

Nucleoredoxin regulates the Wnt/planar cell polarity pathway in *Xenopus*

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The Wnt signaling pathway is conserved across species, and is essential for early development. We previously identified nucleoredoxin (NRX) as a protein that interacts with dishevelled (Dvl) *in vivo* to negatively regulate the Wnt/ β -catenin pathway. However, whether NRX affects another branch of the Wnt pathway, the Wnt/planar cell polarity (PCP) pathway, remains unclear. Here we show that NRX regulates the Wnt/PCP pathway. In *Xenopus laevis*, over-expression or depletion of NRX by injection of NRX mRNA or antisense morpholino oligonucleotide, respectively, yields the bent-axis phenotype that is typically observed in embryos with abnormal PCP pathway activity. In co-injection experiments of Dvl and NRX mRNA, NRX suppresses the Dvl-induced bent-axis phenotype. Over-expression or depletion of NRX also suppresses the convergent extension movements that are believed to underlie normal gastrulation. We also found that NRX can inhibit Dvl-induced up-regulation of c-Jun phosphorylation. These results indicate that NRX plays crucial roles in the Wnt/PCP pathway through Dvl and regulates *Xenopus* gastrulation movements.

Introduction

Signaling pathways stimulated by secreted Wnt ligands are known to be essential for early development in multicellular species (Moon *et al.* 2004; Clevers 2006). The Wnt signaling pathway was first identified in *Drosophila melanogaster*, and it was later shown to be conserved through evolution, from nematodes to mammals. Genetic and biochemical studies have clarified the framework of the Wnt signaling pathway and showed that it can be divided into several branches. The Wnt/ β -catenin pathway, the first identified Wnt pathway (also known as the “canonical” Wnt pathway), is involved in accumulation of β -catenin and subsequent activation of transcription

factor T-cell factor/lymphoid enhancer factor (TCF/LEF). The Wnt/ β -catenin pathway affects cell growth and cell fate through regulation of various target gene expression. Recent studies have shown that the Wnt/ β -catenin pathway is important in stem cell maintenance. It is also widely known that aberrant activation of the Wnt/ β -catenin pathway is a major cause of various types of human tumors, such as colorectal tumors.

Another well-characterized branch of the Wnt signaling pathway, the Wnt/planar cell polarity (PCP) pathway, governs cell polarity and movement. PCP signaling was also first identified in *Drosophila*. A mutant fly *frizzled* (*fz*), with abnormal orientation of cuticular hairs and bristles was first reported (Vinson & Adler 1987). Genetic screening in *Drosophila* identified many of the components of the PCP pathway, including dishevelled (Dvl), Van Gogh/Strabismus (Vang/Stbm), Rho and c-Jun (Theisen

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et al. 1994; Strutt *et al.* 1997; Boutros *et al.* 1998; Taylor *et al.* 1998; Wolff & Rubin 1998). Notably, Dvl is a component of both the Wnt/ β -catenin pathway and the Wnt/PCP pathway and is regarded as a branchpoint between these two pathways (Axelrod *et al.* 1998; Boutros *et al.* 1998). Further studies showed that activation or loss-of-function of PCP pathway components affects various processes in many organisms, such as ommatidia polarity in the *Drosophila* compound eye, neuronal polarity in mammalian neurons, and gastrulation movements in vertebrates (Tada & Smith 2000; Wallingford *et al.* 2000, 2002; Ciani & Salinas 2005). Therefore, it is widely accepted that the Wnt/PCP pathway is conserved across species.

Xenopus has a number of advantages as a brilliant model organism for developmental analyses, such as large embryos, availability for microsurgies and external development. Since the 19th century, numerous researchers have utilized *Xenopus* to clarify the mechanisms of early development. Many developmental processes and underlying signaling pathways, including the Wnt signaling pathway, have been investigated in *Xenopus* embryos. Hyperactivation or suppression of PCP pathway molecules, such as Dvl and Stbm, in *Xenopus* causes severe gastrulation defects and results in a bent-axis phenotype (Wallingford *et al.* 2000; Darken *et al.* 2002). This phenotype is caused by errors in convergent extension, a polarized intercalation movement of the sheet-formed cells, which results in narrowing in one dimension and perpendicular elongation (Keller *et al.* 1985).

In a previous study, we searched for novel interacting partners of Dvl and identified nucleoredoxin (NRX) (Funato *et al.* 2006). NRX is a member of the thioredoxin (TRX) redox-regulating protein family (Funato & Miki 2007; Lillig & Holmgren 2007). TRX family proteins have thiol-oxidoreductase activity, which is often exerted as a disulfide bond reducing reaction. NRX binds to Dvl *in vivo* in a redox-dependent manner. Over-expression and knockdown analyses in mammalian culture cells indicated that NRX inhibits the Wnt/ β -catenin pathway. Developmental analyses of *Xenopus* embryos also supported this conclusion. Therefore, the negative effect of NRX on the Wnt/ β -catenin pathway has been solidly confirmed. In contrast, the effect of NRX on the Wnt/PCP pathway remains unknown.

In the present study, we investigated the possible role of NRX in the Wnt/PCP pathway. We found that NRX can also regulate the Wnt/PCP pathway as well as Wnt/ β -catenin pathway and that NRX is required for proper gastrulation movements in *Xenopus*. Moreover, NRX inhibited Dvl-induced phosphorylation of c-Jun, which is known to be a crucial biochemical mechanism regulating the PCP pathway.

