Lef/Tcf-dependent Wnt/β-catenin signaling during *Xenopus* axis specification

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Received 2 April 2003; revised 13 May 2003; accepted 14 May 2003

First published online 20 June 2003

Edited by Giulio Superti-Furga

Abstract Though the Wnt/β-catenin signaling pathway is known to play key roles during *Xenopus* axis specification, whether it signals exclusively through Lef/Tcf transcription factors in this process remains unclear. To investigate this issue, we generated transgenic frog embryos expressing green fluorescent protein (GFP) driven by a Lef/Tcf-dependent and Wnt/β-catenin-responsive promoter. This promoter is highly sensitive and even detects maternal β-catenin activity prior to the large-scale transcription of zygotic genes. Unexpectedly, *GFP* expression was observed only in some, but not all, known Wnt/β-catenin-positive territories in *Xenopus* early development. Furthermore, ubiquitous expression of dominant Lef-1 protein variants from transgenes revealed that zygotic Lef/Tcf activity is required for the ventroposterior development of *Xenopus* embryos. In summary, our results suggest that endogenous Wnt/β-catenin activity does not result in obligatory Lef/Tcf-dependent gene activation, and that the ventroposteriorizing activity of zygotic Wnt-8 signaling is mediated by Lef/Tcf proteins.

Key words: Axis specification; Wnt; β-Catenin; Lef-1; Tcf; Restriction enzyme-mediated integration transgenesis; *Xenopus*

1. Introduction

Wnt/β-catenin signaling (also known as canonical Wnt signaling) has been demonstrated to play key roles during vertebrate axis specification. In *Xenopus*, maternal β-catenin activity is detected on the future dorsal side of the embryo and induces the formation of the Spemann organizer [1,2]. Once formed at the beginning of gastrulation, the Spemann organizer secretes not only bone morphogenetic protein inhibitors to specify dorsal structures but also Wnt antagonists including Frzb, crescent, Dkk1, and Cerberus to block Wnt/β-catenin activity (presumably initiated by zygotic Wnt-8), which is essential for the formation of anterior structures [3]. Meanwhile, outside the territory of the organizer's activity, zygotic Wnt-8 specifies posterior structures [4]. Later on, Wnt/β-catenin functions again to maintain the midbrain–hindbrain boundary (MHB) that functions as an organizer during brain patterning [5,6].

According to the current model of the canonical Wnt signaling pathway, the binding of class I Wnt ligands to Frizzled receptors activates Dishevelled and it in turn stabilizes cytoplasmic β-catenin via inhibiting glycogen synthase kinase 3β (GSK-3β)-mediated phosphorylation. Consequently, stabilized β-catenin is translocated into nuclei and binds to the Lef/Tcf family of HMG box-containing transcription factors to modulate transcription [7]. To date, three members of this family – XTcf-3, XLef-1 and XTcf-4 – have been found in *Xenopus* with distinct, but partly overlapping, expression domains [8,9]. In addition, several recent reports suggest that DNA binding factors other than Lef/Tcf may mediate canonical Wnt signaling. For example, Pitx2 was found in the transcriptional activation complex with β-catenin on the cyclin D2 promoter in murine C2C12 myoblast cells upon stimulation with LiCl, which inhibits GSK-3β and thus mimics Wnt/β-catenin signaling [10]. Moreover, overexpression of *XSox17*, a member of the Sox family of HMG transcription factors, can inhibit secondary axis formation by β-catenin, presumably by sequestering it from Lef/Tcf proteins [11]. Additionally, Armadillo, the *Drosophila* homologue of vertebrate β-catenin, can interact with the product of the gene *teashirt*, a zinc finger-containing transcription factor, to modulate the responses to Wnt signaling in different regions of the fly larva [12]. These observations raise the possibility that Wnt/β-catenin may signal through transcription factors other than Lef/Tcf proteins to achieve cell-specific responses during *Xenopus* axis specification. Thus, the knowledge of when and where Lef/Tcf-dependent Wnt/β-catenin activity takes place becomes critical for deeper understanding of the mechanism of *Xenopus* axis specification.

