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The Wnt signaling mediator tcf1 is required for expression of *foxd3* during *Xenopus* gastrulation

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ABSTRACT TCF1 belongs to the family of LEF1/TCF transcription factors that regulate gene expression downstream of Wnt/β-catenin signaling, which is crucial for embryonic development and is involved in adult stem cell regulation and tumor growth. In early *Xenopus* embryos, tcf1 plays an important role in mesoderm induction and patterning. *Foxd3* emerged as a potential tcf1 target gene in a microarray analysis of gastrula stage embryos. Because foxd3 and tcf1 are coexpressed during gastrulation, we investigated whether *foxd3* is regulated by tcf1. By using morpholino-mediated knockdown, we show that during gastrulation *foxd3* expression is dependent on tcf1. By chromatin immunoprecipitation, we also demonstrate direct interaction of β-catenin/tcf complexes with the *foxd3* gene locus. Hence, our results indicate that tcf1 acts as an essential activator of *foxd3*, which is critical for dorsal mesoderm formation in early embryos.

KEY WORDS: *foxd3, lef1/tcf, tcf1, Xenopus, Wnt*

Introduction

LEF1/TCF transcription factors perform essential functions in developing embryos and in the maintenance of stem cell compartments in adult tissues (Kléber and Sommer, 2004; Logan and Nusse, 2004). Most vertebrates have four *LEF/TCF* genes: *TCF1 (TCF7)*, *TCF3 (TCF7L1)*, *TCF4 (TCF7L2)* and *LEF1*. Alternative splicing and/or promoter use in these genes can generate a variety of isoforms, which are involved in differential tissue-specific and stage-specific Wnt responses (Arce *et al.*, 2006). All these transcription factors bind DNA via their common, highly conserved HMG box and mediate Wnt signaling in the nucleus by recruiting β-catenin and co-activators to Wnt response elements in the target genes. In the absence of Wnt signaling, LEF/TCF factors can also be bound by groucho factors, mainly to repress target gene transcription (Brantjes *et al.*, 2001). The *Xenopus* embryo is one of the best understood vertebrate model systems for investigating the role of Wnt signaling *via* lef1/tcf factors in dorsoventral axis determination (reviewed in (Hoppler and Kavanagh, 2007)). The early *Xenopus* embryo expresses three maternally inherited lef1/tcf family members: tcf1, tcf3 and tcf4 (Houston *et al.*, 2002; Molenaar *et al.*, 1996; Roël *et al.*, 2003). Zygotic expression of these genes and of *lef1* increases at the start of gastrulation (König *et al.*, 2000; Molenaar *et al.*, 1998; Roël *et al.*, 2003, 2009).

In early *Xenopus* embryogenesis, mesodermal cell fate is induced at the equator of the embryo (also called the marginal zone). During subsequent gastrulation movements, the mesoderm is further specified and moves into the embryo, whereby the ventrolateral zone of the pre-gastrulating embryo will give rise to the somite forming paraxial and ventral mesoderm, and the dorsal organizer zone will form the head mesoderm and notochord.

Abbreviations used in this paper: ChIP: chromatin immunoprecipitation; Dex: dexamethasone; MO: morpholino.

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At the time of gastrulation, tcf1 mRNA is present at high levels in the animal cap and in most of the marginal zone (Roël *et al.*, 2003). Maternal loss-of-function experiments have shown that tcf1 functions as an activator of dorsal genes, but can also repress ectopic expression of these genes on the ventrolateral side of the embryo (Standley *et al.*, 2006). Furthermore, zygotic knockdown experiments revealed that tcf1 plays a role in mesoderm induction and, along with lef1, in subsequent patterning of the ventrolateral mesoderm (Liu *et al.*, 2005). These studies have shown that in the *Xenopus* embryo, tcf1 acts cooperatively with β-catenin to activate target genes that have essential functions in the formation of the dorsal embryonic axis and in mesoderm development.