Results

Over-expression and inhibition of NRX causes bent-axis phenotype through Dvl

In our previous study, we identified NRX as a negative regulator of the Wnt/ β -catenin pathway (Funato *et al.* 2006). To evaluate the role of NRX in the Wnt/PCP pathway in *Xenopus*, we injected NRX mRNA into the dorsoanimal (DA) region of 8-cell-stage fertilized *Xenopus* eggs. The NRX mRNA-injected embryos had a short, bent-axis or open blastopore with gastrulation defects (Fig. 1 A–D,H). The severity of each phenotype increased in a dose-dependent manner. These phenotypes of NRX mRNA-injected tadpoles were similar to those of embryos injected with Dvl mRNA (Fig. 1E–H). Over-expression/loss-of-function of Dvl is reported to perturb the Wnt/PCP pathway and cause gastrulation defects, resulting in a bent-axis phenotype (Sokol 1996; Wallingford *et al.* 2000). The effect of NRX mRNA injection was less severe than that of Dvl mRNA injection.

We previously reported that NRX functions as a negative regulator of the Wnt/ β -catenin pathway by directly inhibiting the function of Dvl (Funato *et al.* 2006). Therefore, we co-injected both Dvl and NRX mRNAs to determine if there is a functional interaction between Dvl and NRX with respect to the bent-axis phenotype in *Xenopus*. Co-injection of NRX mRNA clearly reduced the severity of the bent-axis phenotype in Dvl mRNA-injected embryos in a dose-dependent manner (Fig. 2), suggesting that NRX can attenuate the Wnt/PCP pathway by inhibiting Dvl function.

We carried out loss-of-function analyses with MO against *Xenopus* NRX. First, we examined whether *Xenopus* NRX is expressed during gastrulation. By reverse transcription-polymerase chain reaction (RT-PCR) analyses, we detected positive signals because of the *Xenopus* NRX expression in all areas we analyzed (dorsal, lateral and ventral marginal zones) at stage 10, a critical stage for gastrulation (Fig. 3A). Next, the specificity of NRX-MO was examined by using western blot analyses. We constructed a vector encoding GFP with an additional 5' sequence that is complementary to the sequence of NRX-MO (pCS2-NRX5'UTR-GFP). Embryos were co-injected with the above vector and NRX-MO or control MO, and the lysates were subjected to Western blot analyses with anti-GFP antibody. NRX-MO clearly suppressed the expression of GFP in a dose-dependent manner, whereas control MO did not (Fig. 3B). We then injected NRX-MO into the DA region of the eight-cell embryos. As a result, we observed a significant bent-axis phenotype (Fig. 3C,D,F). This phenotype was similar to that of embryos injected with MO against *Idax*, which