In this study, we traced Lef/Tcf-dependent Wnt activity with p-LEF$_7$-fos-GFP, an artificial Lef/Tcf-dependent and Wnt/β-catenin-responsive reporter construct, in transgenic *Xenopus* embryos. We show that the transcription of the reporter gene occurs during dorsoventral and anteroposterior axis specification, as well as during brain patterning. However, in several other regions with canonical Wnt activity the reporter gene was not activated, suggesting that nuclear

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Abbreviations: GFP, green fluorescent protein; Luc, luciferase; MHB, midbrain–hindbrain boundary; GSK-3β, glycogen synthase kinase 3β; REMI, restriction enzyme-mediated integration; MBT, midblastula transition; NSE, non-specific expression; NE, no expression; PE, patched expression

0014-5793/03/$22.00
doi:10.1016/S0014-5793(03)00639-2

β-catenin activity does not constitutively modulate Lef/Tcf-regulated transcription. In agreement with the observation that Lef/Tcf-dependent transcription occurs in ventroposterior mesoderm, we provide evidence that zygotic inhibition of Lef/Tcf activity impairs posterior development, whereas its zygotic activation blocks head formation. Our results strongly suggest that ventroposterior-promoting Wnt signaling is mediated by Lef/Tcf transcription factors.

2. Materials and methods

2.1. Plasmids construction, DNA injection and luciferase assay

The p-fos-Luc reporter construct was generated by removing the seven Lef-1 binding sites from p-LEF$_7$-fos-Luc provided by Shrikesh Sachdev [13]. For transgenic reporter assays, the *Luciferase* gene in p-LEF$_7$-fos-Luc was replaced by a green fluorescent protein (*GFP*) cDNA [14].

Eggs were obtained from *Xenopus* females, cultured and staged as described previously [15,16]. The p-fos-Luc or p-LEF$_7$-fos-Luc reporter construct was injected into the dorsovegetal marginal zone of four-cell stage embryos. The injected embryos were harvested at stage 12.5 for luciferase assay as described [17]. The p-fos-GFP or p-LEF$_7$-fos-GFP reporter construct was injected radially into the marginal zone of four-cell stage embryos. The injected embryos were cultured to stage 11 and fixed in 1×MEMFA [18] for analysis by in situ hybridization.

2.2. Restriction enzyme-mediated integration (REMI) transgenesis

REMI transgenic embryos were generated as described previously [19]. Co-transgenesis was adopted in our study, in which two different constructs with the same quantity were introduced into the transgenic system. Dominant Lef-1 protein variants were derived from pCS2+LEFdeltaN-HA-VP16 (a gift from Rolf Kemmler) and pCS2+MT-LEF-EnR [14]. *GFP* expression derived from pCS2+GFP served as an indicator of successful insertion of the transgene. Embryos with fluorescence were sorted out at stage 11 and either fixed for analysis of gene expression by in situ hybridization or allowed to proceed to the tadpole stage (approx. stage 29) for morphological analysis. A control experiment was carried out with the plasmid pCS2+GFP alone. Plasmids used for transgenesis were linearized by *Not*I digestion, except pCS2+MTLEF-EnR, which was digested with *Kpn*I.

2.3. In situ hybridization and bleaching

In situ hybridization was performed as described [20]. Whole or bisected embryos were used in this assay. Digoxigenin-labelled antisense RNA probes used were *GFP* [14], *Xpo*, *Xnot* [21], and *XmyoD* [20]. The bleaching was done in methanol/H_2O_2 (37% stock solution) 2:1 in bright light or under UV.

2.4. Lithium treatment

Transgenic *Xenopus* embryos were treated with 0.3 M LiCl for 10 min at the stages indicated in Fig. 2 and subsequently washed six times in 0.1×Marc's modified Ringers [18]. Embryos were left to develop to stage 11, stage 14 or tadpole stage and then fixed in 1×MEMFA.

