A DUAL ACTION OF XTCF-3 IN BODY AXIS FORMATION

MIRANDA MOLENAAR

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A DUAL ACTION OF XTCF-3 IN BODY AXIS FORMATION

EEN DUBBELROL VOOR XTCF-3 BIJ DE VORMING VAN DE LICHAAMSAS
(met een samenvatting in het nederlands voor niet ingewijden)

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CONTENTS

CHAPTER 1	INTRODUCTION: AXIS FORMATION IN XENOPUS LAEVIS	7
CHAPTER 2	XTCF-3 TRANSCRIPTION FACTOR MEDIATES ß-CATENIN–INDUCED AXIS FORMATION IN XENOPUS EMBRYOS	27
CHAPTER 3	DIFFERENTIAL EXPRESSION OF THE HMG BOX TRANSCRIPTION FACTORS XTCF-3 AND XLEF-1 DURING EARLY *XENOPUS* DEVELOPMENT	45
CHAPTER 4	THE *XENOPUS* WNT EFFECTOR XTCF-3 INTERACTS WITH GROUCHO-RELATED TRANSCRIPTIONAL REPRESSORS	53
CHAPTER 5	DIFFERENTIAL EXPRESSION OF THE GROUCHO RELATED GENES 4 AND 5 DURING EARLY DEVELOPMENT OF *XENOPUS LAEVIS*	65
CHAPTER 6	DISCUSSION: A DUAL ROLE FOR XTCF-3 IN PRIMARY AXIS FORMATION	75
	SAMENVATTING VOOR NIET INGEWIJDEN	89
	DANKWOORD	93
	CURRICULUM VITAE	95
	LIST OF PUBLICATIONS	97

CHAPTER 1

INTRODUCTION

AXIS FORMATION IN XENOPUS LAEVIS

AXIS FORMATION

During vertebrate embryonic development, a cellular pattern is established, which specifies the formation of the primary body axis with its anterior to posterior features. In all vertebrates, a group of cells in the pre-gastrula embryo, called the organizer, is believed to be contributory for this patterning event. In this thesis, emphasis lies on the molecular events involved in determining the amphibian vertebrate organizer, the Spemann organizer[106]. In other vertebrates, organizers with similar properties are termed the node in mammals and birds and the shield in fish[2,101,109]. It is assumed that the molecular events, which determine this organizer, are conserved during evolution. Therefore, the outcome of research on organizer function in the amphibian embryo will generate more insight into similar events in other vertebrates.

Embryos of the South African claw-toed frog, *Xenopus laevis*, are favorite objects for the study of the cellular and molecular interactions during vertebrate axis formation. Eggs of *Xenopus laevis* are 1-2 millimeter in diameter, relatively easy to collect and the embryos can grow in plain tap water.

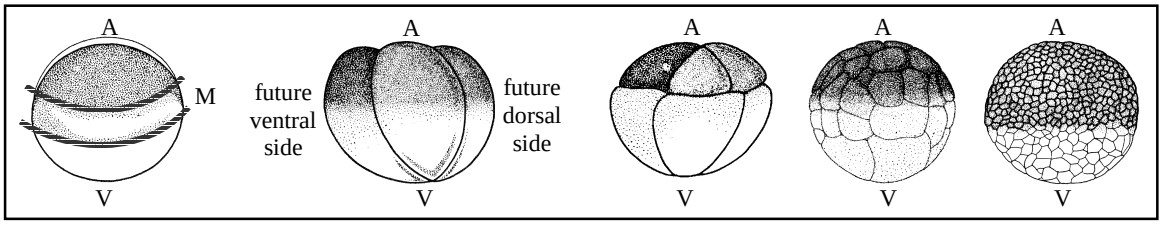

Figure 1. Early cleavage stages of Xenopus laevis. From left to right: one cell stage; four cell stage; eight cell stage; thirty-two cell stage; early blastula. In these early stages, an animal pole (A), a vegetal pole (V) and a marginal zone (M) (a.k.a. equatorial region) can be distinguished.

The *Xenopus laevis* egg is radially symmetric with an animal and a vegetal pole (figure 1). The animal side is highly pigmented and is destined to contribute mostly to the ectoderm. The vegetal part contains most of the energy source, stored in yolk platelets, needed for development up until the embryo is capable of feeding. The vegetal side will contribute mostly to the endoderm. The region in between, the marginal zone or equatorial region, is fated to form the mesoderm. When sperm fertilizes the oocyte, the animal pole turns upward to protect the embryo from damaging UV-irradiation. Opposite to the sperm entry point, the prospective dorsal side is set. Thus, the point at which the

sperm enters the animal pole determines the Dorsal/Ventral axis and at the same time the Left/Right axis (figure 3)[22].

After fertilization a series of synchronous cell divisions takes place (figure 1). The synchronism in cell cycle is gradually lost[72]. Animal cells divide a little faster than vegetal cells. The pattern of divisions results in smaller animal cells, with an underlying blastocoelic cavity, and relatively large vegetal cells (figure 2a). At the end of this so-called blastula stage, cells start to migrate inwards at the prospective dorsal side of the embryo (figure 2B). This area is named the blastopore lip. During this gastrulation process mesodermal cells will involute underneath the ectoderm, pulling the connected endoderm inside. At the same time the ectoderm will cover the entire embryo (figure 2b-d).

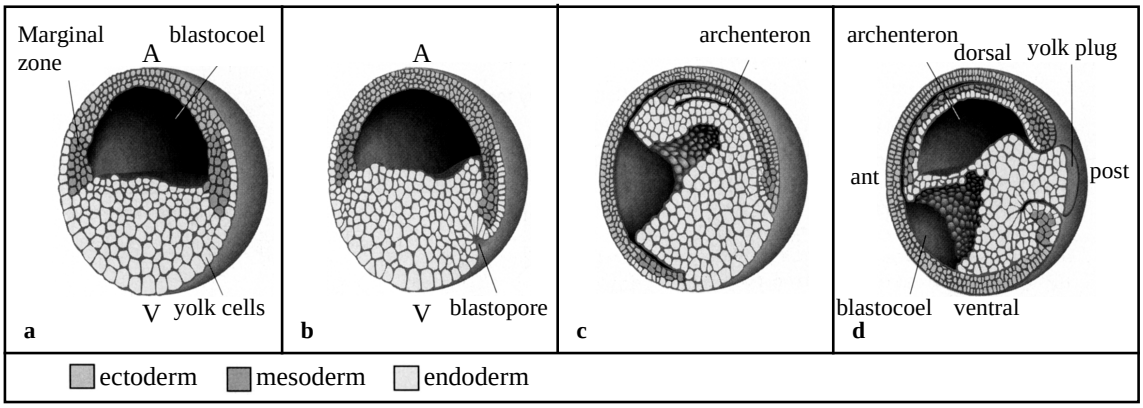

Figure 2. The process of gastrulation of the *Xenopus laevis* embryo. Blastula (a) and early gastrula (b) stages with animal (A), vegetal (V) and marginal regions. During the mid-gastrula (c) and late-gastrula (d) stages the dorsal-ventral and anterior(ant)-posterior(post) axes become apparent. Adapted from L. Wolpert et al., 1998[119]

At the end of gastrulation, the dorsal mesoderm interacts with the overlying competent dorsal ectoderm (figure 2d). This interaction results in induction of neural tissue. The dorsal mesoderm that involutes first will become the prechordal plate. Together with the anterior endoderm, the prechordal plate induces the anterior part of the brain and head structures in the overlying dorsal neurectoderm. The more posterior brain and head structures and the spinal cord are induced by the subsequent dorsal mesoderm, which forms the notochord. These sub-organizers are defined at late blastula/early gastrula stages. Before gastrulation, cells of the prospective anterior endoderm and

prechordal plate lie in the dorsal vegetal-marginal region. In the marginal region the prospective notochord cells reside (figure 2a).

THE ONSET OF EMBRYONIC TRANSCRIPTION

Until the end of the blastula stage (S.9), the development of the embryo relies on maternal mRNAs and proteins present in the egg. Repression of transcription of the embryonic genome is relieved at what is called the Mid Blastula transition (MBT)[71,72]. The limitations in transcriptional capacity before MBT are a result of chromatin-mediated repression and deficiencies in the basal transcription machinery[1,84,116]. High amounts of histone protein assemble the DNA and, in this way, prevent access of the TATA binding protein (TBP) to initiate basal transcription[1,84]. TBP protein levels increase dramatically at the beginning of the blastula stage[116]. At the end of the blastula stage, the competition between histones and TBP for DNA is in favor of TBP. From this time-point on, basal transcription of the embryonic genome can take place. The capacity for activated transcription, through the binding of enhancers to trans-acting elements, is gradually acquired at the beginning of gastrulation[116]. This transition from basal to activated transcription is called the early gastrulation transition (EGT)[107]. From this time-point on, strict regulation of the embryonic genome is the key issue for further development.

THE MOLECULAR NATURE OF SPEMANN ORGANIZER FORMATION

Patterning processes before MBT and EGT pinpoint the subset of dorsal genes responsible for formation of the Spemann organizer. As soon as activated transcription is enabled, the dorsal specific genes are transcribed. Before MBT/EGT, maternal mRNAs

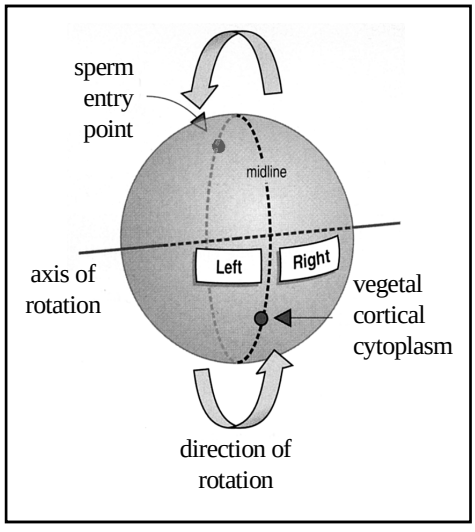

Figure 3. Immediately after fertilization, the sperm entry point defines the future embryonic axes by inducing a rotation of the cortex. During this cortical rotation, vegetal cortical cytoplasm is translocated to the future dorsal side. Adapted from L. Wolpert et al., 1998[119]

and proteins are responsible for the events that mark the dorsal genes.

One of the first dorsalizing genes, which are actively transcribed, is *XSiamois*. XSiamois is a homeo-domain transcription factor and has the potency to induce a complete Spemann organizer[17,50]. In specifying the different sub-domains of the organizer, XSiamois is believed to cooperate with pan-endodermal and pan-mesodermal factors to bring about the sub-marginal and the marginal regions, respectively[14,42].

Maternal factors, which induce ectopic *XSiamois* expression and as a consequence an ectopic axis, act in the WNT signaling pathway (see below). During cortical rotation, the vegetal cortical cytoplasm (VCC) containing activators of this pathway migrates from the vegetal pole towards the future dorsal side (figure 3)[19,30,39,40,57,58,61,93,127]. This occurs about 30 minutes after fertilization, possibly by a microtubule-mediated transport of organelles[47,90]. UV irradiation of a fertilized egg disrupts the cytoskeleton, thereby preventing the migration of these components. UV-irradiated embryos are radially symmetric without any dorsal structures[96,97]. In these embryos *XSiamois* is transcribed, but transcripts are localized vegetally[60]. Together with the fact that e.g. XSiamois can induce an ectopic organizer at the ventral marginal side[50], these results suggest that the WNT signaling pathway and XSiamois must be active at the dorsal marginal side and that only under these "circumstances" the Spemann organizer is induced.

THE WNT SIGNALING PATHWAY

A pathway implicated in the formation of the organizer is the WNT signaling pathway. Introduction of transcripts for WNT or one of its downstream components, in a prospective ventral blastomere of a four-cell stage *Xenopus laevis* embryo, activates dorsal genes immediately after EGT and induce an ectopic Spemann organizer (figure 4).

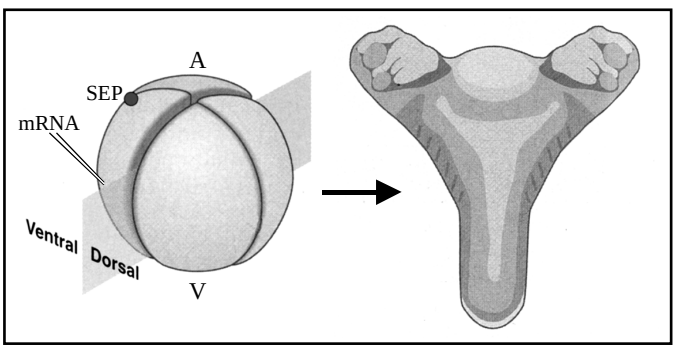

Figure 4. Induction of an ectopic axis by injection of mRNA encoding certain WNTs or one of the downstream components in the pathway. Adapted from L. Wolpert et al., 1998[119]

The WNT signaling pathway is involved in multiple developmental processes, not only in vertebrates but also in, for example, flies and nematodes. Genetic studies in *Drosophila melanogaster* and *Caenorhabditis elegans*, together with biochemical manipulations in early *Xenopus laevis* embryos, have elucidated many of the molecules and their interactions involved in the WNT signaling pathway.

WNT HISTORY

In 1980, Nüsslein-Volhard and Wieschaus reported a *Drosophila* null mutation of the gene *wingless* (*Wg*), first identified as a weak mutation disrupting wing patterning[75,100]. The null mutation leads to embryonic lethality and severe patterning defects. Several other mutants exhibit a similar type of patterning defect. The genes involved were identified and epistatic analysis revealed a novel pathway, the wingless pathway (figure 5)[73,81,102].

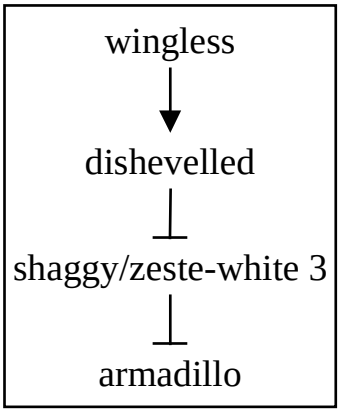

Figure 5. Epistasis of the different components of the so-called wingless pathway originally discovered in a screen for patterning defects. The wingless pathway is an activating pathway in which wingless activates dishevelled, which on its turn inactivates shaggy/zeste white 3. Inactivation of shaggy/zeste white leads to activation of armadillo.

A few years later, Nusse and Varmus discovered that inappropriate activation of the *Int*-1 gene could induce tumors in the murine mammary gland. *Int*-1 turned out to be the ortholog of *Wg* and was later renamed as *Wnt*-1[74,86]. Ectopic expression of *Wnt*-1 mRNA in *Xenopus laevis* embryos causes axis duplication. *Wg*/*Wnt*-1 relatives now form a multigene family with at least 20 different members identified in man, mouse, chicken, *Xenopus laevis*, *Drosophila melanogaster* and *Cenorhabditis elegans* (for the most recent overview, see the Wnt webpage (http://www-leland.edu/~rnusse/wntwindow.html).

In vertebrates, WNT genes are expressed largely in the nervous system and mesoderm derivatives, and appear to be essential players in embryogenesis and carcinogenesis. Dysfunction of WNT genes or of genes in the downstream pathway lead to severe developmental defects and cancer. For example, Wnt-1 knock out mice completely lack a cerebellum. Inappropriate activation of the Wnt-1 gene can induce mammary gland tumors[74].

WNTs can be divided into two main classes, the Wg/Wnt-1 and the Wnt-5A class, based on their different function and downstream signaling pathway[66]. The canonical WNT pathway involves the events downstream of the Wg/Wnt-1 class. Based on the epistatic interactions known in *Drosophila*, several vertebrate orthologs for the different components of the *Wingless* signaling pathway were identified and assayed for their functional interactions in vertebrates. Together with recent findings discussed below, the picture of WNT signaling in vertebrates becomes apparent, but it is still far from complete. The molecules involved in the pathway and their interactions are schematically drawn in figure 6.

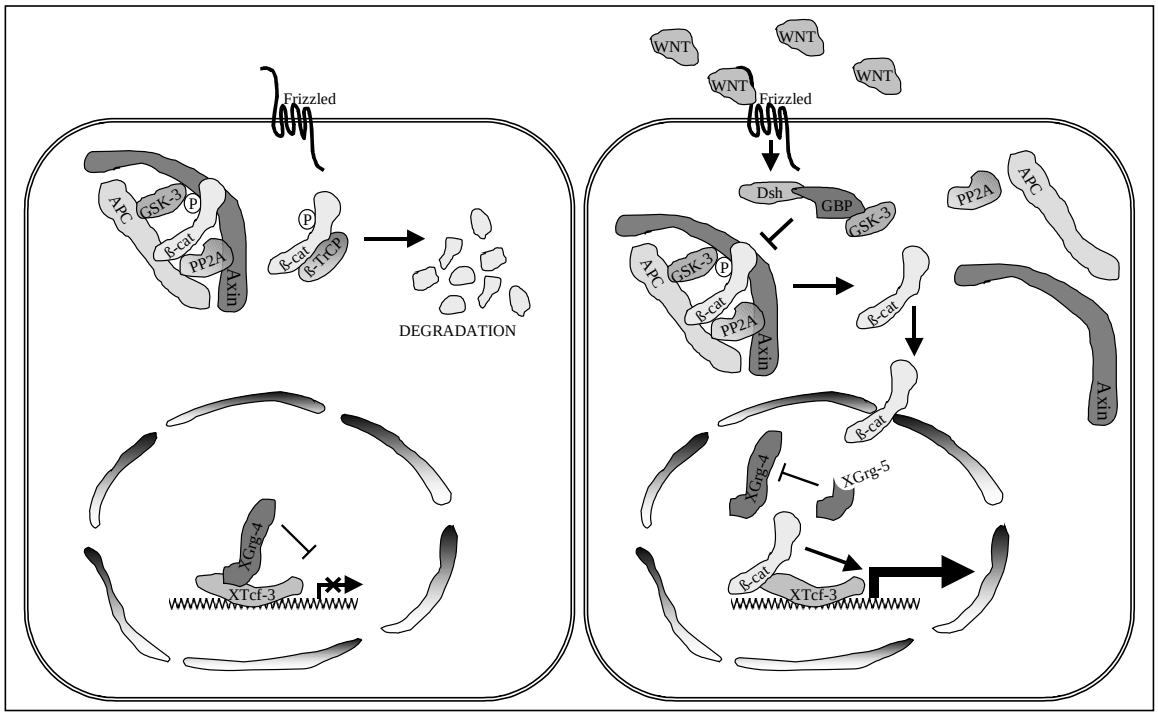

Figure 6. Model for WNT signaling. *Left cell:* when no WNT signal is perceived, GSK-3 phosphorylates cytosolic ß-catenin. Phosphorylated ß-catenin is degraded via the ubiquitination pathway. In the nucleus WNT target genes are repressed by interaction of XGrg-4 to XTcf-3. *Right cell*: when WNT binds the receptor, phosphorylation of ß-catenin by GSK-3 is inhibited, cytosolic ß-catenin levels rise and ß-catenin translocates to the nucleus. In the nucleus ß-catenin interacts with XTcf-3 to activate WNT target genes. Glycogen Synthase Kinase 3 (GSK-3); Adenomatous Polyposis Coli (APC); Protein Phosphatase 2A (PP2A); ß-Transducin repeat Containing Protein (ß-TrCP); ß-catenin (ß-cat); Groucho related gene (Grg); T cell factor (Tcf); Dishevelled (Dsh); GSK-3 Binding Protein (GBP).

WNT PERCEPTION

Genes of the Wnt family encode secreted glyco-proteins, which most likely act as ligands that bind to specific receptors. The candidate receptors for WNTs belong to the family of Frizzled (Fz) proteins[5]. Frizzled genes encode seven trans-membrane proteins with an extra-cellular cysteine-rich domain (CRD), which is responsible for interaction with the WNT ligand. Once secreted, the availability of WNTs for their receptor is regulated. First, glycosaminoglycans are able to enhance WNT activity, possibly by regulating the level of reactive protein and/or adjusting the affinity for Fz[13]. Second, there is a competition for interaction with WNT between Fz receptors and secreted WNT inhibitors, including FRPs (Frizzled-related proteins)[53,117,121], Dickkopf-1[23], Cerberus-1[6] and WIF-1 (WNT inhibitory factor-1)[31]. These WNT inhibitors prevent WNT to interact with its receptor and thereby negatively regulate WNT signaling. It should be noted that these inhibitors appear after Early Gastrulation transition, so they cannot manipulate events concerning Spemann organizer induction. In fact, the encoding genes are expressed in the marginal-vegetal part of the organizer itself.

