Development 133, 1745-1755 (2006) doi:10.1242/dev.02343

XNF-ATc3 affects neural convergent extension

Annette Borchers[1], Yuri Fonar[2], Dale Frank[2] and Julie C. Baker[1,*]

Convergent extension is the primary driving force elongating the anteroposterior body axis. In *Xenopus,* convergent extension occurs in the dorsal mesoderm and posterior neural ectoderm, and is mediated by similar molecular pathways within these tissues. In this paper, we show that activation of NF-AT, a transcription factor known to modulate multiple signaling events, inhibits convergent extension in the dorsal mesoderm and in the posterior neural ectoderm. This is seen in whole embryos, mesodermal explants and posterior neural explants, solidly implicating a role of NF-AT in convergent extension. In the whole embryo, inhibition of NF-AT reveals a more selective function, affecting only convergent extension in the neural ectoderm. This specific activity was further teased apart using a variety of temporal and spatial approaches. Targeted injections of dominant-negative XNF-ATc3, or dosing over time with the calcineurin inhibitor cyclosporin in neural tube explants or in whole embryos, shows that inhibition of NF-AT signaling blocks neural convergent extension. Consistent with a function in neural convergent extension, we show that *XNF-ATc3* is expressed and transcriptionally active within the neural tube. This work identifies XNF-ATc3 as a regulator of neural convergent extension in *Xenopus* and adds to a short list of molecules involved in this process.

KEY WORDS: Convergent extension, NF-AT, *Xenopus*, Neural CE

INTRODUCTION

Cells in the developing embryo undergo complex morphogenetic movements that establish the adult body plan. In *Xenopus*, the dorsal mesoderm and posterior neural ectoderm are the first cell types to display these activities. Movements in these tissues are essential for normal embryonic patterning and defective morphogenesis leads to multiple defects in all vertebrates, including spina bifida in humans (Mitchell et al., 2004). In *Xenopus*, the dorsal mesoderm and posterior neural ectoderm undergo a specialized type of cellular movement called convergent extension (CE), whereby a field of cells narrows (converges) mediolaterally and then lengthens (extends) anteroposteriorly (Keller, 2002; Wallingford et al., 2002). These movements are the driving force that elongates the anteroposterior axis of the embryo and closes the neural tube (Keller and Danilchik, 1988; Keller et al., 1992b).

Two types of non-canonical Wnt pathways have been described that mediate both posterior neural ectodermal and dorsal mesodermal CE; the planar cell polarity (PCP) pathway (Keller, 2002; McEwen and Peifer, 2000; Peifer and McEwen, 2002) and the Wnt/Ca^{2+} pathway (Kuhl et al., 2000). These pathways, under control of the secreted ligands Wnt11, Wnt5a and Wnt4, do not activate β-catenin signaling (Moon et al., 1993; Torres et al., 1996), but rather either function to release calcium (Wnt/Ca^{2+} pathway) (Slusarski et al., 1997a; Slusarski et al., 1997b) or regulate PCP pathway members like strabismus, prickle, JNK, Daam1 or dishevelled (Wallingford et al., 2002). Although the PCP and Wnt/Ca^{2+} pathways instruct morphogenesis in both the neural ectoderm and dorsal mesoderm, the behavior and morphology of cells undergoing CE is different within these cellular populations. It remains unknown how these two pathways are integrated into coordinating distinct cellular movements, although as these pathways share molecules and functions, it is likely that there is extensive crosstalk between the various players as well as possible subtle modifications of downstream targets (Elul and Keller, 2000; Elul et al., 1997; Ezin et al., 2003; Shih and Keller, 1992a; Shih and Keller, 1992b).

The integration of pathways, like the Wnt/Ca^{2+} or PCP, to coordinate cellular movements will most certainly involve the modification and activation of different and diverse downstream transcriptional targets. The nuclear effectors responsible for these events remain to be elucidated, but it is likely that such effectors will need to integrate information from multiple signaling events. NF-AT, a well known transcriptional activator and modulator of Ca^{2+}, is important in several specialized morphological movements (de la Pompa et al., 1998; Graef et al., 2001; Horsley et al., 2001), including neuronal migration (Graef et al., 2003). All NF-AT proteins are characterized by calcineurin and rel-homology binding domains (Rao et al., 1997). Ca^{2+} activates the phosphatase, calcineurin, causing the dephosphorylation of serines within the N terminus of the NF-AT protein, which serves to transport NF-AT into the nucleus (Beals et al., 1997; Crabtree and Olson, 2002). In the nucleus, NF-AT requires additional partners for DNA binding and transcriptional activation (Hogan et al., 2003; Im and Rao, 2004). This mechanism of action allows NF-AT to receive input from multiple signaling pathways, including the Ca^{2+} and PCP pathway (Chen et al., 1998; Macian et al., 2001; Yamanaka et al., 2002). This crosstalk with known players of CE suggests that NF-AT could play a key role in the coordination of cellular movements during early development.

In this paper, we provide evidence that a primary role of NF-AT in early *Xenopus* development is to mediate CE in neural ectoderm. These results are based on *XNF-ATc3* expression and activity within the *Xenopus* embryo, and an extensive analysis aimed at isolating the role NF-AT plays in CE in both the neural ectoderm and dorsal mesoderm. Recent published data support a role for NF-AT in CE movements in *Xenopus* dorsal mesoderm and as a negative regulator of canonical Wnt signaling (Saneyoshi et al., 2002). Here, we add to this work and suggest that although XNF-ATc3 affects dorsal mesodermal CE movements, it is also necessary for these

[1]Department of Genetics, Stanford University Medical School, Stanford, CA 94062, USA. [2]Department of Biochemistry, Rappaport Faculty of Medicine, Technion-Israel Institute of Technology, Haifa 31096, Israel.

*Author for correspondence (e-mail: jbaker@stanford.edu)

Accepted 27 February 2006

movements within the neural ectoderm. This is the first evidence showing that NF-AT signaling has a role in neural CE movements and adds to a short list of molecules involved in this process.

MATERIALS AND METHODS

Injection and manipulation of *Xenopus* embryos

RNA synthesis and *Xenopus* injection experiments were performed as described (Borchers et al., 2002). The plasmids CA XNF-AT, DN XNF-AT and WT XNF-AT used for RNA synthesis are a kind gift of Dr K. Mikoshiba (Saneyoshi et al., 2002). To represent the identity of the constructs better, we refer to them as CA XNF-ATc3, DN XNF-ATc3 and WT XNF-ATc3 in the text. For cyclosporin A (CsA) treatment, embryos were incubated in 4 mM or 400 μM CsA (Bedford Laboratories) at time points indicated in the text.

Cell adhesion assay

For cell adhesion assays stage 12.5-13 embryos were transferred into Ca^{2+}/Mg^{2+}-free medium CMFM (Sive et al., 2000) and neural plates were removed. After removal of the mesoderm, the neural tissue was transferred to a new dish containing CMFM and cells were manually dissociated using an eyebrow knife. If cells did not dissociate they were further agitated for 30 minutes. For re-aggregation single cells were transferred to 1/3 NMR.

β-Galactosidase staining and whole mount in situ hybridization

β-Galactosidase staining and whole-mount in situ hybridization were performed as previously described (Borchers et al., 2002; Harland, 1991). Antisense probes were generated using the following plasmids: p33 En2 for engrailed (Brivanlou and Harland, 1989), pG1s for HoxB9 (Sharpe et al., 1987), XKrox20 for Krox20 (Bradley et al., 1993), psp6nucβgal for *lacZ* (Smith and Harland, 1991), pNPG152 for Nrp1 (Knecht et al., 1995), XPax3 for Pax3 (Bang et al., 1999), XSix1-pBSII for Six1 (Pandur and Moody, 2000), pCS2-Sox2 for Sox2 (Mizuseki et al., 1998), and pXAG-1 for XAG-1 (Sive et al., 1989). For the XNF-ATc3 in situ hybridization, we generated two XNF-ATc3 probes by RT-PCR from a Clontech *Xenopus* MATCHMAKER cDNA library. Probes were designed against regions of the XNF-ATc3 sequence that are most divergent from NF-ATc1-c4. The first probe targets a fragment that includes the N-terminal transcription activation domain (TAD) and the calcineurin docking side (CDS). This fragment was cloned by using the following primers: 5′-CATTGCG-GTTGGGAAGATTTG-3′ and 5′-CAAGATGAAGGCAGAGATGGTCC-3′. The second probe targets a fragment that contains the CDS, two SP boxes and part of the first nuclear localization signal. Primers that were used to clone this fragment are: 5′-CAGTTCAACCCATTCTTCCTGC-3′ and 5′-TGCCGTCTTTTCCCACAAGG-3′. The resulting PCR products were cloned into pCRII-TOPO (Invitrogen). Antisense probes generated from both plasmids produced identical results in whole-mount in situ hybridization.

Analysis of CE in the mesoderm

Ectodermal explants were prepared as described by Wallingford and Harland (Wallingford and Harland, 2001). To induce mesodermal CE, ectodermal explants were incubated in 35 ng/ml of human recombinant Activin A (R&D Systems). For cyclosporin A (CsA) treatment, CsA (Bedford Laboratories) was added at concentrations as stated in Fig. 5. FK506 (Prograf) was used at concentration of 100 ng/ml. Ectodermal explants were cultured in individual agarose wells until control embryos reached stage 20. Mesodermal CE was visible as elongation of the ectoderm.

For Keller explants, embryos were injected dorsally and animally at the four-cell stage using GFP as a lineage tracer. From fluorescent embryos, Keller open face explants (Shih and Keller, 1992b) were prepared at stage 10.5 and cultured in Steinberg solution [60 mM NaCl, 0.67 mM KCl, 0.34 mM $Ca(NO_3)_2$, 0.83 mM $MgSO_4$, 1 mM HEPES pH 7.4]. CE was assessed when unmanipulated sibling control embryos had reached stage 16.