3. Results and discussion

3.1. p-LEF$_7$-fos-GFP/Luc is active in Xenopus embryos

Though Wnt/β-catenin is known to play key roles during *Xenopus* axis specification, whether it signals exclusively through Lef/Tcf transcription factors in this process remains unclear. Aiming to address this issue, we used artificial Lef/Tcf-dependent and Wnt-responsive reporter constructs, p-LEF$_7$-fos-Luc and p-LEF$_7$-fos-GFP, for further investigation. These constructs contain seven consensus Lef-1 binding sites and a minimal Fos promoter, driving *GFP* or luciferase (*Luc*) reporter gene expression (Fig. 1A). These constructs have been demonstrated to be transcriptionally active in the presence of both stabilized β-catenin and Lef/Tcf proteins in vitro [13]. To test whether these reporter constructs are also active in vivo, we injected p-LEF$_7$-fos-Luc into the lower part of the dorsal marginal zone at the four-cell stage, i.e. the Nieuwkoop center, where harbors strong β-catenin-mediated dorsalizing activity [22,23]. Luciferase activities of reporter constructs were quantified at the gastrula stage. p-fos-Luc, which lacks seven Lef-1 binding sites (Fig. 1A), was used as a negative control. As shown in Fig. 1B, the luciferase activity of p-LEF$_7$-fos-Luc was three times higher than that of p-fos-Luc (Fig. 1B), indicating that p-LEF$_7$-fos-Luc is active in *Xenopus* embryos. Furthermore, *GFP* expression derived from sibling constructs, p-fos-GFP and p-LEF$_7$-fos-GFP, revealed that this comparatively modest increase in total luciferase activity was caused by a small region of reporter gene transcription in the Spemann organizer (Fig. 1C,D). Thus we conclude p-LEF$_7$-fos-Luc/GFP is active in *Xenopus* embryos and its activation depends on Lef/Tcf binding sites.

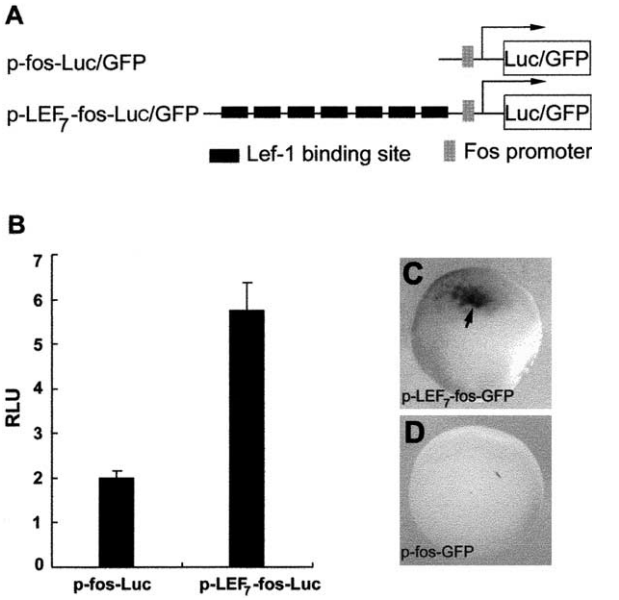

Fig. 1. p-LEF$_7$-fos-Luc/GFP reporter construct is active in *Xenopus* embryos. A: Diagrams of reporter constructs used in this study. B: Luciferase activities of the indicated constructs injected into both of the dorsal marginal zone cells at the four-cell stage, measured at stage 12.5. RLU, relative light unit. C,D: *GFP* expression in embryos radially injected with indicated reporter constructs into the marginal zone at the four-cell stage, assayed by in situ hybridization at stage 11. The black arrow indicates the *GFP* expression domain, vegetal view, with dorsal up.

3.2. LiCl treatment activates p-LEF$_7$-fos-GFP reporter construct during blastula and gastrula stages

Previous studies have shown that endogenous β-catenin/Lef-regulated Wnt promoters respond to canonical Wnt signaling according to developmental contexts. For example, the *Siamois* promoter responds to maternal, but not zygotic, Wnt/β-catenin activity at the mid-blastula stage [24]; in contrast, the *Engrailed-2* promoter is only active upon stimulation of zygotic Wnt signaling in the neural ectoderm [25]. Therefore, we asked whether the p-LEF$_7$-fos-GFP construct reports nuclear β-catenin activity constitutively, or whether its readout is temporally restricted like that of endogenous genes.

To answer this question, p-LEF$_7$-fos-GFP was stably introduced into *Xenopus* embryos via REMI transgenesis [19]. The