REGULATION OF CYTOSOLIC ß-CATENIN LEVELS

One of the key players in the WNT signaling pathway is ß-catenin. ß-Catenin was first identified as a molecule assisting cadherin in cell-cell adhesion[69,78]. The core region of ß-catenin contains 12 copies of a 42 amino acid sequence motif known as the armadillo repeat. These repeats form a superhelix of helices that features a long positively charged groove. The domains of molecules known to interact with ß-catenin are acidic and are proposed to interact with this groove[33].

At the dorsal side of an early cleavage *Xenopus laevis* embryo, ß-catenin accumulates in the cytoplasm, and by the end of the 16-32 cell stages, it is also detected in nuclei of dorsal but not ventral cells[48,98]. XSiamois is expressed within the domain of nuclear ß-catenin accumulation[51]. The introduction of ß-catenin mRNA in ventral blastomeres of *Xenopus laevis* embryos results in induction of an ectopic axis[20] (figure 2). *Xenopus* embryos depleted of maternal ß-catenin mRNA develop without dorsal structures[28]. In these ß-catenin depletion experiments, cell-cell adhesion is not altered,

only cytosolic ß-catenin is depleted[43]. These results suggest that ß-catenin can act as a signaling molecule, which is necessary for Spemann organizer formation.

CONSTITUTIVE TARGETED BREAKDOWN OF ß-CATENIN

A key event in WNT signaling pathway is the regulation of cytoplasmic levels of ß-catenin. In the absence of a WNT signal, the serine/threonine Glycogen Synthase Kinase-3ß (GSK3ß) phosphorylates the N terminal serine residues of ß-catenin (figure 4), directing ß-catenin towards ß-TrCP[27,126]. The *ß-TrCP* gene encodes a conserved F-box/WD40 repeat protein, which targets serine phosphorylated ß-catenin for degradation by the ubiquitin proteasome pathway[56]. In order to phosphorylate ß-catenin, GSK3ß needs to be incorporated in a multi-molecular complex. This complex consists of Adenomatous Polyposis Coli (APC)[91,92], Axin [29,35,41,70,95] (also known as Conductin[3] or Axil[123]) and protein phosphatase 2A (PP2A)[32,99]. In this complex, APC and axin function as scaffold proteins. These relatively large molecules embody domains for interaction with GSK3ß, ß-catenin, PP2A and for each other[3,25,29,32,35,36,41,70,91,92,95,99,123]. Mutation of either APC or Axin result in elevated levels of ß-catenin, indicating that both molecules are necessary for the function of this multi-molecular complex[45,67,68,128]. In this complex, GSK3ß phosphorylates APC and thereby facilitates the binding of ß-catenin[91]. GSK3ß also phosphorylates Axin, which prevents Axin from WNT induced degradation[122]. How the phosphatase PP2A can facilitate ß-catenin degradation remains to be elucidated.

Taken together, in the absence of a WNT signal, the multi-molecular complex is constitutively active in the phosphorylation of N-terminal serine residues of ß-catenin. As a consequence all cytosolic ß-catenin is targeted for breakdown (figure 5, *left cell*).

WNT ACTIVATED STABILISATION OF CYTOSOLIC ß-CATENIN

When WNT molecules bind to their receptors (Fz), the cytosolic phospho-protein Dishevelled (Dsh) becomes active, resulting in inactivation of GSK3ß. ß-catenin is no longer phosphorylated by GSK3ß and as a consequence the cytosolic levels of ß-catenin increase (figure 5, *right cell*). Overexpression of Dsh, in a ventral blastomere of a four-cell stage *Xenopus* embryo, can induce a complete ectopic axis[105]. How Dsh is activated

by the Frizzled receptor is still unclear. Recent evidence implied that Dsh might be activated by Casein kinase-Iε (CKIε)[83]. Like Dsh, overexpression of this serine kinase in *Xenopus laevis* embryos leads to axis duplication[83,94]. CKIε interacts with and increases phosphorylation of Dsh. Dsh phosphorylation increases in response to WNT signaling[124]. The level at what CKIε functions in the pathway is still unclear.

How Dsh, on its turn, inactivates GSK3ß is unknown. Preliminary results suggest that Dsh can bind to Axin, giving Axin the function as a bridge between Dsh and GSK3ß [54,103]. Moreover, Dsh also interacts with FRAT-1[37,38,54]. FRAT-1 is the mammalian homolog of GSK3ß binding protein (GBP). GBP can interact with and inhibit GSK3ß, which results in activation of the WNT pathway[125]. So, Dsh might inhibit GSK3ß via GBP.

INTERACTION OF ß-CAT WITH TCF

When a WNT signal causes elevation of cytosolic ß-catenin, ß-catenin accumulates in the nucleus[48,98]. In the nucleus, ß-catenin interacts with transcription factors of the TCF/LEF family, to activate transcription of WNT target genes (CHAPTER 2[4,34,65]). The TCF/LEF family defines a small subfamily of High Mobility Group (HMG) box genes[9]. To date, four different homologs called TCF-1, LEF-1, TCF-3 and TCF-4 have been described (CHAPTER 4)[15,21,46,64,76,82,111,114,118]. Due to alternative splicing and dual promoter usage, TCF protein isoforms exist with different transcriptional properties[88,112]. In general, a TCF factor consists of a DNA binding domain, the HMG box, which is highly conserved between the different homologs and orthologs. This HMG box is flanked by two putative Nuclear Localization Signals (NLS), which are most likely responsible for the nuclear translocation of TCFs. Furthermore, the first part of the N-terminus is highly conserved between the different homologs, while the C-terminus is very diverse. TCF factors were originally identified as lymphoid-specific transcription factors and were later on shown to be present in many different tissues during murine embryogenesis[77]. Although *Tcf* genes encode DNA binding proteins, when transfected together with a reporter gene, they fail to activate transcription[115].

In *Xenopus*, the maternally expressed *XTcf*-3 was found to act directly downstream of ß-catenin in embryonic axis specification (CHAPTER 2)[65].

Transcriptional activation of reporter genes containing recognition sites for TCF, depended on complex formation between XTcf-3 and ß-catenin. Armadillo repeats of ß-catenin interact with the N-terminal part of XTcf-3. Deletion of the N-terminus of XTcf-3 abrogated formation of this complex. This dominant negative ΔNXTcf-3, inhibited the activation of transcription mediated by the ß-catenin/XTcf-3 complex, resulting in suppression of axis formation in *Xenopus* embryos (CHAPTER 2)[65].

To date, XTcf-3 is the only TCF factor known to be expressed maternally in *Xenopus laevis* embryos (CHAPTER 3)[64]. Transcripts of other family members, XLef-1 and XTcf-4, are found in developmental stages after Mid Blastula transition. Therefore, XTcf-3 is likely to be the TCF factor involved in dorsal mesoderm specification. As was found for murine TCF factors, XTcf-3 and XLef-1 are broadly expressed throughout further development (CHAPTER 3)[24,64].

Results obtained by loss of function genetics, confirmed TCF to be downstream of ß-catenin in the WNT pathway[8,55,113]. Null mutations of *Drosophila dTcf* (ortholog of *Tcf-1*), also named *pangolin* (*pan*), showed a *wg*-like segment polarity phenotype. This indicates that dTCF is genetically downstream of armadillo[8,113]. *Pop-1*, the *Tcf-1* ortholog in *C. elegans*, is involved in WNT dependent asymmetric cell division of the EMS blastomere[55]. However, unlike *dTcf* in *Drosophila*, *Pop-1* has the opposite phenotype to that of the WNT components of the *Mom* class[87,110].

WNT TARGET GENES

After the identification of TCFs as a class of transcriptional regulators acting downstream of WNTs, a search for direct target genes of WNTs began. The previously identified consensus motif for TCF binding is found in a large number of genes[12]. Studies in *Drosophila* and *Xenopus* identified several potential WNT target genes with functional TCF sites in their promoter region. In *Drosophila*, a minimal wingless response element in the midgut enhancer of *Ultrabithorax* (*Ubx*) is recognized by mLef-1. In complex with armadillo, mLef-1 can stimulate transcription of this *Ubx* enhancer[85].

In *Xenopus laevis*, directly after Early Gastrula Transition, a number of genes is induced, which are responsible for the specification of dorsal mesoderm. In the promoter regions of three of these genes, *XSiamois*, *XTwin* and *Xnodal-related*-3[49,51,104], functional binding

sites for TCF factors were identified[7,16,49,59]. The *XSiamois* promoter, for example, contains three TCF sites capable of regulating its transcription in response to ß-catenin[7,16]. Mutation of these sites eliminated the ß-catenin/XTcf-3 activation of a reporter. The promoter was much more active in dorsal than in ventral blastomeres. Ventral expression of ß-catenin eliminates this difference in transcriptional activity, dependent on the presence of functional TCF/LEF sites. Interestingly, mutating the TCF sites elevated the ventral expression of the reporter gene, compared to the non mutated version[7]. In *Drosophila*, this de-repression by mutating TCF sites, in the absence of the ß-catenin homolog Armadillo, is also shown for the *Ubx* enhancer[85]. Thus TCF factors can mediate both repression and activation of the same promoter.

TCF AS A REPRESSOR OF TRANSCRIPTION

Recently, binding partners have been identified, both in *Drosophila* and *Xenopus*, which are proposed to be responsible for the repressive effects of TCF factors on transcription. Besides ß-catenin, another binding partner of TCF was identified performing a yeast two hybrid screen for proteins interacting with human TCF-1 (CHAPTER 4)[89]. This TCF partner is the product of the murine *Groucho-related gene* 5 (*mGrg*-5) and belongs to the Groucho family of transcriptional repressors. *Drosophila* Groucho is a widely expressed co-repressor, proposed to be involved in numerous developmental processes[18,26,79,80]. In vertebrates, different homologs of *groucho* have been identified with similar domain structures[44,52,62,108 120]. These are termed Transducin Like Enhancer of split (TLE) genes or Groucho related genes. *mGrg*-5 encodes a naturally truncated product containing only the amino-terminal two domains of the long forms of Grg[62]. Using *mGrg*-5 as a probe, the *Xenopus* orthologs of *Grg*-5 (*XGrg*-5 or *XAES*[11]) and *Grg*-4 (*XGrg*-4) were cloned. Like *XTcf*-3, these *Xenopus Grg* genes are expressed maternally and throughout embryogenesis (CHAPTER 5)[63]. Both XGrg-4 and 5 interact with XTcf-3, in a region upstream of the HMG box, but downstream of the ß-catenin binding domain. In transfection assays, the long form (XGrg-4) inhibited ß-Cat/XTcf-3 induced activation of transcription of a synthetic TCF reporter and the *XSia* promoter. In contrast, the short version (XGrg-5) enhanced the ß-Cat/XTcf-3 induced transcriptional activation. Dorsal injection of XGrg-4 into 4-cell stage *Xenopus* embryos

repressed transcription of *XSia* and *Xnr*-3 and suppressed formation of the endogenous axis. Ectopic axis formation, induced by a dominant positive ARM-XTcf-3 fusion protein, was inhibited by XGrg-4 and enhanced by XGrg-5 (CHAPTER 4)[89].

The functional relevance of this interaction was also shown for *Drosophila* Tcf and Groucho[10]. dTcf and Groucho physically interact. A reduction in both dTcf and Groucho expression caused a suppression of the *wg* and *arm* segment polarity phenotype. Hence, dTcf in complex with Groucho acts as a repressor of transcription, in the absence of Armadillo.

Therefore, the actual transcription of WNT target genes depends, amongst others, on the balance between the constitutive repressive effects mediated by long forms of Grg (possibly counteracted by the short form of Grg) and the activating effects of ß-catenin. This dual function of TCF may also explain the observation that the *C. elegans* homolog, Pop-1, has opposite effects to that of WNT. Pop-1 possibly functions as a repressor of transcription in the asymmetric cell division of the EMS blastomere. Removal of this transcription factor results in de-repression, and subsequently activation of WNT target genes.

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CHAPTER 2

XTCF-3 TRANSCRIPTION FACTOR MEDIATES ß-CATENIN–INDUCED AXIS FORMATION IN *XENOPUS* EMBRYOS

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ABSTRACT

XTcf-3 is a maternally expressed Xenopus homolog of the mammalian HMG box factors Tcf-1 and Lef-1. The N-terminus of XTcf-3 binds to ß-catenin. Microinjection of *XTcf*-3 mRNA in embryos results in nuclear translocation of ß-catenin. The ß-catenin-XTcf-3 complex activates transcription in a transient reporter assay, while XTcf-3 by itself is silent. N-terminal deletion of XTcf-3 (ΔN) abrogates the interaction with ß-catenin, as well as the consequent transcription activation. This dominant-negative ΔN mutant suppresses the induction of axis duplication by microinjected ß-catenin. It also suppresses endogenous axis specification upon injection into the two dorsal blastomeres of a four-cell stage embryo. We propose that signaling by ß-catenin involves complex formation with XTcf-3, followed by nuclear translocation and activation of specific target genes.

INTRODUCTION

Tcf-1 and *Lef*-1 define a small subfamily of vertebrate high mobility group (HMG) box transcription factor genes[3,24,33-35,41]. This family also includes the less well characterized, embryonally expressed genes *Tcf*-3 and *Tcf*-4 (V.K. and H.C., unpublished data)[4]. Tcf-1 and Lef-1 were originally defined as lymphoid-specific transcription factors, but were later found to be expressed in a largely overlapping, complex pattern during murine embryogenesis[25]. Tcf-1 and Lef-1 perform differential functions, as evidenced in gene disruption experiments. *Tcf*-1$^{-/-}$ mice have a severe defect in T lymphopoiesis, but are otherwise normal[39]. *Lef*-1$^{-/-}$ mice lack hair follicles and other skin appendages, teeth, and the trigeminal nucleus and die perinatally[37]. Lef-1 can play an architectural role in the activity of the T cell receptor α enhancer[2,10]. Lef-1 affects spacial enhancer structure, enabling contacts between factors that are bound elsewhere in the enhancer. Tcf-1 and Lef-1 do not behave as "classical" transcription factors, in that they do not activate transcription from reporter gene constructs that contain multimerized Tcf/Lef-binding sites[36].

ß-catenin was originally identified as a 92-94 kDa protein associated with the cytoplasmic tail of cadherin cell adhesion molecules[22,26]. ß-catenin is the vertebrate homolog of the *Drosophila* segment polarity gene product, Armadillo[19,28]. The central portion of these two proteins is made up of 13 Armadillo repeats, putative protein-protein interaction domains found in a range of proteins of diverse functions[27].

The biological activities of ß-catenin are not yet fully understood. In addition to a role in adhesion, ß-catenin is also involved in a signaling pathway during Xenopus development. Antibody-mediated perturbation[20] or injection of ß-catenin mRNA[8] induces axis duplication, while depletion of ß-catenin inhibits dorsal mesoderm induction, thus abrogating this axis specification[13]. These signaling properties of ß-catenin in the vertebrate embryo are not unexpected, given the firmly established participation of armadillo in the wingless cascade in Drosophila[23,29].

Thus the manipulation of ß-catenin levels in the Xenopus embryo affects axis specification. This likely occurs as a result of the perturbation of a signaling cascade initiated by products of the *Wnt* genes, vertebrate homologs of *wingless*. Wnt factors can induce axis duplication in Xenopus[21]. Vertebrate homologs of other components of the Wingless cascade are also implied in the Wnt dorsal induction pathway in the early *Xenopus* embryo. This has specifically been demonstrated for glycogen-synthase-kinase-3 (GSK-3), the vertebrate homolog of Zeste-white-3 kinase[12], and for vertebrate Dishevelled[32]. Thus, a Wingless/Wnt cascade appears to be conserved between *Drosophila* and vertebrates. It is currently unknown, however, how signaling through ß-catenin or Armadillo would affect the execution of downstream genetic programs.

Here we describe the in vitro and in vivo interactions between ß-catenin and a *Xenopus* member of the Tcf/Lef family of transcription factors, XTcf-3. Based on our data, we propose that the ß-catenin–XTcf-3 complex is responsible for activation of target genes in response of upstream (*e.g.* Wnt) signal that allow cytoplasmic ß-catenin to interact with XTcf-3.

METHODS

Cloning of XTcf-3

A stage-17 Xenopus embryo cDNA library in GT10[17] was screened at low stringency with murine *Tcf*-1[24] and *Lef*-1[33] cDNA probes according to standard procedures; 12 positive clones were subcloned into pBluescript SK and sequenced. RT–PCR was performed on cDNA prepared from two-cell stage embryos with the primers ATGAAG/AGAG/AATGC/AGG/A/TGCG/A/T/CAAT/GG and TCC/TCGT/CGCG/ACTG/CG/CG/AG/ACCG/AGGATA based on the highest conserved HMG box sequences of human and mouse *Tcf*-1 and *Lef*-1. The approximately 120 bp PCR products were subcloned and sequenced.

Two-Hybrid Screen

A fragment encoding amino acids 4–359 of human Tcf-1 was inserted into the bait vector pMD4 (a gift of Dr. L. van 't Veer). A mouse brain cDNA library in pGADRx was obtained from Dr. van 't Veer. A thymus cDNA library in pGAD10 was purchased from Clontech. pMD4-Tcf and the pertinent cDNA libraries were transformed into the HF7c yeast strain (Clontech). Plasmids were recovered from His+/LacZ+ clones, tested for the absence of interaction with irrelevant baits, and sequenced. Of 363 recovered cDNA clones, 40 were found to encode ß-catenin.

Northern Blotting

RNA isolation and Northern blot hybridizations were performed as described previously by Destrée et al., 1992[7] with a full-length *XTcf*-3 probe or a control actin probe.

In Situ Hybridization

Whole-mount in situ hybridization of albino Xenopus laevis embryos of different stages was performed as described elsewhere using antisense digoxigenin-labeled RNA[11]. The *XTcf*-3 cDNA in pBluescript SK was linearized with NcoI (base pair 1360 of cDNA sequence), resulting in a probe spanning the terminal 118 amino acids of the unique C-terminus, as well as 3' untranslated sequences. goosecoid cDNA was prepared as described previously[6]. Methods of egg collection, fertilization, microinjection, and culture of embryos have been described previously[9].

Whole-Mount Immunohistochemistry

Embryos were processed as described previously[1,38]. Myc-tagged ß-catenin[14] was detected using a monoclonal antibody (9E10; Santa Cruz Biotechnology). HA-tagged XTcf-3 was detected using the monoclonal 12CA5. As secondary antibody, Cy5-conjugated donkey anti-mouse antibody (Jackson Laboratory) was used. Nuclear staining was visualized using BO-PRO-3 (Molecular Probes). Embryos

were cleared and stored in 33% benzyl alcohol, 67% benzyl benzoate. Image analysis was performed on a Bio-Rad confocal laser scanning microscope.

Gel Retardation Analysis

XTcf-3, ß-catenin, and their derivatives were individually transcribed and translated from the pT7TS vector using T7 RNA polymerase in the Promega T7 TnT rabbit reticulocyte lysate–coupled transcription/translation system according to the instructions of the manufacturer. Gel retardation analysis was performed as described elsewhere[35]. In brief, the double-stranded gel retardation probe derived from the T cell receptor enhancer[33] (cccagagcTTCAAAGGgtgccctacttg, binding motif capitalized) was radiolabeled by T4 DNA kinase. Oligonucleotides were from Isogen (Amsterdam). For a typical binding reaction, the pertinent combinations of extracts (1 µl of each) and 1 µg of poly(dI–dC) were preincubated for 5 min at room temperature in 15 µl of 10 mM HEPES, 60 mM KCl, 1 mM EDTA, 1 mM DTT, and 12% glycerol. Probe (1 ng) was added and incubated for 20 min. The nondenaturing 4% polyacrylamide gel was run in 0.25× TBE at room temperature.

CAT Assays

Assay is described in detail elsewhere[35]. In short, 2 × 106 IIAI.6 B cells were transfected by electroporation with the various combinations of plasmids. CAT vectors were $(pTK(56)_7$ and $pTK(56Sac)_7$[35]. cDNAs encoding tagged versions of -catenin and XTcf-3 were inserted into the mammalian expression vector pCDNA (Invitrogen). After 48 hr, CAT values were determined as pristane/xylene-extractable, radiolabeled, butyrylated chloramphenicol.