Analysis of CE in neural tissue

To analyze NF-AT activity in neuralized tissue, animal caps were neuralized in two different ways. Either XBF-2 RNA (Mariani and Harland, 1998) or dominant-negative BMP receptor RNA (Graff et al., 1994) were injected in the animal pole of one-cell stage embryos. Animal caps were cut at the blastula stage and cultured to stage 16/17 when CE was assessed and marker expression was analyzed by RT-PCR. RT-PCR primers for *EF1a*, *HoxB9*, *Krox20* and *NCAM* have been described elsewhere (Hemmati-Brivanlou and Melton, 1994). The primers for *HoxD1* have been published by Kolm and Sive (Kolm and Sive, 1995), the primers for XE10 (EphA2 receptor) by Weinstein et al. (Weinstein et al., 1996).

To assess neural CE in the neural plate, embryos were cultured until stage 12-12.5. Embryos were devitellinized and neural plate, including the underlying mesoderm and endodermal epithelium, was removed. Neural plate explants were cultured in Steinberg solution in petri dishes, which had been blocked with 1% BSA (Fisher Scientific). The explants were immobilized using glass coverslides (5×5 mm) with silicon grease-coated edges. To analyze how CsA affects neural CE, explants were incubated in 400 nM CsA in Steinberg solution. As CsA is dissolved in 33.2% ethanol, additional control explants were incubated in 0.33% ethanol in Steinberg solution to test for toxicity or other effects. Immediately after immobilizing the explants, images were taken at 1-hour intervals using a Leica MZFLIII microscope and Leica DC500 camera (Leica Fire Cam 1.2.0 software for Macintosh). Explants were monitored over 4-7 hours, until a closed neural tube was visible in control explants. The area and lengths of the transplants was measured using the public domain ImageJ program (developed at the US National Institute of Health and available at http://rsb.info.nih.gov/ij/). The change in length (ΔL) was calculated as $\Delta L=L_t: L_o$, whereby L_o is the length at the start of the experiment and L_t is the length at the different time points (t=1 to 7). The change in area (ΔA) and the change in neural tube length (ΔNT) were calculated in a similar way. The change in width (ΔW) was calculated by $\Delta W=\Delta A: \Delta L$. The control versus CsA experiment was repeated three times with a total of 18 explants. The control versus DN XNF-ATc3 experiment was performed twice with a total of 18 explants. In two of our experiments, we analyzed a total of six explants for ethanol-induced effects. These data were not combined, as individual experiments continued over different time spans. However, the results of the different experiments were identical and one representative experiment is presented. Stars in the graphs indicate time points in which the CsA-treated or DN XNF-ATc3 explants are significantly different in ΔL or ΔW from the control and ethanol-treated explants in an unpaired Student's *t*-test.

RESULTS

Temporal and spatial inhibition of XNF-AT signaling results in neural CE defects

NF-AT is a well-studied transcription factor, which functions downstream of both Ca^{2+} and PCP signaling components, the latter including JNK (Crabtree and Olson, 2002; Hogan et al., 2003). Therefore, we tested whether NF-AT signaling plays a role in CE movements during *Xenopus* neural development. Our analysis of *Xenopus* genomic and EST databases identified four *Xenopus* NF-AT genes (XNF-ATc1-4), each of which is orthologous to the four mouse NF-AT genes (NF-ATc1-4; data not shown). The previously published *Xenopus* NF-AT molecule (XNF-AT) is most closely related to mouse NF-ATc3 (Saneyoshi et al., 2002). For clarity we will refer to XNF-AT as XNF-ATc3 (kind gift from K. Mikoshiba) throughout this paper.

XNF-ATc3 has recently been shown to be involved in dorsoventral patterning of the *Xenopus* embryo by inhibiting canonical Wnt signaling. Ventral injection of a dominant-negative XNF-ATc3 (DN XNF-ATc3) induced the formation of secondary axis or dorsalized embryos in over 80% of embryos (Saneyoshi et al., 2002). We saw a much smaller induction of dorsal fates and instead observed a severe failure in neural tube formation. In our experiments, ventral injection of 1 ng DN XNF-ATc3 (kind gift of K. Mikoshiba) resulted in 2.3% partial axes and 3.4% double axes (0% axes in the control, n=194). Higher concentrations of 2 ng or 4 ng DN XNF-ATc3 gave 27.6% partial and 5.2% double axes, and 28.2% and 5.1% double axes, respectively (2.7% double axes in the control, n=171).

In order to determine the role of XNF-AT during *Xenopus* neurulation in the absence of any potential effects on earlier dorsoventral patterning, we blocked XNF-ATc3 activity in a spatial and temporal manner. *Xenopus* expresses three NF-AT paralogs (c1, c2 and c3) during gastrulation (see Fig. S1 in the supplementary material). These molecules are functionally redundant in mouse (Crabtree and Olson, 2002), and therefore knockdown approaches using morpholinos are not suitable in this instance. Therefore, to inhibit NF-AT signalling, we used DN XNF-ATc3 or cyclosporin A (CsA), a calcineurin inhibitor (Lin et al., 1991; Liu et al., 1991) known to effectively and specifically block NF-AT signaling (Graef et al., 2001; Graef et al., 2003). First, we targeted injections of *DN XNF-ATc3* specifically into the prospective ectoderm. Second, we exposed embryos to CsA at gastrula stages, long after the dorsoventral axis has been specified. These spatial and temporal approaches bypass the effects of XNF-AT on dorsoventral patterning and mesodermal specification, allowing the study of XNF-AT signaling only in the neural ectoderm. DN XNF-ATc3 expressing or CsA-treated embryos were cultured until neurula stage (19-21) when they were fixed and analyzed by whole-mount in situ hybridization. To ensure that CsA treatment was effective, control embryos were also cultured in CsA and analyzed for heart and gut defects characteristic for CsA treatment (Yoshida et al., 2004). Independent of the method used, embryos showed wider neural tubes than the controls. This was seen along the whole anteroposterior neural axis as markers were expanded, but not misplaced (Fig. 1). Dorsoventral patterning was not affected and we observed normal ventral marker patterning (*globin*) in *DN XNF-ATc3*-injected embryos (data not shown). This indicates that although neural tube morphogenesis was seriously affected, cell fate decisions have remained intact. Furthermore, embryos injected with *DN XNF-ATc3* had few gastrulation defects when compared with controls (4.7% n=85 for DN XNF-ATc3; 2.3% n=127 for controls).

To confirm that the *Xenopus* CsA-phenotype we observe is specific for an inhibition of endogenous NF-AT signaling, we performed rescue experiments using wild-type XNF-ATc3 (WT XNF-ATc3, gift of K. Mikoshiba). CsA is known to inhibit NF-AT signaling specifically in vitro and during mouse development (Graef et al., 2001; Graef et al., 2003). Indeed, injection of 2 ng *WT XNF-ATc3* could rescue the CsA-induced neural tube closure defect (Fig. 1B).

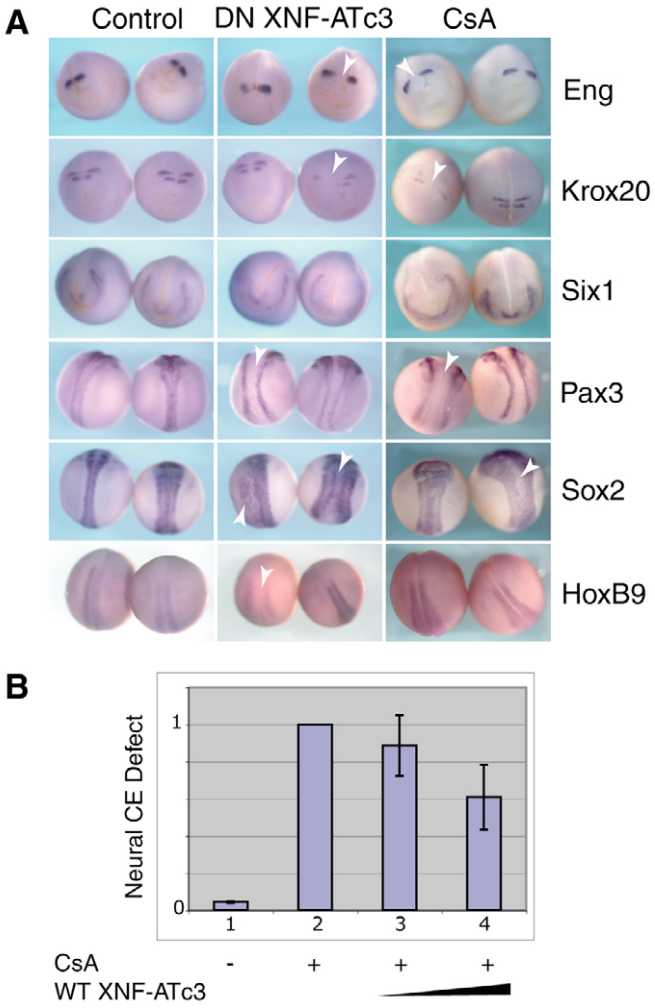

Fig. 1. Inhibition of XNF-ATc3 affects neural tube closure. (**A**) Embryos were either injected in the prospective ectoderm at the one-cell stage with 2 ng *DN XNF-ATc3* or treated with 4 mM cyclosporin A (CsA) at stage 10 for 1 hour. Both sets were analyzed for *Eng, Krox20, Six1, Pax3, Sox2* and *HoxB9* expression at late neurula stages (19-21) by in situ hybridization. Neural tube closure defects were seen at concentrations as low as 250 pg *DN XNF-ATc3*. White arrowheads indicate expansions in the neural marker expression. (**B**) WT XNF-ATc3 rescues neural CE defects caused by CsA treatment. The graph represents three different experiments (n=731), which were normalized by the percentage of neural CE defects in embryos that were incubated in 400 μM CsA starting at the 64-cell stage. Co-injection of 2 ng *WT XNF-ATc3*(4) lead to a rescue of neural CE defects.