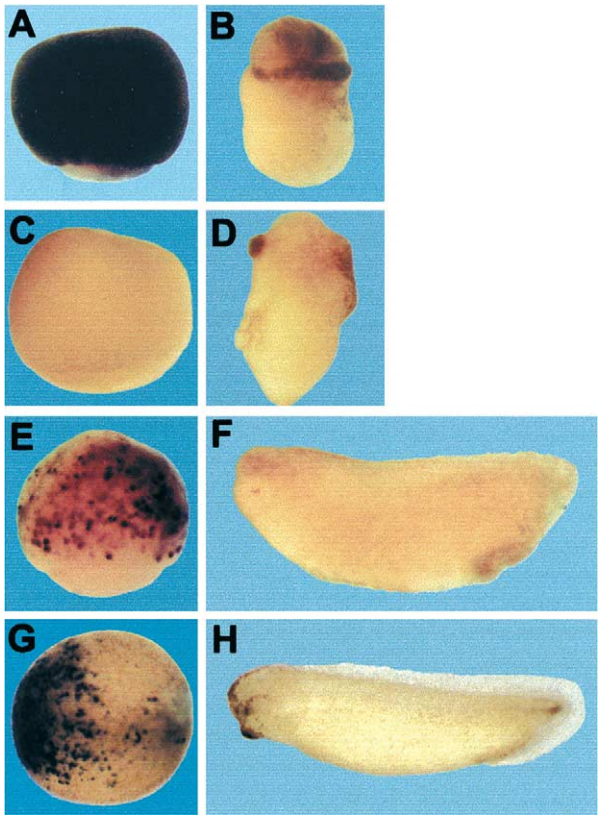

Fig. 2. LiCl treatment activates the p-LEF$_7$-fos-GFP reporter construct. p-LEF$_7$-fos-GFP transgenic embryos were treated with LiCl for 10 min at stage 5 (A,B), stage 10 (E,F) or stage 13 (G,H). These embryos were analyzed for *GFP* expression at stage 11 (A,E) or stage 14 (G) and for morphology at stage 29 (B,F,H). Ectopic *GFP* expression can be detected regardless of the time point of LiCl treatment (A,E,G). As a control, negligible *GFP* expression is observed in p-fos-GFP transgenic embryos treated with LiCl at stage 5 (C,D). Typical phenotypes for LiCl treatment at different stages are shown. B,D: Dorsoanteriorized embryo. F: Ventroposteriorized embryo. G: Embryo with forebrain defects. A,C,E: Lateral view. G: Dorsal view, with anterior to the left. B,D,F,H: Lateral view, with anterior to the left.

integration efficiency of REMI transgenesis was evaluated using the reporter construct driven by the promoter of *Xmyf-5*, whose expression has been shown to be stable [14]. An average of 66% integration efficiency was observed in the transgenesis (66% ventrolateral mesoderm expression, 9% non-specific expression (NSE), 25% no expression (NE), $n = 35$), which was very similar to that reported previously [19]. Transgenic embryos with p-LEF$_7$-fos-GFP were treated with LiCl for 10 min at different stages, which activates β-catenin by inhibiting its phosphorylation through GSK-3β [26]. At the tadpole stage, these embryos showed typical phenotypes for LiCl treatment at the relevant stages [21,27], verifying the effectiveness of the treatment. At the mid-gastrula stage, intense, pan-embryonic *GFP* expression was detected in transgenic embryos, which were treated with LiCl either prior to mid-blastula transition (MBT, the time point when large-scale transcription of zygotic genes commences) (73%, 7% patched expression (PE), 20% NE, $n = 49$; Fig. 2A) or after it (72%, 12% PE, 16% NE, $n = 25$; Fig. 2E). In contrast, little if any *GFP* expression could be detected in p-fos-GFP transgenic embryos treated with LiCl at stage 5 ($n = 71$, Fig. 2C), demonstrating that the LiCl-stimulated reporter gene activity depends on Lef/Tcf binding sites. Even when transgenic embryos were LiCl-treated at stage 13 and assayed for reporter gene expression at stage 14, ectopic *GFP* mRNA was detected in 25% of the embryos (75% NE, $n = 56$). Interestingly, although LiCl inhibits GSK-3β in all cells, more than half of these embryos expressed *GFP* predominantly in the dorsoanterior region (14%, $n = 56$, Fig. 2G), where high levels of *XTcf-3* mRNA are detected at this stage [8]. Since Lef/Tcf proteins are considered to be ubiquitously expressed as a family, i.e. cells express at least one member of this family, the observed correlation between reporter gene expression and *XTcf-3* mRNA levels suggests that the absolute cellular levels of Lef/Tcf proteins could be important for β-catenin-dependent gene activation.

From these experiments we conclude that p-LEF$_7$-fos-GFP is suitable for tracing endogenous Lef-1/Tcf-dependent Wnt signaling during *Xenopus* early development. This conclusion is supported by a recent study in which Lef/Tcf-dependent Wnt signaling was successfully traced with a similar reporter construct in transgenic fish throughout zebrafish development [28].