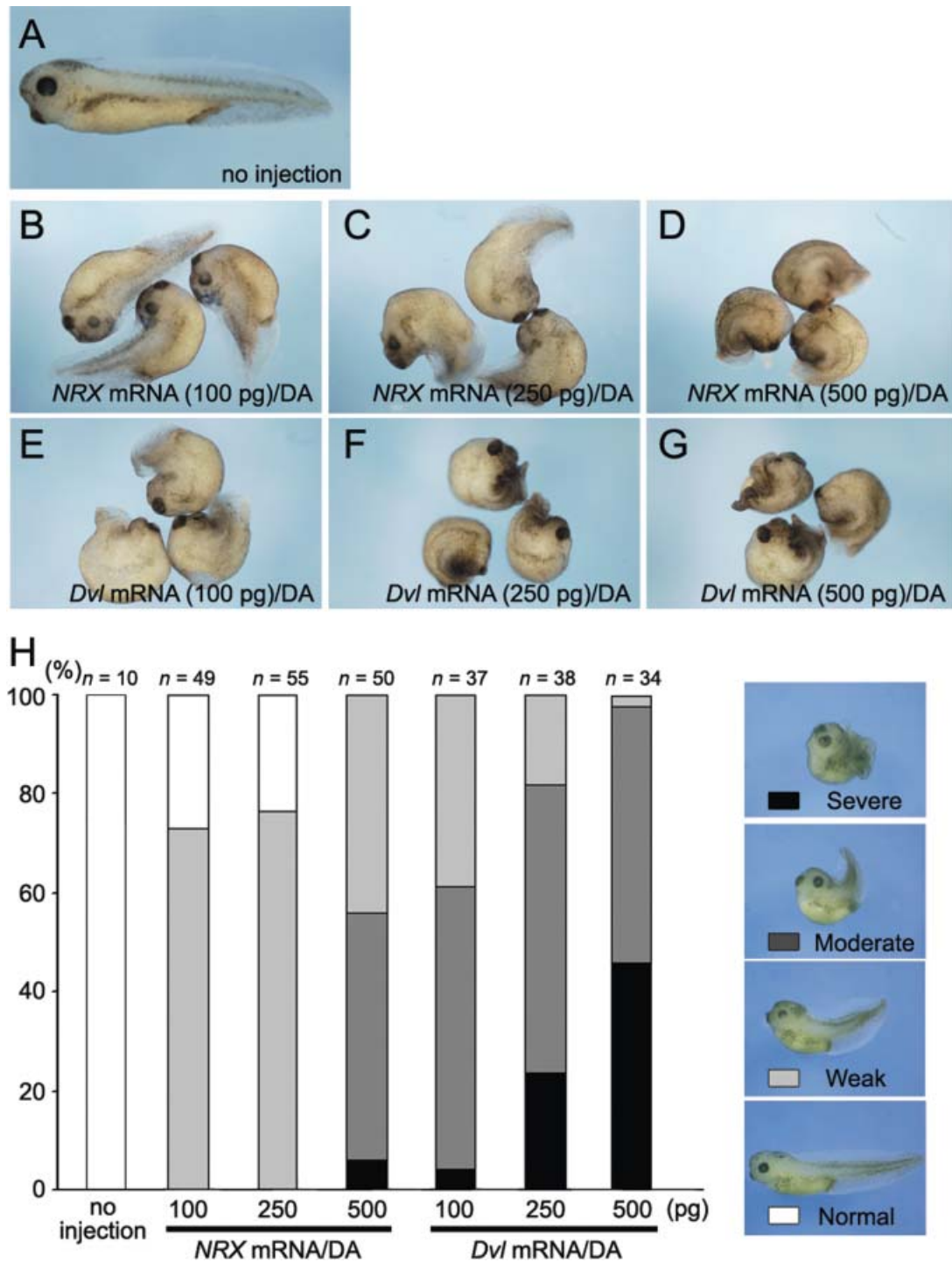


Figure 1 Bent-axis phenotype caused by *NRX* mRNA injection. Representative embryos with (A) no injection, (B–D) *NRX* mRNA injection (100, 250 or 500 pg), and (E–G) *Dvl* mRNA injection (100, 250 or 500 pg). (H) The ratio of bent-axis phenotype caused by *NRX* or *Dvl* mRNA injection. Representative embryos with “severe”, “moderate”, “weak” and “normal” phenotypes are also shown.

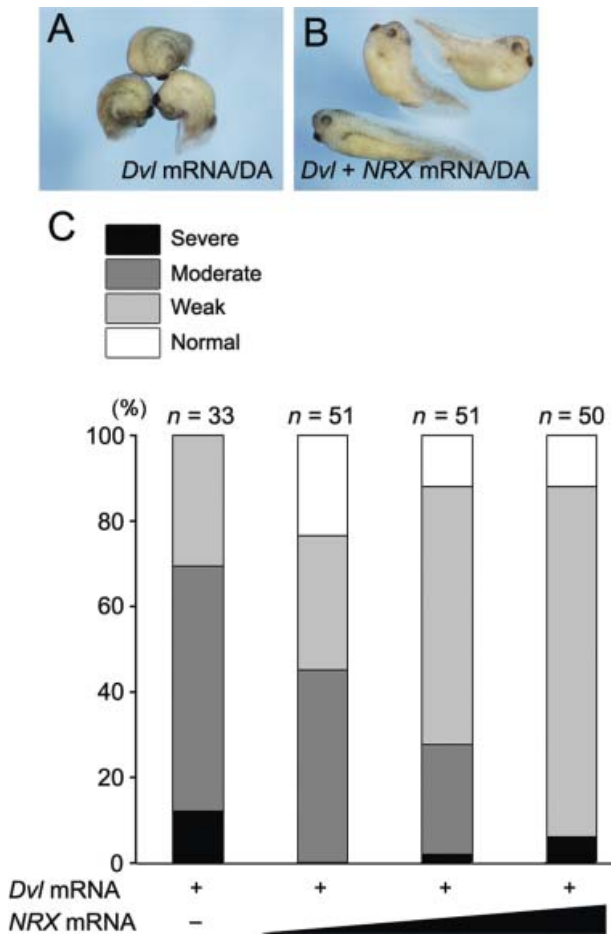


Figure 2 The effect of *NRX* mRNA co-injection with *Dvl* mRNA on bent-axis phenotypes. Representative embryos with (A) *Dvl* mRNA injection (100 pg) and (B) *Dvl* mRNA (100 pg) and *NRX* mRNA (100, 500 or 1000 pg) co-injection. (C) The ratio of bent-axis phenotype by injection of *Dvl* mRNA alone, or with *NRX* mRNA.

also appears to regulate the Wnt/PCP pathway (Fig. 3E,F; Michiue *et al.* in preparation). As mentioned earlier, the bent-axis phenotype in *Xenopus* is caused both by the gain- and loss-of-function of PCP components (Wallingford *et al.* 2000; Darken *et al.* 2002). Therefore, the results obtained by injection of *NRX* mRNA or MO are consistent with the notion that *NRX* is probably involved in the Wnt/PCP pathway.

We next carried out co-injection experiments with *NRX* mRNA and *NRX*-MO. The co-injection of *NRX*-MO clearly rescued the bent-axis phenotype observed in embryos injected with *NRX* mRNA alone, as well as *Dvl* mRNA co-injection (Fig. 4A–D). However, co-injection of β -catenin or GSK3 β , well-characterized selective activator or inhibitor of the Wnt/ β -catenin

pathway, respectively, did not significantly alter the bent-axis phenotype induced by *NRX* mRNA injection (Fig. 4E–G). These results strongly suggest that *NRX* directly affects the Wnt/PCP pathway, but not indirectly through the suppression of the Wnt/ β -catenin pathway. We also examined the expression of mesoderm marker *Xenopus brachyury* (*Xbra*) and confirmed that injection of neither *NRX* mRNA nor *NRX*-MO has significant effect on the expression pattern of *Xbra* (data not shown), suggesting the importance of *NRX* in the Wnt/PCP pathway.