CE defects mediated by cell adhesion changes cause the embryonic neural tube closure defects

Proper neural tube closure is dependent on a variety of morphogenetic movements, including neural fold formation and CE movements (Schoenwolf and Smith, 1990). To distinguish whether inhibition of XNF-ATc3 is involved in either neural fold formation or CE movements, we analyzed whether neural fold hinge points exists in embryos either expressing DN XNF-ATc3 or treated with CsA. This was achieved by staining effected embryos with phalloidin, which detects f-actin within the hinge points of the neural folds. Our analysis showed that hinge point and neural fold formation were not affected by inhibition of NF-AT signaling (Fig. 2). However, the neural plate area was wider in these embryos and neural tube closure was delayed, strongly indicating a defect in neural CE. Therefore, we will refer to this phenotype as neural CE defect throughout the text.

As CE is known to be mediated by the Wnt/Ca^{2+} pathway, and as NF-AT is a crucial regulator of Ca^{2+} signals, we hypothesized that DN XNF-ATc3 might inhibit CE by changing the adhesive properties of the neural ectodermal cells. To this end, neural ectoderm injected with DN XNF-ATc3 was dissected at stage 12.5-13 and dissociated in Ca^{2+}/Mg^{2+}-free medium (CMFM). Neural tissue expressing DN XNF-ATc3 immediately dissociated into single cells (Fig. 2I), while the control neural cells needed further agitation to dissociate (Fig. 2H). To induce re-aggregation, single cells were transferred to a Ca^{2+}-containing medium. The control cells aggregated well (Fig. 2K), while only some DN XNF-ATc3-expressing neural cells showed aggregation (Fig. 2L). This data supports the hypothesis that the convergent extension defect is a consequence of a change in cell adhesion and points towards an involvement in cell adhesion processes regulated by the Wnt/Ca^{2+} pathway.

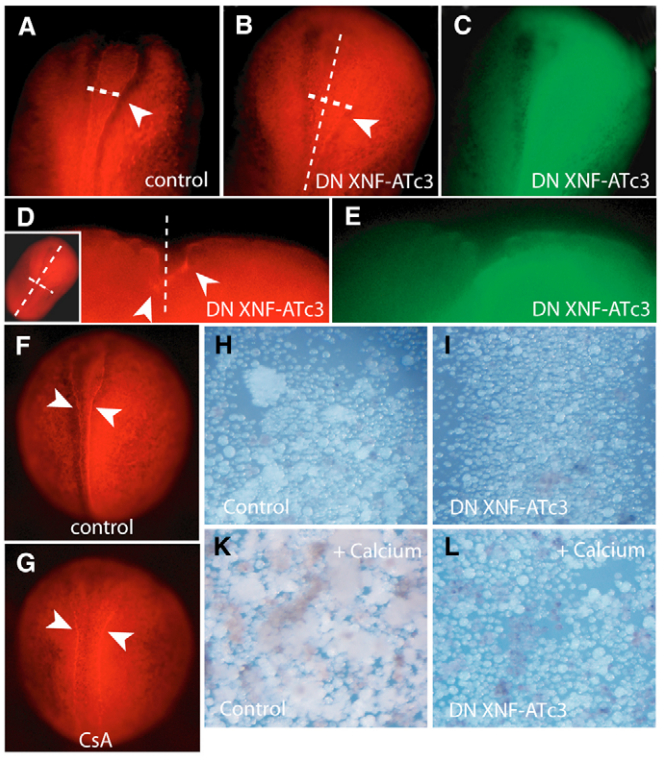

Fig. 2. Inhibition of NF-AT signaling by DN XNF-ATc3 or CsA does not affect hinge point formation. (**A**) Phalloidin (red) was used to stain the accumulated apical actin in hingepoints (white arrowhead) of control embryos. White line shows width of the neural tube. (**B**) Embryos were injected with 2 ng *DN XNF-ATc3* and 500 pg *EGFP*-RNA at the two-cell stage. Although the neural plate appears wider (horizontal line) on the injected side, phalloidin staining (white arrowhead) is not disrupted. Vertical line sketches the midline of the embryo. (**C**) GFP fluorescence marking the injected side. (**D**) Transverse section of the embryo in B showing phalloidin staining (arrowheads). (**E**) GFP fluorescence of D. (**F**) Phalloidin staining of a control embryo and (**G**) of an embryo treated with 400 μM CsA at the gastrula stage. Arrowheads in F,G indicate normal phalloidin staining. (**H-L**) Cell aggregation assay. (H) Uninjected control neural cells dissociated in CMFM. (I) Neural cells injected with 2 ng DN XNF-ATc3 dissociate faster and more completely than controls. (K) The reaggregation of control neural cells in calcium containing medium (1/3 NMR). (L) Only a few DN XNF-ATc3 expressing cells re-aggregate in 1/3 NMR.

Dosage regulation of XNF-AT is critical for correct formation of the neural tube

Molecules involved in CE movements typically display similar defects in morphogenesis when overexpressed or inhibited, indicating that dose is crucial for the correct movements to ensue. This dose effect has been observed for several PCP molecules involved in mesodermal CE, including dishevelled, frizzled 7 and NRH1 (Djiane et al., 2000; Sasai et al., 2004; Wallingford and Harland, 2001; Wallingford et al., 2000). Therefore, we tested whether overexpression of XNF-ATc3 also affects neural CE. We employed a calcineurin-independent active mutant of XNF-ATc3 (CA XNF-ATc3) to address this issue. To avoid effects on mesoderm, *CA XNF-ATc3* was targeted by injection into the prospective ectoderm of one-cell stage *Xenopus* embryos. Activation of XNF-ATc3 signaling resulted in neural CE defects. Although CA XNF-ATc3 leads to severe neural CE defects, the embryos remained normal throughout gastrulation, with the exception that at the highest concentrations (500 pg RNA) there was a slight delay in closure of the blastopore lip. This is seen in Fig. 3A, which shows embryos expressing different concentrations of CA XNF-ATc3 during gastrula and neurula stages.

To analyze the effect of NF-AT activation on neural marker expression, we injected very low doses of *CA XNF-ATc3* (50 pg RNA) to avoid severe neural CE defects. The embryos were analyzed by in situ hybridization with probes specific for different neural regions. The expression of the cement gland marker *XAG-1*, the forebrain marker *otx2* (data not shown), the midbrain-hindbrain boundary marker *engrailed*, and the hindbrain and neural crest marker, *Krox20*, were reduced (Fig. 3B). The expression of the neural tube markers *Pax3*, *Sox2* and *HoxB9* was wider, indicating mild neural CE defects even at this low concentration. At higher doses of CA XNF-ATc3 we see the same severe neural CE defects as seen with the DN XNF-ATc3 (data not shown). These observations suggest that activation of XNF-ATc3 causes defects in anteroposterior patterning and in neural CE.

Tissue-autonomous requirement of XNF-AT for neural CE

The temporal and spatial inhibition experiments performed above strongly indicate that XNF-ATc3 does affect the morphology of the neural ectoderm. As we only found gastrulation defects when using higher concentrations of CA XNF-ATc3, we analyzed to what degree XNF-ATc3 influences dorsal mesodermal movements. We activated and inhibited XNF-ATc3 signals specifically in either the neural ectoderm or the dorsal mesoderm by targeting *CA XNF-ATc3* or *DN XNF-ATc3* with *GFP* specifically either into the presumptive neural ectoderm or the dorsal mesoderm (see cartoon in Fig. 4). At gastrula stages, we analyzed the GFP fluorescence and confirmed that the constructs were targeted correctly (Fig. 4A). When overexpressed in the neural ectoderm, both *CA XNF-ATc3* and *DN XNF-ATc3* resulted in neural CE defects at concentrations as low as 50 pg and 250 pg, respectively. Overexpression at the same concentrations in the dorsal mesoderm did not affect either neural CE or dorsal mesodermal movements (Fig. 4B). However, expression of higher concentrations of CA XNF-ATc3 in the dorsal mesoderm led to delays in blastopore lip closure (100 pg *CA XNF-ATc3* shows 9% delay, 500 pg *CA XNF-ATc3* results in 36% delayed embryos).

Inhibition of XNF-AT signaling does not inhibit mesodermal CE

We observed that activation, but not inhibition, of XNF-ATc3 within the dorsal mesoderm can alter gastrulation movements. To confirm this finding, we tested whether XNF-ATc3 was necessary and/or sufficient to affect dorsal mesodermal movements in a rigorously defined explant system. Treatment of ectodermal explants with activin leads to the formation of dorsal mesoderm, which elongates as a result of CE (Fig. 5A,B). Inhibition of this elongation, without a change in cell fate, has been the gold standard by which to judge whether a protein can alter CE movements in the mesoderm. Therefore, we repeated the activin-treated ectoderm assay to ascertain whether our observations with XNF-ATc3 in the whole embryo were consistent with those of the explant system. *CA XNF-ATc3* and *DN XNF-ATc3* were injected into the prospective ectoderm of one-cell *Xenopus* embryos. Ectoderm was explanted at stage 9 and cultured in the presence or absence of activin. As in the whole embryos, we did observe inhibition of elongation in activin-

treated explants expressing active CA XNF-ATc3 (Fig. 5C,D). This activity is consistent with that previously described (Saneyoshi et al., 2002). Inhibiting XNF-ATc3, either with DN XNF-ATc3 or CsA, never inhibited activin-induced elongation of explants (Fig. 5E,F), even at high concentrations and in combination with the calcineurin inhibitor FK506 (Flanagan et al., 1991; Liu et al., 1991) (data not shown).