3.3. Region- and stage-specific transcription of p-LEF$_7$-fos-GFP reporter construct

3.3.1. Blastula stages. In p-LEF$_7$-fos-GFP transgenic embryos, the earliest transcripts of the reporter gene were detected at early stage 8 (around 2000 cells per embryo), i.e. before MBT. At this stage, *GFP* mRNA could be detected in a patch of vegetal cells adjacent to the border of pigmentation (56%, 8% NSE, 36% NE, $n = 59$; Fig. 3A). *GFP* mRNA was still nuclear at this time (Fig. 3A, white arrowheads), indicating that reporter gene transcription had started just a short while ago. After MBT, *GFP* mRNA has shifted to the cytoplasm, and the signal has increased in this region by stage 9 (57%, 10% NSE, 33% NE, $n = 30$; Fig. 3B). This asymmetric expression domain was confined to the prospective dorsal side of the embryo, which can be distinguished from the ventral side by cell size and pigment distribution [16]. Thus, the expression domain of the reporter gene at blastula stages is most probably co-localized with the Nieuwkoop center, the blastula organizer, where maternal β-catenin activity exists [22,23]. As mentioned above, it is generally accepted that maternal Wnt/β-catenin activity specifies dorsal fates via inducing the formation of the Spemann organizer. Consistent with previous studies [21,29,30], our results indicate that Lef/Tcf transcription factors mediate maternal canonical Wnt signaling in *Xenopus* dorsal axis specification.

The observed pre-MBT transcription of the reporter gene is in agreement with a recent study, in which β-catenin/Tcf-regulated transcription of endogenous *Xnr5* and *Xnr6* genes was detected by reverse transcription polymerase chain reaction in the dorsovegetal region of *Xenopus* embryos prior to MBT, and Lef/Tcf-dependent pre-MBT transcription was shown to be essential for dorsoventral patterning in *Xenopus* [31]. In this study, however, the results from the luciferase assay of a microinjected Wnt-responsive reporter construct similar to p-LEF$_7$-fos-Luc indicated that the reporter gene was not transcribed prior to MBT. This discrepancy raises the possibility that chromosomal transgenes are more susceptible to pre-MBT transcription than episomal DNA templates. However, it is also possible that a lower sensitivity of the luciferase

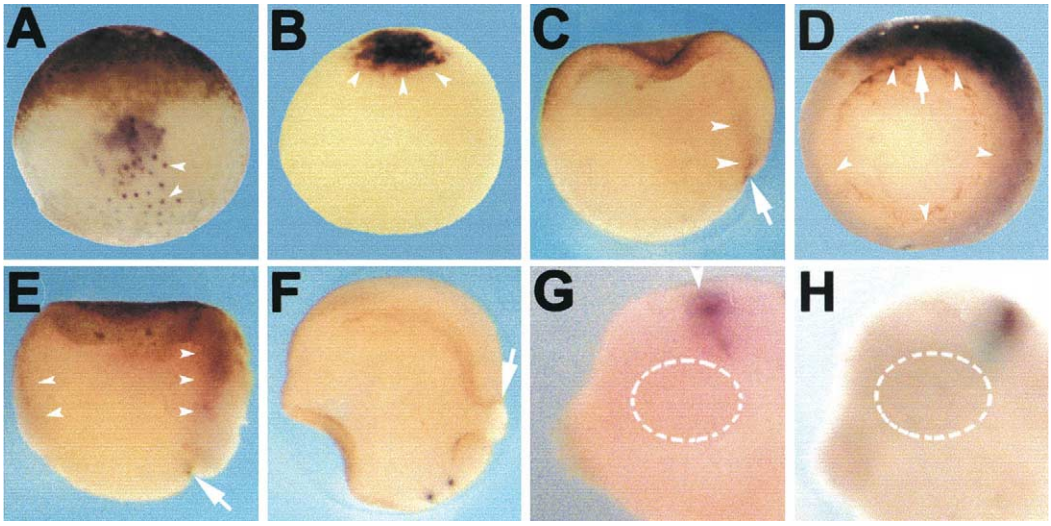

Fig. 3. Lef/Tcf-dependent transcription occurs during *Xenopus* axis specification. Transcripts of the reporter gene were detected by in situ hybridization. A–G: Expression pattern of *GFP* reporter gene at stage 8 (A), stage 9 (B), stage 10.25 (C), stage 11 (D,E), stage 13 (F) and stage 29 (G). White arrowheads indicate *GFP* expression domains. White arrow indicates dorsal blastopore. H: Endogenous *Engrailed-2* expression marks the MHB of *Xenopus* tadpole. G,H: Dashed circle indicates the position of the eye; white arrowhead indicates the location of *GFP* or *En-2* expression. A: Dorsal view, with pigmented animal pole up. B,D: Vegetal view, with dorsal side up. C,E: Bisected embryos, with dorsal to the left. F: Bisected embryo, with dorsal up. G,H: Lateral view, with anterior to the left.

assay than RNA in situ hybridization or quick silencing of microinjected plasmids [32] caused the difference. On technical grounds, the pre-MBT *GFP* expression emphasizes the speed and sensitivity with which the reporter construct senses the accumulation of nuclear β-catenin.