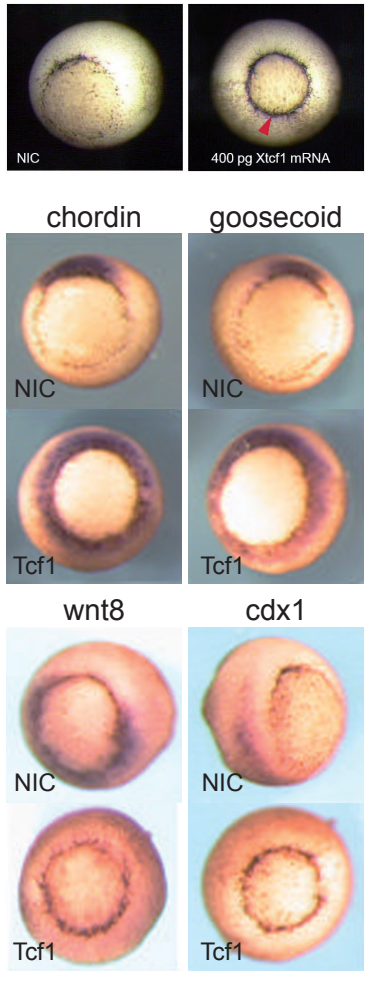
We previously investigated the role of tcf1 as a downstream regulator of Wnt signaling in early gastrula stages by performing microarray analysis to identify genes differentially regulated upon tcf1 overexpression (van den Broek and Destrée, personal observations). Many genes expressed on the dorsal side of the embryo were found to be upregulated by overexpression of tcf1, including known direct β-catenin/TCF target genes such as *siamois* (*sia1*), *xnr3*, and *dkk1* (Brannon *et al.*, 1997; Chamorro *et al.*, 2005; McKendry *et al.*, 1997). Most of the downregulated genes are known to be expressed in the ventrolateral zone of the embryo, such as *cdx1* (*Xcad*) and *wnt8* (Christian *et al.*, 1991; Pillemer *et al.*, 1998). One of the identified upregulated genes was *foxd3*, which is an essential regulator of dorsal mesoderm development (Steiner *et al.*, 2006) and at later stages has a role in the formation of neural crest cells (Kos *et al.*, 2001; Lister *et al.*, 2006; Pohl and Knöchel, 2001; Sasai *et al.*, 2001). Furthermore, *Foxd3* has also been shown to be involved in regulating pluripotency in mouse embryonic stem cells (Liu and Labosky, 2008).

Because *foxd3* expression overlaps with that of *tcf1* at the dorsal blastopore lip during *Xenopus* gastrulation, we investigated whether tcf1/β-catenin complexes regulate *foxd3* expression during mesoderm formation. By using morpholino injections, chromatin immunoprecipitation and other methods, we demonstrated that during gastrulation, endogenous tcf1 is necessary and sufficient for activating the expression of *foxd3* and confirmed that this dorsal mesoderm regulating transcription factor is a Wnt/β-catenin target gene.

Results

Overexpression of tcf1 dorsalizes Xenopus *embryos and induces* foxd3

To determine the function of tcf1 in early gastrulation, *Xenopus tropicalis* embryos were injected with synthetic Xtcf1 mRNA at the two-cell stage. When non-injected embryos had reached stage 10.5, these tcf1-overexpressing embryos displayed premature mesodermal involution around the entire marginal zone, consistent with a dorsalized phenotype (Fig. 1A). To confirm the dorsalized phenotype of the embryos overexpressing tcf1, we analyzed the expression patterns of the dorsal organizer genes *chordin* (Sasai *et al.*, 1994) and *goosecoid* (Cho *et al.*, 1991) and of the ventrolateral markers *wnt8* (Christian *et al.*, 1991) and *cdx1* (Pillemer *et al.*, 1998) by *in situ* hybridization (Fig. 1B). Upon tcf1 overexpression, *chordin* and *goosecoid* were expressed all around the premature circular blastopore and expression of *wnt8* and *cdx1* became undetectable, indicating an expansion of organizer tissue at the expense



