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Anterior neural development requires Del1, a matrix-associated protein that attenuates canonical Wnt signaling via the Ror2 pathway

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SUMMARY

During early embryogenesis, the neural plate is specified along the anterior-posterior (AP) axis by the action of graded patterning signals. In particular, the attenuation of canonical Wnt signals plays a central role in the determination of the anterior brain region. Here, we show that the extracellular matrix (ECM) protein Del1, expressed in the anterior neural plate, is essential for forebrain development in the *Xenopus* embryo. Overexpression of *Del1* expands the forebrain domain and promotes the formation of head structures, such as the eye, in a *Chordin*-induced secondary axis. Conversely, the inhibition of Del1 function by a morpholino oligonucleotide (MO) represses forebrain development. *Del1* also augments the expression of forebrain markers in neuralized animal cap cells, whereas *Del1-MO* suppresses them. We previously reported that Del1 interferes with BMP signaling in the dorsal-ventral patterning of the gastrula marginal zone. By contrast, we demonstrate here that Del1 function in AP neural patterning is mediated mainly by the inhibition of canonical Wnt signaling. Wnt-induced posteriorization of the neural plate is counteracted by *Del1*, and the *Del1-MO* phenotype (posteriorization) is reversed by *Dkk1*. *Topflash* reporter assays show that *Del1* suppresses luciferase activities induced by *Wnt1* and β-*catenin*. This inhibitory effect of Del1 on canonical Wnt signaling, but not on BMP signaling, requires the Ror2 pathway, which is implicated in non-canonical Wnt signaling. These findings indicate that the ECM protein Del1 promotes forebrain development by creating a local environment that attenuates the cellular response to posteriorizing Wnt signals via a unique pathway.

KEY WORDS: Del1, Wnt, Anterior-posterior (AP) patterning, Ror2, *Xenopus*

INTRODUCTION

During early embryogenesis, vertebrate embryos are patterned along the dorsal-ventral (DV) and anterior-posterior (AP) axes. The embryonic regions are subdivided along the AP axis into three major domains: the head, trunk and tail. The classic 'Einsteck' studies in amphibian embryos demonstrated that these three domains are distinctly induced in a temporally controlled fashion by the actions of the early, intermediate and late Spemann organizers (reviewed by Gerhart, 2001; Mangold, 1933; Spemann, 1931).

In the two-signal model proposed by Nieuwkoop (Nieuwkoop, 1952a; Nieuwkoop, 1952b) (reviewed by Sasai and De Robertis, 1997), the forebrain is the default fate induced by the neural inducer, whereas more caudal neural tissues are secondarily transformed by caudalizing factors. Signaling factors such as Wnt, FGF, Nodal and retinoic acid cause the posteriorization of neural tissues and suppress head formation (reviewed by Rallu et al., 2002). In particular, canonical Wnt signaling has a crucial and conserved role in suppressing anterior embryonic development in vertebrate embryos (reviewed by Niehrs, 2004; Lewis et al., 2008). Although the microinjection of Wnt or β-*catenin* mRNA, which mimics maternal Wnt activation before the mid-blastula transition (MBT), induces the formation of a secondary axis (Molenaar et al., 1996; Sokol et al., 1991; Steinbeisser et al., 1993), the activation of Wnt signaling after the MBT (e.g. via plasmid injection) causes a strong posteriorization in the embryo and also in the neuralized animal cap (Christian and Moon, 1993; Kiecker and Niehrs, 2001). Moreover, the inhibition of endogenous Wnt signals by extracellular Wnt antagonists, such as Dkk1, Frzb and WIF1, induces substantial anteriorization, resulting in the formation of a large head (Glinka et al., 1998; Hsieh et al., 1999; Leyns et al., 1997). Wnt signaling in the head is also attenuated by non-secreted factors. Shisa, an anteriorly produced protein located in the endoplasmic reticulum (ER), inhibits the transport of the Wnt receptor Frizzled from the ER to the plasma membrane (Yamamoto et al., 2005). The *headless* mutant in zebrafish is caused by a mutation in the *tcf3* gene, which is expressed in the head and inhibits Wnt signals (Kim et al., 2000). In *Xenopus*, *Tcf3* expression in the head is regulated by the forebrain-specific zinc-finger protein XsalF (Onai et al., 2004).