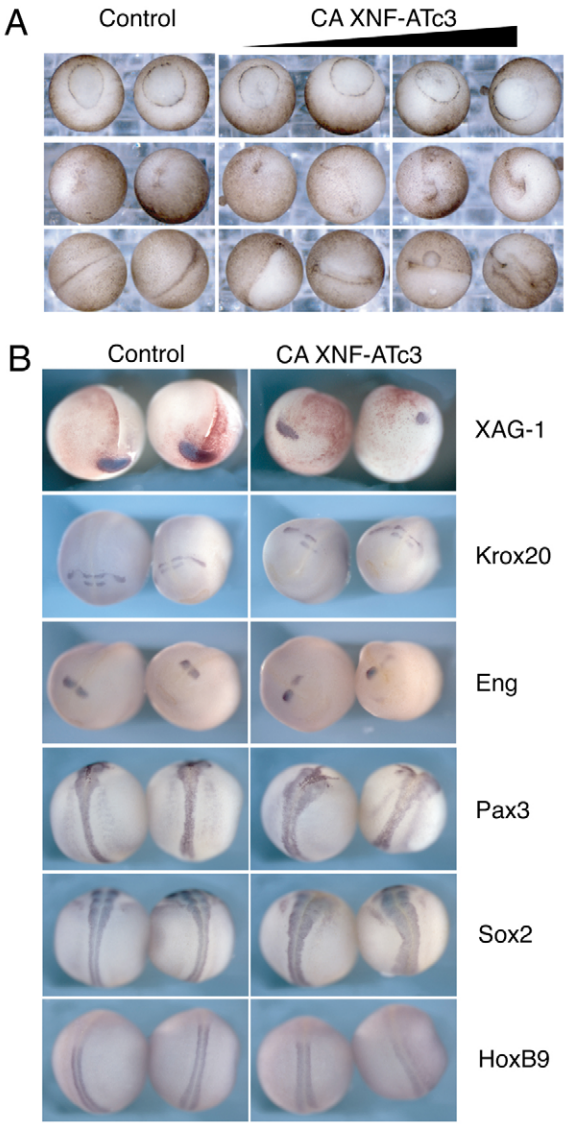

Fig. 3. Activation of XNF-ATc3 generates neural CE defects and anterior truncations. (**A**) *Xenopus* embryos were injected at the one-cell stage with 100 pg (middle panels) or 500 pg (right panels) of *CA XNF-ATc3*. Embryos injected with 100 pg *CA XNF-ATc3* resembled control embryos during gastrula stages (first row, vegetal view of stage 11; second row, vegetal view of stage 12.5), but developed neural tube CE defects in 31% of embryos at neurula stages (third row: dorsal view of stage 17). Embryos injected with 500 pg *CA XNF-ATc3* exhibited slight defects at stage 12.5 and neural CE defects in 81% of injected embryos. Control embryos developed no neural CE defects. (**B**) *Xenopus* embryos were injected at the one-cell stage with 50 pg *CA XNF-ATc3* and analyzed for *Krox20, Eng, Pax3, Sox2* and *HoxB9* expression at the neurula stage. For *XAG-1* in situ hybridization embryos were injected with 250 pg *CA XNF-ATc3* and 100 pg *lacZ* (red) for lineage tracing.

To confirm the observation that DN XNF-ATc3 could not inhibit mesodermal elongation we also used another approach. Keller explants, which are dissected and cultured dorsal mesoderm, are more representative of the endogenous state than activin-treated ectoderm. Therefore, Keller explants were cut from embryos that were injected with *DN XNF-ATc3* and *GFP*. Like in ectodermal explants, we did not see an inhibition of mesodermal CE by DN XNF-ATc3 (Fig. 5G,H). Thus, although activation of XNF-ATc3 is sufficient to inhibit elongation of dorsal mesoderm in explants, blocking XNF-ATc3 signaling does not.

NF-AT activity affects CE in neuralized explants

In whole embryos, NF-AT is necessary for CE in the neural ectoderm. As NF-AT appears to also play a role in elongation of dorsal mesoderm, we sought to further tease apart neural and mesodermal morphogenesis by examining NF-AT signaling in neural explants. To this end, we expressed active or dominant-negative XNF-ATc3 in explants composed of different types of neural ectoderm, which are void of mesoderm.

To determine whether XNF-ATc3 can affect the CE of posterior neural tissue, we overexpressed the transcription factor XBF-2 in ectodermal explants. XBF-2 induces general neural, hindbrain and spinal cord cell fates without inducing mesoderm. These explants elongate as a result of CE in the posterior neural ectoderm (Mariani and Harland, 1998; Wallingford and Harland, 2001). As expected, two-thirds of the XBF-2 expressing animal caps elongated in our experiments, while the uninjected controls did not (Fig. 6A). Repressors of neural CE, like Xdsh-D2, have been shown to inhibit elongation of XBF-2 expressing explants (Wallingford and Harland, 2001). Indeed, co-expression of CA XNF-ATc3 blocks XBF-2 induced CE. RT-PCR analysis on these explants confirmed that the effects are not the result of a dramatic cell fate change (Fig. 6B). Surprisingly, even though inhibition of XNF-ATc3 results in neural tube closure defects in the whole embryo, explants co-expressing DN XNF-ATc3 and XBF-2 elongate at least as well as those expressing only XBF-2 (Fig. 6A).

Furthermore, we analyzed the effect of activating and inhibiting XNF-ATc3 only within anterior neural tissue. To this end, we generated anterior neural tissue by expressing the dominant-negative BMP receptor (BMP DNR) (Graff et al., 1994) in ectodermal explants. As these explants do not contain posterior neural ectoderm, they do not undergo CE. Co-expression of CA XNF-ATc3 and BMP DNR did not lead to elongation of explants. However, if DN XNF-ATc3 was co-expressed with BMP DNR these explants showed some elongation (Fig. 6C). Analysis of neural marker expression by RT-PCR showed an increase in HoxB9 expression compared to explants expressing only BMP DNR or co-expressing both BMP DNR and CA XNF-ATc3 (Fig. 6D). This indicates an increase in posterior marker expression in the DN XNF-ATc3 expressing explants and thus may explain their ability to elongate.

Inhibition of XNF-AT signaling blocks neural CE

Our data shows that CA XNF-ATc3 and DN XNF-ATc3 affect neural CE in opposite ways. Activation of XNF-ATc3 always inhibits CE, whether dorsal mesoderm or neural ectoderm, in vivo or in vitro. Inhibition of XNF-ATc3 is a much more complex story and its effects on neural CE are contradictory. In vivo, DN XNF-ATc3 blocks neural CE. In vitro, it stimulates extension of neuralized explants. We hypothesized that this difference was the result of neighboring non-neural cells within the whole embryo that, when present, can change the competence of the neural tube to respond to inhibition of NF-AT signaling. Therefore, we analyzed

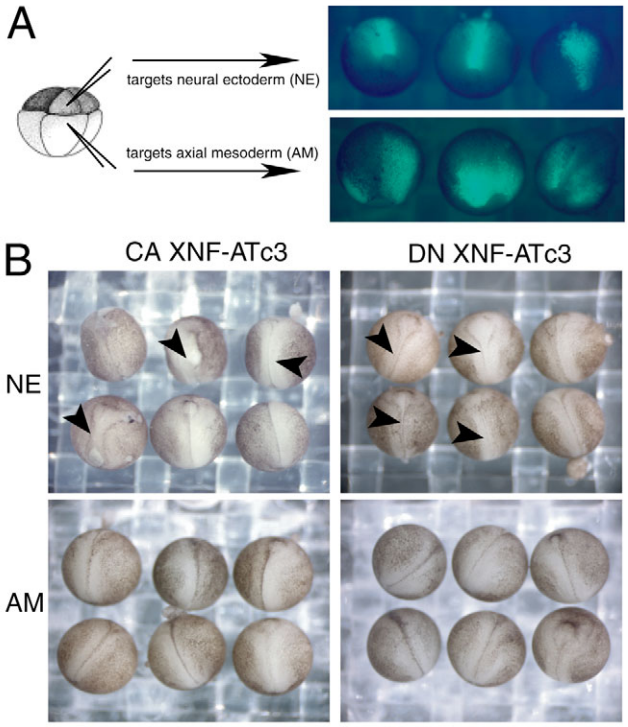

Fig. 4. XNF-ATc3 is necessary for neural ectoderm morphogenesis. (**A**) *CA XNF-ATc3* (50 pg) or 250 pg *DN XNF-ATc3* were co-injected with 50 pg *GFP*. Dorsal animal injections target the posterior neural ectoderm while dorsal vegetal injections target the axial mesoderm. The right panel shows the GFP fluorescence in stage 12 *Xenopus* embryos, demonstrating correct targeting of tissues. (**B**) Only embryos where the RNA was targeted into the neural ectoderm (NE) show neural CE defects at neurula stages (16-18), while embryos from axial mesoderm (AM) injections appear normal, with the exception that high concentration (500 pg) of *CA XNF-ATc3* cause gastrulation delays (data not shown). Neural CE defects are marked with black arrowheads.