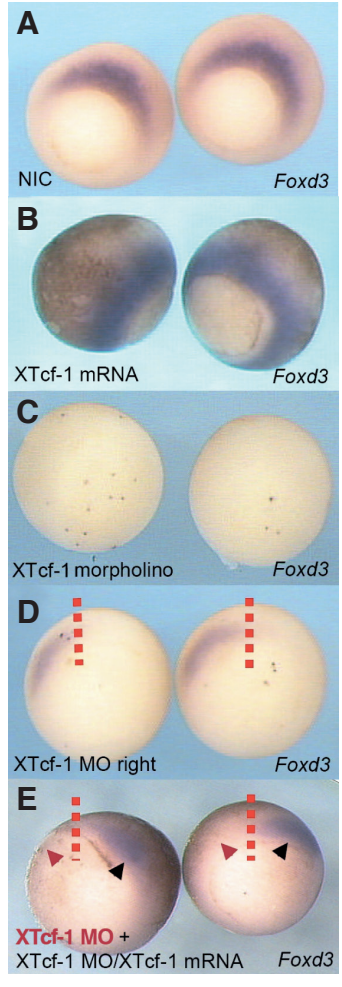


Fig. 1 (left). Overexpression of tcf1 induces a dorsalized phenotype in *Xenopus* embryos. (A) Xenopus tropicalis *embryos overexpressing tcf1 display premature mesodermal involution. Stage 10.5 embryos, marginally injected in both blastomeres at the two-cell stage with 180 pg of tcf1 RNA, show premature mesodermal involution around the entire marginal zone (81%, n=62; red arrowhead, ventral).* **(B)** *Overexpression of tcf1 induces dorsalization. Expression of* chordin *(83%, n=58) and* goosecoid *(83%, n=59) has expanded all around the blastophore in* Xenopus laevis *embryos injected with tcf1 RNA, while expression of* wnt8 *(73%, n=64) and* cdx1 *(89%, n=55) is lost.*

Fig. 2 (right). Tcf1 regulates the expression of *foxd3* in the early mesoderm. (A) *Endogenous expression of* foxd3 *at the dorsal blastoporus lip.* **(B)** *Injection of tcf1 RNA in each blastomere (total of 400 pg) in two-cell stage* Xenopus laevis *embryos leads to the expression of* foxd3 *all around the blastopore (75%, n=61).* **(C)** *Injection of 10 ng of tcf1 morpholino (MO) in both blastomeres at the two-cell stage abrogates* foxd3 *expression in* Xenopus laevis *(50%, n=86).* **(D)** *Unilateral injection of tcf1 MO (right blastomere) at the two-cell stage inhibits* foxd3 *expression only on the injected side (51%, n=84).* **(E)** *Coinjection of Xtltcf1 RNA (right blastomere) with tcf1 MO (both blastomeres) rescues* foxd3 *expression (right blastomere) in* Xenopus laevis*; left side is indicated by a red arrowhead, right side is indicated by a black arrowhead. (NIC, non-injected stage 10.5 control embryo).*

of ventrolateral tissue. These results show that overexpression of tcf1 during blastula and early gastrula stages dorsalizes *Xenopus* embryos, which is in line with the previously reported effects of *tcf1* overexpression in oocytes (Standley *et al.*, 2006).

One other gene affected by tcf1 overexpression was *foxd3* (*XFD-6*). This transcription factor is implicated in mesoderm development and subsequent embryonic axis formation as well as in later neural crest development (Kos *et al.*, 2001; Lister *et al.*, 2006; Pohl and Knöchel, 2001; Sasai *et al.*, 2001; Steiner *et al.*, 2006). Steiner *et al.,* showed that foxd3 is required as a transcriptional repressor at the *Xenopus* Spemann organizer to maintain expression of nodal-related genes essential for dorsal mesoderm formation (Steiner *et al.*, 2006). However, *foxd3* has not yet been identified as a downstream target of tcf1.