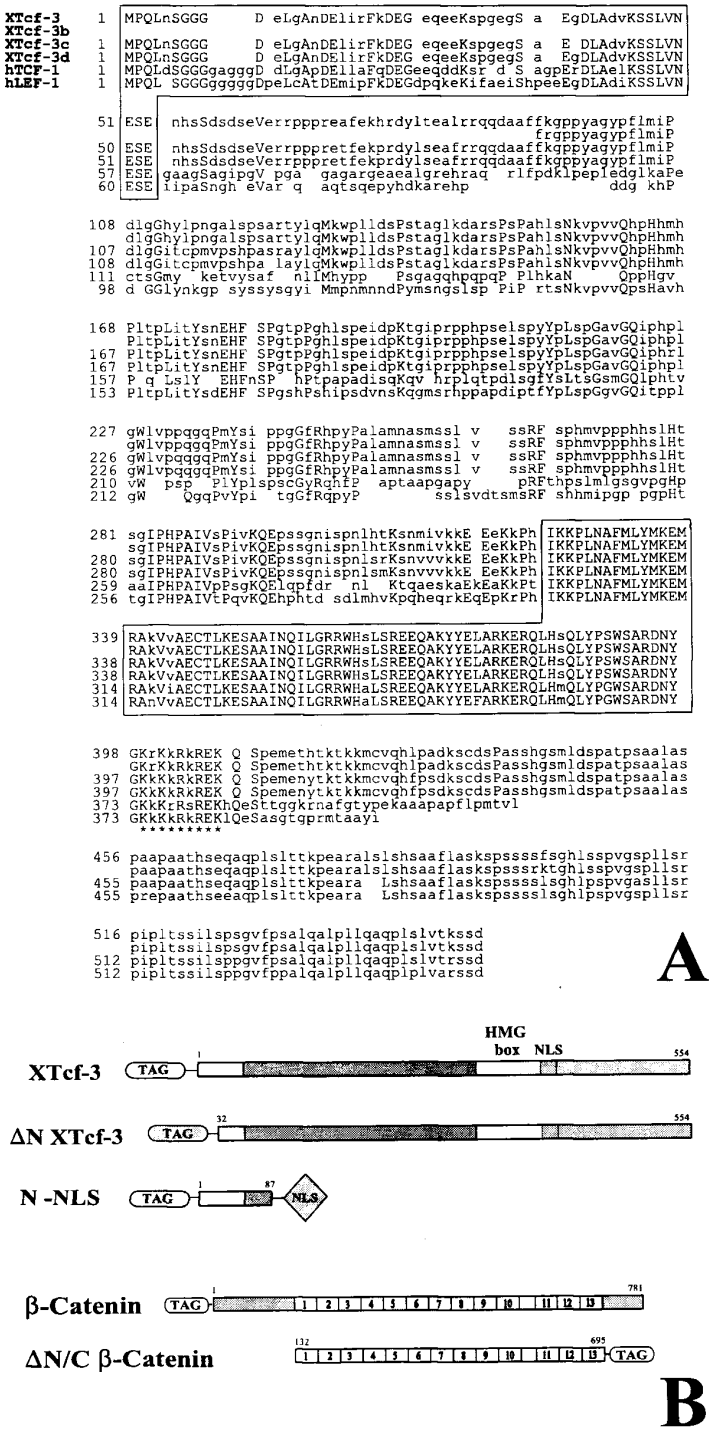

Figure 1. Four novel T*cf/Lef*-like genes expressed in *Xenopus* embryos.
(A) Four highly homologous gene products (*XTcf*-3, *XTcf*-3b, *XTcf*-3c, and *XTcf*-3d) were identified in a stage-17 embryonic cDNA library. These are compared with human *Tcf*-1 and *Lef*-1. The HMG domains are boxed, as is the conserved N-terminal region. The sequences diverge in the region C-terminal to the HMG box. A putative nuclear localization signal (NLS) overlaps with the C-terminus of the HMG box (asterisks)[30]. (B) XTcf-3 and ß-catenin constructs used in this study.

RESULTS

Identification of four Tcf/Lef homologs in Xenopus

Screening of a library of stage 17 Xenopus embryos with a *Tcf*-1/*Lef*-1 probe yielded 12 cDNA clones, which defined four virtually identical genes (figure 1A). These genes belonged to the *Tcf/Lef* family, but diverged from *Tcf*-1 and *Lef*-1 in the region C-terminal to the HMG box. Based on high similarity to mammalian *Tcf*-3 (V.K. and H.C., unpublished data), the Xenopus genes were termed *XTcf*-3, *XTcf*-3b, *XTcf*-3c, and *XTcf*-3d. Independently, 19 cDNA clones were obtained from two-cell stage embryos by reverse transcription-polymerase chain reaction (RT-PCR) using degenerate primers for the conserved *Tcf/Lef* HMG box region. All clones were derived from the *XTcf*-3 genes, which are identical in this region. This implied that the *XTcf*-3 genes represent the predominant maternally expressed *Tcf/Lef* family members in the early *Xenopus* embryo. *XTcf*-3 was arbitrarily chosen for further analysis.

The expression of *XTcf*-3 during *Xenopus* embryogenesis was first documented by Northern blot analysis (figure 2A). Given their virtual identity, expression of all four *XTcf*-3 genes was visualized simultaneously with the *XTcf*-3-derived probe. A prominent band of approximately 3.5 kb was observed in mRNA derived from unfertilized eggs and early cleavage stages, confirming the maternal expression of *XTcf*-3. The mRNA remained present during later cleavage stages and was also found after the mid blastula transition (MBT). By *in situ* hybridization (figures 2B and 2C), we observed *XTcf*-3 mRNA in the blastocoel roof in stage 7 embryos and in the marginal zone. Much lower levels of expression were observed in vegetal cells

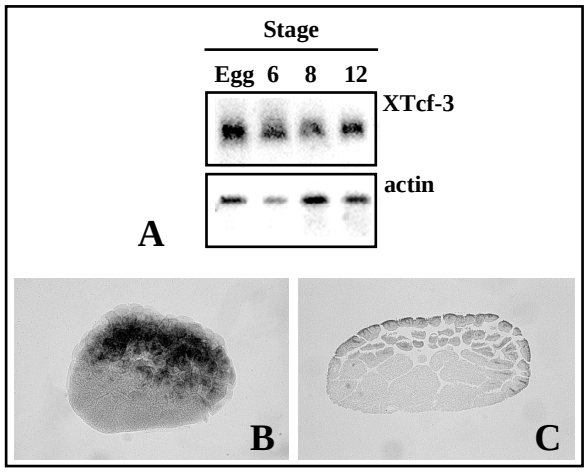

Figure 2. Expression pattern of *XTcf*-3 in early embryonic stages. (A) Northern blot analysis of RNA extracted from unfertilized eggs (lane 1) and embryos of the stages 6, 8, and 12. The blot was probed for the 3.5 kb *XTcf*-3 mRNA (top) and subsequently for actin (bottom). (B and C) In situ hybridization for *XTcf*-3 expression in early embryonic stages. (B) shows a stage-7 cleared embryo, viewed laterally. (C) shows a section of a stage-7 embryo.

of the embryo, which might relate to a general difficulty in visualizing mRNAs in this region by whole-mount *in situ* hybridization[31].

XTcf-3 binds to ß-catenin in vitro

In a yeast two-hybrid screen for proteins interacting with an N-terminal fragment of human Tcf-1, we retrieved multiple cDNA clones encoding ß-catenin. The shortest clone started at amino acid 252, while all clones encoded the complete C-terminus. Base on these findings, we wished to assess whether XTcf-3 could also physically interact with ß-catenin. In vitro translated XTcf-3 bound to a gel retardation probe containing the *Tcf/Lef* consensus motif (figure 3). In vitro translated ß-catenin bound to XTcf-3, resulting in a supershift. A deletion clone of ß-catenin that contained all the 13 Armadillo repeats, but lacked the unique N- and C-terminus, still bound to XTcf-3. A small deletion removing the N-terminal 31 amino acids of XTcf-3 (ΔN) did not interfere with DNA

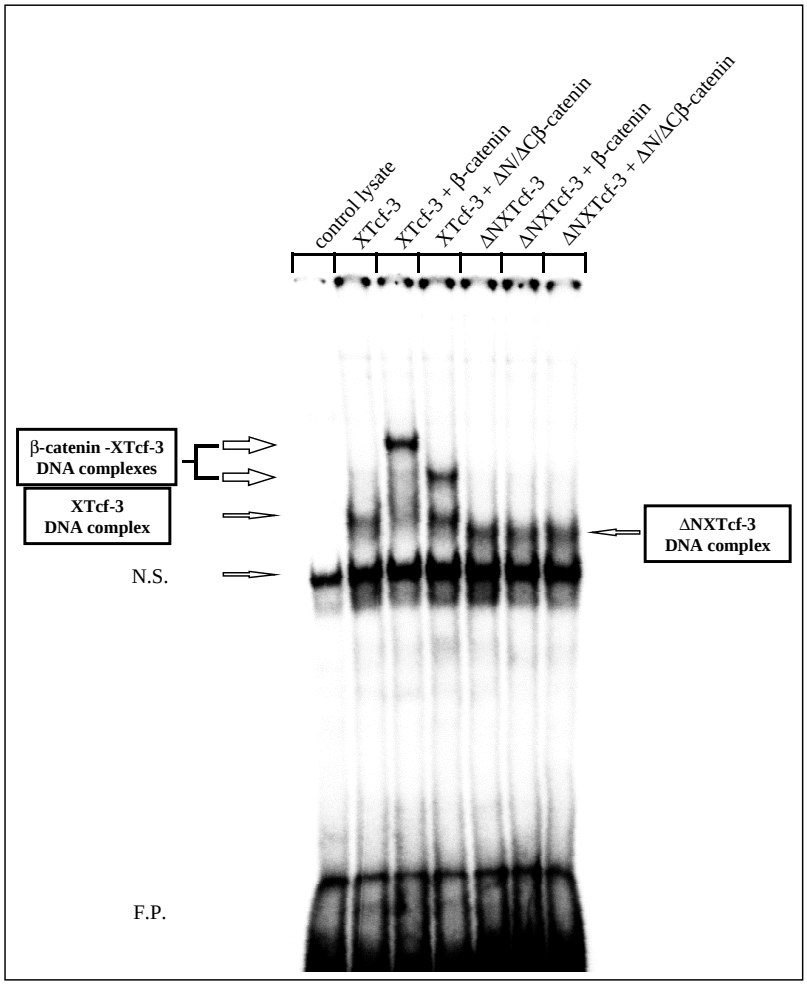

Figure 3. Physical interaction of *XTcf*-3 with ß-catenin.
In vitro translated XTcf-3 was subjected to gel retardation with an optimal *Tcf/Lef* probe (see Methods). A nonspecific band (N. S.) resulted from an endogenous protein in the transcription/translation lysate (lane 1). F. P., free probe. Cotranslated full-length ß-catenin and the deletion mutant N/C -catenin yielded supershifted bands (lanes 2–4). Deletion of the first 31 amino acids of XTcf-3 (ΔN) abrogated the interaction with ß-catenin (lanes 5–7).

binding but abrogated the ß-catenin interaction. The 60 N-terminal amino acids of Tcf/Lef proteins are conserved and likely constitute the interaction domain (figure 1A).

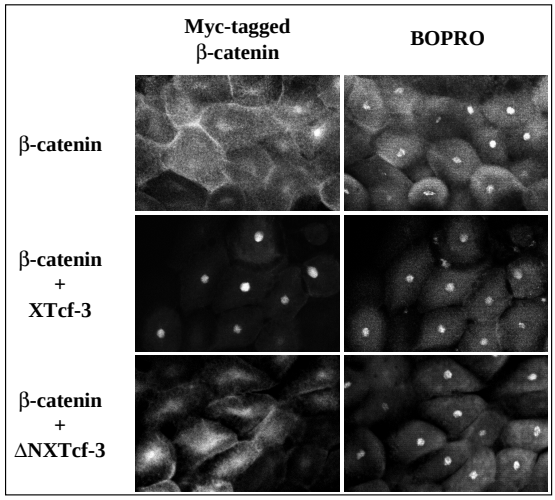

Figure 4. Nuclear translocation of injected ß-catenin by coinjected XTcf-3.
Stage-7 pre-MBT *Xenopus* embryos analyzed by whole-mount immunohistochemistry. Three optical sections (Z-step, 5 µm) were projected onto each other for each picture. At left are the following: Myc-tagged ß-catenin mRNA was injected alone (500 pg of RNA) (top) or in combination with 250 pg of RNA encoding wild-type *XTcf*-3 (middle) or ΔN (bottom). ß-catenin was visualized by staining for the Myc tag. Corresponding BO-PRO-3 nuclear staining is shown to the right.

XTcf-3 translocates ß-catenin to the nucleus

The interaction of XTcf-3 with ß-catenin in vivo was then analyzed by microinjection of mRNAs encoding epitope tagged versions of XTcf-3 and ß-catenin into *Xenopus* embryos. Like Tcf-1[5] and Lef-1[33], XTcf-3 and its deletion mutant ΔN accumulated in the nucleus (data not shown). Overexpressed, epitope-tagged human ß-catenin accumulated in the cytoplasm before MBT (figure 4), but was also occasionally seen in or near nuclei, as reported previously[8]. Coinjection with XTcf-3 resulted in translocation of the tagged ß-catenin to the nucleus in pre-MBT blastomeres. As expected, coinjection with ΔN (incapable of interacting with ß-catenin) did not result in nuclear translocation of ß-catenin. By contrast, coinjection of ß-catenin with a mRNA encoding amino acids 1-87 of XTcf-3 with a synthetic C-terminal nuclear localization signal (PKKKRKV; N-NLS in figure 1B) translocated ß-catenin to the nucleus (data not shown). The latter experiment mapped the interaction domain to the N-terminus of XTcf-3.

The ß-catenin-XTcf-3 complex activates transcription

In a transient reporter gene assay, we tested whether XTcf-3 was capable of activating transcription. To this end, we performed transfections with a CAT reporter plasmid containing a multimerized cognate motif for Tcf/Lef proteins ($pTK(56)_7$)[35]. A

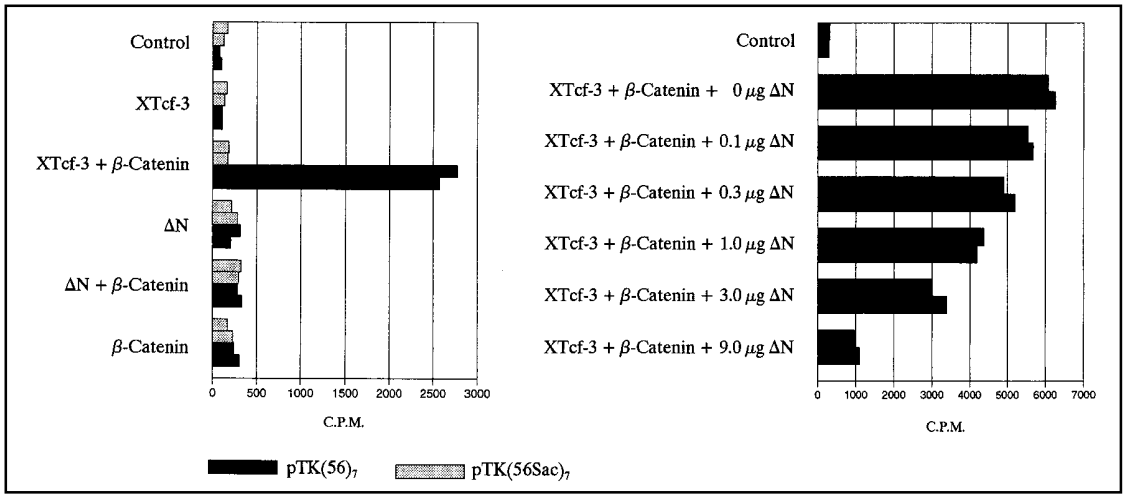

Figure 5. ß-catenin–XTcf-3 complex transactivates transcription.
Cells were transfected either with a CAT vector containing a minimal herpesvirus thymidine kinase promoter and an upstream concatamer of the *Tcf/Lef* cognate motif ($pTK(56)_7$) or with the negative control vector ($pTK(56Sac)_7$). Left, cotransfections were performed with the indicated plasmids: 1 µg of CAT reporter plasmid; 2 µg of XTcf-3 expression vectors; 4 µg of ß-catenin expression vector; and empty pCDNA vector as stuffer. Right, cotransfection of 1 µg of XTcf-3 with 4 µg of ß-catenin and the indicated amounts of ΔN expression plasmids; pCDNA was used as stuffer. Relative CAT activity is presented in counts per minute. Both values of duplicate transfections are given.

murine B lymphoid cell line was chosen because it lacks endogenous *Tcf/Lef* mRNAs and contains low levels of endogenous ß-catenin.

As observed previously for Tcf-1 and Lef-1 (see Introduction), XTcf-3 or ΔN alone did not activate transcription above background levels. However, cotransfection of XTcf-3 with ß-catenin potently activated transcription. No effect was seen when ΔN was cotransfected with ß-catenin. As expected, a control reporter CAT plasmid carrying mutated versions of the Tcf/Lef-binding motif ($pTK(56Sac)_7$)[35] always scored negative. A typical experiment is depicted in figure 5. Staining for XTcf-3 and ß-catenin in the transfected lymphocytes recapitulated the observations made by microinjected embryos: in brief, transfected ß-catenin was predominantly cytoplasmic, but was translocated to the nucleus upon cotransfection with intact XTcf-3. Again, the ΔN protein itself was nuclear, but it failed to transport ß-catenin to the nucleus (data not shown).

Thus, XTcf-3 and the ΔN protein in isolation accumulated in the nucleus, presumably bound to their cognate DNA motifs, but were transcriptionally inert. By contrast, the ß-catenin-XTcf-3 complex potently activated transcription. The ΔN mutant failed to interact with ß-catenin and consequently remained inert when cytoplasmic ß-

catenin was available. Cotransfection of increasing amounts of the ΔN mutant with activating amounts of ß-catenin and wild-type XTcf-3 in the CAT assay resulted in a progressive suppression of the induced transcriptional activation (figure 5). This indicated that the ΔN mutant could act as a dominant-negative mutant the proposed pathway, providing a tool for probing the function of XTcf-3 in vivo.

Table 1.ΔN suppresses ß-catenin-induced axis formation

RNA injected	Amount (pg)	Second axis (%)	n
ß-catenin ß-Gal	500 250	52	50
ß-catenin ΔN	500 250	0	50
ß-catenin ΔN ß-Gal	500 100 100	2	50
ß-catenin ΔN XTcf-3	500 100 100	25	48
Noggin ß-Gal	200 250	19	98
Noggin ΔN	200 250	35	100

Embryos were injected at the four-cell stage in the equatorial region of one ventral blastomere and screened for secondary axis induction at stages 18-22. Percentages include complete and partial secondary axis. n, number of embryos.

The ΔN Mutant of XTcf-3 Suppresses Axis Formation

We analyzed the in vivo function of XTcf-3 in the well-established axis formation assay in Xenopus embryos. Injection of full-length XTcf-3 RNA in Xenopus embryos did not lead to severe axial perturbations, nor did it facilitate axis duplication upon coinjection of suboptimal amounts of -catenin RNA (data not shown). This was likely due to the relatively large pools of endogenous XTcf-3, and suggested that the amount of endogenous XTcf-3 is not a limiting factor in axis specification.

As reported previously[8], injection of -catenin in the vegetal ventral region of early cleavage-stage embryos leads to axis duplication. We found that injection of 500 pg of -catenin RNA (together with 250 pg of control ß-galactosidase [ß-gal] RNA)

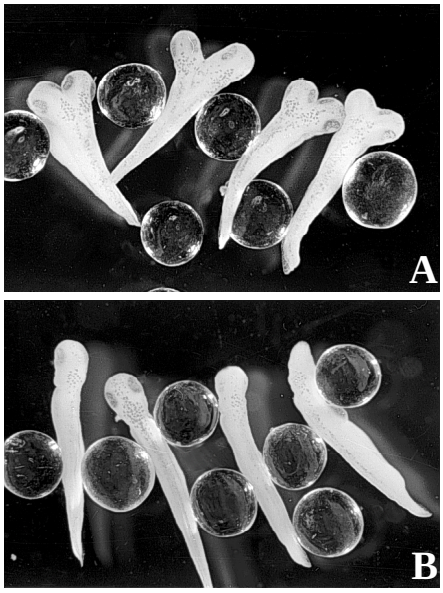

Figure 6. Suppression of ß-catenin-induced axis formation in *Xenopus* embryos
Stage-35 embryos injected at the four-cell stage in a single ventral blastomere with 250 pg of ß-catenin RNA alone (A) or in combination with 250 pg of ΔN RNA (B).

consistently induced axis duplication in >50% of the embryos (table 1; figure 6A). Coinjection with comparable amounts of XTcf-3-encoding RNA did not modify this ß-catenin effect (data not shown). However, ΔN was found to be a potent suppressor of ß-catenin-induced axis duplication (table 1; figure 6B). Coinjection of 500 pg of ß-catenin RNA with 250 pg of ΔN RNA strongly suppressed axis duplication and in most cases led to normal development of the embryos. The frequency of axis duplication was still reduced upon coinjection with 50 pg of ΔN RNA (data not shown). The ΔN-mediated suppression could partially be relieved by coinjection of wild-type XTcf-3 (table 1). Axis duplication induced by injection of *noggin* mRNA, which presumably acts in a parallel pathway, could not be suppressed by ΔN (table 1). This demonstrated the specificity of ΔN-mediated suppression.