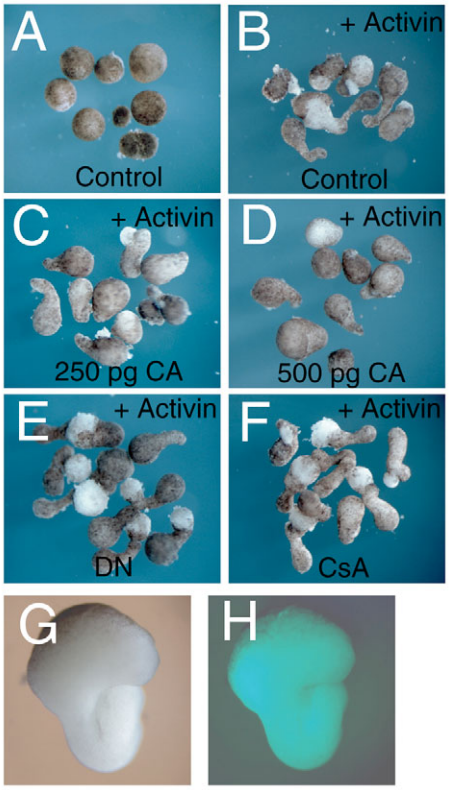

Fig. 5. Inhibition of XNF-ATc3 signaling does not inhibit CE in the mesoderm. Animal caps were treated as follows: (**A**) uninjected control, (**B**) uninjected control treated with activin, (**C**) injected with 250 pg *CA XNF-ATc3* (CA) and treated with activin, (**D**) injected with 500 pg *CA XNF-ATc3* and treated with activin, (**E**) injected with 1 ng *DN XNF-ATc3* (DN) treated with activin, and (**F**) treated with 400 nM CsA and activin. Injection of DN XNF-ATc3 did not inhibit CE even at concentrations of 4 ng *DN XNF-ATc3*. The same was seen in animal caps treated with 4 mM CsA or 400 nM CsA plus FK506, a calcineurin inhibitor that is know to show synergistic effects with CsA (Flanagan et al., 1991; Liu et al., 1991). (**G**,**H**) *DN XNF-ATc3* also failed to inhibit CE in Keller explants overexpressing 2 ng *DN XNF-ATc3* and 200 pg *GFP*. All seven explants from two experiments elongated. (G) Bright-field of an elongating Keller explant. (H) GFP fluorescence of G.

how inhibition of NF-AT signaling shapes CE in explants of the neural plate that contain the underlying mesoderm (Elul and Keller, 2000). We injected one-cell stage embryos with *DN XNF-ATc3* and *GFP* RNA, and removed the GFP-fluorescent neural plates at late gastrula stages (Fig. 7A). These DN XNF-ATc3 expressing neural plates and control neural plates were cultured in vitro until complete neural tube closure was visible in control explants. Images were taken in hourly intervals and CE was evaluated by the change in length (ΔL) and the change in width (ΔW) over the course of the experiment. The length of control explants increased faster than the length of the *DN XNF-ATc3* injected explants. Additionally, DN XNF-ATc3 expressing neural plates remained wider than the controls. We also noted that the DN XNF-ATc3 explants developed a curled shape and their neural tubes were significantly shorter than the ones in control explants. The curling may result from an unequal expression of DN XNF-ATc3. To circumvent this problem, we removed the neural plates of late gastrula stage embryos and treated them with CsA (Fig. 7B) or ethanol as a vehicle control. As the DN XNF-ATc3 explants, the CsA-treated neural plates showed an inhibition in CE compared with the control and ethanol-treated explants. In contrast to the DN XNF-ATc3-overexpressing explants, the CsA-treated neural plates did not curl up and the whole explant appears evenly shorter and wider than the controls. To verify that inhibition of NF-AT signaling is not the result of major cell fate changes, we also analyzed the CsA-treated explants by in situ hybridization using the cement gland marker XAG-1, the rhombomere r3 and r5 marker Krox20, and the spinal cord marker HoxB9. Although XAG-1 expression varied depending on the amount of anterior tissue that was included in the explants, the expression pattern of the CsA-treated explants was identical to the controls (Fig. 7C).

XNF-ATc3 is expressed in the neural tube and anterior neural structures

Xenopus neural morphogenesis is strongly affected by active or dominant-negative XNF-ATc3 or by chemical inhibitors of XNF-AT signaling. However, no *XNF-AT* RNA or protein has ever been spatially localized within the *Xenopus* embryo. Thus, we sought to determine whether *XNF-ATc3* was expressed at the right time and place to have an in vivo role in neural morphogenesis. To localize

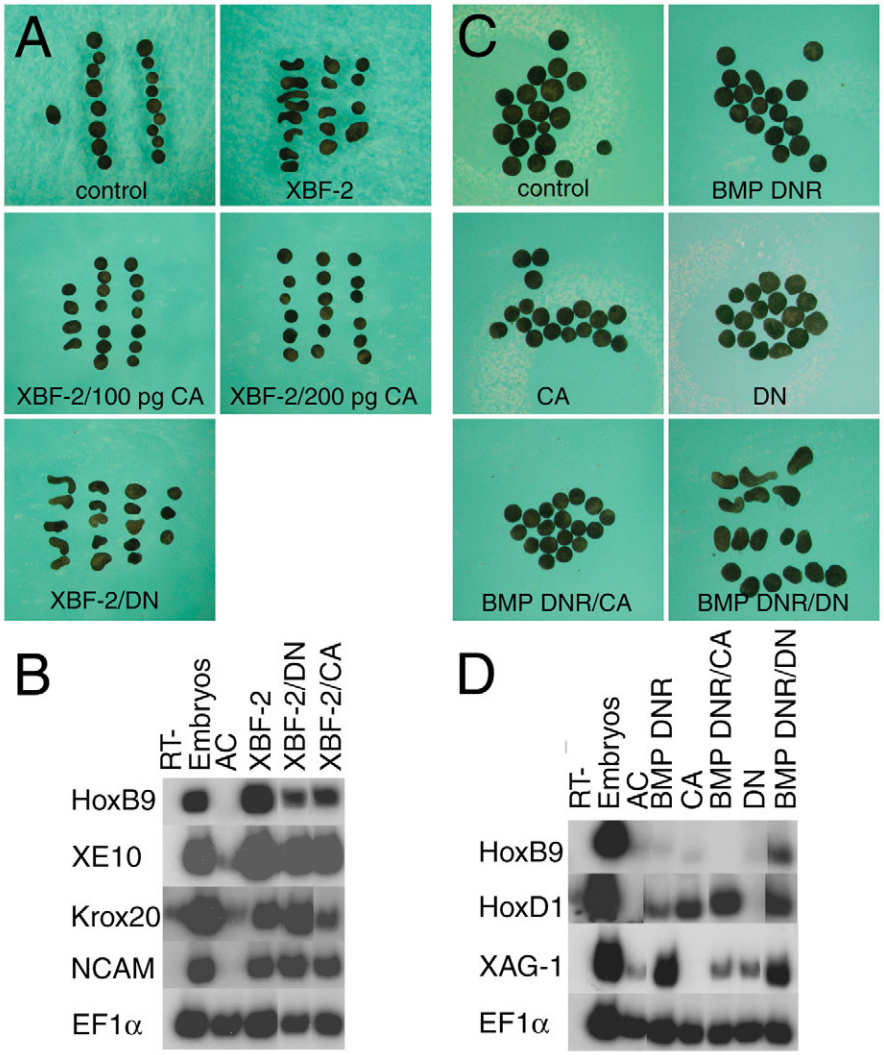

Fig. 6. NF-AT activity in neuralized animal caps. (**A**) One-cell stage embryos were co-injected in the animal hemisphere with 20 pg of *XBF2* and 0.1 ng or 0.2 ng of CA XNF-ATc3 (CA) or 1 ng of DN XNF-ATc3 (DN) encoding RNAs. (**B**) RT-PCR analysis was performed with the markers *HoxB9*, *XE10* (EphA2 receptor), *Krox20*, *NCAM* and EF1α (loading control). (**C**) One-cell stage embryos were injected into the animal hemisphere with 0.2 ng of *BMP DN receptor* (BMP DNR), 0.2 ng of CA XNF-ATc3- or 1 ng of DN XNF-ATc3. BMP-DNR was also co-injected with either CA XNF-ATc3 or DN XNF-ATc3 at the same concentrations. (**D**) RT-PCR analysis was performed with the markers *HoxB9, HoxD1*, *XAG-1* and EF1α (loading control).

XNF-ATc3 expression, we cloned fragments of *XNF-ATc3* that were the most divergent from mouse NF-ATc1-c4 sequences and performed in situ hybridization throughout *Xenopus* development. From the one-cell stage to gastrulation, *XNF-ATc3* mRNA is detected in the animal hemisphere (Fig. 8A-C). At neurula stages, *XNF-ATc3* is predominantly expressed in the neural tube and the anterior region (Fig. 8D,E). Beginning at stage 21, we observe *XNF-ATc3* transcripts in the migrating neural crest, the cement gland, the brain (forebrain to hindbrain), the eye, the otic placode and the somites (Fig. 8E-H). Thus, *XNF-ATc3* is expressed within the early developing neural tube consistent with a role in morphogenesis of this structure.

As NF-AT requires co-factors to be active, simply being present does not imply function (Hogan et al., 2003; Im and Rao, 2004). To determine whether XNF-AT signaling is active within the neural tube, we expressed a reporter plasmid containing a minimal IL-2 promoter followed by green fluorescent protein (GFP) in *Xenopus* embryos [kind gift of G. Crabtree (Graef et al., 1999)]. The reporter is correctly activated by NF-AT and repressed by CsA in *Xenopus* embryos (see Fig. S3 in the supplementary material). As NF-AT binding sites control the expression of GFP, we were able to visualize NF-AT-induced transcription in regions of the embryo that contained any active, dephosphorylated NF-AT, along with the relevant co-activator. This reporter assay provides a read-out for all NF-AT activity, including *XNF-ATc3* and other *Xenopus NF-AT* genes. To ensure that we obtained a read-out for all embryonic regions, we targeted the reporter plasmid to animal, medial and vegetal regions of one-cell stage embryos. The first detectable GFP fluorescence was seen at stage 12 in the developing neural plate (see Fig. S2A, parts a-c in the supplementary material). During neurula stages (15-21), fluorescence increased significantly with the most prominent staining present in the anterior neural regions and in the neural tube (see Fig. S2A, parts d-i in the supplementary material). As development progressed, we also observed fluorescence within the somites and ventral regions (see Fig. S2A, parts j-n in the supplementary material). Therefore, although NF-AT activity occurs throughout the embryo, it is clearly active within the neural tissue at the time of neural tube closure. This supports an endogenous function for XNF-AT signaling within the neural ectoderm.