Tcf1 is necessary and sufficient for regulating foxd3 *expression*

To confirm that *foxd3* is a target of tcf1, we manipulated *tcf1* expression in *Xenopus laevis* embryos and studied the effects on *foxd3* expression by *in situ* hybridization. At stage 10.5, endogenous expression of *foxd3* was highest in the dorsal lip (Fig. 2A), as reported before (Pohl and Knöchel, 2001; Steiner *et al.*, 2006). Overexpression of tcf1 by injection of 400 pg of synthetic tcf1 mRNA at the two-cell stage resulted in ectopic expansion of *foxd3* expression all around the circular blastopore (Fig. 2B).

Conversely, inhibition of *tcf1* expression by injection of a translation blocking morpholino (MO) in both blastomeres at the two-cell stage abolished *foxd3* expression (Fig. 2C), indicating that early *foxd3* expression is dependent on endogenous tcf1. Furthermore, unilateral inhibition of *tcf1* expression resulted in loss of *foxd3* only on the injected side of the embryo (Fig. 2D), demonstrating that induction of *foxd3* by tcf1 is likely cell-autonomous and that secreted inducing factors are not involved in the response to tcf1. The effect of tcf1 morpholino (MO) could be rescued by co-injection of modified, MO-unsusceptible XtlTcf1 mRNA (Liu *et al.*, 2005) (Fig. 2E, 44 of 80 affected embryos rescued), showing that the loss of *foxd3* is specifically related to the inhibition of *tcf1* expression. These experiments collectively reveal that transcription of *foxd3* in the earliest phases of its zygotic expression is dependent on tcf1 at the dorsal lip of the blastopore.

Foxd3 *is controlled by Wnt/β-catenin signaling during gastrulation*

In early *Xenopus* embryos, *tcf1* is strongly expressed in almost the entire marginal zone, except for a narrow region around the blastopore (Roël *et al.*, 2003) and this pattern overlaps with the presence of nuclear β-catenin during gastrulation (Schohl and Fagotto,