Table 2. Equatorial injection of ΔN suppresses endogenous axis formation

Position	n	DAI	Percent
Animal	86	4/5	90
		1/2/3	8
		0	2
Equatorial	99	4/5	25
		1/2/3	8
		0	67
Vegetal	80	4/5	63
		1/2/3	21
		0	16

Embryos were injected at the four-cell stage in each dorsal blastomere with 250 pg ΔN RNA at the position indicated. The embryos were scored according to the standard dorso-anterior index scale (DAI[16]. A normal embryo is assigned DAI 5, while embryos lacking dorso-anterior structures are assigned DAI 0.

Depletion of ß-catenin suppresses endogenous axis formation[13]. To test the effects of our dominant-negative XTcf-3 mutant on endogenous axis formation, we injected ΔN RNA in both dorsal blastomeres of four-cell-stage embryos. This consistently led to failure of axis formation (table 2; figure 7). The effect was maximal when injections were performed in the equatorial region, much weaker when injected in the vegetal region, and virtually absent when injected in the animal region (table 2). ΔN-injected embryos developed normally through the cleavage and blastula stages and completed gastrulation in most cases. Subsequently, the embryos failed to form neural folds and at a sibling stage 27 lacked visible head–tail and dorsal–ventral axes (figure 7). A closed blastopore was present in these embryos, identifying an antero-posterior axis. In some cases, the presence of a vestigial dorsal fin (figure 7C) indicated a rudimentary dorsal–ventral axis. Histological sectioning of the embryos revealed no obvious notochord, somites, or neural tube, nor were any other clear structures (e.g., cement gland) present (figure 7B).

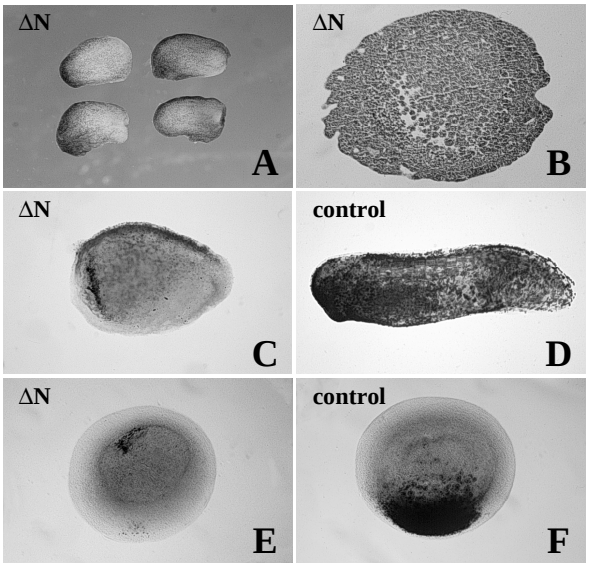

Figure 7. Suppression of endogenous axis formation.
(A–D) Embryos allowed to develop until sibling stage 27. Anterior is to the left. (A) shows phenotypes of embryos injected with 250 pg of ΔN RNA in both dorsal blastomeres at the four-cell stage. (B) shows a horizontal section of an embryo as in (A), with blastopore to the right. (C and D) Embryos injected with a combination of 250 pg of ΔN RNA and 250 pg of ß-gal RNA (C) or ß-gal RNA alone (D). (E and F) In situ hybridization with goosecoid on embryos injected with 250 pg of N (E) or 250 pg of -gal (F) analyzed at stage 11. Dorsal view of cleared embryos with the dorsal blastopore lip to the bottom. Some nonspecific staining occurs in the blastocoel cavity in (E).

Embryos showed a three tissue-layer configuration. A very short archenteron was found just inside the blastopore. In most embryos, including those with a rudimentary fin, the anterior ectoderm was thickened and showed disordered folding. The phenotype resembled that generated by reduction of ß-catenin levels reported by Heasman et al., 1994[13]. The expression of the dorsal marker goosecoid[6] was very low or absent in stage-11 embryos (figure 7E). Injection of equivalent amounts of ß-gal RNA had no effect on axis specification (figure 7D). Coinjection of ß-gal RNA with ΔN RNA and subsequent staining with X-Gal revealed that the injected cells actively participated in early embryonic morphogenetic events, but that proper axis specification did not occur (figure 7C).

DISCUSSION

The present study supports the notion that the novel Xenopus transcription factor XTcf-3 acts directly downstream of -catenin in embryonic axis specification. We originally found evidence for the interaction of Tcf/Lef transcription factors with ß-catenin in a yeast two-hybrid screen using human Tcf-1 as bait. Since the Xenopus embryo provides the best-characterized model system for vertebrate ß-catenin signaling, we studied the in vitro and in vivo interaction of ß-catenin with the maternally expressed Xenopus Tcf/Lef homolog, XTcf-3. The XTcf-3 protein binds to the consensus Tcf/Lef

DNA motif, while the N-terminus of XTcf-3 associates with the Armadillo repeat region of ß-catenin, as evidenced in a gel retardation assay. Microinjection of XTcf-3 mRNA reveals that the protein accumulates in the nucleus. ß-catenin, expressed in the absence of XTcf-3, is mostly cytoplasmic. Coexpression of ß-catenin with XTcf-3 results in translocation of ß-catenin to the nucleus. A small deletion of the N-terminus of XTcf-3 abrogates the in vitro association of ß-catenin, as well as the nuclear translocation of ß-catenin in injected Xenopus embryos.

XTcf-3 by itself binds DNA in vitro, but is inert in a transient reporter gene assay. By contrast, cotransfection of XTcf-3 with ß-catenin potently activates transcription. Again, N-terminal deletion of XTcf-3 abrogates the ß-catenin-dependent activation of transcription. Thus, the transcriptional activation by XTcf-3 is dependent on complex formation between XTcf-3 and ß-catenin. The ΔN deletion mutant of XTcf-3 represents a dominant-negative mutant in the proposed pathway, in that it suppresses the activation of transcription mediated by the ß-catenin–wild-type XTcf-3 complex.

The ΔN mutant provides a tool for probing the in vivo function of XTcf-3. ΔN suppresses the formation of an ectopic axis induced by microinjected ß-catenin, indicative of an inhibitory effect downstream in the ß-catenin pathway. In addition, injection of the XTcf-3 deletion mutant into the equatorial region of the two dorsal blastomeres of a four-cell stage embryo also suppresses endogenous axis specification. The phenotypes of the resulting embryos are very similar to those observed by Heasman et al., 1994[13], who depleted the endogenous ß-catenin pool by an antisense strategy or by overexpression of cadherins.

Based on these data, we propose the following model. Signaling through the wingless/Wnt pathway results in the generation of cytoplasmic ß-catenin in a form that allows the Armadillo repeat region to interact with the N-terminus of Tcf/Lef factors. XTcf-3 is the most likely candidate to mediate ß-catenin signaling in the Xenopus embryo, although we cannot formally rule out the involvement of other, yet to be cloned, factors from this family. The nuclear localization signal in XTcf-3 mediates the translocation of the ß-catenin–XTcf-3 complex to the nucleus. The HMG box of XTcf-3 binds in a sequence-specific fashion to the regulatory sequences of specific target genes. Other regions in the XTcf-3 protein might contribute to the specificity of target gene

selection. In our model, activation of transcription of target genes only occurs when XTcf-3 is complexed to ß-catenin. When no signals are transduced through the wingless/Wnt pathway, XTcf-3 (which is not complexed to ß-catenin in that situation) may also occupy the regulatory sites of the target genes, but will fail to activate transcription.

Embryonic axis formation initiates before major activation of the zygotic genome. Likely target genes of XTcf-3 are some of the earliest zygotic genes activated after MBT, which themselves are capable of inducing a secondary axis, e.g., siamois[18] or goosecoid[6]. It has been shown that both Xenopus[40] and zebrafish[15] goosecoid promoters can be activated in vivo by Wnt-1.

In conclusion, the current data place the products of the XTcf-3 genes directly downstream from ß-catenin and indicate the mechanism by which the axis-specification signal of ß-catenin is transduced to specific genes in the nucleus. The existence of multiple Tcf/Lef transcription factors and ß-catenin homologs implies that the type of interaction described in this study occurs in multiple developmental processes.

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Genbank accession numbers

The accession numbers for the XTcf-3 clones described in this paper are X99308, X99309, X99310, and X99311.

CHAPTER 3

DIFFERENTIAL EXPRESSION OF THE HMG BOX TRANSCRIPTION FACTORS XTCF-3 AND XLEF-1 DURING EARLY *XENOPUS* DEVELOPMENT.

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ABSTRACT

The recent discovery that the HMG box transcription factor XTCF-3 is involved in early axis specification in *Xenopus laevis*[10] led us to search for other members of the TCF/LEF family in this species. A newly identified HMG box factor was cloned with highest homology to human LEF-1, called XLEF-1. Unlike *XTcf*-3, *XLef*-1 is not expressed maternally, but its transcripts become detectable directly after the Mid Blastula Transition (MBT). At later stages, both genes are expressed in the central nervous system (CNS), eyes, otic vesicles, head mesenchyme, neural crest and derivatives, branchial arches, developing heart, tailbud and limb buds. The expression pattern of *Lef*-1 during later stages of development is evolutionarily conserved.

INTRODUCTION

The TCF/LEF family forms a small subfamily of vertebrate High Mobility Group (HMG) box transcription factor genes, including *Tcf*-1, *Lef*-1, *Tcf*-3 and *Tcf*-4[9,16,17,19]. TCF-1 and LEF-1 were originally defined as lymphoid-specific transcription factors, but were later found to be expressed in largely overlapping complex patterns during murine embryogenesis[13]. At embryonic day 7.5, expression of *Lef*-1 was observed in the posterior part, while *Tcf*-3 mRNA is present in the anterior part of the embryo[9]. At later stages, murine *Lef*-1 mRNA is found in the mesencephalon, the dermatome and in tissues that require epithelial-mesenchymal interactions (e.g. neural crest, branchial arches, limb buds)[13,18]. Members of the Tcf family function downstream of ß-catenin/armadillo in the WNT/wingless signaling pathway, both in normal development and in cancer (reviewed by Clevers and van de Wetering, 1997)[3]. Overexpression of dominant negative *XTcf*-3[10] or *Lef*-1[1,8] suppresses ß-catenin induced primary axis formation in *Xenopus* embryos. During these early stages of *Xenopus* development, only *XTcf*-3 was shown to be expressed[10]. WNTs play a role in many other aspects of development, *e.g.* of the central nervous system (CNS), neural crest, heart and limbs (reviewed by Moon et al., 1997; Cadigan and Nusse, 1998)[2,11]. To obtain a better insight in the possible roles of different TCF/LEF family members, during early vertebrate development, we compared the

expression patterns of *XTcf*-3 and of the newly identified *Xenopus* TCF/LEF family member, *XLef*-1.

METHODS

Cloning of XLef-1

An adult *Xenopus* brain cDNA library[15] was screened at low stringency with a mixture of degenerate cDNA probes (i.e. murine *Tcf*-1, *Lef*-1, *Tcf*-3 and *Tcf*-4, *dTcf* and *XTcf*-3) according to standard procedures. A Xenopus stage 30 library (R. Morgan, unpublished data) was screened at high stringency with the partial *XLef*-1 cDNA as a probe. The 5' most 135 bp were cloned by PCR using specific *XLef*-1 and anchor primers on stage 30 cDNA.

Embryo manipulation and microinjection

Methods of egg collection, fertilization and embryo culture were as described by Gao et al., 1994[5]. Developmental stages are assigned according to the normal table of *Xenopus laevis*[12].

Northern blotting

RNA isolation and Northern blot hybridization were performed as described by Destrée et al., 1992[4] with randomly labeled .probes not containing the HMG box sequences.

```
                10        20        30        40        50        60        70
XLef-1   MPQLSGaGGGnGvGGDPELCATDEMIPFKDEGDPQKEKIysEISnPEEEGDLADIKSSLVNEtEIIPsSN
hLef-1   MPQLSGgGGG-G-GGDPELCATDEMIPFKDEGDPQKEKIfaEIShPEEEGDLADIKSSLVNEsEIIPaSN

                80        90       100       110       120       130       140
XLef-1   sHEisRrrldS---YHEKsREHPeDaGKHPDGGLYsKGPSYtgYpsYIMMPNMNNePYMSNGSLSPPIPR
hLef-1   gHEvaRqaqtSqepYHEKaREHPdD-GKHPDGGLYnKGPSYssYsgYIMMPNMNNdPYMSNGSLSPPIPR

               150       160       170       180       190       200       210
XLef-1   TSNKVPVVQPSHAVHPLTPLITYSDEHFaPGvHPSHIPSDiNtKQGMhRHPqaPDlPTFYPmSPGsVGQm
hLef-1   TSNKVPVVQPSHAVHPLTPLITYSDEHFsPGsHPSHIPSDvNsKQGMsRHPphPDiPTFYPlSPGgVGQi

               220       230       240       250       260       270       280
XLef-1   TPPLGW----------------------------ypHHMvsGPPGPHaTGIPHPAIVnPQVKQEHPHnDn
hLef-1   TPPLGWqgqpvypitggfrqpypsslsvdtsmsrfsHHMipGPPGPHtTGIPHPAIVtPQVKQEHPHtDs

               290       300       310       320       330       340       350
XLef-1   DLMHmKPhHEQRKEQEPKRPH**IKKPLNAFMLYMKEMRANVVAECTLKESAAINQILGRRWHALSREEQsK**
hLef-1   DLMHvKPqHEQRKEQEPKRPH**IKKPLNAFMLYMKEMRANVVAECTLKESAAINQILGRRWHALSREEQaK**

               360       370       380       390       400
XLef-1   **YYElARKERQLHMQLYPGWSARDNY**GKKKKkKREKLQEStSGaGPRMTAAYI
hLef-1   **YYEfARKERQLHMQLYPGWSARDNY**GKKKKrKREKLQESaSGtGPRMTAAYI
```

Figure 1. Amino acid sequence alignment of a newly identified *Xenopus* HMG box factor XLEF-1 and human LEF-1. The HMG box is shown in bold.

In situ hybridization

Whole mount In situ hybridization of *Xenopus* embryos was performed as described by Harland, 1991[7] with slight modifications: after antibody incubation embryos were washed in MaNaT (100mM Maleic acid pH 7.5, 150mM NaCl, 0.1% Tween-20). Embryos were processed using a robot (Abimed[14]) and stained in BM Purple (Boerhinger Mannheim).

RESULTS AND DISCUSSION

By screening a *Xenopus* brain cDNA library with a mixture of degenerate probes we isolated a partial cDNA sequence with high homology to *hLef*-1, called *XLef*-1. The complete coding sequence for *XLef*-1 (figure 1) was obtained by screening a *Xenopus* stage 30 cDNA library using the partial *XLef*-1 cDNA as a probe (see Methods). Northern blot analysis (figure 2) revealed that *XTcf*-3 mRNA is present at a high level from the egg stage onward, while *XLef*-1 mRNA is detectable only after MBT with increasing levels during subsequent stages. At stage 9, both genes are expressed in the animal as well as the vegetal half. Whole mount *in situ* hybridization showed that maternal *XTcf*-3 mRNA is ubiquitously present in early embryos (figure 3A). As gastrulation proceeds, a strong zygotic expression appears in the anterior region of the late gastrula (similar to murine *Tcf*-3 mRNA at E7.5[9]). In contrast, *XLef*-1 mRNA is not detected before MBT nor in early gastrula, but is first seen at low levels in the anterior and dorsal regions of the late gastrula (figure 3B). Thus, in contrast to *XTcf*-3, *XLef*-1 is not expressed maternally, but its transcripts start to be detectable directly after the MBT.

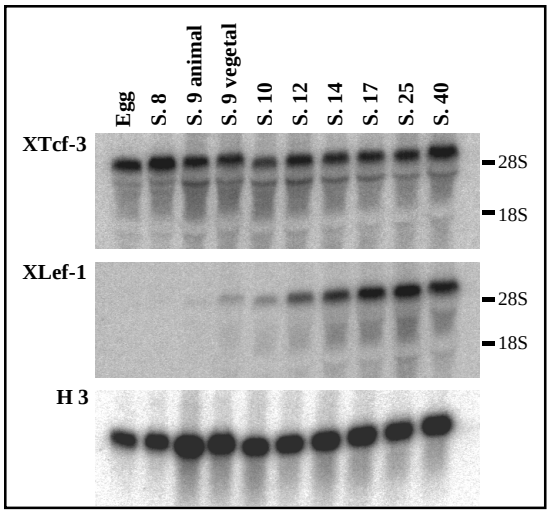

Figure 2. Northern blot analysis of different stages of *Xenopus* development

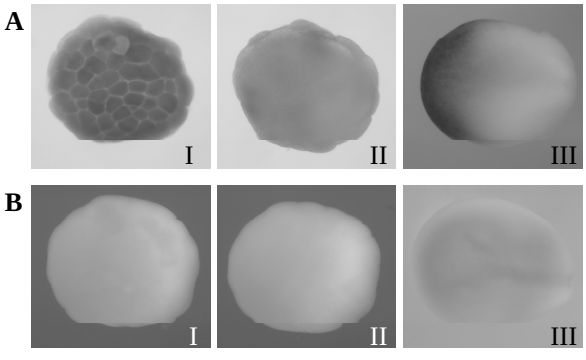

Figure 3. Whole mount *in situ* hybridization of blastula and gastrula stage embryos with antisense *XTcf*-3 (A) and *XLef*-1 (B) probes. Of stage 7 (I + II) both the animal (I) and vegetal (II) sides are shown. Stage 12½ (III) is viewed from the dorsal side.

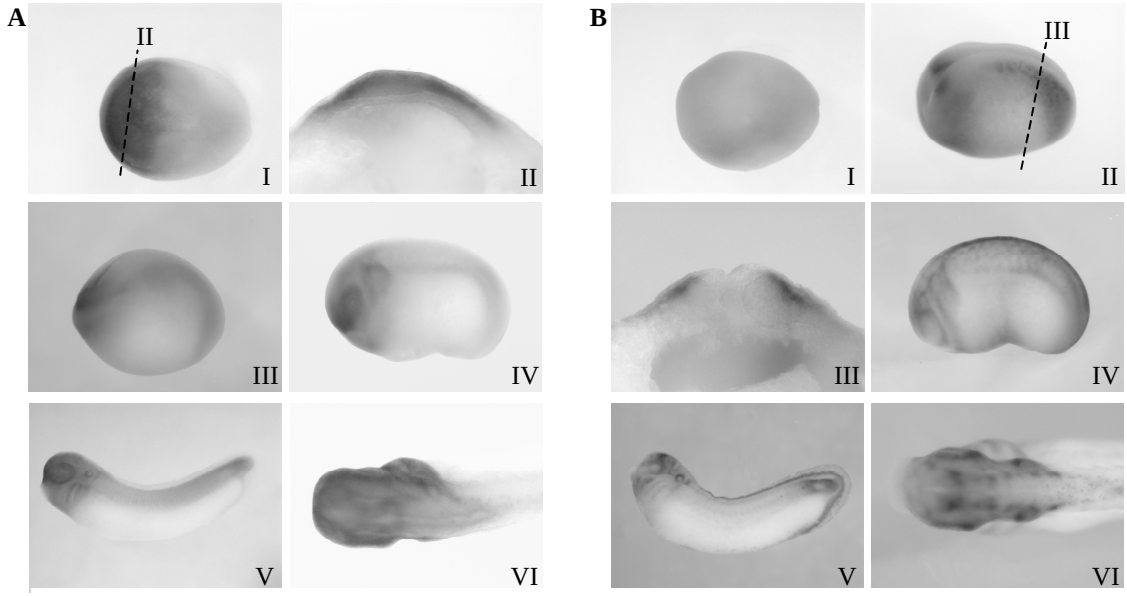

Figure 4. Whole mount *in situ* hybridization of neurula, tailbud and tadpole stage embryos with antisense *XTcf*-3 (A) and *XLef*-1 (B) probes. A: S.14 (I,II), S.18 (III), S.22 (IV), S.31 (V,VI). B: S.14 (I), S.16 (II,III), S.22 (IV), S.31 (V,VI).

In the early neurula, *XTcf*-3 mRNA is present in the mesoderm and, with a sharp posterior boundary, in the sensorial layer of the neurectoderm (figure 4A I, II). In the late neurula, anterior and posterior levels are similar (figure 4A III). At early tailbud stages, different regions with higher expression appear, which become more apparent during later stages (figure 4A IV-VI). High expression of *XTcf*-3 is seen in the CNS, the eyes, the otic vesicles, the head mesenchyme (*e.g.* above the cement gland) and the branchial arches (incl. the endoderm (figure 5A II)). Sectioning reveals expression of *XTcf*-3 in neural crest cells and their derivatives (not shown). Interestingly, a high expression is found in the tailbud, a region containing organizer like capacities [6].