DISCUSSION

In this paper, we demonstrate that NF-AT signaling is involved in neural CE. We show that activation of NF-AT signaling using constitutively active XNF-ATc3 inhibits CE movements in both the neural tube and dorsal mesoderm, and does so in all contexts studied. Thus, these results solidly implicate NF-AT as an effector of

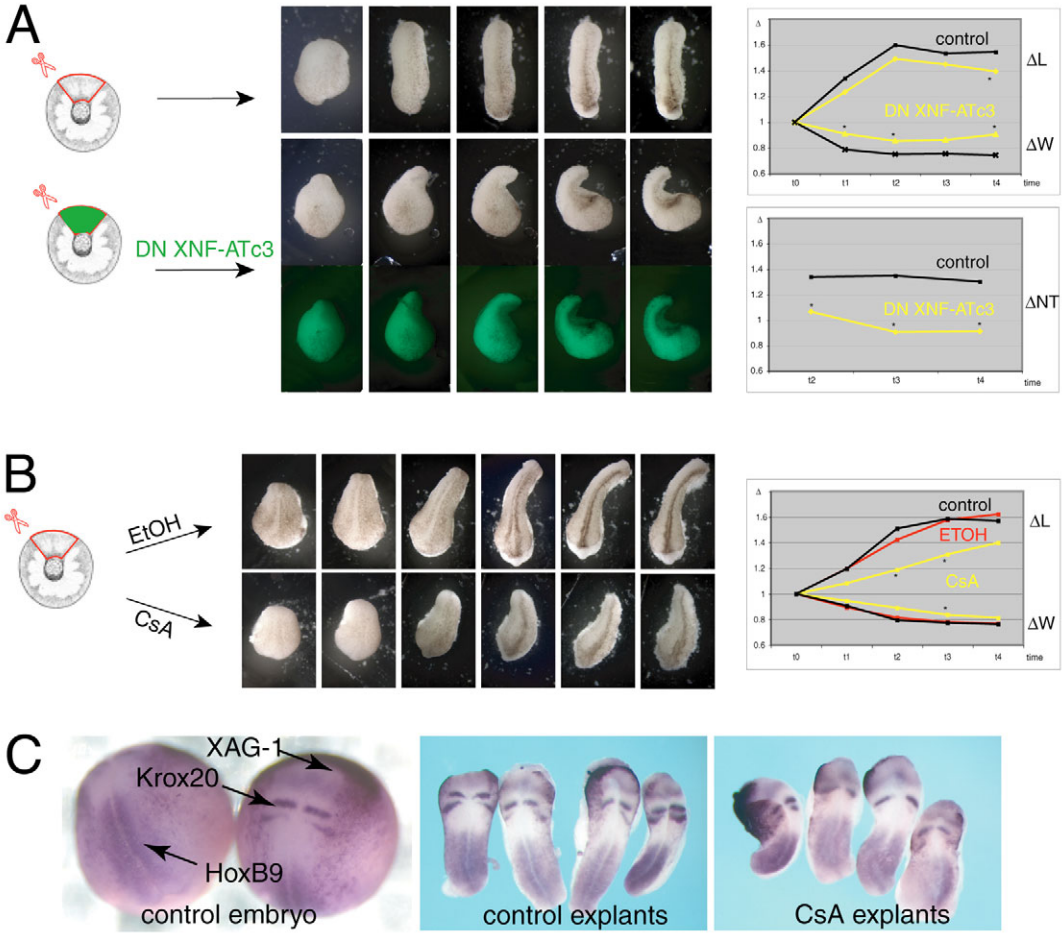

Fig. 7. Inhibition of XNF-ATc3 blocks CE in neural plate explants. (**A**) Neural plate explants from stage 12-12.5 were cultured and photographed hourly. Upper panels show a control neural plate explant. The middle panels show a neural plate explant of an embryo injected with 2 ng *DN XNF-ATc3* and 200 pg *GFP*. The lower panels show GFP fluorescence. The upper graph illustrates the change in length (ΔL) and the change in width (ΔW) over time. The lower graph shows the change in neural tube length (ΔNT) between 2 and 4 hours. Stars indicate where the *DN XNF-ATc3* injected explants are significantly different than the controls in an unpaired Student's *t*-test. (**B**) Neural plate explants incubated in 400 nM CsA (lower panels). As a vehicle control, neural plate explants were incubated in 0.33% ethanol (upper panels). The graph describes the change in length (ΔL) and change in width (ΔW) over time. Stars indicate where the CsA-treated explants were significantly different from the control and ethanol treated explants using an unpaired Student's *t*-test. (**C**) CsA treatment does not lead to cell fate changes in neural plate explants. In situ hybridization using antisense probes for *XAG-1, Krox20* and *HoxB9*. Left panel shows two control embryos used to stage the explants (left, posterior view; right, anterior view). Middle panel presents control neural plate explants, while the right panel shows explants incubated in 400 nM CsA.

convergence and extension. The function of NF-AT in neural CE is a significant finding because very few molecules are known to regulate morphogenesis of the neural tube. As NF-AT signaling is known to be an integrator of multiple varied signals throughout development, we propose that NF-AT is acting downstream of several pathways to mediate these effects.

Several clues suggest that indeed XNF-ATc3 could act downstream of both Wnt/Ca^{2+} and PCP pathways. For example Wnt5a, a known regulator of mesodermal CE (Moon et al., 1993), leads to nuclear localization of XNF-ATc3 in ectodermal explants (Saneyoshi et al., 2002), resulting in neural tube closure defects in whole embryos. Additionally, we know that disruption of Ca^{2+} signaling, which activates NF-AT-dependent transcription, also blocks CE (Wallingford et al., 2001), suggesting that XNF-ATc3 may mediate Wnt/Ca^{2+} signals within the nucleus during neural CE. Furthermore, multiple members of the PCP pathway are known regulators of NF-AT in other contexts. NRH1, a recently identified modulator of PCP signaling, leads to nuclear localization of XNF-ATc3 (Sasai et al., 2004). Furthermore, JNK is known to phosphorylate c-Jun, a component of AP-1, which is a well-studied NF-AT co-activator. Therefore, XNF-ATc3 may be a key downstream regulator of neural CE by modifying both Wnt/Ca^{2+} and PCP signals.

Several intriguing results need to be addressed concerning inhibition of NF-AT signaling within the neural ectoderm. First, overexpression of active NF-AT and dominant-negative NF-AT results in the same effect on neural tube morphogenesis. From the literature on PCP signaling and its role in CE, it is not surprising that inhibition and activation of NF-AT result in the same phenotypic effect. Molecules involved in CE movements typically display similar defects in morphogenesis when overexpressed or inhibited, indicating that dose is absolutely crucial for the correct movements to ensue. This dose effect has been observed for most of the known PCP molecules involved in mesodermal CE,

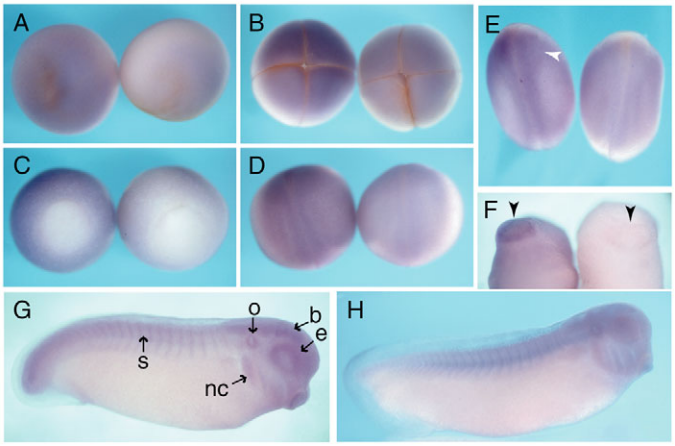

Fig. 8. XNF-ATc3 is expressed in neural tissue. *Xenopus* embryos analyzed for *XNF-ATc3* expression by in situ hybridization: (**A**) One cell, (**B**) four cell, (**C**) stage 11.5, (**D**) stage 15, (**E**) stage 21, (**F**,**G**) stage 26 and (**H**) stage 28. Embryos hybridized with the antisense probe are shown on the left, sense control is shown on the right (A-F). Image perspective: (A,B) animal, (C) vegetal, (D,E) dorsal and (G,H) lateral views; (F) ventral view of the head. White arrowhead in E indicates the cranial neural crest; black arrowheads in F mark the cement glands. Abbreviations: b, brain; e, eye; nc, neural crest; o, otic vesicle; s, somites.

including dishevelled, frizzled 7 and NRH1 (Djiane et al., 2000; Sasai et al., 2004; Wallingford and Harland, 2001; Wallingford et al., 2000).