3.3.2. Gastrula stages. In early gastrula, localized *GFP* expression was observed in the Spemann organizer (53%, 9% NSE, 38% NE, $n = 34$; Fig. 3C), confirming the dorsal expression of *GFP* in blastula. In a significant percentage of transgenic embryos, *GFP* expression extended ventrally and formed a ring in the mesoderm at the mid-gastrula stage with dorsally higher levels than on the ventral and lateral sides (21%, $n = 66$; Fig. 3D,E). The rest of the *GFP*-positive transgenic embryos showed a restricted dorsal expression only (36%, $n = 66$; data not shown), which was stronger than that in early gastrula. Probably, the difference in *GFP* expression between the two subgroups of transgenic embryos reflects a slight developmental retardation of the second subgroup, as transgenic embryos generally develop less synchronously than normal embryos. The *GFP* expression in the ventrolateral mesoderm fits well with the crescent of zygotic *Wnt-8* transcripts (see Fig. 3D and [33]), raising the possibility that Lef/Tcf proteins also mediate zygotic Wnt-8 signaling, in addition to the dorsalizing maternal β-catenin activity. This is addressed experimentally below.

Though zygotic Wnt/β-catenin activity was expected to be

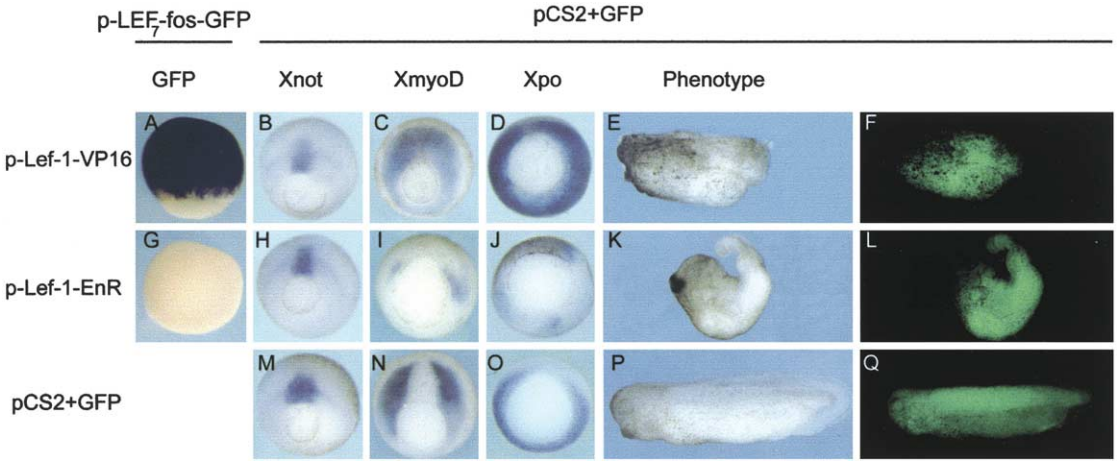

Fig. 4. Loss or gain of zygotic Lef-1 activity results in severe developmental defects. A: Strong *GFP* expression is detected in p-LEF$_7$-fos-GFP and p-Lef-1-VP16 co-transgenic embryos at the mid-gastrula stage by in situ hybridization. B–F: pCS2+GFP and p-Lef-1-VP16 co-transgenic embryos were analyzed for marker gene expression at stage 11 (B–D) and morphology at stage 29 (E). F: The same embryo as in E under fluorescent optics. Little if any *GFP* expression, however, is detected in p-LEF$_7$-fos-GFP and p-Lef-1-EnR co-transgenic embryos at the mid-gastrula stage (G). The molecular marker expression and the morphology of pCS2+GFP and p-Lef-1-EnR co-transgenic embryos are shown (H–L). L: The same embryo as in K under fluorescent optics. As a control, transgenic embryos expressing *GFP* alone show normal expression patterns of marker genes and develop into well-organized embryos (M–Q). A,G: Lateral view. B–D,H–J,M–O: Vegetal view, with dorsal up. E,F,K,L,P,Q: Lateral view, with anterior to the left.