The initial pattern of expression of *XLef*-1, *i.e.* in late gastrula stages, differs from that of *XTcf*-3 (figure 3). Later in development their patterns overlap, but *XLef*-1 mRNA is much more restricted (*e.g.* in the CNS, figure 4A,B VI). In the early neurula, weak expression is found in the dorsal mesoderm (figure 4B I).

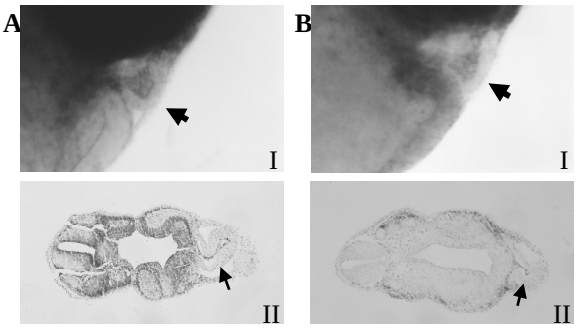

Figure 5. Expression of *XTcf*-3 (A) and *XLef*-1 (B) in the developing heart (arrows). Stage 33/34 embryos cleared in BABB (33% benzylalcohol, 67% benzylbenzoate) (I) and sectioned (II).

Higher levels of expression appear in the prospective mesencephalon, in the mesoderm of the prospective pharyngeal region and posterior in the intermediate mesoderm (figure 4B II, III). In tailbud stages, *XLef*-1 mRNA, compared to *XTcf*-3, is present at high levels in the neural crest extending to the posterior most end (including the crest cells which have migrated into the fin (figure 4B IV-VI)). Expression of *XLef*-1 in the branchial arches is less prominent than that of *XTcf*-3, since *XLef*-1 is not detected in the meso- and endodermal part (figure 5B II). An additional, more anterior, region of *XLef*-1 expression appears in the paraxial mesoderm of the tailbud, at stage 25 (not shown, compare figure 4B V).

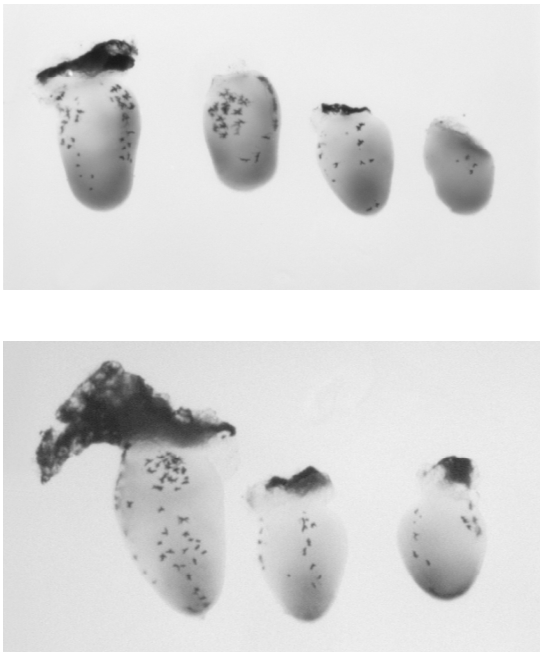

Figure 6. Dorsal view of *XTcf*-3 (A) and *XLef*-1 (B) expression in developing hind limb buds of stage 50-52 tadpoles.

Besides the patterns described above, later in development we find expression of both *XTcf*-3 and *XLef*-1 in the prospective endocard (figure 5) and both mRNAs are present in a graded fashion in isolated hind limb buds (figure 6).

Although no expression was detected for murine *Tcf*-3 after E10.5[9], we find a continuous and wide expression of *XTcf*-3 during *Xenopus* development. In contrast, the pattern of expression of *Lef*-1 in the *Xenopus* embryo is strikingly similar to that in the mouse (*e.g.* mesencephalon, neural crest, branchial arches and limbs).

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Genbank Accession number

The accession number for the coding sequence of XLef-1 described in this paper is AF063831.

CHAPTER 4

THE *XENOPUS* WNT EFFECTOR XTCF-3 INTERACTS WITH GROUCHO-RELATED TRANSCRIPTIONAL REPRESSORS

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ABSTRACT

Tcf/Lef transcription factors mediate signaling from Wingless/Wnt proteins by recruiting Armadillo/ß-catenin as a transcriptional co-activator[1,4,5,11,20,25,30]. However, studies of *Drosophila*, *Xenopus* and *Caenorhabditis elegans* have indicated that Tcf factors may also be transcriptional repressors[2,3,8,25,26,29]. Here we show that Tcf factors physically interact with members of the Groucho family of transcriptional repressors. In transient transfection assays, the *Xenopus* Groucho homologue XGrg-4 inhibited activation of transcription of synthetic Tcf reporter genes. In contrast, the naturally truncated Groucho-family member XGrg-5 enhanced transcriptional activation. Injection of *XGrg-4* into *Xenopus* embryos repressed transcription of *Siamois* and *Xnr-3*, endogenous targets of ß-catenin-Tcf. Dorsal injection of *XGrg-4* had a ventralizing effect on *Xenopus* embryos. Secondary-axis formation induced by a dominant-positive Armadillo-Tcf fusion protein was inhibited by XGrg-4 and enhanced by XGrg-5. These data indicate that expression of Tcf target genes is regulated by a balance between Armadillo and Groucho.

METHODS

Two-hybrid

Experiments were performed as described[20]. Plasmid pVA3 encodes a murine p53-Gal4 binding domain hybrid in pGBT9 (Clontech). Preys mGrg-5, XGrg-5, and ß-catenin were inserted in frame with the Gal4 activation domain in pGADGH (Clontech) or pGADRX (Stratagene). pTD1 encodes SV40 large T antigen in pGAD3F (Clontech). Baits and preys were transformed into the *Saccharomyces cerevisiae* strain HF7C (Clontech).

***In vitro* binding assays.**

Radiolabelled hTCF-1 and Armadillo were produced in the PROTEINscript kit (Ambion). Translated products were diluted in 0.5 ml ELB buffer (150 mM NaCl, 50 mM HEPES, pH 7.5, 5 mM EDTA, 0.1% NP40, 10 mM ß-glycerophosphate, 5 mM NaF, 1 mM PMSF, 10 mg ml^{-1} trypsin inhibitor, 20 U ml^{-1} aprotinin and 1 mM sodium orthovanadate). Amylose-sepharose beads loaded with MBP-Grg5 fusion protein or control MBP were incubated at 4 °C for 2 h. Washed beads were analysed by gel electrophoresis and autoradiography.

Cloning of *XGrg-5* and *XGrg-4*.

A *Xenopus* brain complementary DNA library in λgt11[20] was screened at low stringency with murine *Grg-5* cDNA probes according to standard procedures. *XGrg-5* was previously described as *XAES*[6] (GenBank accession number U18776). The accession number of *XGrg-4* is AJ224945.

COS cell transfections.

COS cells were transfected with *Tcf* and *XGrg* constructs in a ratio of 10:1 using standard diethyl aminoethyl (DEAE)-dextran transfection. After 24 h, cells were methanol-fixed and stained using an anti-Myc-tag antibody.

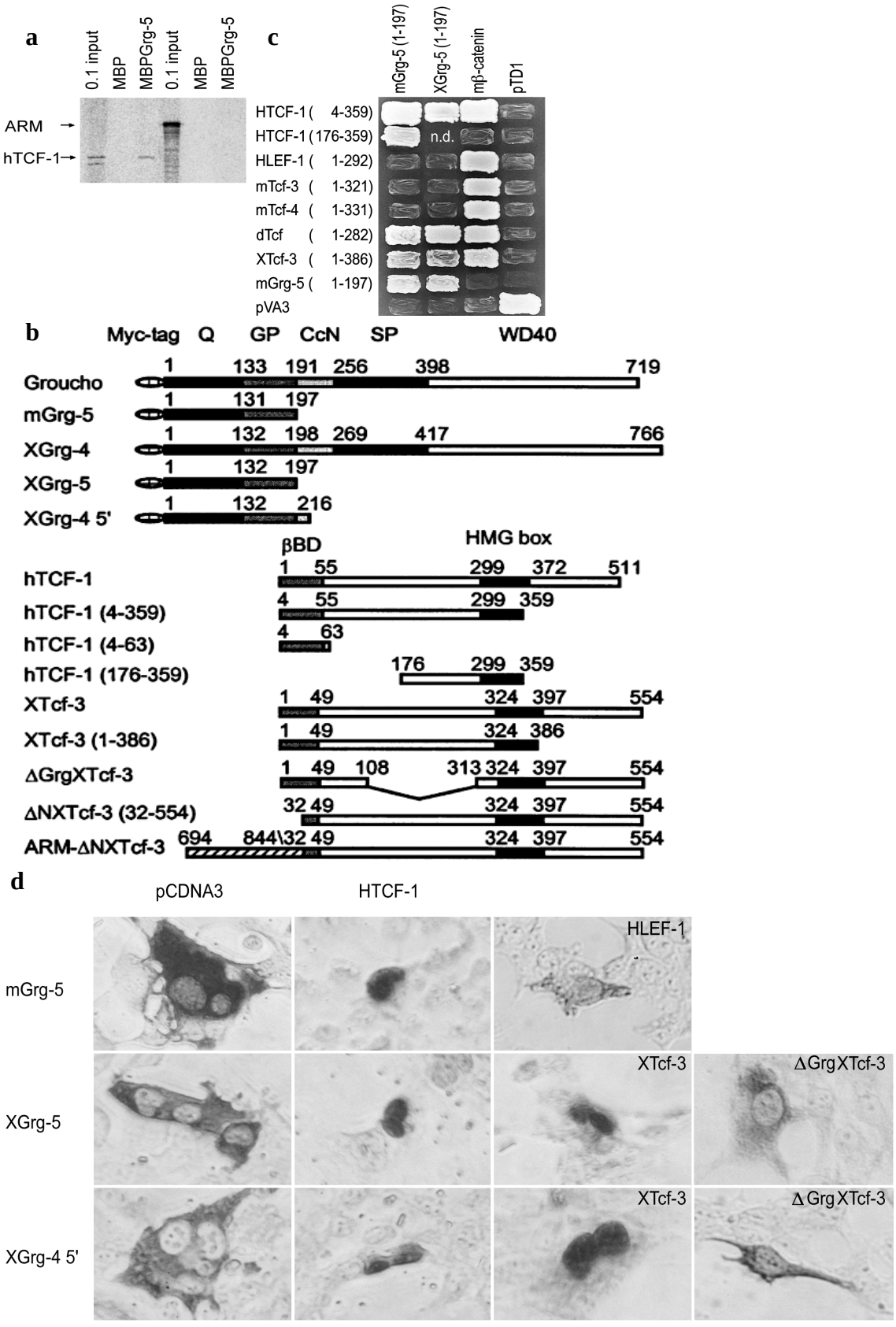
Luciferase assays and CAT assays.

2 x 10^6 IIAI.6 B cells were electroporated with a luciferase reporter plasmid containing three optimal Tcf sites upstream of the minimal HSV-TK promoter (1 µg pTKTOP) or its negative control containing mutated Tcf sites (pTKFOP), the internal transfection control (0.5 µg pSV40CAT), 20 µg *Tcf* expression vectors, and 0.5 or 5.0 µg Gro expression plasmids. For *XGrg-5* experiments, 0.5 µg *Arm* was used; for *XGrg-4* experiments, 5.0 µg pCDNA, was used as a stuffer when appropriate. cDNAs encoding tagged versions of *XTcf-3*, Δ*Grg-XTcf-3*, *XGrg-4* and *XGrg-5* were inserted into pCDNA3. Luciferase and CAT activities were separately determined 24 h after transfection as described[30].

Reverse transcription (RT)-PCR analysis.

Total RNA for detection of endogenous *Siamois* mRNA by RT-PCR was isolated from stage 10 embryos[20]. Oligo-d(T)-primed cDNA from total RNA was prepared using standard techniques. cDNA was quantified by PCR analysis for histone H4 and products were compared after 12, 14, 16, 18 and 20 cycles. The product after 20 cycles is shown in figure 3c. RT-PCR was carried out as described[16].

Figure 1. Physical interaction between Groucho (Gro)-related proteins and Tcf. **a**, *In vitro* binding assay for hTCF-1 and Grg-5. *In vitro*-transcribed and translated hTCF-1 binds to an MBP-Grg-5 fusion protein (lane 3), but not to control MBP protein (lane 2). *In vitro*-translated Armadillo does not bind to these MBP proteins (lane 5, 6). Input hTCF-1 protein is run in lane 1, and input Armadillo protein in lane 4. **b**, Domains of Gro and Tcf constructs. Like Gro, XGrg-4 contains five distinct domains: mGrg-5 and XGrg-5 contain only the Q and GP domains[18]. hTCF-1 and XTcf-3 contain a centrally located DNA-binding HMG box and the N-terminal ß-catenin-binding domain (ßBD). ΔGrg-XTcf-3 was constructed by deleting amino acids 109-312 of XTcf-3. A dominant-positive version of XTcf-3 (bottom) was created in which its N-terminus is replaced by the C-terminal transactivation domain of Arm (amino acids 694-844). **c**, Two-hybrid assay for the interaction of Tcfs, Grgs and ß-catenin. All tested Tcf-family members bind to ß-catenin. In contrast, only hTCF-1, dTcf, and XTcf-3 interact with Grg-5. The Grg5-interaction domain of Tcf proteins (amino acids 176-359) is separable from the domain that interacts with ß-catenin (amino acids 4-63)[15]. **d**, Tcf transports Gro to the nucleus. Tagged, truncated Gro proteins localize to the cytoplasm of COS cells. Introduction of hTCF-1 results in nuclear translocation of mGrg-5, XGrg-5 and XGrg-4 5'. In the same way, XTcf-3 interacts with XGrg-5 and XGrg-4 5'. ΔGrg-XTcf-3, however, did not cause nuclear translocation of XGrg-5 and XGrg-4 5'. Introduction of hLEF-1 does not result in nuclear translocation of mGrg-5.


a
0.1 input
MBP
MBPGrg-5
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MBP
MBPGrg-5
ARM
hTCF-1
c
mGrg-5 (1-197)
XGrg-5 (1-197)
mβ-catenin
pTD1
HTCF-1 (4-359)
HTCF-1 (176-359)
HLEF-1 (1-292)
mTcf-3 (1-321)
mTcf-4 (1-331)
dTcf (1-282)
XTcf-3 (1-386)
mGrg-5 (1-197)
pVA3
n.d.
b
Myc-tag Q GP CcN SP WD40
Groucho 1 133 191 256 398 719
mGrg-5 1 131 197
XGrg-4 1 132 198 269 417 766
XGrg-5 1 132 197
XGrg-4 5' 1 132 216
βBD
HMG box
hTCF-1 1 55 299 372 511
hTCF-1 (4-359) 4 55 299 359
hTCF-1 (4-63) 4 63
hTCF-1 (176-359) 176 299 359
XTcf-3 1 49 324 397 554
XTcf-3 (1-386) 1 49 324 386
ΔGrgXTcf-3 1 49 108 313 324 397 554
ΔNXTcf-3 (32-554) 32 49 324 397 554
ARM-ΔNXTcf-3 694 844\32 49 324 397 554
d
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XTcf-3
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RESULTS AND DISCUSSION

In our yeast two-hybrid screen for proteins interacting with human (h) TCF-1, which led to the identification of ß-catenin[20], roughly 60 out of 300 clones encoded the murine (Gro)-related gene *Grg-5*[18]. We confirmed independently that TCF-1 and Grg-5 interact in a binding assay using a recombinant maltose-binding protein (MBP)-Grg5 fusion protein and *in vitro*-translated hTCF-1(figure 1a).

Groucho (*Gro*) is a broadly expressed *Drosophila* corepressor, and may be involved in segmentation, sex determination and neurogenesis[7,10,22,23]. Hairy and Enhancer of Split-like (HES) helix-loop-helix factors interact with the non-DNA-binding Gro protein to repress transcription of their target genes[12,23]. In mammals, multiple homologues with a similar overall domain structure have been identified. These are termed *TLE 1-4* in man, and *mGrg-1*, *-3* and *-4* in mouse (figure 1b). *Grg-5* encodes only the two amino-terminal domains of these proteins (figure 1b). In a search for maternally expressed Gro proteins in *Xenopus*, we cloned *XGrg-5* (or *XAES*[6]) and a *Grg-4* orthologue (figure 1b). Both were abundantly and ubiquitously expressed in oocytes and in embryos undergoing the pre-mid-blastula transition (results not shown).

In yeast two-hybrid assays, we found that mGrg-5 and XGrg-5 interacted with *Drosophila* (d) Tcf, XTcf-3 and, as reported previously[24], with mGrg-5 itself (figure 1c), but not with hLEF-1, mTcf-3 and mTcf-4. Deletion analysis defined a minimal region in hTCF-1 (amino acids 176-359) that was capable of binding to Grg-5; this domain was separable from the Armadillo (Arm)-interaction domain (amino acids 4-63)[15]. No conclusive data were obtained in yeast for the interactions between Tcfs and the 'long' Gro homologues, which we attribute to transcriptional repression of the selection gene (histidine). To circumvent this problem, tagged N-terminal fragments of Gro proteins (collinear with full-length Grg-5; figure 1b) were expressed in COS cells. Although the full-length proteins were nuclear (not shown), these N-terminal fragments localized to the cytoplasm. Co-transfection of such truncated complementary DNA clones with various *Tcf* expression constructs allowed us to visualize interaction between Tcfs and the long Gro homologues by nuclear translocation. In this assay, hTCF-1 interacted with mGrg-5, XGrg-4 and XGrg-5, whereas XTcf-3 interacted with XGrg-4 and XGrg-5. Removal of

the Grg-interaction domain from XTcf-3 (figure 1b; ΔGrg-XTcf-3) abrogated the interaction of XTcf-3 with Gro homologues and nuclear translocation of the complexes (figure 1d).

In transient transfections using a previously established ß-catenin-Tcf reporter gene assay[30], we found that XGrg-4 repressed the activation of transcription by Arm and XTcf-3 (Arm-XTcf3 complexes) (figure 2a). The repression was specific, as we did not observe effects on the mutant Tcf reporter gene (figure 2a), nor on the co-transfected control chloramphenicol acetyltransferase (CAT) vector. In contrast, XGrg-5, which lacks the carboxy-terminal WD40 repeats of the longer Grg proteins[18], enhanced the transcriptional activity of suboptimal amounts of Arm-XTcf-3 complexes(figure 2b). mGrg-5 had no intrinsic transactivation properties when fused to a Gal4 DNA-binding domain (not shown). The enhancement of transcription by XGrg-5 could probably be attributed to its interference with the repressive effects of endogenous Gro proteins. We note that each line in a large and diverse cell panel expressed multiple *Grg/TLE* genes (H. B., J. R. and H. C., unpublished observations); the B-cell line used in our reporter assay

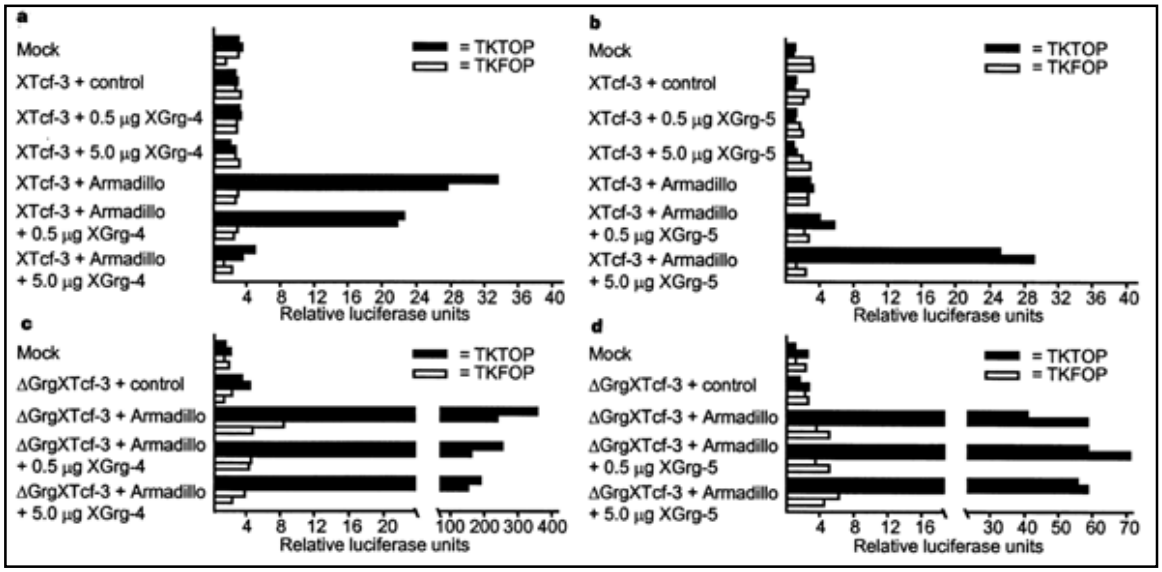

Figure 2. Gro represses Arm-Tcf-driven transactivation of a Tcf reporter. **a**, IIAI.6 B cells were transfected with optimal amounts of the indicated plasmids. XGrg-4 represses transactivation by Arm-XTcf-3. Tenfold transactivation is induced by 5 μg *Arm* plasmid. **b**, XGrg-5 enhances transcription in the presence of a suboptimal amount of Arm (0.5 μg). **c**, 5 μg *Arm* plasmid induces a 100-fold transactivation, with ΔGrg-XTcf-3 as an effector protein. The transactivation cannot be repressed by introduction of XGrg-4. **d**, XGrg-5 does not enhance transcription induced by ΔGrg-XTcf-3 and Arm (0.5 μg). In all figures, both values of duplicate transfections are given (corrected for transfection efficiency based on an internal CAT control). TKTOP, Tcf OPtimal reporter; TKFOP, Tcf Far-from-OPtimal reporter (see Methods).

expressed moderate levels of both *Grg-1* and *Grg-4* (results not shown). A deletion mutant of XTcf-3 that lacked the Grg-interaction domain was a tenfold more potent transcriptional activator than its wild-type counterpart (figure 2c), confirming the activity of endogenous co-repressors of Tcf factors in our assay. As expected, this ΔGrg-XTcf-3 mutant was not subject to repression by XGrg-4 or anti-repression by XGrg-5 (figure 2d).