To further confirm the physiological importance of *NRX* in the Wnt/PCP pathway, we also examined whether *NRX* functions cooperatively with *Wnt11*, a known Wnt ligand that governs the Wnt/PCP pathway (Heisenberg *et al.* 2000; Tada & Smith 2000). We injected *Wnt11* mRNA and examined the effect of *NRX* co-injection. As reported previously, *Wnt11* mRNA injection showed only weak dorsalizing effect (Glinka *et al.* 1996; Tada & Smith 2000). Actually, embryos injected with *Wnt11* mRNA alone showed only weak bent-axis (Fig. 4H). However, *NRX*-MO co-injection with *Wnt11* mRNA resulted in moderate or severe bent-axis phenotype even at the concentration of *NRX*-MO that can only induce weak bent-axis phenotype (Fig. 4I,J). This synergistic effect between *Wnt11* mRNA and *NRX*-MO also strongly supports our idea that *NRX* is a component of the Wnt/PCP pathway.

NRX is required for convergent extension movements

Abrogation of the Wnt/PCP pathway in *Xenopus* is known to cause convergent extension errors, resulting in defects in gastrulation and the bent-axis phenotype (Wallingford *et al.* 2002). To evaluate the function of *NRX* in *Xenopus* convergent extension, we co-injected Alexa Fluor 488 with *NRX* mRNA or *NRX*-MO into the DA region. At stage 11, ectodermal cells were migrating toward the vegetal region with the involution of the mesoderm. At this stage, all embryos including *NRX*-MO-injected embryos showed a broad pattern of fluorescence throughout the presumptive ectoderm to the blastopore lip, and differences between embryos were not evident (Fig. 5, left column). After stage 11, migration of mesoderm had proceeded and, at stage 13, epiboly was almost complete, and the ectodermal cells had converged midiolaterally. As a result, control embryos injected with Alexa Fluor 488 alone showed a very narrow fluorescence pattern (Fig. 5, right column). However, *NRX* mRNA- or *NRX*-MO-injected embryos still showed a broad distribution of fluorescence, suggesting that there are defects in convergent

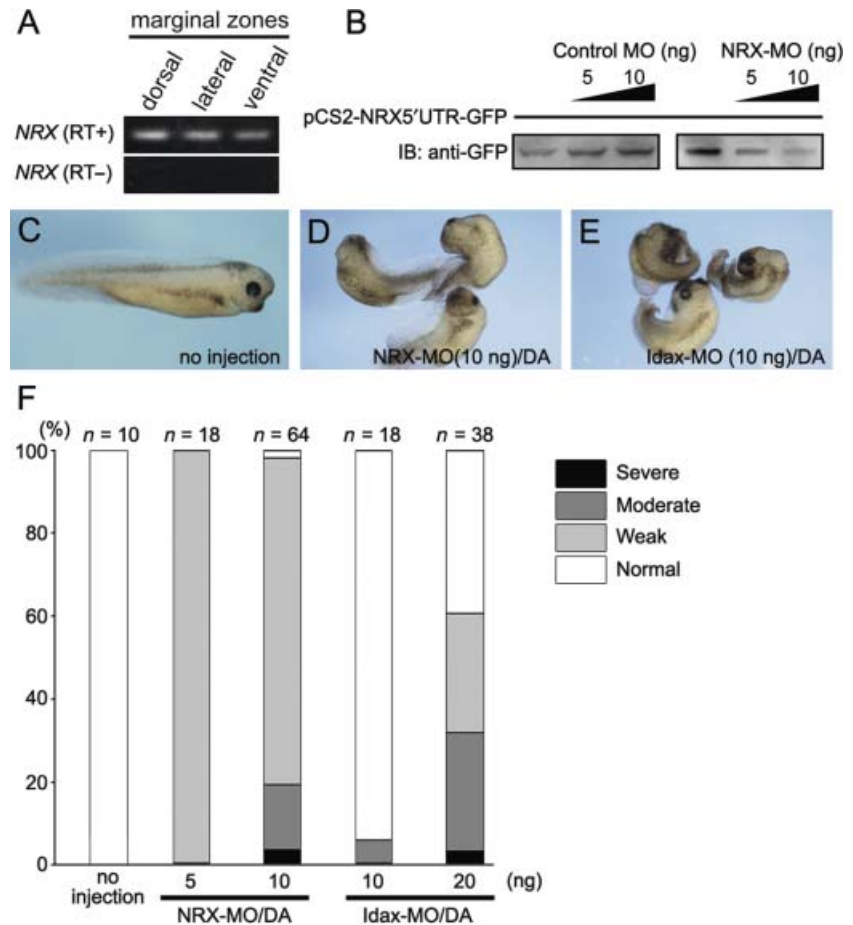


Figure 3 Bent-axis phenotype caused by NRX-MO injection. (A) Total RNAs were collected from dissected stage 10 *Xenopus* embryos, and RT-PCR analyses for NRX were carried out (B) pCS2-NRX5'UTR-GFP (50 pg) and NRX-MO (5, 10 ng) or control MO (5, 10 ng) were injected, and the embryos were harvested and subjected to Western blot analyses. (C–E) Representative embryos with (C) no injection, (D) Idax-MO injection (10 ng), and (E) NRX-MO injection (10 ng). (F) The ratio of bent-axis phenotype by Idax- or NRX-MO injection.

extension of ectodermal cells in these embryos. NRX-MO-injected embryos showed open blastopores. Taken together, these results suggest that NRX is involved in epiboly.

