed Tcf4, in the early zebrafish embryo; or (3) an additional non-Tcf related mechanism by which zebrafish restricts AP axis formation.

Studies with XTcf3 knockdowns revealed greater similarities to our mouse knockout. Knockdown of XTcf3 causes an induction of dorsal blastomere-specific genes by a VegT-dependent mechanism (Houston et al., 2002). Many of the dorsal-specific genes upregulated by loss of XTcf3 are involved in formation of the Spemann organizer. In mouse Tcf3\(^{-/-}\) embryos, we also find expansions of node cells and the upregulation of Foxa2, a Nodal target gene required for specification of node cell types. However, Tcf3\(^{-/-}\) mouse embryos displayed distinct duplications of the AP axis not seen in the Xenopus knockdown embryos.

Curiously, no other Lef/Tcf gene has been directly implicated in AP axis induction despite the requirement of Wnt3 and \(\beta\)-catenin for this process (Huelsken et al., 2000; Liu et al., 1999). In mouse, Lef1\(^{-/-}\), Tcf1\(^{-/-}\) and Lef1\(^{-/-}\) Tcf1\(^{-/-}\) embryos are all competent at inducing an AP axis, and Tcf4 is not expressed in pregastrula embryos (Galceran et al., 1999; Korinek et al., 1998a; Korinek et al., 1998b). Our studies with TOPGal embryos provide evidence that Wnt3 functions through Lef/Tcf-\(\beta\)-catenin activation of target genes during AP axis induction. Furthermore, the induction of the AP axis coupled with the persistence of TOPGal expression in Tcf3 knockout embryos reveals a previously unrealized level of functional redundancy between Lef/Tcf factors in transducing this signal.

The putative redundant function of Tcf3 in promoting AP axis induction in Lef1\(^{-/-}\) Tcf1\(^{-/-}\) embryos becomes particularly interesting when one considers its opposing, non-redundant function in early embryogenesis. The fact that TOPGal activity in Tcf3 mutant embryos is both maintained in the expanded organizer cells and not ectopically expressed in other cells offers important insights into how Tcf3 must be exerting its non-redundant effects. Together, these findings indicate that Tcf3 acts alone in restricting the activation of target genes that are positively regulated by other transcription factors. The simplest model consistent with these data is that the primary function of Tcf3 is essential for repressing the expression of genes that promote AP axis induction and formation of axial mesoderm.

Given the requirement for Nodal signaling during AP axis
induction (Conlon et al., 1994) and axial mesoderm patterning (Hoodless et al., 2001; Vincent et al., 2003; Yamamoto et al., 2001) and the VgT-XTcf3 interaction elucidated in Xenopus organizer formation (Houston et al., 2002), it is attractive to speculate that Tcf3 might be repressing transcriptional targets of Nodal signaling. In support of this model, ectopic primitive streak formation in chick embryos requires the combined action of both Wnts and a Nodal-like molecule, Vg1 (Skromme and Stern, 2001). As the transcriptional activation of Foxa2 is directly downstream of Nodal signaling (Hoodless et al., 2001; Yamamoto et al., 2001), the ectopic expression of Foxa2 in the gastrulating Tcf3/–/- embryo lends further support to this view. We also noted that a Foxa2 promoter element harboring multiple, conserved Lef1/Tcf-binding sites directs transgenic expression in axial mesoderm and node (Sasaki and Hogan, 1996).

The phenotype of Tcf3 mutant embryos is similar to that reported for the loss of Axin or Apc, two other inhibitors of Wnt signaling (Ishikawa et al., 2003; Popperl et al., 1997; Zeng et al., 1997) and ectopic expression of Wnt8c (Popperl et al., 1997). Previously, these effects were attributed to the ectopic expression of target genes normally regulated by β-catenin/Tcf transactivating complexes. However, the lack of ectopic TOPGal activity in Tcf3/–/- embryos suggests that β-catenin/Tcf transactivating complexes are not required for the axis duplication phenotypes. An alternative mechanism consistent with our data is that Tcf3 functions as a transcriptional repressor in the absence of β-catenin, and that ectopic stabilization of β-catenin results in relief of Tcf3-mediated repression at sites that are not reflected here by TOPGal activity. Precedence for a role for β-catenin in derepression of Tcf genes has been provided in lower eukaryotic systems (Cavallo et al., 1998).

In conclusion, the potent effects of loss of the repressor function of Tcf3 illustrate the requirement for the coalescence of transcriptional activators and repressors to properly define the temporal and spatial pattern of expression of AP axis inducing gene products. Although in vertebrates, the temporal and spatial pattern of expression of AP axis function of Tcf3 illustrate the requirement for the coalescence (Cavallo et al., 1998).

References


Liu, P., Wakiyama, M., Shea, M. J., Albrecht, U., Behringer, R. R. and