We then determined whether the Gro-Tcf interaction was involved in the *in vivo* regulation of ß-catenin-Tcf target genes in *Xenopus* embryos. Tcf-binding sites in the *Siamois* promoter have been proposed to mediate ventral repression of this ß-catenin-

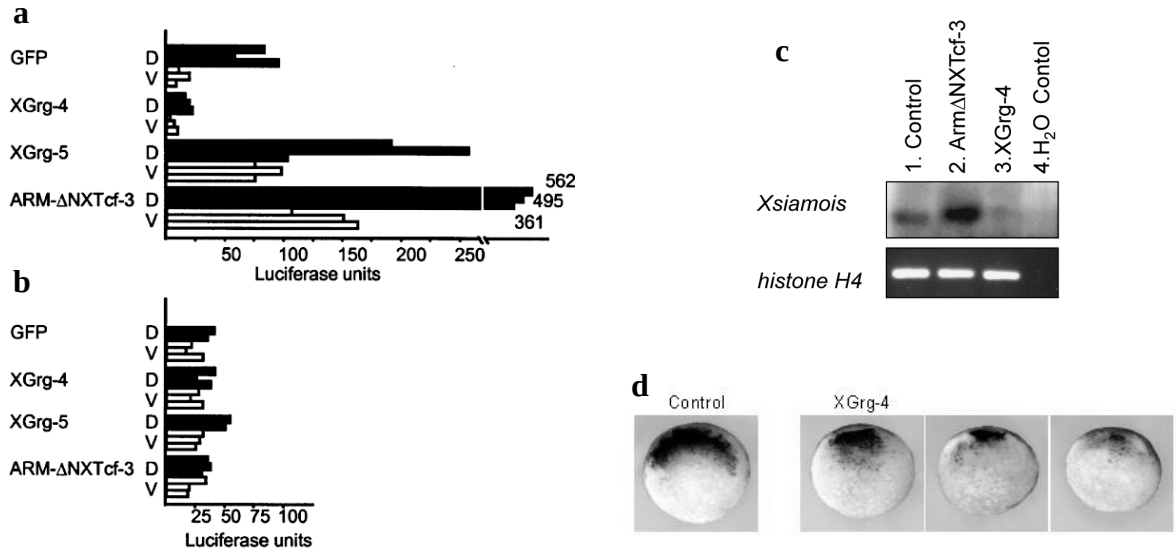

Figure 3 XGrg-4 represses *Siamois* promoter activity *in vivo*. **a**, **b**, For *Siamois* reporter assays two different constructs, S01234 and S24[3], were injected in the equatorial region of the two dorsal (D) or the two ventral (V) blastomeres at the four-cell stage. S01234 is a luciferase reporter construct consisting of 0.8 kilobases of the wild-type *Siamois* promoter, containing three Tcf consensus sites that are ß-catenin-responsive; these three sites are mutated in S24[3]. **a**, 265 pg S01234 were injected together with equimolar amounts of capped synthetic mRNA, encoding green fluorescent protein (GFP) (750 pg per embryo), ArmΔNXTcf-3 (500 pg), XGrg-4 (3,000 pg) or XGrg-5 (3,000 pg). Luciferase activities from two to three pools of five stage-10 embryos were determined for each combination. XGrg-4 represses dorsal *Siamois* promoter activation, whereas XGrg-5 enhances the activity. **b**, No effects are seen when the different RNAs are injected together with S24. **c**, RT-PCR analysis of *Siamois* expression. Embryos were injected at the four-cell stage in the equatorial region of each blastomere with either 500 pg *ArmΔNXTcf-3* (ventral) or 3,000 pg *XGrg-4* (dorsal). Twenty embryos were pooled for each data point. Dorsal injection of *XGrg-4* RNA results in a strong reduction of *Siamois* expression (lane 3), whereas *Siamois* expression is enhanced on injection of *ArmΔNXTcf-3* (lane 2). The amount of cDNA per sample was standardized for histone H4 expression (bottom). Nearly identical results were obtained in each of three independent experiments. Control embryos raised until stage 42 showed typical phenotypes (figure 4)[20]. **d**, *In situ* hybridization with *Xnr-3* for stage-9 embryos injected dorso-equatorially with 3,000 pg *XGrg-4* RNA at the four-cell stage. Left to right: non-injected embryo, in which expression of *Xnr-3* is seen at the future dorsal side, and three embryos injected with increasing amounts of *XGrg-4*, showing increasingly reduced levels of *Xnr-3* RNA.

regulated gene in *Xenopus* embryos[3,17]. When we injected this promoter into *Xenopus* embryos, we obtained results that were nearly identical to those obtained when expressing the synthetic reporters in mammalian cells: XGrg-4 repressed promoter activity, whereas XGrg-5 enhanced the activity (figure 3a). These effects depended on the presence of the three Tcf-binding sites in the promoter (figure 3b). We then studied the consequence of injection of *XGrg-4* on the expression of the endogenous *Siamois* gene. Because it is expressed at low levels, we used the semiquantitative polymerase chain reaction (PCR) to amplify *Siamois* RNA purified from pooled, XGrg4-injected embryos[16]. Injection of *XGrg-4* at the four-cell stage led to a strong reduction of transcription of the endogenous *Siamois* gene at stage 10, whereas injection of dominant-positive XTcf-3 (see below) enhanced transcription of *Siamois* (figure 3c).

Position	n	DAI	Percent
Animal	216	5	32
		4	60
		3	1
Equatorial	272	5	18
		4	37
		3	29
		2	1
		1	1
Vegetal	201	5	38
		4	50
		3	1

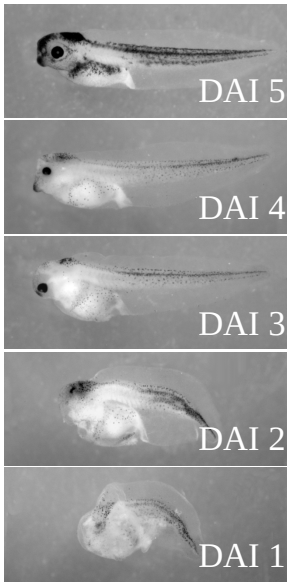

Figure 4. Dorsal injection of *XGrg-4* suppresses endogenous axis formation. Embryos were injected, at the four-cell stage, in each dorsal blastomere with 1,500 pg *XGrg-4* at the position indicated. The embryos were scored at stages 33-40 according to the standard dorso-anterior index scale (DAI[13]). A normal embryo is assigned DAI 5, whereas embryos lacking dorso-anterior structures are assigned DAI 0. A typical range of phenotypes found in one experiment is shown at the right. No effects were observed upon injection of *XGrg-5* or of control *GFP* or ß-galactosidase mRNA.

To test the effect of XGrg-4 on another direct target of XTcf-3, *Xnr-3*[19], we performed whole-mount *in situ* hybridization[9] on stage 9 embryos. As expected, dorsal injections of *XGrg-4* RNA at the four-cell stage markedly reduced the *Xnr-3* signal (figure 3d).

We have shown previously that XTcf-3 in *Xenopus* embryos mediates the axis-inducing ß-catenin signal[20]. As XGrg-4 and XGrg-5 both interact with XTcf-3 and are expressed maternally, we analysed the effect of dorsal injections of these two proteins on axis formation. Injection of *XGrg-4* inhibited formation of the endogenous axis, resulting in ventralization of the embryos, whereas *XGrg-5* had no effect. Effects were strongest after injection in the equatorial region of the two dorsal blastomeres of four-cell-stage embryos. Phenotypes ranged from complete lack of a head in combination with a shortened tail, to microcephaly and cyclopia (figure 4).

We also tested the effects of Gro on axis duplication. To avoid perturbations of pools of endogenous Wnt cascade components, we designed a dominant-positive version of XTcf-3 in which the N terminus is replaced by the C-terminal transactivation domain of Arm[30] (figure 1b). Although ventrally injected *XTcf-3* is essentially inert and Δ*N-XTcf-3* is a potent dominant-negative mutant[20], the chimaeric protein potently induced secondary axes (table 1). This showed that recruitment of the transactivating C terminus of Arm to Tcf sites constitutes the primary nuclear event upon signalling. Injection of the chimaeric protein with *XGrg-4* inhibited this activity, but injection of the chimaera with *XGrg-5* potentiated the activity (table 1). This result concurred with the repressive effects of XGrg-4 and the enhancing effects of XGrg-5 on transcription in mammalian cells. Ventral injections of *XGrg-5* RNA alone had no effect on axis duplication (results not shown), indicating that derepression alone is not sufficient

Table 1. XGrg-4 suppresses, XGrg-5 potentiates axis duplication by Arm

RNA injected (pg)	Incomplete secondary axis (%)	Secondary axis incl. eye and cement gland (%)	Total number of embryos injected
500 Arm-ΔNXTcf-3 + 1,500 XGrg-4	17	0	334
500 Arm-ΔNXTcf-3 + 375 GFP	40	18	357
25 Arm-ΔNXTcf-3 + 1,000 XGrg-5	25	4	408
25 Arm-ΔNXTcf-3 + 1,000 GFP	9	2	384
100 XNoggin + 1,500 XGrg-4	46	6	282
100 XNoggin + 375 GFP	46	8	269

Embryos were injected at the four-cell stage in the equatorial region of one ventral blastomere and screened for secondary axis induction at stages 25-30. As a negative control, equimolar amounts of green fluorescent protein (GFP) mRNA were injected.

for the biological effect. Axis duplication induced by injection of *noggin* messenger RNA[28] could not be blocked by *XGrg-4* (table 1).

We propose that the transcription of Tcf target genes is the result of a balance between the constitutive, repressive effects mediated by Gro (possibly counteracted by Grg5-like proteins) and the activating effects of Arm. In non-signalling cells, the repressive activities will dominate. Following Wnt activation, ß-catenin will associate with Tcfs and will activate transcription of genes such as *Xnr-3* and *Siamois* in *Xenopus*, or *Engrailed* and *Ubx* in flies. The active repression by Gro secures tight control over the Wingless/Wnt-driven developmental decisions. The dual activities of Tcf factors may explain the puzzling observation that mutation of the *C. elegans Tcf* homologue *Pop-1* has opposite effects on E versus MS-cell specification to those resulting from mutation of Wnt and Arm[8]. Pop-1 probably functions mainly as a repressor of target gene transcription. Our results predict a role for Gro in Pop1-controlled cell-fate decisions.

Constitutive activation of Tcf-mediated transcription occurs in melanoma and colon carcinoma after loss of APC or gain-of-function mutations in ß-catenin[14,21,27]. As Gro proteins repress Tcf activity, it will be interesting to study the status of *TLE* genes in human cancers in which Tcf transcription is inappropriately activated.

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CHAPTER 5

DIFFERENTIAL EXPRESSION OF THE GROUCHO RELATED GENES 4 AND 5 DURING EARLY DEVELOPMENT OF *XENOPUS LAEVIS*

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ABSTRACT

Recently, we demonstrated that the *Xenopus* Wnt effector XTcf-3 interacts with Groucho-related transcriptional repressors[13]. A long form of the Groucho-related genes, *XGrg*-4, was shown to repress axis formation in the *Xenopus* embryo, whereas a short form, *XGrg*-5, acted as a potentiator.

In this study, the temporal and spatial expression of XG*rg-4* and *XGrg-5* is described in *Xenopus laevis* embryos. Both genes are maternally expressed. In the gastrula, transcripts of both genes are present in the animal as well as the vegetal region. At later stages, *XGrg-4* and *XGrg-5* show specific patterns of expression in the central nervous system (CNS), cranial ganglia, eyes, otic vesicles, stomodeal-hypophyseal anlage, cement gland, head mesenchyme, branchial arches, neural crest and derivatives, somites, pronephros, pronephric duct, heart and tailbud. Differences in the expression of *XGrg-4* and *XGrg-5* were found in the CNS, cranial ganglia, olfactory placodes, stomodeal-pharyngeal anlage, cement gland, head mesenchyme and ectoderm.

INTRODUCTION

Vertebrate homologues of *Drosophila Groucho* (*Gro*)[7] were isolated from human[16], mouse[8-11], rat[14], *Xenopus*[1,13] and zebrafish[17]. A naturally truncated form, termed Grg-5, consists of the two amino-terminal domains of Gro, and is expressed in man[11] mouse[11], rat[14] and *Xenopus*[1,13]. The developmental expression of *Gro*-related genes in vertebrates was studied by Northern blot[1,9,11,16,17] and/or *in situ* hybridization [3,8-10,17,18].

Xenopus Grg-4 and Grg-5 both interact with the *Xenopus* member of the Tcf family of transcription factors, XTcf-3[13]. XGrg-4 represses both Armadillo-XTcf-3-driven transcriptional activation of a Tcf reporter and Arm-XTcf-3-mediated axis duplication. In contrast, XGrg-5 de-represses a Tcf reporter and potentiates Arm-XTcf-3-mediated secondary axis formation. To obtain a better insight in the possible roles of different Grg family members during the earliest stages of vertebrate development, we studied the expression patterns of *XGrg-4* and *XGrg-5* by Northern blot and whole mount *in situ* hybridization.

METHODS

XGrg-4 and *XGrg*-5 cDNAs and probes.

A X*enopus* brain cDNA library in λgt11[15] was screened at low stringency with murine *Grg-4* and *Grg-5* cDNA probes as described by Roose et al., 1998[13]. *XGrg*-5 was previously described as XAES by Choudhury et al. (1997)[1], (GenBank accession number U18776). *XGrg-4* encodes full length XGrg-4 and is identical to the partial cDNA described as XESG2 by Choudhury et al. (1997)[1]. The accession number for *XGrg-4* is AJ224945. For Figs as shown we used a *XGrg*-4 probe covering nucleotides –270 to + 650, and for *XGrg*-5, +105 to + 368.

Northern blotting

RNA isolation and Northern blot hybridization were performed as described by Destrée et al. (1992)[4].

Embryo manipulation and *in situ* hybridization

Albino embryos were cultured and collected as described by Molenaar et al. (1998)[12]. The *XGrg*-4 and *XGrg*-5 cDNA's were used to generate digoxigenin labeled antisense RNA probes. The whole mount ISH procedure was adapted and modified from Harland (1991)[6].

RESULTS AND DISCUSSION

Both *XGrg-4* and *XGrg-5* are maternally expressed, as shown before by Choudhury et al. (1997)[1], and transcripts of both genes are present throughout early

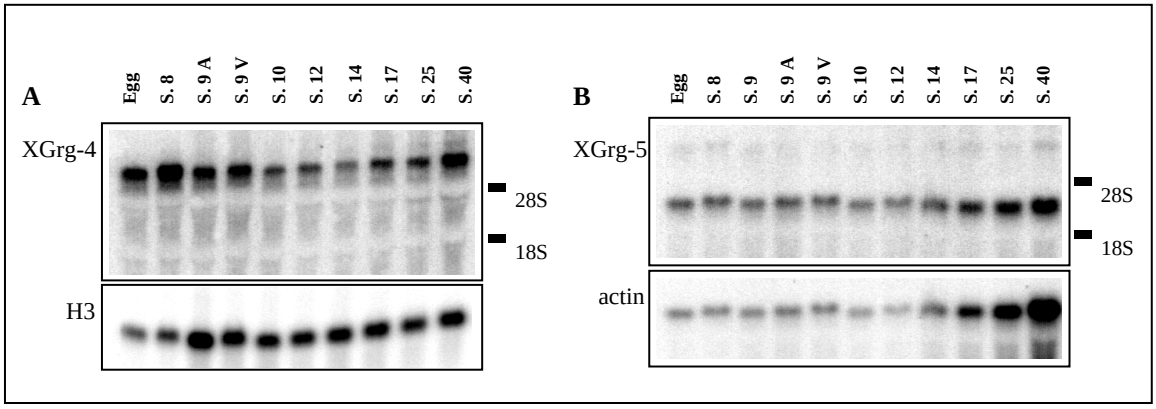

Fig 1. Northern blot analysis of *XGrg-4* and *X-Grg-5* transcripts in *Xenopus laevis* embryos at different stages. (A) *XGrg-4* and histone H3 control, (B) *XGrg-5* and actin control. Expression of XGrg-4 and XGrg-5 is found both in the animal (A) and the vegetal (V) parts of stage 9 embryos.

development (figure 1). Whole mount *in situ* hybridization shows that at the end of gastrulation, relatively high levels of *XGrg-4* and *XGrg-5* are present in the prospective neural plate region (figure 2). Posteriorly, *XGrg-5* expression is restricted to two sharp lines along the midline (figure 2).

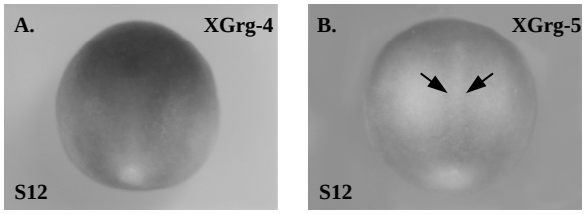

Fig 2. Whole mount *in situ* hybridization of gastrula stage embryos with antisense *XGrg-4* and *XGrg-5* probes. Dorsal views of stage 12 embryos (A, B). *XGrg-5* expression along the midline is indicated by arrows (B).