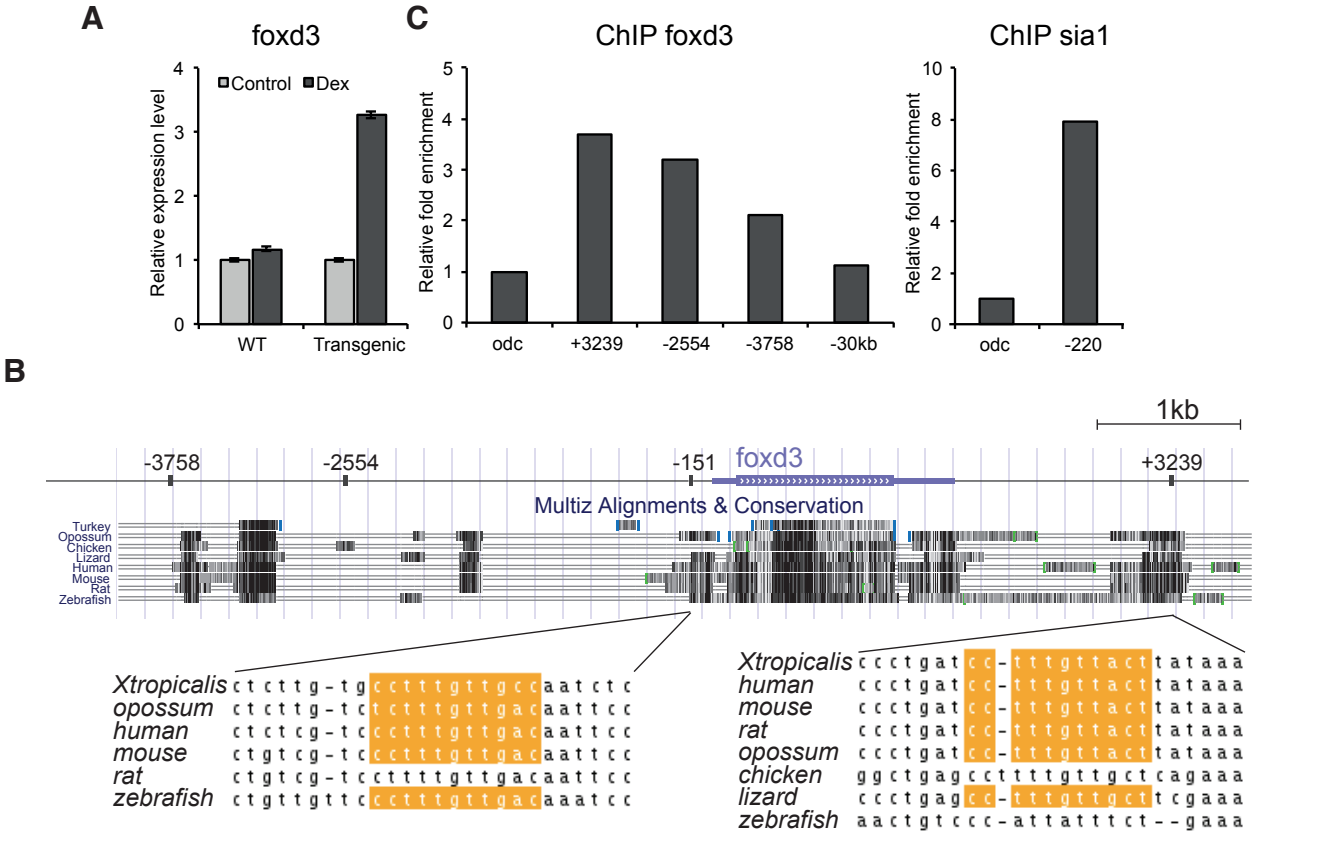


Fig. 3. Quantitative RT-PCR and chromatin immunoprecipitation (ChIP) indicate that *foxd3* is a direct target of Wnt/β-catenin signaling during gastrulation. (A) Foxd3 *is induced upon artificial Wnt activation during gastrulation.* Xenopus tropicalis *wild type embryos (WT) and embryos transgenic for a Dexamethasone (Dex) inducible Wnt activating transgene were subjected to Dex or control treatment for 2.5 h during gastrulation.* **(B)** *Schematic overview of LEF/TCF consensus sites at the* X. tropicalis foxd3 *gene locus. Evolutionary conservation around the position -151 and +3239 relative to the transcription initiation site is shown.* **(C)** *ChIP assay on WT* X. tropicalis *gastrulating embryos detects binding of βcatenin/lef/tcf complexes to the* foxd3 *locus.* Sia1 *was included as a positive control and a region in the* odc *gene served as a negative control. Data are shown as fold enrichment relative to the negative control.*

2002). Consequently, we tested whether *foxd3* is directly regulated by endogenous Wnt/β-catenin signaling during gastrulation.

First, we investigated if *foxd3* expression is under the control of Wnt/β-catenin signaling during mesoderm formation. We used transgenic *X. tropicalis* embryos that contain an optimized hormone-inducible system allowing activation of the Wnt pathway at the transcriptional endpoint (Denayer *et al.*, 2008). The activator part of this transgenic system consists of a chimeric fusion of the LEF/TCF DNA binding HMG box of murine LEF1, fused to the transactivation domain of the *Herpes simplex* virus VP16 gene. To make this transgene inducible, the hormone binding domain of the human glucocorticoid receptor was added, resulting in a dexamethasone (Dex) inducible system for activating LEF/TCF target genes.