We next carried out elongation assays with animal cap explants. In this *in vitro* culture model, elongation of animal cap explants in response to activin occurs as a result of convergent extension movements (Cunliffe & Smith 1992). Animal cap assays have been used to evaluate the possible effect on convergent extension. Animal cap cells were dissected from injected embryos and treated with 10 ng/mL activin A. We observed a significant elongation of animal caps derived from control (uninjected) embryos or *LacZ* mRNA- or control MO-injected embryos (Fig. 6A). However, animal caps derived from *Dvl* mRNA-, NRX mRNA- or NRX-MO-injected embryos elongated only slightly, indicating the importance of NRX in convergent extension movements. Animal cap elongation can also be affected by mesoderm induction (Cunliffe & Smith 1992). To exclude the possibility that the effect of NRX on animal cap elongation is due to mesoderm induction, we examined the expression of

mesoderm marker genes by RT-PCR analysis of RNAs from animal cap explants. We found no significant difference in activin-induced expression of well-known mesoderm marker genes *goosecoid* (*gsc*) and *Xbra* after the injection of NRX mRNA or NRX-MO (Fig. 6B). Our data strongly suggest that NRX affects *Xenopus* gastrulation movements via regulation of convergent extension.

To further examine the functional relationship between Dvl and NRX in convergent extension, we carried out animal cap explant assays using mRNA of Xdd, the central PDZ domain-lacking mutant form of *Xenopus* Dvl that shows a strong dominant-negative effect on convergent extension movements (Sokol 1996; Wallingford *et al.* 2000). As reported, Xdd mRNA-injected animal caps failed to elongate (Fig. 6C). However, we observed that the animal caps injected with both Xdd mRNA and NRX-MO elongated to the level comparable to that of noninjected animal caps (Fig. 6C). Considering that the injection of NRX-MO alone inhibits animal cap elongation (Fig. 6A), these data strongly support our notion that NRX functions antagonistically against Dvl in convergent extension movements.