In neurula stages, *XGrg-4* and *XGrg-5* transcript levels remain highest in the sensorial layer of the neurectoderm (figure 3A-E and sections, not shown), decreasing from anterior to posterior. A broad transverse band of high *XGrg*-4 expression is seen just anterior to the border of the neural groove (figure 3A, B). Sections demonstrate that this staining is localized in the anterior region of the entoderm, in a thin layer of adjacent mesenchyme and in the ectoderm; an area corresponding to the stomodeal-hypophyseal anlage (figure 3C). Within the anterior neural plate relatively high expression of *XGrg-4* is detected in localized areas, i.e. anteriorly in the floor of the neural groove (figure 3A, B), at the level of the presumptive pros-mesencephalic boundary and in the anterior

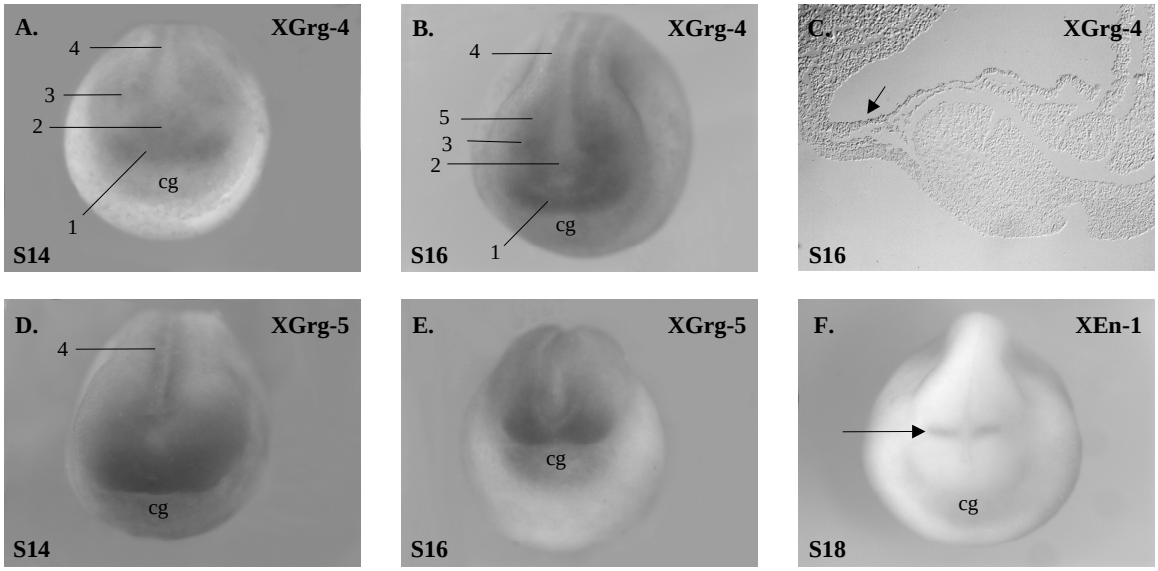

Fig 3. *XGrg*-4 and *XGrg*-5 expression in neurula stages. A, B, D, E and F: antero-dorsal views. C: Sagittal section of a stage 16 embryo. F: *XEn*-1 marks the mid-hindbrain border region. cg, cement gland, (1) stomato-hypophyseal anlage, (2) anterior neural plate, (3) prospective pros-mesencephalic boundary, (4) rim along the neural groove, (5) prospective anterior rhombencephalon.

prospective rhombencephalon (figure 3A, B; cf Eagleson and Harris (1990)[5]). A speckled pattern of *XGrg-4* expression in the epidermal ectoderm, appears in stage 14 embryos (figure3A and figure 4) and disappears at stage 35. This might represent expression in precursors of ciliated epidermal cells[2]. *XGrg-4* and *XGrg-5* are expressed in the anlage of the cement gland at stage 14 (figure 3A, B). *XGrg-5* transcripts are mainly located in the area of the prospective telencephalon and between the eye anlagen.

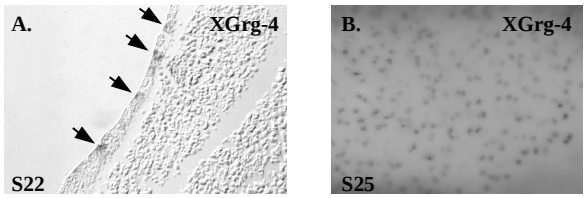

Fig 4. Expression of *XGrg*-4 in the ectoderm. Speckled expression of *XGrg*-4 in the ectoderm in stage 25 (B) and transverse section of a stage 22 embryo (A).

At stage 22, *XGrg-4* and *XGrg-5* transcripts are abundantly present in the eye vesicles, the ear placodes and the prospective branchial arches (figure 5). In sections, intensive staining is also observed in cells between the olfactory placodes, in the lateral mesenchyme of the branchial arches and around the optic vesicles and cement gland (not shown). *XGrg-4* and *XGrg-5* transcripts are present in the pronephric anlage, the prospective pronephric duct region, the presomitic mesoderm and the somites (figure 5). Pronounced differences between *XGrg-4* and *XGrg-5* expression are found in the cement

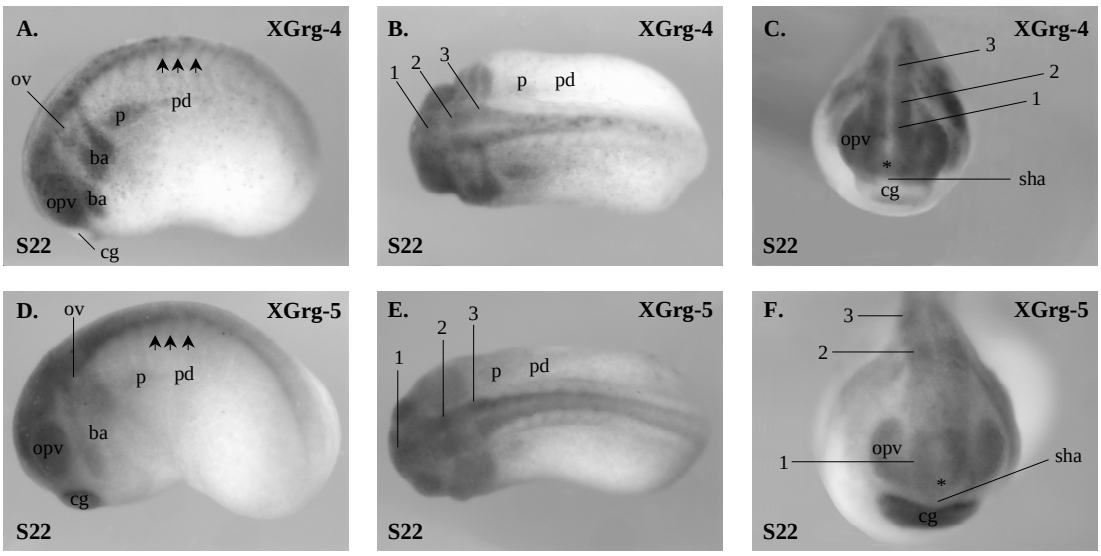

Fig 5. Localization of *XGrg-4* and *XGrg-5* transcripts in early tailbud stage (22). A, B, C: High *XGrg-4* expression at the pros-mesencephalic boundary (1), at the mes-rhombencephalic boundary (2) and in the rhombencephalon (3). D, E, F: High *XGrg-5* expression in the prosencephalon (1), in the anterior rhombencephalon (2) and in the posterior rhombencephalon (3). Arrows in A and D: somites. Asterisks in C and F: mesenchyme between the prospective olfactory placodes. ba, branchial arches; opv, optic vesicle; ov, otic vesicle; p, pronephros; pd, pronephric duct; sha, stomodeal-hypophyseal anlage. Embryos are viewed from lateral (A, D), dorsal (B, E) or anterior (C, F).

gland, the head mesenchyme, the brain, the spinal cord and the ectoderm. In the developing brain, *XGrg-4* and *XGrg*-5 transcripts are present throughout; *XGrg-4* is most abundant in the mesencephalon and posterior rhombencephalon, while *XGrg-5* is more pronounced in the prosencephalon and and two distinctive bands in the rhombencephalon (figure 5B-F). High *XGrg-4* expression is detected at the pros-mesencephalic and mes-rhombencephalic boundaries (figure 5B, C). In the spinal cord, both *XGrg-4* and *XGrg-5* are expressed in an anterior to posterior decreasing gradient with *XGrg-4* in an asymmetric, scattered pattern (figure 5B, E).

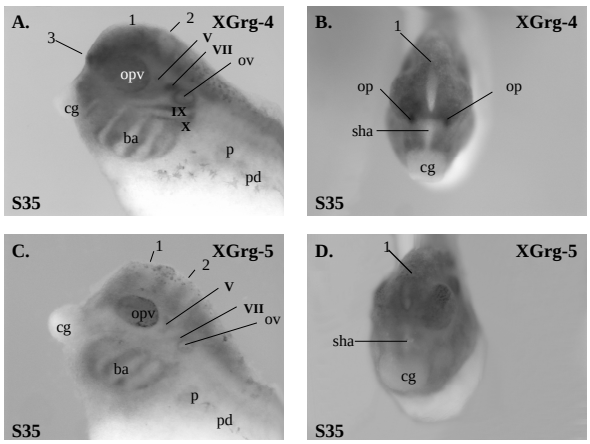

Fig 6. Localization of *XGrg-4* and *XGrg-5* expression at tailbud stage 35. A, B: *XGrg-4*. C, D: *XGrg-5*. (1) Pros-mesencephalic boundary, (2) mes-rhombencephalic boundary. V-X: cephalic ganglia V-X. Embryos are viewed from lateral (A, C) or anterior (B, D).

At stage 25, *XGrg-4* and *XGrg-5* expression patterns are similar to those at stage 22. In the cement gland, expression of *XGrg-5* is high, while *XGrg-4* RNA is detected only in the basal region (not shown). A strong signal of *XGrg-5* expression is observed at the pros-mesencephalic boundary. At later tailbud stages, *XGrg-5* is strongly expressed in the keel of the foregut (sections, not shown). In the brain, expression of *XGrg-4* and *XGrg-5* is very dynamic. Both *XGrg-4* and *XGrg-5* are weakly expressed in the epiphysis (sections not shown) and *XGrg-5* in the infundibulum (not shown).

At stage 35, *XGrg-4* and *XGrg-5* expression is high in the future choroid plexus of the telencephalon and in the ependymal layer (sections, not shown). In the brain, both *XGrg-4* and *XGrg-5* expression is highest laterally. Throughout the neural tube, *XGrg-4* and *XGrg-5* are expressed in a dorsal to ventral gradient (sections not shown). In the canalis neurentericus, *XGrg-4* has a scattered expression, while XGrg-5 RNA is evenly distributed. *XGrg-4* and *XGrg-5* display a similar expression pattern in the developing eye: in the prospective ganglion cell layer, the ciliary marginal zone and the lens (sections, not shown). At stage 30, *XGrg-4* expression is found in cephalic ganglia V and VII (not shown) and in stage 35, also IX and X (figure 6A). *XGrg-5* is only weakly expressed in ganglia V and VII (figure 6C). Both *XGrg-4* and *XGrg-5* are expressed in

the future endocardium and pericardium, while *XGrg-5* is additionally expressed in the future myocardium (sections not shown).

Both in mouse and *Xenopus*, *Grg-5*[10] is expressed in the heart and foregut. While *mGrg*-5 is expressed in the liver primordium and the ventral spinal cord floor of the developing brain, no expression of *XGrg*-5 is detected at these positions at the stages analyzed in *Xenopus*. The expression pattern of *XGrg-4* is strikingly similar to that of *mGrg-4*[8], suggesting functional conservation.

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CHAPTER 6

DISCUSSION

A DUAL ROLE FOR XTCF-3 IN PRIMARY AXIS FORMATION

The role of the TCF family of transcriptional regulators in primary axis formation is addressed by studying the mechanisms of action of XTcf-3 in *Xenopus laevis* embryos. The early events of primary axis induction involve activation through the WNT signaling pathway. As a result of activation of the pathway the cytoplasmic level of ß-catenin increases at the future dorsal side of the early cleavage stage embryo. Around the 16-32 cell stage, ß-catenin becomes apparent in the nuclei. The presence of nuclear ß-catenin causes several hours later the activation of specific target genes, like e.g. *XSiamois*. Since ß-catenin does not contain a DNA binding region, DNA binding proteins must mediate this transactivation. Ectopic expression of ß-catenin causes activation of dorsal genes and results in the induction and differentiation of a secondary axis. (See introduction).

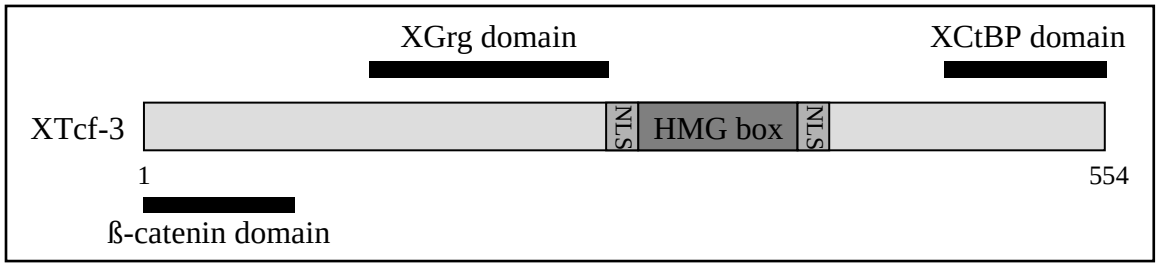

Figure 1. Domain structure of XTcf-3.

Three different homologs of the *Tcf/Lef* family of transcription factors have been cloned in *Xenopus laevis*, *XTcf*-3, *XLef*-1 and *XTcf*-4 (Chapter 3)[13,26]. Only transcripts of XTcf-3 are present maternally and are therefore likely to be involved in axis specification. As described in chapters 2 and 4, during axis induction in *Xenopus laevis* embryos, XTcf-3 can act both as an activator as well as a repressor of transcription.

ß-catenin interacts with the N-terminus of XTcf-3 (figure 1; Chapter 2)[27]. A ß-catenin/XTcf-3 complex can activate transcription of an artificial and natural TCF reporter. An N-terminal deletion mutant of XTcf-3 acts as a dominant-negative. ΔNXtcf-3 suppresses both the transactivation induced by ß-catenin/XTcf-3 as well as the axis induction in *Xenopus laevis* embryos. Endogenous XTcf-3 is likely to function as a key transcriptional regulator that, in a complex with ß-catenin, transduces WNT signals to activation of specific target genes.

Two different factors interacting with XTcf-3 belong to the Groucho family of transcriptional repressors, XGrg-4 and XGrg-5 (Chapter 4)[38]. The interaction domain of XTcf-3 for XGrg lies between the ß-catenin binding domain and the HMG box (figure 1).

Transcripts of XGrg-4 and XGrg-5 are present maternally (Chapter 5)[18]. XGrg-4 embodies the five different domains identified in *Drosophila* Groucho, while XGrg-5 only contains the N-terminal two domains (figure 2)[34]. In complex with XGrg-4, XTcf-3 acts as a transcriptional repressor. XGrg-4 can strongly suppress the ß-catenin/XTcf-3 induced transactivation of TCF reporters and dorsal injection of XGrg-4 transcripts strongly suppresses endogenous axis formation in *Xenopus laevis* embryos. Most likely, XGrg-5 acts as a de-repressor by interfering with these actions of XGrg-4.

DORSAL NUCLEAR LOCATION OF ß-CATENIN

Ectopic myc-tagged ß-catenin is located at the membrane, in the cytoplasm and occasionally in the nucleus of pre-MBT *Xenopus laevis* embryos. Injection of Myc-ß-catenin together with XTcf-3 results in a complete nuclear localization of Myc-ß-catenin. Deletion of the ß-catenin-interaction domain of XTcf-3 abrogates the increased nuclear localization. Similar results have been reported using murine Lef-1 and ΔNLef-1[3]. Therefore, the interaction of ß-catenin with endogenous XTcf-3 might be necessary for its nuclear localization. Possibly, XTcf-3 can mediate the nuclear translocation of the ß-catenin/XTcf-3 complex through its Nuclear Localization signal. However, mutants in the *Drosophila* homolog armadillo that are defective in dTcf binding still can enter the nucleus[31,46]. Furthermore, ß-catenin can freely cross the nuclear envelope in an importin-independent fashion[11,37]. Nuclear translocation via a classical NLS sequence is always mediated by importins[1,30]. Interestingly, like ß-catenin, importins also contain ARM repeat domains and their three-dimensional structure resembles that of the crystal structure found for ß-catenin[9,10,47]. It is therefore possible that ß-catenin is imported in the nucleus in a novel fashion similar to the way by which soluble transport receptors are translocated into the nucleus[11]. If this is the case, ß-catenin and XTcf-3 enter the nucleus independent of each other. When XTcf-3 is present in the nucleus, it can binds to ß-catenin and in this way ß-catenin is trapped in the nucleus. The mere presence of both factors in the nucleus might not be enough to activate transcription, since the transactivation of TCF reporters can be antagonized by activation of a TAK1-NLK-MAPK-related pathway[16,23].

If this conclusion is extrapolated to the pre-MBT *Xenopus laevis* embryo, two events are essential to induce the transcription of dorsal factors. Both an increase in cytoplasmic levels of ß-catenin at the dorsal side and a nuclear localization of XTcf-3 are necessary to evoke activation of transcription of target genes.

Already at the two-cell stage, an increase in cytosolic ß-catenin is seen at the future dorsal side[20]. This dorsal-ventral difference in cytosolic ß-catenin is dependent on the movement of the Vegetal Cortical Cytoplasm (VCC) to the future dorsal side (see introduction). The exact molecular nature of the VCC is still a mystery. Recently, Marikawa and Elinson reported that XAPC mRNA behaved similarly to the dorsal factor in the VCC with regard to the response to several WNT-interfering constructs[22]. This is unexpected, since APC is known to be a protein necessary for the degradation of cytoplasmic ß-catenin and as such functions as a tumor suppressor (reviewed in[35,36]).

Deletions in the APC gene were found to be the cause of several human colorectal adenomas[24]. In cell lines from these adenomas, a ß-catenin/Tcf-4 complex constitutively activates TCF reporters[19,28]. The introduction of wt APC in these cell lines rescues the constitutive activation. APC constitutes many different interaction domains for various different proteins (reviewed in [36]). The ß-catenin interaction domains are believed to be of central importance in its tumor suppression function[39,41,45]. Somatic deletions found in APC typically eliminate five of the seven ß-catenin binding domains and the axin binding domains. These mutants are still able to bind ß-catenin, but fail to induce its degradation[40]. Mice with spontaneous intestinal adenomas carry a similar mutation in the APC gene (APC^{min} mice)[44]. Furthermore, the mutant protein APC1638T, which retains three ß-catenin binding domains and one axin binding domain, supports a tumor free life in transgenic mice[43]. Deletion of the axin domain of APC1638T impairs its ability to downregulate ß-catenin in embryonic stem cells. These data are a strong indication that not only the binding of ß-catenin to APC, but also the interaction with axin are critical for its tumor suppressor function.

Surprisingly, the phenotype of a complete null mutation for mAPC is quite dramatic[29]. The embryos die before gastrulation, implying the loss of signal transduction. This indicates that the complete loss of APC might result in the loss of a ß-catenin pool that is normally required for WNT signaling. Together with the fact that XAPC

functionally resembles the VCC dorsal factor, one might assume that besides a role in ß-catenin degradation, APC is also required for sequestering the signaling pool of ß-catenin. This ß-catenin pool might represent newly synthesized ß-catenin or might be derived from the cadherin-bound pool.

At the 16-32 cell stage, ß-catenin becomes nuclear at the future dorsal side[20,42]. It is not known how ß-catenin is tethered in these nuclei, it might be that from this time-point on XTcf-3 becomes nuclear. There are several possibilities: 1, Polyadenylation of XTcf-3 mRNA is regulated. 2, The protein is actively retained in the cytoplasm. 3, Nuclear import via the nuclear pore complex is still impaired before these stages (see also Kaffmann and O'Shea 1999[17]). The whereabouts of the XTcf-3 protein during these early cleavage stages should be carefully monitored by studying the localization with specific antibodies against XTcf-3.

MECHANISMS OF ACTIVATION OF TRANSCRIPTION BY ß-CATENIN/XTCF-3 COMPLEXES

XTcf-3 by itself is transcriptionally inert. Activation of transcription of a TCF reporter requires the additional presence of a co-activator. ß-catenin can interact with XTcf-3 through its ARM repeat region. Genetic and biochemical data suggest that ß-catenin contains sequences that mediate transcriptional activation (figure 3).

Genetically removing the carboxyl terminus of armadillo results in impairment of wg action in *Drosophila*[32]. Chimeric fusion proteins containing the C-terminus of armadillo and the ΔNXTcf-3 or the C-terminal part of ß-catenin together with the HMG

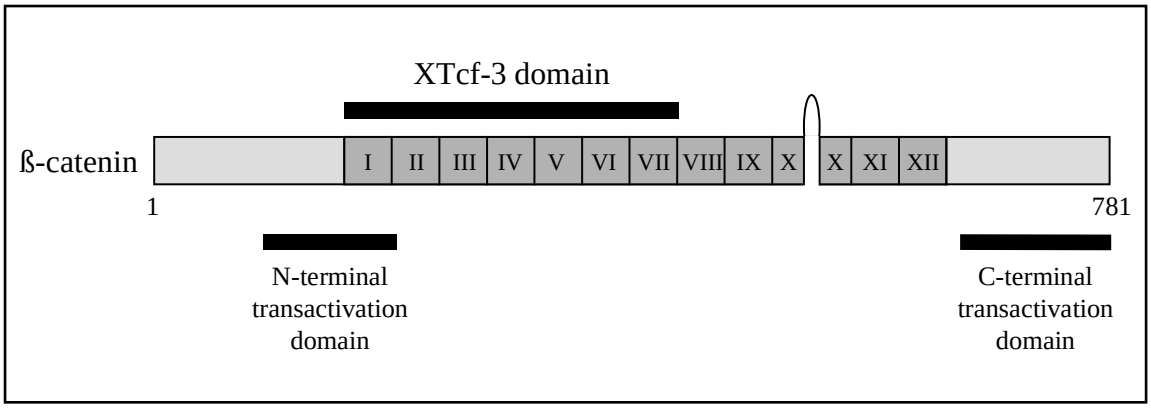

Figure 2. Domain structure of ß-catenin.

box of Lef-1 can activate transcription and induce an ectopic axis in *Xenopus laevis*[38,48]. Moreover, the N- and C-terminal parts of ß-catenin fused to the GAL4 DNA binding domain can activate transcription[15,46]. Recently, Hecht et al. (1999) have shown that the two transactivation domains posses a modular structure, consisting of multiple sub-elements that cover broad regions at its N and C termini, and extend considerably into the Armadillo repeat region (figure 3)[14]. Furthermore, transactivating elements of ß-catenin interact specifically and directly with the TATA-binding protein (TBP) *in vitro*[14]. Apparently, the ultimate function of ß-catenin in WNT signaling is to recruit the basal transcription machinery to promoter regions of specific target genes.