Second, dominant-negative XNF-ATc3 inhibits neural CE in whole embryos and neural plate explants, but not in neuralized ectodermal explants. A possible explanation for this finding is that, as the ectodermal explants do not contain mesoderm and therefore lack important instructive cues, their elongation may not reflect the endogenous situation. Elul and Keller (Elul and Keller, 2000) and subsequently Ezin et al. (Ezin et al., 2003) have analyzed the protrusive activity of the neural plate in detail. Following gastrulation, the cells of the neural ectoderm develop an organized monopolar character with medially directed motility that is dependent upon the underlying mesoderm (Elul and Keller, 2000). Upon further dissection, this dependence was found to reside within the notochord/notoplate region in the midline. Neural plate explants that lack the midline fail to generate a monopolar character, having a bipolar and mediolateral directed motility. These cells do not converge and extend as well as explants that either contain the midline or have an ectopic midline added (Ezin et al., 2003). These results strongly suggest that signals from the midline instruct the overlying neural ectoderm to obtain monopolar medially directed activities that lead to coordinated and effective CE. Little is known on a cellular level about the mechanisms of CE in neuralized explants. As no mesoderm is contained within these explants, one cannot assume that the movements observed in elongating ectoderm are similar to that of the endogenous situation. Indeed the explants may reflect the weaker CE movements seen within neural plate explants that lack mesoderm and contain bipolar mediolaterally directed protrusive activities. This would be consistent with the observation that explants of posterior neural tissue (including XBF-2 expressing explants) have typically weak effects on elongation.

Why then do neuralized ectodermal explants converge and extend in the presence of DN XNF-ATc3? One explanation is that NF-AT inhibits canonical Wnt signaling. XNF-ATc3 has previously been implicated as a downstream target of both the Wnt/Ca^{2+} pathway and as an inhibitor of canonical Wnt signaling. It has been proposed that DN XNF-ATc3 could indeed activate canonical Wnt signaling within ventral mesoderm (Saneyoshi et al., 2002). Although we do not observe the robust dorsalizing activity of DN XNF-ATc3 reported by Saneyoshi et al., we cannot completely discount this claim. In our experiments, we do see a low level of dorsalization in whole embryos and can also detect a slight induction of Xnr3 in ectoderm expressing DN XNF-ATc3 (data not shown). Thus, we believe that a mild activation of canonical Wnt signaling does exist in response to DN XNF-ATc3. As GSK3β is known to interact directly with and actively phosphorylates NF-AT, this is the most likely target of NF-AT within the canonical Wnt pathway. According to this model, activating canonical Wnt signaling via GSK3β within anterior neural tissue would lead to posteriorization. This newly generated posterior neural tissue would then converge and extend. Our data supports this hypothesis in that we observe posteriorization and elongation of ectoderm exposed to DN XNF-ATc3 (Saneyoshi et al., 2002).

The question remains why this posteriorization does not occur within the whole embryo or within the neural plate explant environment. We propose two answers to this question. First, endogenous regulators, possibly secreted from the notochord, compete for the use of NF-AT as a downstream target and do not allow for an interaction with GSK3β used for inhibition of canonical Wnt signaling. In the neuralized explants, these endogenous regulators are not present simply because mesoderm is not present and therefore XNF-ATc3 signaling may function in a distinctly different manner because of its own promiscuous nature. Second, the neuralized explants and whole embryos have been exposed to vastly different signals and certainly the whole embryo response is a superior read-out of function. Our explants were cut at blastula stage well before the complex cascades of signals sent and received during gastrulation and neurulation in *Xenopus*. It is simply possible that DN XNF-ATc3 is sufficient to posteriorize anterior ectoderm via the Wnt pathway within explants that have only responded to selective signals, whereas within the embryo, posterior patterning proceeds appropriately because the correct signals are present and do not co-opt other players. Certainly, the role of NF-AT is best demonstrated within these intact embryos, which receive appropriate endogenous signals and NF-AT signaling does indeed play an important role in regulating neural morphogenesis within this milieu.

Although XNF-AT can function as an inhibitor of the canonical Wnt pathway – at least in neuralized ectodermal explants, the effects observed in whole embryos on neural CE presented in this paper are not due to inhibition of the canonical Wnt pathway. First, inhibition of NF-AT signaling with CsA after dorsoventral and anteroposterior pattern has already been established, still results in neural tube closure defects. Second, it is unlikely that Wnt signaling is responsible for the neural tube defects generated by DN XNF-ATc3, as they are not accompanied by posteriorization or cell fate changes within the whole embryo. Third, the fate changes we observe after expression of CA XNF-ATc3 are not consistent with an inhibition of canonical Wnt signaling by NF-AT. Canonical Wnt signaling is known to posteriorize neural tissue. Therefore, activation of NF-AT signaling, which according to the published model (Saneyoshi et al., 2002) inhibits canonical Wnt signaling, should anteriorize neural tissue. However, overexpression of CA XNF-ATc3 in whole embryos decreases the expression of both forebrain and midbrain markers, and in explants can induce ectopically the posterior marker HoxD1. Taken together, we hypothesize that although XNF-ATc3 can use the canonical Wnt pathway, this function cannot explain the

effects of XNF-ATc3 on neural tube morphogenesis. Indeed this specific phenotypic effect is most surely caused by mediating PCP or Wnt/Ca^{2+} pathways.

Cellular movements in neural and mesodermal CE are morphologically similar (Keller et al., 1992a) and mediated by conserved signaling pathways. However, there are differences in cell behavior and cell polarity in these two events (Elul and Keller, 2000; Elul et al., 1997; Ezin et al., 2003), which may be controlled by different mechanisms and flavors of signaling targets. So far, only mesodermal CE has been studied in depth at the molecular level and only a few molecules have been shown to function in neural CE. This paper adds XNF-ATc3 to the short list by demonstrating that XNF-ATc3 has a function in neural CE. We suggest that XNF-AT plays a crucial role in the detection of diverse signaling pathways and serves to activate downstream targets that will determine morphogenetic movements within the neural tube. Certainly the molecular nature of NF-AT is well suited to integrate information of multiple signaling events, including Ca^{2+}. The next challenge will be to dissect these pathways and deduce how NF-AT relays the PCP and Wnt/Ca^{2+} pathway to specify movements within the neural plate.

The authors thank G. R. Crabtree, J. W. Gestwicki, I. A. Graef, A. L. Hufton, K. J. Liu, X. Lu and T. Saneyoshi for critical discussion; I. A. Graef, M. Hattori, K. Mikoshiba and T. Saneyoshi for supplying reagents. This work was supported by grants from the National Institute of Health (R03 HD045593 and R01 HD41557).

Supplementary material

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/133/9/1745/DC1

References

Bang, A. G., Papalopulu, N., Goulding, M. D. and Kintner, C. (1999). Expression of Pax-3 in the lateral neural plate is dependent on a Wnt-mediated signal from posterior nonaxial mesoderm. *Dev. Biol.* **212**, 366-380.

Beals, C. R., Clipstone, N. A., Ho, S. N. and Crabtree, G. R. (1997). Nuclear localization of NF-ATc by a calcineurin-dependent, cyclosporin-sensitive intramolecular interaction. *Genes Dev.* **11**, 824-834.

Borchers, A. G., Hufton, A. L., Eldridge, A. G., Jackson, P. K., Harland, R. M. and Baker, J. C. (2002). The E3 ubiquitin ligase GREUL1 anteriorizes ectoderm during Xenopus development. *Dev. Biol.* **251**, 395-408.

Bradley, L. C., Snape, A., Bhatt, S. and Wilkinson, D. G. (1993). The structure and expression of the Xenopus Krox-20 gene: conserved and divergent patterns of expression in rhombomeres and neural crest. *Mech. Dev.* **40**, 73-84.

Brivanlou, A. H. and Harland, R. M. (1989). Expression of an engrailed-related protein is induced in the anterior neural ectoderm of early Xenopus embryos. *Development* **106**, 611-617.

Chen, L., Glover, J. N., Hogan, P. G., Rao, A. and Harrison, S. C. (1998). Structure of the DNA-binding domains from NFAT, Fos and Jun bound specifically to DNA. *Nature* **392**, 42-48.

Crabtree, G. R. and Olson, E. N. (2002). NFAT signaling: choreographing the social lives of cells. *Cell* **109**, S67-S79.

de la Pompa, J. L., Timmerman, L. A., Takimoto, H., Yoshida, H., Elia, A. J., Samper, E., Potter, J., Wakeham, A., Marengere, L., Langille, B. L. et al. (1998). Role of the NF-ATc transcription factor in morphogenesis of cardiac valves and septum. *Nature* **392**, 182-186.

Djiane, A., Riou, J., Umbhauer, M., Boucaut, J. and Shi, D. (2000). Role of frizzled 7 in the regulation of convergent extension movements during gastrulation in Xenopus laevis. *Development* **127**, 3091-3100.

Elul, T. and Keller, R. (2000). Monopolar protrusive activity: a new morphogenic cell behavior in the neural plate dependent on vertical interactions with the mesoderm in Xenopus. *Dev. Biol.* **224**, 3-19.

Elul, T., Koehl, M. A. and Keller, R. (1997). Cellular mechanism underlying neural convergent extension in Xenopus laevis embryos. *Dev. Biol.* **191**, 243-258.

Ezin, A. M., Skoglund, P. and Keller, R. (2003). The midline (notochord and notoplate) patterns the cell motility underlying convergence and extension of the Xenopus neural plate. *Dev. Biol.* **256**, 100-114.

Flanagan, W. M., Corthesy, B., Bram, R. J. and Crabtree, G. R. (1991). Nuclear association of a T-cell transcription factor blocked by FK-506 and cyclosporin A. *Nature* **352**, 803-807.

Graef, I. A., Mermelstein, P. G., Stankunas, K., Neilson, J. R., Deisseroth, K., Tsien, R. W. and Crabtree, G. R. (1999). L-type calcium channels and GSK-3 regulate the activity of NF-ATc4 in hippocampal neurons. *Nature* **401**, 703-708.