absent from the organizer for notochord formation [33], the observed *GFP* expression in the organizer of transgenic gastrula is consistent with several recent reports. For example, Schohl and Fagotto reported the detection of nuclear accumulation of β-catenin in the organizer during gastrulation [34]. Consistently, Tcf-dependent zygotic Wnt activity localized in the organizer is suggested to be required for normal expression of *Xbra*, whose product plays a key role in mesoderm specification [35]. While these reports, together with the observed increase in *GFP* expression from late blastula stage to gastrula stages, support ongoing transcription of the reporter gene in the organizer region during gastrulation, it is also possible that the dorsal *GFP* mRNA persisted from the previous burst of maternal β-catenin-dependent transcription. Unfortunately, our assay system does not allow us to distinguish clearly between these two possibilities.

3.3.3. Neurula through tadpole stages. *GFP* expression was not observed at the late gastrula stage ($n = 17$; Fig. 3F) and remained undetectable during *Xenopus* neurulation (data not shown). This observation was unexpected, given that (i) class I Wnt ligands and Frizzled receptors (e.g. Wnt-1, Wnt-3A, Xfz-7 and Xfz-9; for review see [36]), as well as Lef/Tcf proteins [8] are expressed during these developmental stages, and (ii) forced β-catenin activation by LiCl ascertained the principal responsiveness of the reporter gene at the gastrula/neurula transition (see Fig. 2G). Therefore, this finding raises the possibility that canonical Wnt signaling may regulate gene expression through transcription factors other than Lef/Tcf proteins during neurulation. In support of this hypothesis, a hormone-inducible activating Tcf variant has been found to posteriorize neuroectoderm in cap assay only if hormone was applied at or prior to late gastrula, but not later [37]. At the tadpole stage, the *GFP* transgene became de novo activated in a small part of the brain (37%, 8% NSE, 55% NE, $n = 59$; Fig. 3G). In comparison to *Engrailed-2* mRNA, which marks the MHB [6], the reporter gene is transcribed anterior to the MHB (compare Fig. 3G with Fig. 3H). Based on published data, this part of the midbrain is likely to receive inputs from several canonical Wnt ligands, including Wnt-1, -2B, and -3A [38].

In summary, at least four known domains exhibiting Wnt/β-catenin activity are reported by p-LEF$_7$-fos-GFP in a correct spatio-temporal manner. These domains include dorsally the pre-MBT Nieuwkoop center and the post-MBT Spemann organizer, both of which require maternal β-catenin activity [2,22,23], the ventrolateral mesoderm, which receives zygotic Wnt-8 activity [33], and part of the midbrain [38]. The reporter gene activity indicates that cells in these territories utilize Lef/Tcf proteins, at least in part, to read out Wnt/β-catenin activity. Notably, cells in several other regions did not activate the reporter gene, although they express class I Wnt ligands and receptors (for review see [36]) as well as Lef/Tcf proteins [8]. These regions include eye, otic vesicle, spinal cord, MHB and tailbud [35,39]. Though we cannot fully exclude the possibility that the lack of reporter gene expression in these regions is caused by technical limits, such as the relatively low sensitivity of reporter construct and lower responsiveness of the transgene in older embryos due to epigenetic silencing, several observations argue strongly for a faithful reporting of β-catenin/Lef/Tcf activity by this reporter construct. These include its rapid response to LiCl treatment (less than 90 min, between stages 13 and 14; Fig. 2G), the observed de novo activation late in development in the midbrain, and finally the very good correlation between reporter gene activity and known domains of canonical Wnt/β-catenin activity, including pre-MBT transcription in the Nieuwkoop center.

3.4. *Lef-1/Tcf proteins mediate early ventroposterior patterning in* Xenopus

During early gastrulation, the p-LEF$_7$-fos-GFP reporter transgene was co-expressed in the ventral and lateral equatorial region (prospective ventroposterior tissues), with zygotic Wnt-8, consistent with the assumption that Lef/Tcf proteins mediate at least some of the functions of Wnt-8. Direct target genes of Wnt-8 are currently not known. Hamilton and colleagues have demonstrated that Wnt-8 signals through β-catenin, however, without requiring XTcf-3 [21]. Therefore we decided to test whether Lef/Tcf activity is required for ventroposterior development. As transgene-derived proteins are synthesized as early as at stage 10 [19], endogenous Lef/Tcf activity can be interfered with from gastrulation onwards via transgenic expression of dominant variants of Lef-1.