Transgenic and wild type embryos were obtained by natural mating and induced during gastrulation by the addition of 10 μM Dex to the cultivation buffer for 2.5 h. Afterwards, embryos were lysed, RNA was extracted and quantitative RT-PCR was performed to analyze *foxd3* expression. As Fig. 3A shows, when artificial Wnt/β-catenin was activated, *foxd3* mRNA was upregulated three-fold.

To investigate whether regulation of *foxd3* by tcf1 is a direct effect of the binding of β-catenin/tcf1 to *foxd3* regulatory sequences, we first performed an *in silico* screening of the *foxd3* locus of *X. tropicalis* for the presence of potential tcf1 binding sites. This uncovered five regions in the *foxd3* locus, at positions +3239, -151, -2554 and -3758 and one site more upstream of the *foxd3* transcription start site, at -30 kb (Fig. 3B).

To determine whether tcf1 is associated with chromatin at these conserved TCF binding sites in the *foxd3* locus, we wanted to perform chromatin immunoprecipitation (ChIP). Unfortunately, antibodies recognizing *Xenopus* tcf1 usable for ChIP assays are missing. However, β-catenin is the nuclear interaction partner of LEF1/TCF factors necessary for transmitting Wnt/β-catenin signaling. Hence, we performed ChIP assays on cross-linked chromatin of gastrula stage *X. tropicalis* embryos with a polyclonal antibody against β-catenin. A β-catenin/ChIP assay for the known Wnt target gene *siamois* (*sia1)* was included as a positive control (Brannon *et al.*, 1997). Immunoprecipitated DNA was then used for qPCR with primers that flank the putative lef1/tcf binding sites. To correct for the background signal, we normalized the data against DNA immunoprecipitated with rabbit IgG. Several independent experiments revealed enrichment in the *foxd3* locus at positions +3239, -2554 and -3758. The region located farther upstream at 30 kb was not immunoprecipitated (Fig. 3C). Unfortunately, the predicted lef1/tcf binding site at position -151 is located in a highly conserved region that is resistant to PCR amplification, as reported before by Nelms *et al.,* (Nelms and Labosky, 2011). Consequently, the binding of β-catenin/tcf complexes to this site could not be evaluated. Hence, we could confirm binding of tcf/β-catenin complexes at least at three lef/tcf binding sites surrounding the *foxd3* locus.

Discussion

Tcf1 is required for expression of foxd3

We found that tcf1 overexpression is correlated with the induction of dorsal mesoderm and with the ectopic expression of foxd3 during *Xenopus* gastrulation. Previous tcf1 loss-of-function experiments have documented its role in the induction of mesodermal markers (Liu *et al.*, 2005), and Pohl *et al.,* reported that *foxd3* can be ectopically activated on the ventral side of the gastrulating embryo by experimentally activated Wnt signaling (Pohl and Knöchel, 2001). Consistent with the overlap of tcf1 expression (Pohl and Knöchel, 2001; Roël *et al.*, 2003; Steiner *et al.*, 2006) with the earliest zygotic expression of *foxd3* (*i.e.*, in the dorsal blastopore lip), we could confirm that *foxd3* is directly regulated by Wnt/β-catenin signaling in the dorsal mesoderm. Using β-catenin ChIP experiments, we identified three Wnt responsive lef1/tcf binding sequences at the *foxd3* gene locus. Of these, the +3239 site, as well as its surrounding sequences, shows high evolutionary conservation (Fig. 3B). The other two sites are positioned in regions of the *foxd3* promoter that show virtually no evolutionary conservation. Notice that the PCR-resistant -151 lef1/tcf site is also highly conserved (Fig. 3B). ChIP enrichment of the *foxd3* locus was not as strong as that of the *sia1* promoter, which served as a positive control. Nonetheless, we could confirm enrichment at the *foxd3* locus in every β-catenin ChIP experiment we performed. Furthermore, it is possible that β-catenin interacts predominantly at the PCR-resistant -151 site, or that the high GC content and consequent secondary structures that form at the *foxd3* promoter (Nelms and Labosky, 2011) can interfere with the ChIP assay.