Graef, I. A., Chen, F., Chen, L., Kuo, A. and Crabtree, G. R. (2001). Signals transduced by Ca(2+)/calcineurin and NFATc3/c4 pattern the developing vasculature. *Cell* **105**, 863-875.

Graef, I. A., Wang, F., Charron, F., Chen, L., Neilson, J., Tessier-Lavigne, M. and Crabtree, G. R. (2003). Neurotrophins and netrins require calcineurin/NFAT signaling to stimulate outgrowth of embryonic axons. *Cell* **113**, 657-670.

Graff, J. M., Thies, R. S., Song, J. J., Celeste, A. J. and Melton, D. A. (1994). Studies with a Xenopus BMP receptor suggest that ventral mesoderm-inducing signals override dorsal signals in vivo. *Cell* **79**, 169-179.

Harland, R. M. (1991). In situ hybridization: an improved whole-mount method for Xenopus embryos. *Methods Cell Biol.* **36**, 685-695.

Hemmati-Brivanlou, A. and Melton, D. A. (1994). Inhibition of activin receptor signaling promotes neuralization in Xenopus. *Cell* **77**, 273-281.

Hogan, P. G., Chen, L., Nardone, J. and Rao, A. (2003). Transcriptional regulation by calcium, calcineurin, and NFAT. *Genes Dev.* **17**, 2205-2232.

Horsley, V., Friday, B. B., Matteson, S., Kegley, K. M., Gephart, J. and Pavlath, G. K. (2001). Regulation of the growth of multinucleated muscle cells by an NFATC2-dependent pathway. *J. Cell Biol.* **153**, 329-338.

Im, S. H. and Rao, A. (2004). Activation and deactivation of gene expression by Ca2+/calcineurin-NFAT-mediated signaling. *Mol. Cells* **18**, 1-9.

Keller, R. (2002). Shaping the vertebrate body plan by polarized embryonic cell movements. *Science* **298**, 1950-1954.

Keller, R. and Danilchik, M. (1988). Regional expression, pattern and timing of convergence and extension during gastrulation of Xenopus laevis. *Development* **103**, 193-209.

Keller, R., Shih, J. and Sater, A. (1992a). The cellular basis of the convergence and extension of the Xenopus neural plate. *Dev. Dyn.* **193**, 199-217.

Keller, R., Shih, J., Sater, A. K. and Moreno, C. (1992b). Planar induction of convergence and extension of the neural plate by the organizer of Xenopus. *Dev. Dyn.* **193**, 218-234.

Knecht, A. K., Good, P. J., Dawid, I. B. and Harland, R. M. (1995). Dorsal-ventral patterning and differentiation of noggin-induced neural tissue in the absence of mesoderm. *Development* **121**, 1927-1935.

Kolm, P. J. and Sive, H. L. (1995). Regulation of the Xenopus labial homeodomain genes, HoxA1 and HoxD1: activation by retinoids and peptide growth factors. *Dev. Biol.* **167**, 34-49.

Kuhl, M., Sheldahl, L. C., Park, M., Miller, J. R. and Moon, R. T. (2000). The Wnt/Ca2+ pathway: a new vertebrate Wnt signaling pathway takes shape. *Trends Genet.* **16**, 279-283.

Lin, C. S., Boltz, R. C., Siekierka, J. J. and Sigal, N. H. (1991). FK-506 and cyclosporin A inhibit highly similar signal transduction pathways in human T lymphocytes. *Cell Immunol.* **133**, 269-284.

Liu, J., Farmer, J. D., Jr, Lane, W. S., Friedman, J., Weissman, I. and Schreiber, S. L. (1991). Calcineurin is a common target of cyclophilin-cyclosporin A and FKBP-FK506 complexes. *Cell* **66**, 807-815.

Macian, F., Lopez-Rodriguez, C. and Rao, A. (2001). Partners in transcription: NFAT and AP-1. *Oncogene* **20**, 2476-2489.

Mariani, F. V. and Harland, R. M. (1998). XBF-2 is a transcriptional repressor that converts ectoderm into neural tissue. *Development* **125**, 5019-5031.

McEwen, D. G. and Peifer, M. (2000). Wnt signaling: moving in a new direction. *Curr. Biol.* **10**, R562-R564.

Mitchell, L. E., Adzick, N. S., Melchionne, J., Pasquariello, P. S., Sutton, L. N. and Whitehead, A. S. (2004). Spina bifida. *Lancet* **364**, 1885-1895.

Mizuseki, K., Kishi, M., Matsui, M., Nakanishi, S. and Sasai, Y. (1998). Xenopus Zic-related-1 and Sox-2, two factors induced by chordin, have distinct activities in the initiation of neural induction. *Development* **125**, 579-587.

Moon, R., Campbell, R., Christian, J., McGrew, L., Shih, J. and Fraser, S. (1993). Xwnt-5A: a maternal Wnt that affects morphogenetic movements after overexpression in embryos of Xenopus laevis. *Development* **119**, 97-111.

Pandur, P. D. and Moody, S. A. (2000). Xenopus Six1 gene is expressed in neurogenic cranial placodes and maintained in the differentiating lateral lines. *Mech. Dev.* **96**, 253-257.

Peifer, M. and McEwen, D. G. (2002). The ballet of morphogenesis: unveiling the hidden choreographers. *Cell* **109**, 271-274.

Rao, A., Luo, C. and Hogan, P. G. (1997). Transcription factors of the NFAT family: regulation and function. *Annu. Rev. Immunol.* **15**, 707-747.

Saneyoshi, T., Kume, S., Amasaki, Y. and Mikoshiba, K. (2002). The Wnt/calcium pathway activates NF-AT and promotes ventral cell fate in Xenopus embryos. *Nature* **417**, 295-299.

Sasai, N., Nakazawa, Y., Haraguchi, T. and Sasai, Y. (2004). The neurotrophin-receptor-related protein NRH1 is essential for convergent extension movements. *Nat. Cell Biol.* **6**, 741-748.

Schoenwolf, G. C. and Smith, J. L. (1990). Mechanisms of neurulation: traditional viewpoint and recent advances. *Development* **109**, 243-270.

Sharpe, C. R., Fritz, A., De Robertis, E. M. and Gurdon, J. B. (1987). A homeobox-containing marker of posterior neural differentiation shows the importance of predetermination in neural induction. *Cell* **50**, 749-758.

Shih, J. and Keller, R. (1992a). Cell motility driving mediolateral intercalation in explants of Xenopus laevis. *Development* **116**, 901-914.

Shih, J. and Keller, R. (1992b). Patterns of cell motility in the organizer and dorsal mesoderm of Xenopus laevis. *Development* **116**, 915-930.
Sive, H. L., Hattori, K. and Weintraub, H. (1989). Progressive determination during formation of the anteroposterior axis in Xenopus laevis. *Cell* **58**, 171-180.
Sive, H. L., Grainger, R. M. and Harland, R. M. (2000). *Early Development of* Xenopus Laevis*: A Laboratory Manual.* Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.
Slusarski, D. C., Corces, V. G. and Moon, R. T. (1997a). Interaction of Wnt and a Frizzled homologue triggers G-protein-linked phosphatidylinositol signalling. *Nature* **390**, 410-413.
Slusarski, D. C., Yang-Snyder, J., Busa, W. B. and Moon, R. T. (1997b). Modulation of embryonic intracellular Ca^{2+} signaling by Wnt-5A. *Dev. Biol.* **182**, 114-120.
Smith, W. C. and Harland, R. M. (1991). Injected Xwnt-8 RNA acts early in Xenopus embryos to promote formation of a vegetal dorsalizing center. *Cell* **67**, 753-765.
Torres, M. A., Yang-Snyder, J. A., Purcell, S. M., DeMarais, A. A., McGrew, L. L. and Moon, R. T. (1996). Activities of the Wnt-1 class of secreted signaling factors are antagonized by the Wnt-5A class and by a dominant negative cadherin in early Xenopus development. *J. Cell Biol.* **133**, 1123-1137.
Wallingford, J. B. and Harland, R. M. (2001). Xenopus Dishevelled signaling regulates both neural and mesodermal convergent extension: parallel forces elongating the body axis. *Development* **128**, 2581-2592.
Wallingford, J. B., Rowning, B. A., Vogeli, K. M., Rothbacher, U., Fraser, S. E. and Harland, R. M. (2000). Dishevelled controls cell polarity during Xenopus gastrulation. *Nature* **405**, 81-85.
Wallingford, J. B., Ewald, A. J., Harland, R. M. and Fraser, S. E. (2001). Calcium signaling during convergent extension in Xenopus. *Curr. Biol.* **11**, 652-661.
Wallingford, J. B., Fraser, S. E. and Harland, R. M. (2002). Convergent extension: the molecular control of polarized cell movement during embryonic development. *Dev. Cell* **2**, 695-706.
Weinstein, D. C., Rahman, S. M., Ruiz, J. C. and Hemmati-Brivanlou, A. (1996). Embryonic expression of eph signalling factors in Xenopus. *Mech. Dev.* **57**, 133-144.
Yamanaka, H., Moriguchi, T., Masuyama, N., Kusakabe, M., Hanafusa, H., Takada, R., Takada, S. and Nishida, E. (2002). JNK functions in the non-canonical Wnt pathway to regulate convergent extension movements in vertebrates. *EMBO Rep.* **3**, 69-75.
Yoshida, Y., Kim, S., Chiba, K., Kawai, S., Tachikawa, H. and Takahashi, N. (2004). Calcineurin inhibitors block dorsal-side signaling that affect late-stage development of the heart, kidney, liver, gut and somitic tissue during Xenopus embryogenesis. *Dev. Growth Differ.* **46**, 139-152.