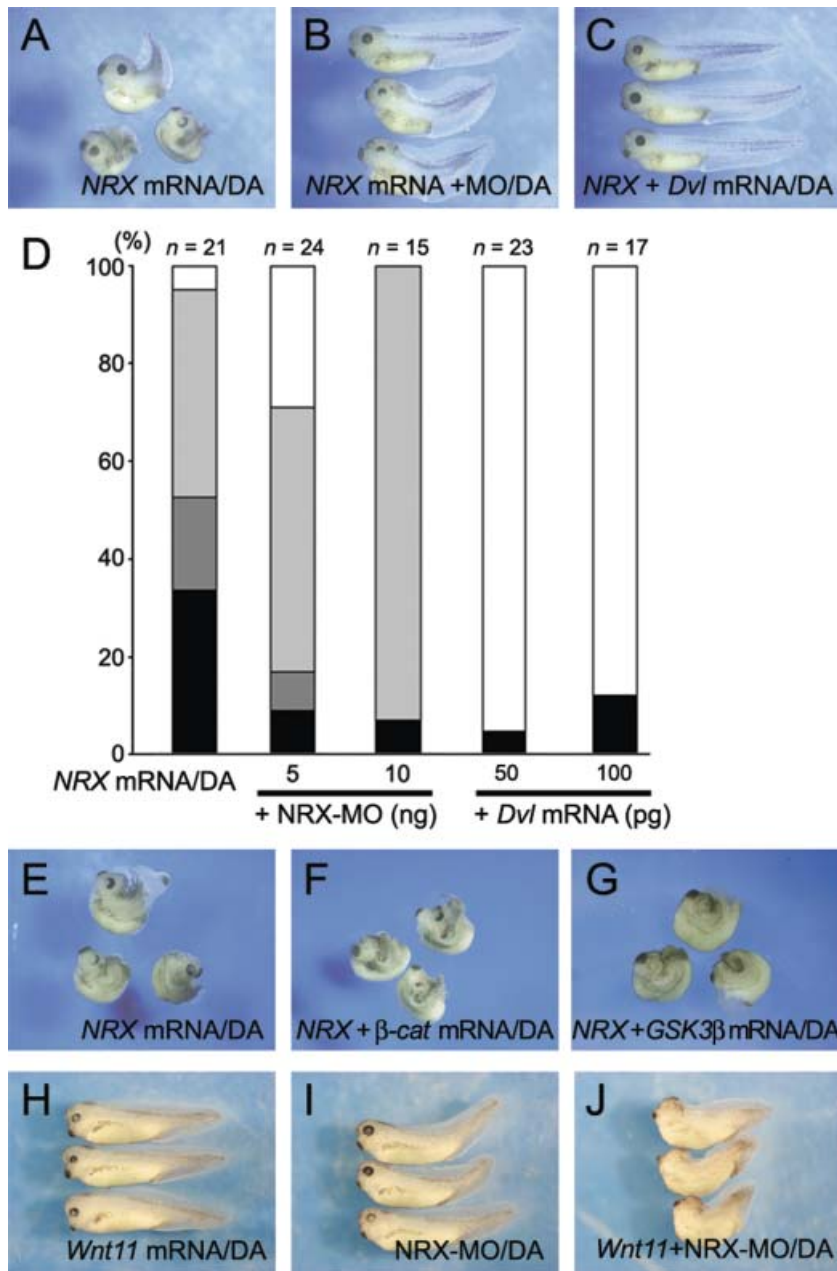


Figure 4 Co-injection experiments with NRX mRNA and NRX-MO. (A–C) Representative embryos injected with (A) NRX mRNA alone (200 pg), (B) NRX mRNA and NRX-MO (5 ng), (C) NRX mRNA and *Dvl* mRNA (100 pg). (D) The ratio of bent-axis phenotype by injection of NRX mRNA alone, or with NRX-MO or *Dvl* mRNA. (E–G) Embryos injected with (E) NRX mRNA (500 pg) alone, (F) NRX (500 pg) and β -catenin (500 pg) mRNA, and (G) NRX (500 pg) and GSK3 β (500 pg) mRNA. (H–J) Embryos injected with (H) *Wnt11* mRNA alone (500 pg), (I) NRX-MO alone (5 ng), (J) *Wnt11* mRNA (500 pg) and NRX-MO (5 ng).

Suppression of Dvl-induced c-Jun phosphorylation by NRX

Activation of the Wnt/PCP pathway induces JNK activation and subsequent phosphorylation of c-Jun (Boutros *et al.* 1998; Li *et al.* 1999; Moriguchi *et al.* 1999). Therefore, we examined the effect of NRX on c-Jun phosphorylation. When we expressed Dvl in mammalian cells, we observed a significant increase in phosphorylation of c-Jun (Fig. 7A, approximately 4.8-fold compared with

cells expressing c-Jun alone). Expression of NRX reduced the phosphorylation of c-Jun.

We next carried out co-expression experiments with Dvl and NRX. We found that Dvl-induced phosphorylation of c-Jun was reduced significantly by NRX (from 5.3-fold to 1.9-fold) as well as Par1b, a reported inhibitor of Dvl-induced c-Jun phosphorylation (Sun *et al.* 2001) (Fig. 7B).

Phosphorylation of c-Jun occurs downstream of Rac activation (Habas *et al.* 2003). To confirm that NRX