XTCF-3 INTERACTS WITH MEMBERS OF THE GROUCHO GENE FAMILY

In chapter 4 we show that a homolog of the *Drosophila* Groucho gene product (XGrg-4) and a naturally truncated version (XGrg-5) can physically interact with XTcf-3 and as such repress or de-repress activation of transcription. Similar mechanisms of repression by Groucho or Groucho related factors and TCFs were described, *in vivo*, for *Drosophila* (Groucho and dTCF)[5] and, *in vitro*, for human TLE1 and LEF-1[21]. The interaction domain for Groucho or GRGs on TCF lies in between the ß-catenin/Armadillo interaction domain and the HMG box. Further examination of the capacity for TCF interaction by the different mammalian GRGs shows no clear discrimination and analysis of the expression patterns in different cell systems did not imply distinct Tcf-Grg combinations (Brantjes, H., Roose, R. and Clevers, H., in preparation). The expression patterns of the different Grg and Tcf genes show a high degree of overlap (Chapter 5[25]. Therefore, repression of TCF, mediated by members of the Groucho gene family might be a general mechanism throughout development.

In addition to repression mediated by XGrg-4 interaction, interaction of the C-terminus of XTcf-3 with the co-repressor XCtBP adds yet another type of repression via TCF[4]. Functional analysis excludes a role for XCtBP in primary axis induction, but support a role for XCtBP in the regulation of head and notochord development. XCtBP mediated repression by XTcf-3 might also be a general mechanism throughout

development, since the expression pattern of XCtBP also has a striking overlap with the pattern of expression of XTcf-3.

MECHANISMS OF REPRESSION OF TRANSCRIPTION BY XGRG-4/XTCF-3 COMPLEXES

The mechanisms by which XGrg-4 exerts its repressive effects are as yet unknown. The mechanism of repression by *Drosophila* Groucho has been analyzed to some detail (figure 3). Native Groucho is a tetramer in solution[8]. This tetramerization is mediated by two putative amphipathic a-helices (Leucine zipper-like motifs). Formation of the tetramer is required for Groucho mediated repression.

Several studies have implicated a connection between Groucho action and silencing of transcription by chromatin formation. Binding of histone proteins to the DNA leads to compaction into chromatin. The acetylation state of core histones (H3 and H4) reflects the transcriptional state of a gene. Hyper-acetylated histones are usually associated with an active transcriptional state, whereas hypo-acetylated histones are usually associated with the repressed state[2,49]. Groucho family proteins make specific

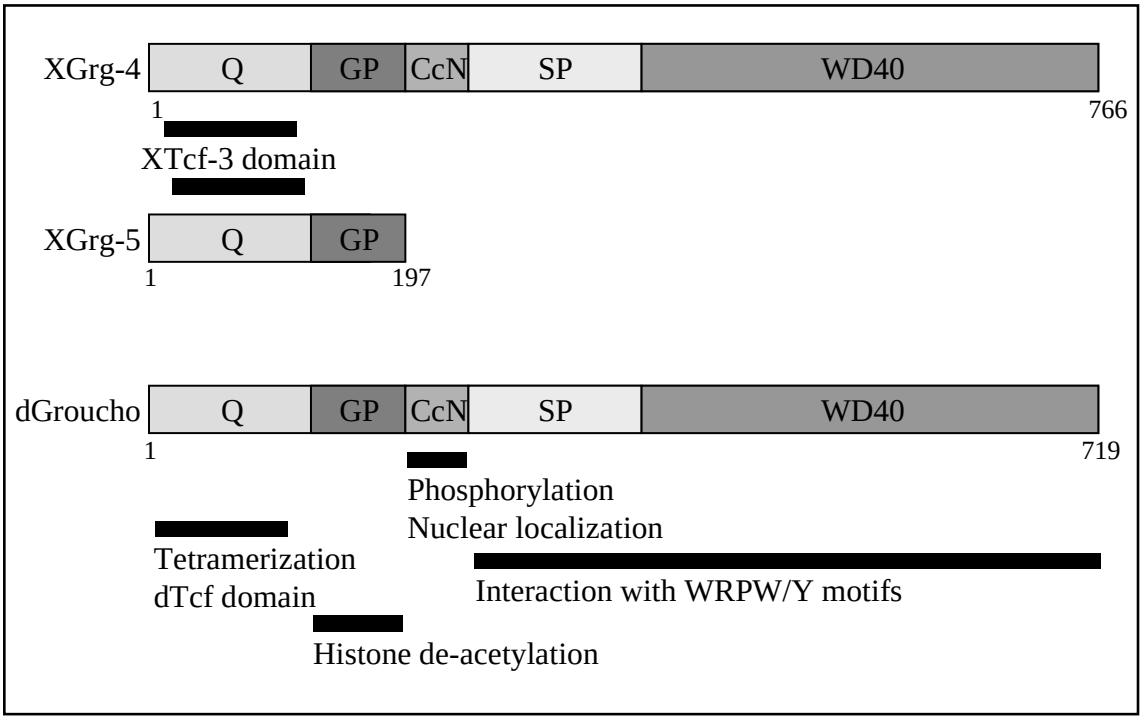

Figure 3. Domain structure of XGrg-4, XGrg-5 and dGroucho.

contacts with the N-terminal tails of the core histone, H3[7,33]. Furthermore, the glycine/proline-rich domain (GP domain) can recruit the histone deacetylase Rpd3[6]. These findings support the view that Groucho mediates repression by the direct recruitment of histones and histone deacetylase to the DNA, where it can modulate local chromatin structure. Inhibiting the Rpd3 activity does not result in complete inhibition of the repression activity of Groucho. In addition, the WD40 repeat domain of Groucho functions as a weak repression domain independent of Rpd3[7,12]. One of the main mechanisms in silencing the zygotic genome before MBT is by chromatin mediated repression (see introduction). Most likely, a similar mechanism of repression holds true for XGrg-4 in complex with XTcf-3.

An additional level of complexity in XGrg-4 mediated repression is revealed by the manipulation of XGrg-5 function (Chapter 4)[38]. XGrg-5 contains only the N-terminal Q- and GP-domains. It potentates ß-catenin/XTcf-3 mediated transactivation of natural and synthetic reporters as well as axis induction in *Xenopus laevis* embryos. XGrg-5 might act as a squelching factor by interfering with the polymerization of XGrg-4, the binding of XGrg-4 to XTcf-3 as well as with the possible interaction with histone deacetylases. Interestingly, a clear NLS sequence is not present in this naturally truncated Grg. Yet XGrg-5 seems to be always nuclear. An interaction of XGrg-5 to XTcf-3 and/or XGrg-4 might already occur in the cytoplasm. Again, a study of the localization of the endogenous proteins should give insight into the intriguing mechanism of repression versus de-repression.

A MODEL FOR THE DUAL ROLE OF XTCF-3 IN PRIMARY AXIS FORMATION

The way by which XTcf-3 might function in the patterning of the blastula mesoderm is illustrated in the model shown in figure 4. When XTcf-3 enters the nucleus, it will bind to the promoter of axis specifying target genes. At the ventral side, XTcf-3 will bind to XGrg-4. XGrg-4 induces local chromatin formation, thereby silencing transcription of the target genes. At the dorsal side, nuclear ß-catenin will compete with XGrg-4 by interacting with XTcf-3. ß-catenin might recruit histone acetylase to open the chromatin and subsequently recruit the basal transcription machinery to activate

transcription of otherwise silenced genes. The role of XGrg-5 in this ying-yang situation might be to facilitate the transcriptional activation of ß-catenin in cells where the concentration of nuclear ß-catenin is relatively low.

The combined repression and de-repression/activation through XTcf-3 secures a tight control over the transactivation of specific XTcf-3 target genes. This dual function for XTcf-3 might constitute a general mechanism throughout development, since the expression patterns of *Xenopus* XTcf-3, XGrg4 and XGrg-5 largely overlap.

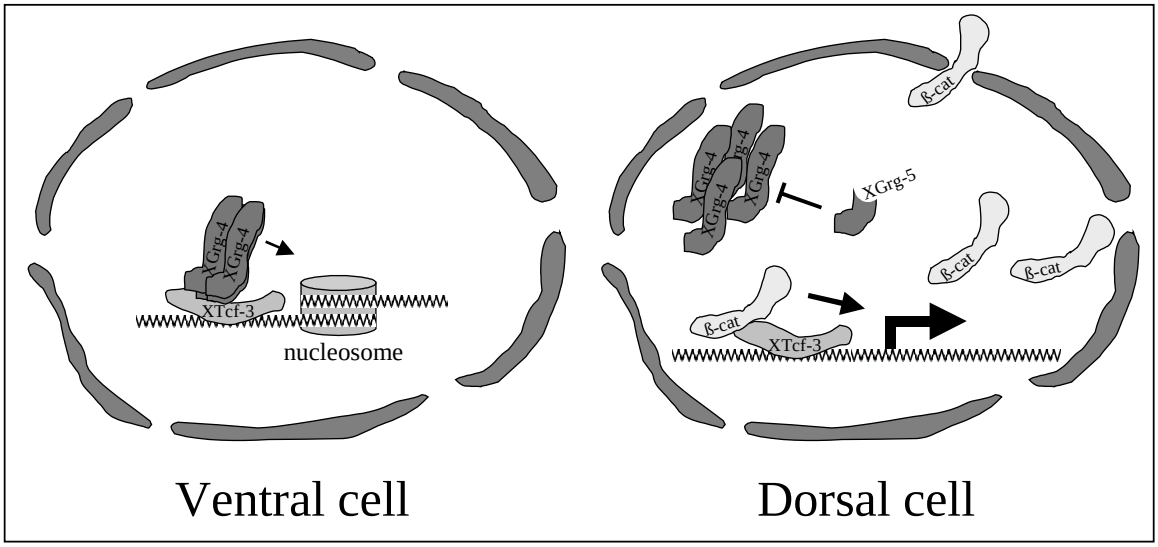

Figure 4. Model for the action of XTcf-3 during axis induction.

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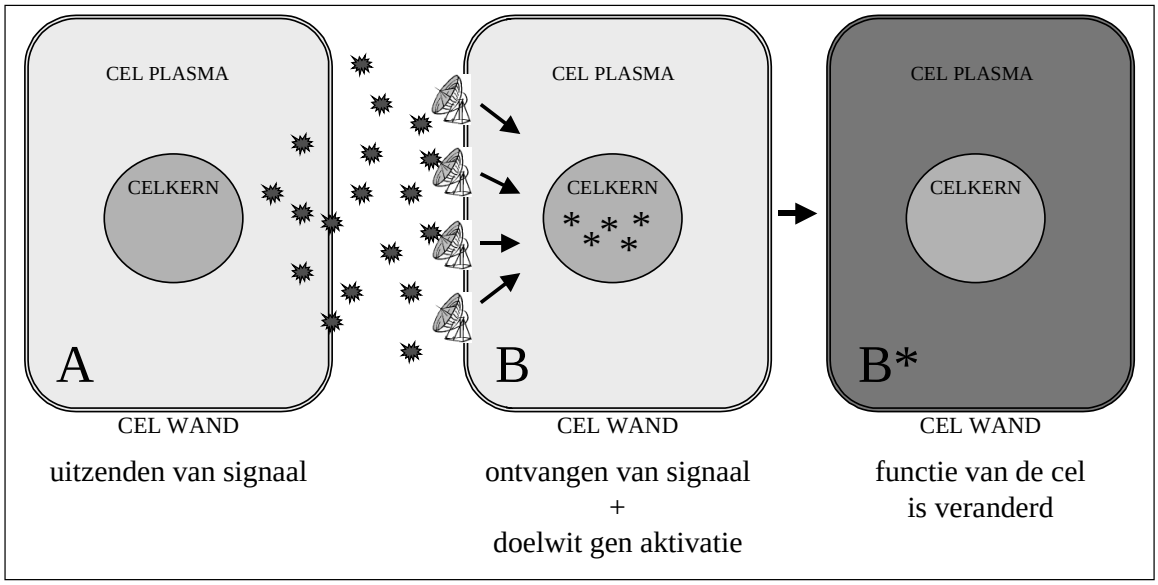
SAMENVATTING IN HET NEDERLANDS

(Voor niet ingewijden)

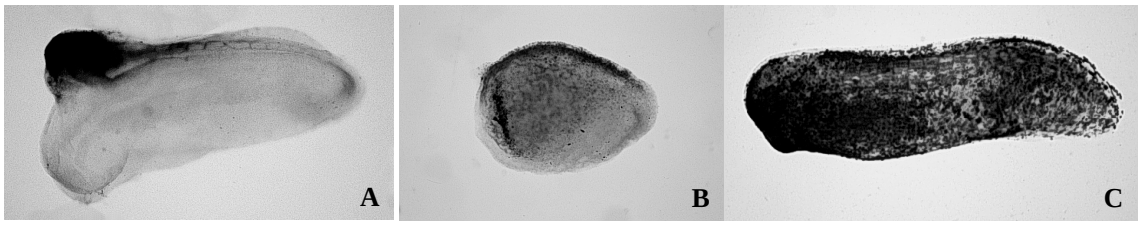
Het vakgebied waarin dit onderzoek heeft plaats gevonden wordt ontwikkelingsbiologie genoemd. In de ruimste zin van het woord laat ontwikkelingbiologie zich omschrijven als dat vakgebied binnen de biologie, dat zich bezig houdt met het verkrijgen van kennis over de ontwikkeling van meercellige levende wezens. Met name de eerste periode van de ontwikkeling worden onderzocht omdat deze bepalend is voor de verdere ontwikkeling.



Figuur 1. Schematische voorstelling van een signaal pad.

De doelstelling van dit onderzoek was het verkrijgen van meer inzicht in een aantal sturende processen die zich afspelen tijdens de eerste periode van de ontwikkeling. Eén van die vroege sturingsprocessen is bepalend voor de vorming van de lichaamsas. Dat wil zeggen , de positionering van de verschillende lichaamsdelen ten op zichte van elkaar in de voor-achterwaartse en buik-rug richting. Verschillende eiwitten die betrokken zijn bij de vroege vorming van de lichaamsas behoren tot het zogeheten WNT (spreek uit “wint”) signaal pad. Een signaal pad beslaat een route van een uitgezonden signaal van cel A naar de kern van cel B (zie figuur 1). Dit signaal heeft als gevolg dat cel B verandert, in die zin dat cel B bepaalde specifieke eigenschappen verkrijgt. Zo’n eigenschap is bijvoorbeeld het betrokken zijn bij de bepaling van de lichaamsas. Het uitgezonden signaal van cel A wordt door zogeheten receptoren op de buitenkant van cel B opgepikt en doorgegeven naar de binnenkant van cel B. Via verschillende eiwitten wordt het signaal omgezet in

een ander signaal dat in de celkern genen kan aktiveren. Aktivatie van genen wil zeggen dat informatie opgeslagen als DNA-code in het gen wordt vertaald in een eiwit dat zijn werking heeft in de cel, waardoor deze zich anders gaat gedragen. Voor het aflezen van de DNA-code zijn zogenaamde transcriptie/overschrijvings factoren nodig. Het ontvangen signaal bepaalt welke genen worden overgeschreven via het inschakelen van transcriptie factoren die alleen bepaalde genen kunnen aktiveren. Dit zijn de doelwit genen van een uitgezonden signaal.



Figuur 2. De vorming van de lichaamsas in kikkerembryos. A) Een embryo met een dubbele kop. De additionele as is onstaan door het inbrengen van een positief werkend eiwit uit het WNT signaal pad. B) In dit embryo ontbreken herkenbare lichaamsas structuren door het inbrengen van mutant Tcf-3. C) Een normaal kikkerembryo. Ter controle is een kleurstof meegespoten.

In geval van het WNT signaal pad is het uitgescheiden WNT eiwit het signaal dat verantwoordelijk is voor de aktivatie van specifieke genen in cellen die voorlopers zijn bij de vorming van een correcte lichaamsas. Voor aanvang van dit promotie onderzoek was nog niet bekend hoe dit WNT signaal uiteindelijk in de kern leidde tot de aktivatie van specifieke doelwit genen en welke trancriptie factoren hierbij betrokken zijn. Uit onderzoek aan de vorming van de lichaamsas in kikkerembryos is gebleken dat de transcriptie factor Tcf-3 (T-cel factor) betrokken is bij het omzetten van WNT signalen in aktivatie van de specifieke doelwit genen.

De experimenten in dit onderzoek zijn voornamelijk gedaan in embryos van de Afrikaanse klauwkikker. De Latijnse naam voor deze kikker is *Xenopus laevis*. Voorafgaand aan dit onderzoek was al bekend dat inbrengen van verschillende geïdentificeerde eiwitten van het WNT signaal pad kan leiden tot de vorming van een extra lichaamsas. Het resultaat hiervan was dat er embryos onstonden met twee koppen en twee rompen (zie figuur 2A). Het omgekeerde kon ook worden bereikt, door het inbrengen van eiwitten die dit aktivatie proces kunnen remmen. In dit geval resulteerde dat in embryos zonder kop of andere te herkennen structuren (o. a. ruggegraat, staart) (zie figuur 2B). Embryos waarin Tcf-3 kunstmatig is toegevoegd ontwikkelen zich normaal

(zie figuur 2C). Maar als een voor de normale werking belangrijk deel van het Tcf-3 eiwit wordt weggehaald heeft dit grote gevolgen. Dit zogeheten mutant Tcf-3 remt namelijk de vorming van de lichaamsas (zie figuur 2B). Hieruit kan geconludeerd worden dat het volledige eiwit als een positieve factor in het WNT signaal pad functioneert. Zo is op het niveau van de aktivatie van trancriptie van doelwit genen de relatie tussen WNT en Tcf-3 dus aangetoond. Een belangrijk gat in de kennis over de vorming van de lichaamsas is hiermee ingevuld. Een ander belangrijk resultaat van het onderzoek is dat XTcf-3 ook een rol speelt bij het voorkomen van ongeoorloofde aktivatie van genen. Aan de ene kant kan Tcf-3 onder sturing van een WNT signaal genen aktiveren en aan de andere kant kan het in afwezigheid van dit signaal dezelfde genen beschermen voor ongeoorloofde aktivatie. Tcf-3 speelt dus een dubbelrol in de sturing en vorming van de lichaamsas.

Veel van de genen die de ontwikkeling van de lichaamsas sturen blijken ook betrokken te zijn bij het onstaan van kanker. In bijvoorbeeld dikke darm kanker is inmiddels aangetoond dat ook hier WNT signalering een rol speelt en dat een humane variant van Tcf-3 betrokken is bij de aktivatie van doelwit genen. Aktivatie van deze genen leidt tot een ongecontroleerde groei van cellen en resulteert uiteindelijk in de vorming van een kwaadaardig gezwel. De kennis die is opgedaan in dit promotie onderzoek heeft dus ondermeer geleid tot een beter inzicht in de mechanismen die een rol spelen bij het ontstaan van kanker. Dit inzicht kan in de toekomst leiden tot de ontwikkeling van nieuwe behandelingsmethoden ter genezing en/of voorkoming van kanker.

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Miranda.

CURRICULUM VITAE

Miranda Molenaar werd geboren op 2 november 1970 in Silvolde. Na het behalen van het VWO diploma aan het Isala College in Silvolde begon zij in 1989 met de studie Medische Biologie aan de Universiteit Utrecht. Het doctoraal examen werd behaald in augustus 1995 met als afstudeervakken Medische farmacologie (begeleiding Dr. M. Verhage, Universiteit Utrecht), Neurofarmacologie (begeleiding Dr. Th. de Boer, N. V. Organon Oss) en Fysiologische Chemie (begeleiding Dr. L. H. Schrama, Universiteit Utrecht). In september 1995 startte zij als onderzoeker in opleiding aan het Hubrecht laboratorium (Nederlands Instituut voor Ontwikkelingsbiologie) te Utrecht. Onder begeleiding van Dr. O. H. J. Destrée en Prof. Dr. H. C. Clevers werd gedurende $4^1/_2$ jaar gewerkt aan het in dit proefschrift beschreven promotie onderzoek.

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