There are no ChIP compatible antibodies described that cross-react with *Xenopus* lef1/tcf factors. Consequently, we could not identify whether endogenous tcf1, lef1, tcf3 or tcf4 binds to these sites. Nevertheless, tcf3 acts as a transcriptional repressor of organizer genes (Brannon *et al.*, 1999; Houston *et al.*, 2002), tcf4 transcripts are present at levels below the detection limit of *in situ* hybridization (König *et al.*, 2000; Standley *et al.*, 2006), and lef1 is primarily expressed at the ventrolateral marginal zone (Roël *et al.*, 2009). On the basis of these findings and on the enhancement of *foxd3* expression by tcf1 overexpression, we conclude that tcf1 is probably the lef1/tcf factor responsible for activation of *foxd3* in the Spemann organizer.

Tcf1 regulates foxd3 *in dorsal mesoderm formation*

Cooperative Wnt and FGF signaling is crucial for dorsal mesoderm development (Christian *et al.*, 1992; Keenan *et al.*, 2006). Also, foxd3, a transcriptional repressor required for dorsal mesoderm formation (Steiner *et al.*, 2006), is downregulated when FGF signaling is blocked during gastrulation (Branney *et al.*, 2009). These results, complemented with our data, indicate that regulation of the endogenous dorsal expression of *foxd3* relies on combined activation through Wnt and FGF dependent mechanisms. A recent model proposes that FGF signaling can stimulate Wnt-mediated mesodermal gene activation via MAPK phosphorylation of the groucho/TLE protein groucho-related 4 (*grg4*), relieving its repressive interaction with lef1/tcf proteins (Burks *et al.*, 2009). In addition, grg4 is co-expressed with tcf1 and enriched at the dorsal mesoderm during gastrulation (Molenaar *et al.*, 2000; Roël *et al.*, 2003). Based on this mechanism and the observation that *foxd3* can be positively regulated by FGF signaling during *Xenopus* gastrulation (Branney *et al.*, 2009), it is possible that *foxd3* expression is regulated through a balance between tcf1/β-catenin activating complexes and tcf1/groucho repressing complexes, the latter of which can be modulated by MAPK phosphorylation via FGF signaling.

Interestingly, foxd3 can bind to grg4. This recruitment of grg4 seems to enhance the transcriptional repressor function of foxd3, which is essential for dorsal mesoderm development. This sug-

TABLE 1

SEQUENCES OF PRIMERS USED FOR qRT-PCR AND ChIP

Gene	Forward primer (5' to 3')	Reverse primer (5' to 3')
foxd3 (cDNA)	GAGGACATGTTCGACAATGG	CAAAGCTTTGCATCATGAGAG
tubb (cDNA)	GACCCCACTGGCACCTATCA	TCGAGGGACATATTTACCACCTGT
odc (cDNA)	TTTGGTGCCACCCTTAAAACA	GCCACTGCCAACATGGAAAC
foxd3 (+3239)	CTGATCCTGTGCTTCTAAATGAC	TGCTATGTGCAGAAATATTCGG
foxd3 (-2554)	CCAACTGAAGGCTCCATCAAG	AAAGGGGAATCAAAGGTGTCCT
foxd3 (-3758)	TGGGAAGAGCACAAAGTGTGAT	TTCACAGCCCAGAGCGATTA
foxd3 (-30kb)	TGTGGAAGTCAAAGGAGAAAAAAA	GGAACAGCGTTCAATAGCTTGT
sia1 (-220)	AAGATCAAGGGAACCAGGTG	TTGCACCCTACAAACATGGG
odc genomic	GTGCACGCCTGAATTCTTTCT	GGCTCAGCAATGATGGTCACT

gests potential re-use of grg4 by foxd3 after its own activation through Wnt signaling.