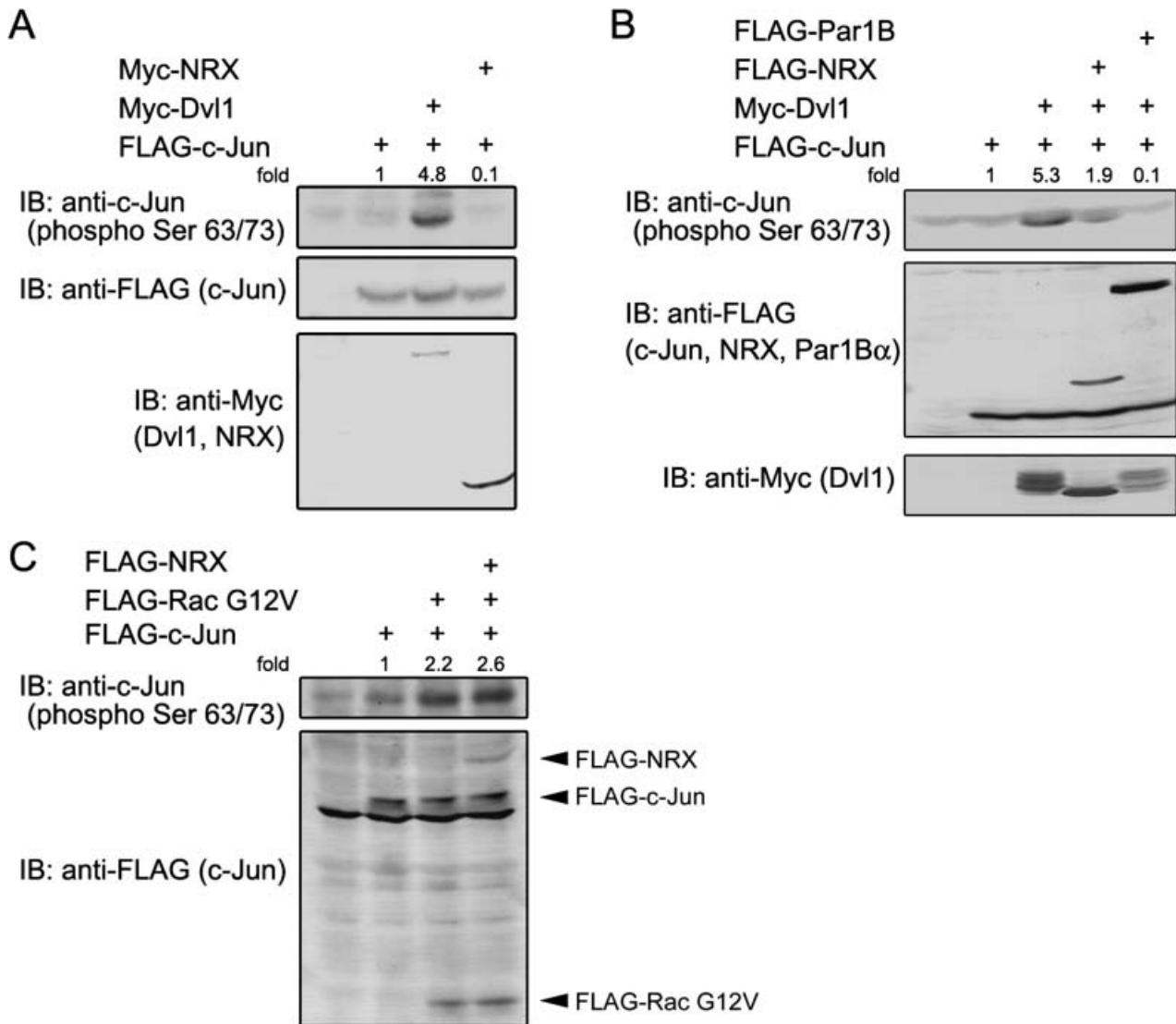


Figure 7 NRX suppresses c-Jun phosphorylation. NIH3T3 cells transfected with FLAG-c-Jun and indicated constructs were harvested, and their lysates were subjected to immunoblotting (IB) with indicated antibodies. The signal intensities of IB with anti-c-Jun (phospho Ser 63/73) were determined by densitometry and their relative values are indicated.

Discussion

In the present study, we investigated whether NRX influences the Wnt/PCP pathway as well as the Wnt/ β -catenin pathway. On the basis of our results of all experiments (gross morphological analyses of embryos, visualization of convergent extension by Alexa Fluor dye injection, animal cap elongation assays in *Xenopus* and c-Jun phosphorylation experiments in mammalian culture cells), we concluded that NRX is a novel component in the Wnt/PCP pathway. Our previous characterization of NRX as a direct interacting molecule against Dvl

(Funato *et al.* 2006), and the results from co-injection (Figs 2 and 4) and co-expression (Fig. 7B) experiments of Dvl and NRX suggest that NRX exerts its effect on the Wnt/PCP pathway via inhibition of Dvl activity. This idea is also supported by our findings that (i) animal cap elongation defect caused by inhibition of Dvl-function is rescued by NRX-MO co-injection (Fig. 6C), (ii) β -catenin or GSK3 β mRNA co-injection did not rescue the bent-axis phenotype caused by NRX mRNA injection, whereas Dvl mRNA co-injection did (Fig. 4A–G), and (iii) NRX expression cannot suppress c-Jun phosphorylation induced by active form of Rac, Rac G12V (Fig. 7C).

We previously suggested the possibility that NRX functions as a selective inhibitor of the Wnt/ β -catenin pathway because we could not detect any significant difference in Dvl-stimulated JNK-kinase activity even when NRX was over-expressed (Funato *et al.* 2006). However, the Dvl over-expression activated JNK-kinase activity only 2-fold, which may have masked the possible effect of NRX. In the present study, we examined activation of the PCP pathway by direct detection of phosphorylated c-Jun inside cells. With this method, we obtained much clearer activation of the Wnt/PCP pathway in response to Dvl over-expression. We found that c-Jun phosphorylation is increased approximately 4.8-fold over mock transfected cells (Fig. 7A), and therefore, we could observe the moderate but significant inhibitory effect of NRX (Fig. 7B). The data, along with the results of various analyses in *Xenopus*, support our conclusion that NRX participates in the Wnt/PCP pathway.

Among numerous Dvl-interacting proteins, Dapper/Frodo is reported to suppress both the Wnt/ β -catenin signaling and the Wnt/PCP signaling (Cheyette *et al.* 2002). However, further examination of Dapper/Frodo showed that it functions as either a positive or negative regulator of the Wnt signaling pathway in a context-dependent manner (i.e., when expressed at low levels, Dapper/Frodo activates Wnt/ β -catenin signaling Gloy *et al.* 2002; Hikasa & Sokol 2004). In contrast, NRX consistently inhibits both the Wnt/ β -catenin and Wnt/PCP pathways irrespective of the expression level (Fig. 1 and Funato *et al.* 2006). Furthermore, Zhang *et al.* reported that Dapper/Frodo promotes Dvl degradation (Zhang *et al.* 2006), but NRX expression increases the amount of Dvl (Funato *et al.* 2006). Therefore, it appears that both Dapper/Frodo and NRX can negatively regulate both the Wnt/ β -catenin and Wnt/PCP pathways, but they function in different manners.

How does NRX function as a negative regulator of the Wnt/PCP pathway? One possibility is that NRX may control phosphorylation of Dvl. Dvl phosphorylation is linked to Wnt/PCP pathway activation (Cong *et al.* 2004). In our previous study, we showed that NRX can suppress phosphorylation of Dvl (Funato *et al.* 2006). How this dephosphorylation or inhibition of phosphorylation occurs remains unclear; however, Lechward *et al.* reported that NRX can bind and activate protein phosphatase 2A (PP2A) (Lechward *et al.* 2006). Widerborst, a regulatory subunit of PP2A, is reported to activate PP2A phosphatase activity and to participate in Wnt/PCP signaling in both *Drosophila* and zebrafish *in vivo* (Hannus *et al.* 2002; Creighton *et al.* 2005). Therefore, we speculate that NRX regulates the Wnt/PCP pathway by enhancing PP2A phosphatase activity and inducing dephosphorylation of Dvl.

We reported previously that NRX binds to the basic/PDZ domain of Dvl and competes out Frat, an activator of the Wnt/ β -catenin pathway, from Dvl (Funato *et al.* 2006). Several molecules responsible for Wnt/PCP signaling, such as Vang/Stbm and Daam, are reported to bind to the PDZ domain of Dvl ((Habas *et al.* 2001; Park & Moon 2002). Therefore, it is also possible that NRX competes out these Dvl-PDZ domain-binding proteins, which should perturb the Wnt/PCP pathway. It will be interesting to investigate the mechanism by which NRX regulates the Wnt/PCP pathway in detail, which should be our future theme.