In this study, we reveal an essential role for the extracellular matrix (ECM) protein Developmental endothelial locus-1 [Del1 (Hidai et al., 1998); also known as EGF-like repeats and discoidin I-like domains 3 (Edil3)] in the negative control of zygotic Wnt signaling to permit proper anterior development of the early *Xenopus* embryo. Del1 is a secreted protein that belongs to the Discoidin domain family (Kiedzierska et al., 2007); it is localized to the ECM and the basement membrane (Aoka et al., 2002). Previous studies have shown that mammalian Del1 is important in vascular development (Zhong et al., 2003), hair regrowth (Hsu et

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al., 2008), leukocyte-endothelial adhesion (Choi et al., 2008) and phagocytosis (Hanayama et al., 2004). Most of these activities of Del1 require its integrin-binding RGD motif, which is located in the second EGF domain.

We previously found that Del1 interferes with BMP signaling in the DV patterning of the gastrula marginal zone, albeit moderately (Arakawa et al., 2007). Interestingly, during our subsequent study of the effects of Del1 on ectodermal development, we noticed that some of the gain- and loss-of-function phenotypes of *Del1* in *Xenopus* could not be explained simply by BMP modulation. In this study, we demonstrate that Del1 strongly regulates intracellular Wnt signaling during the anterior specification of the neuroectoderm. Del1 is required for forebrain development and interferes with the canonical Wnt pathway downstream of the nuclear accumulation of β-catenin. In addition, we provide evidence that the Ror2 (Receptor tyrosine kinase-like orphan receptor 2) pathway, which is implicated in the non-canonical Wnt pathway, plays an essential role in the inhibitory effects of Del1 on canonical Wnt signaling. Thus, Del1 is an ECM protein that induces a unique intracellular signaling pathway that is antagonistic to both the Wnt and BMP pathways.

MATERIALS AND METHODS

Embryonic manipulations, explant experiments and luciferase assays

Xenopus laevis embryos were obtained, cultured, microinjected and subjected to whole-mount in situ hybridization as described previously (Arakawa et al., 2007). As needed, *Venus* mRNA (100 pg) was co-injected as a tracer. For all animal cap assays, embryos were injected animally at the 4- to 8-cell stage. Unless indicated otherwise, animal caps were excised at stage 8 and cultured in 1× LCMR (low calcium/magnesium Ringer's) containing 0.2% BSA until siblings reached stage 11. For dissociated animal cap assays, excised animal caps were dissociated in CMFM (calcium/magnesium-free medium) containing 0.2% BSA. After removal of the outer layer, the dissociated inner layer cells were cultured in CMFM. For the *Topflash* assays, embryos were co-injected with 25 pg of *Topflash* reporter (Upstate) and 1 pg of *phRL-null* (Promega) plasmids, and subjected to luciferase assays using the Dual Luciferase Reporter Assay system (Promega), according to the manufacturer's protocol. All explant experiments were repeated at least three times. All recombinant proteins were obtained from R&D Systems.

RT-PCR and quantitative RT-PCR

Total RNA was isolated from embryos or explants using the RNeasy Mini Kit (Qiagen). RT-PCR and quantitative RT-PCR (qPCR) were performed as described previously (Inomata et al., 2008; Onai et al., 2004; Schambony and Wedlich, 2007; Sasai et al., 1995). Additional primers used in this study were (5′ to 3′): *Six3* fwd CTCTCTCC-CTTTTACTTTCTCACAC and rev GATAGAGGGTTAAACA-AAGTTGCAT; *Krox20* fwd GATTCAGATGAGCGGAGTGA and rev CAAGGGGTAGTTGGACGAGT; *En2* fwd GTGTCAGCAAA-GAGGACAAGAG and rev CCTCTGCTCAGTCAAATACCTG; *NCAM* fwd ATTCCCCAACTGGTGAGAAG and rev CCTTCAGAT-TCTCCCTCTGC; *Rx2a* fwd CAAACTTGAAACTAGGTCTCTGTGA and rev TGTGTTGATCTCCAGCTTTATTTAG; *Otx2* fwd TTTCA-CCAGAGCTCAACTGG and rev CTGGACTCGGGTAGGTTGAT; *HoxD1* fwd TTCTGCGTAAAACCTCCACA and rev TCTCTAGGGTGAAGCGTCCT; *Ror2* fwd TGCAACTGGA-GTCCTCTTTG and rev GGCTGACAAAACCCATCTTT; *MyoD* fwd CCTGAAGCGATACACCTCAA and rev GTTCCAGAACCGGG-TAGAAA; *Xnot* fwd GCCCAGACCCTACCTGTAAA and rev TGCGGATTCTCTTCATCTTG; *alpha-actin* fwd GCTGACAGAA-TGCAGAAGGA and rev CCGATCCAGACGGAGTATTT. Gene expression levels were normalized to that of *ODC* (*ornithine decarboxylase*). Unless indicated otherwise, the expression level of each gene in the control embryo was defined as 100.