In conclusion, our results show that *foxd3* is a direct target of tcf1 during *Xenopus* gastrulation and that Wnt/β-catenin mediated activation of *foxd3 via* tcf1 is essential for dorsal mesoderm induction and axis formation during early embryonic development.

Materials and Methods

In situ *hybridization*

Whole-Mount RNA *in situ* hybridization was performed as described in Molenaar *et al.,* (Molenaar *et al.*, 1998), except that hybridization was carried out at 65°C. Digoxygenin-labeled antisense probes were synthesized with the Ambion Maxiscript kit and digoxigenin-UTP from Roche. The digoxgenin-labelled antisense RNA probes used were *Xenopus* chordin (Sasai *et al.*, 1994), *Xenopus* goosecoid (Cho *et al.*, 1991), *Xenopus* wnt8 (Christian *et al.*, 1991), *Xenopus* cdx1 (Pillemer *et al.*, 1998) and *Xenopus* foxd3 (Dirksen and Jamrich, 1995). All experiments were repeated at least once.

Morpholino and RNA injections

Capped Xtcf1 (tcf1) mRNA was synthesized with the Ambion mMessage mMachine kit after *Xba*I linearization of the plasmid described in Liu *et al.,* (Liu *et al.*, 2005). *Xenopus laevis* embryos were injected with 400 pg of Xtcf1 mRNA in both cells at the two-cell stage in the marginal zone. *Xenopus tropicalis* embryos were injected with a dose of 180 pg of Xtcf1 mRNA. The tcf1 morpholino and modified tcf1 rescue construct have been described in Liu *et al.,* (Liu *et al.*, 2005). Morpholino was injected in *Xenopus laevis* at the two-cell stage into the marginal zone at 10 ng per blastomere. Embryos were fixed in MEMPFA (100 mM MOPS, 2 mM EGTA, 1 mM $MgSO_4$, 4% paraformaldehyde) and dehydrated in methanol.

Chromatin immunoprecipitation

Consensus lef1/tcf binding sites were determined and evaluated for conservation by using the UCSC Genome Browser (genome.ucsc.edu) (Kent *et al.*, 2002), the Match tool of TRANSFAC (Biobase) (Kel *et al.*, 2003) and the alignment tool Contra (Hooghe *et al.*, 2008). ChIP assays were performed according to the method described before (Blythe *et al.*, 2009) but with minor modifications: we used gastrula *X. tropicalis* embryos at stage 11 and cross-linking time was 45 min. After homogenization, embryos were sonicated with a Brandson cell disruptor to obtain DNA fragments smaller than 1000 bp. Immunoprecipitation was performed with rabbit anti-β-catenin polyclonal antiserum (a kind gift from Dr. Barry Gumbiner (McCrea *et al.*, 1993)). Purified normal rabbit IgG was purchased from Santa Cruz (SC-2027). Instead of sepharose beads, protein G Dynabeads (Invitrogen) were used.

Real-time quantitative RT-PCR analysis

Total RNA was isolated using the Aurum Total RNA fatty and fibrous tissue kit (Biorad). For each RNA sample, at least 20 embryos were pooled. cDNA was prepared with oligo (dT) and random hexamer primers using the iScript cDNA Synthesis Kit (Biorad) according to the manufacturer's instructions.

Real-time qPCR analysis was performed by using the SYBR green PCR master mix (Roche) on a LightCycler® 480 Real-Time PCR System (Roche). Primers for measuring gene expression levels (as well as for ChIP experiments) were designed by using Primer express 1.0 software (Perkin-Elmer applied Biosystems). The primer sequences are listed in Table 1. A "no-template control" was included for all primer pairs. When measuring gene expression, a "no amplification control" was included and all values were normalized to the level of the housekeeping genes ornithine decarboxylase (*odc*) and tubulin beta (*tubb*).

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