First of all, the activity of Lef-1-VP16 protein (a dominant active form of Lef-1) was confirmed by co-transgenesis with p-Lef-1-VP16 and p-LEF$_7$-fos-GFP. Strong pan-embryonic *GFP* expression was observed at the mid-gastrula stage (57%, 18% PE, 25% NE, $n = 28$; Fig. 4A), indicating that the transgene-derived Lef-1-VP16 protein is capable of inducing reporter gene transcription even before the mid-gastrula stage, i.e. when zygotic Wnt-8 is believed to pattern the dorsoventral marginal zone [21]. Then, we generated p-Lef-1-VP16 and pCS2+GFP co-transgenic embryos. In this case, *GFP* expression was used to sort out the *Lef-1-VP16*-expressing embryos. The morphology of these embryos was scored at the tadpole stage with the dorsoanterior index (DAI, 0 representing embryos lacking dorsoanterior structures and 10 standing for embryos with the most extremely enhanced dorsoanterior structures [18]). As shown in Fig. 4E, these embryos developed with severe anterior defects and shortened dorsal axes (average DAI = 2.8, $n = 37$). When such embryos were assayed at the mid-gastrula stage for marker gene expression, most of them showed ectopic expression of ventrolateral mesodermal markers such as *XmyoD* and *Xpo* in the organizer territory (Fig. 4C,D, compared with control embryos in Fig. 4N,O). However, the expression of the notochord-specific dorsal mesodermal marker *Xnot* was unchanged (Fig. 4B, compared with Fig. 4M).

In contrast, expression of a dominant negative form of Lef-1, Lef-1-Engrail [14], resulted in decreased *GFP* expression (66% NE, 34% PE, $n = 29$; Fig. 4G). Strikingly, *Lef-1-EnR*-expressing embryos develop with severe posterior defects but normal head development (average DAI = 7.0, $n = 107$; Fig. 4K). In terms of marker gene expression, Lef-1-EnR repressed the expression of *XmyoD* and *Xpo* (Fig. 4I,J, compared with Fig. 4N,O), while *Xnot* expression was again unaffected (Fig. 4H, compared with Fig. 4M). These results were strictly dependent on loss – or gain – of -Lef-1/Tcf activity, because transgenic embryos expressing *GFP* only were normal (average DAI = 4.8, $n = 68$; Fig. 4M–Q).

In summary, the post-blastula expression of a dominant negative variant of Lef-1 affected predominantly the development of ventroposterior structures, while head development was largely normal. From these results, we conclude that Lef-1/Tcf activity is essential for ventral and posterior development in *Xenopus* embryos. Our results are in agreement

with reports establishing an essential Lef-1 function in the formation of posterior structures in mouse and zebrafish [28,40]. During preparation of this article, Roel and colleagues published an elegant morpholino knock-down study, which suggests a switch in Lef/Tcf functions: from Tcf-3, mediating maternal β-catenin activity during the establishment of the dorsal axis, to Lef-1, mediating zygotic Wnt-8 signaling in specifying ventroposterior structures [41]. While the work presented here does not distinguish between Lef-1 and Tcf-3 functions, it argues for an even more complex regulation of nuclear β-catenin activity. Indeed, the developmental expression profile of *GFP* in p-LEF$_7$-fos-GFP transgenic embryos suggests either that the canonical Wnt pathway can be inactive in some cells despite the expression of its major agonists, or alternatively, that Lef/Tcf transcription factors are not constitutive mediators of this pathway. Either model merits further investigation, as this could reveal important insights into the cellular interpretation of Wnt signaling activity.

Acknowledgements: The authors are grateful to Drs. R. Grosschedl, R. Kemmler and S. Hoppler for sharing plasmids. We thank Dr. W. Mei for discussion of the project. This work is supported by grants of the National Foundation of Natural Science (30170111, 30270650) and National Key Project for Basic Research of China (2001CB50991) to X.D.; L.X. was supported by a grant of the Human Frontiers of Science Program (RG0039/1999-M) to R.R., which was also used to support X.G.'s work at the Adolf-Butenandt-Institute. In addition, X.G. received a visitors fellowship from the Max-Planck-Society.

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