Experimental procedures

Constructs

cDNA constructs of human *Dvl1*, mouse *NRX*, human *Par1b* and human *Rac1* are previously described (Funato *et al.* 2004, 2006; Terabayashi *et al.* 2007). Human *c-Jun* cDNA was generated from HEK293 cells mRNA with a standard RT-PCR method. pSP64T-Xwnt11 was a kind gift from Dr. Smith (Tada & Smith 2000). Xdd was generated from pCS2-Xdsh as described in the previous report (Sokol 1996). pCS2-NRX5'UTR-EGFP was constructed by inserting oligonucleotides into pCS2-EGFP, which was generated by insertion of EGFP fragment into EcoRI/XhoI site of pCS2.

Antibodies and materials

Anti-Myc rabbit polyclonal antibody, anti-GFP rabbit polyclonal antibody and anti-phospho c-Jun (Ser 63/Ser 73) rabbit polyclonal antibody were from Santa Cruz Biotechnology. Anti-FLAG mouse monoclonal antibody was from Sigma-Aldrich. Alexa 488 dye was from Invitrogen.

Injection experiments in *Xenopus*

Xenopus injection experiments were carried out according to the previous studies (Michiue *et al.* 2004; Funato *et al.* 2006). Briefly, *in vitro* fertilized *Xenopus* eggs were injected at the 4-cell to 8-cell stages with mRNAs or MOs. mRNAs were synthesized with mMessage mMachin Kit (Applied Biosystems). MOs were purchased from Gene tools. The sequences of MOs utilized were NRX-MO (against the 5'-UTR region of MGC84045, of which product protein shows 77% identity with mouse NRX protein): GCCTGGCCCCACCTCTCTTCTGTGT, Idax-MO: GCCTCTGGGAGTCATTTCTGTGCAT (The validity of Idax-MO is shown in the previous study (Michiue *et al.* 2004)), Control MO: CCTCTTACCTCAGTTACAATTATA. These sequences do not match any other known *Xenopus* mRNAs. The observed bent-axis phenotypes are separated into "weak" (bent for less than 90 degrees), "moderate" (bent for 90 degrees or more) and "severe" (bent for 90 degrees or more and with spina bifida), respectively (Representative embryos are shown in Fig. 1H). Developmental stage was followed by normal table described by Nieuwkoop & Faber (1956).

Animal cap elongation assays

Animal cap assays were carried out according to Kobayashi *et al.* (Kobayashi *et al.* 2005). mRNAs or MOs were injected into the animal pole of eight-cell stage eggs. The animal caps were dissected from stage-8.5 embryos and cultured in 10 ng/mL activin A in 0.1% bovine serum albumin (BSA)/1 X Steinberg's solution. Activin A is prepared as previously described (Eto *et al.* 1987). Animal caps with elongation of more than its own diameter were counted as elongated.

RT-PCR

RT-PCR experiments were carried out as described previously (Michiue *et al.* 2004; Kobayashi *et al.* 2005). Total RNAs were prepared with Isogen (Wako Pure Chemical Industries, JAPAN), and cDNAs were synthesized with Superscript II (Invitrogen). The following primers were used: *ornithine decarboxylase (ODC)*, 5'-GCCATTGTGAAGACTCTCTCCATTC-3' and 5'-TTCGG GTGATTCCCTTGCCAC-3'; *goosecoid (gsc)*, 5'-CACACAAAGT CGCAGAGTCTC-3' and 5'-GGAGAGCAGAAAGTTGGGGCCA-3'; *Xbrachyury (Xbra)*, 5'-AGCCTGTCTGTCAATGCTCC-3' and 5'-ACTGAGACACTGGTGTGATGG-3'; *NRX* (MGC84045), 5'-TCCCATACAGTGACGAAGCAAG-3' and 5'-ACAGGGTC CCTCATTTAATTGCAC-3'.

Cell culture and transfection

NIH3T3 murine fibroblasts were routinely maintained in our laboratory in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% calf serum and antibiotics. Cells were transfected with LipofectAmine2000 (Invitrogen) according to the manufacturers' instructions.

Detection of c-Jun phosphorylation via immunoblotting

For detection of phosphorylated c-Jun, NIH3T3 cells were transfected with FLAG-tagged c-Jun together with various expression constructs and harvested 24 h later. The cells were rinsed once with ice-cold Tris-buffered saline (TBS) and harvested with ice-cold lysis buffer (20 mM Tris-HCl [pH 7.5], 150 mM NaCl, 2 mM EDTA, 0.5% Triton X-100 and 1 mM phenylmethanesulfonyl fluoride). The lysates were centrifuged at 15 000 rpm for 10 min at 4 °C, and the supernatants were mixed with SDS sample buffer. Samples were separated by SDS-PAGE, and transferred to PVDF membranes (Millipore). Blocking was carried out with 10% BSA in TBS for 1 h at room temperature, and anti-phospho-c-Jun (Ser 63/Ser 73) antibody was diluted 1:2000 in TBS and incubated with the blot overnight at 4 °C. c-Jun phosphorylation was quantified with NIH Image software.

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