Expression constructs and morpholinos (MOs)

Del1, *Del1-delC*, *Del1-delN* and *Del1(RGE)* were subcloned into the expression vector *pCS2* (Arakawa et al., 2007). To generate V5-tagged constructs, the V5 tag sequence was added to the C-terminus of each coding sequence. For microinjection, capped mRNAs were synthesized using the SP6 mMessage Machine Kit (Ambion). *Del1-MO* (5′-GACCCCCTTTAGGATCATGCTTGA-3′), *Del1* five-base-mismatched 5mis-*MO* (5′-cACCCgTTTAcGATCtTGCTTGt-3′), *Ror2-MO* (5′-GTCAGGCGAGGTAAGGGGCAACACT-3′) and standard control-*MO* were obtained from Gene Tools. The total amount of injected mRNAs or MOs was equalized with *lacZ* mRNA or control-*MO*, respectively.

Immunostaining of animal cap cells

Cryosections of animal caps were prepared as described previously (Onai et al., 2007) and immunostained with an anti-β-catenin antibody (Sigma, 1/500). For adhesion culture, dissociated animal cap cells were prepared with BSA-free medium, cultured on chamber slides (Nunc) coated with 100 μg/ml human plasma fibronectin (Invitrogen) until the siblings reached stage 13, then fixed with 1× MEMFA [0.1 M MOPS (pH 7.4), 2 mM EGTA, 1 mM $MgSO_4$, 3.7% formaldehyde] at 4°C for 15 minutes and subjected to immunostaining with an anti-V5 antibody (Invitrogen, 1/1000). The membrane components were visualized by co-injecting mRNA for membrane monomeric Cherry (Lyn-mCherry) protein. For permeabilization, the fixed cells were incubated with PBS containing 0.3% Triton X-100 at room temperature for 15 minutes, then washed five times with PBS before immunostaining. *z*-stack images were taken using a Zeiss LSM710 confocal microscope.

Immunoblotting

Animal cap cells were lysed with TNE buffer (50 mM Tris-HCl pH 7.8, 1% NP40, 20 mM EDTA, 150 mM NaCl) containing Protease Inhibitor Cocktail (Nacalai). For the detection of phospho-Erk, Phosphatase Inhibitor Cocktail II (Sigma) was added to the buffer. The primary antibodies were anti-phospho-Erk, anti-Erk (Cell Signaling, 1/500), anti-β-catenin (Sigma, 1/500) and anti-Hsc-70 (Santa Cruz, 1/500), and the secondary antibodies were anti-mouse IgG and anti-rabbit IgG (GE Healthcare, 1/50,000). Signals were detected with ECL Plus reagents (GE Healthcare) and captured with a LAS-3000UVmini (Fuji Film).

Statistical analysis

For statistical analysis, we used the Prism4 program (GraphPad). Two-group and multiple-group analyses were performed with Student's *t*-test and one-way ANOVA (Dunnett or Tukey post-hoc test), respectively.

RESULTS

Del1 promotes the formation of head structures via its Discoidin domains

In our previous report (Arakawa et al., 2007), the equator zone injection of *Del1* mRNA into all four blastomeres of the 4-cell *Xenopus* embryo caused a moderate dorsalization in marginal zone (mesodermal) tissues. These phenotypes were explained, at least in part, by the anti-BMP effect of Del1 on mesodermal patterning.

In the early phase of the present study, we sought to analyze the effects of Del1 on ectodermal development by microinjection of *Del1* mRNA into the four animal blastomeres of the 8-cell embryo, which contribute mainly to the ectodermal derivatives. Interestingly, this resulted in a phenotype distinct from that observed with the equator injection at the 4-cell stage: the formation of an enlarged head (52%, *n*=27; Fig. 1A-D), including large eyes (Fig. 1D, arrow) and an expanded cement gland (Fig. 1D, arrowhead), without other obvious signs of general dorsalization of the body. These phenotypes were considered to reflect the anteriorizing effect of *Del1* on the pattern of the ectoderm rather than mesoderm.

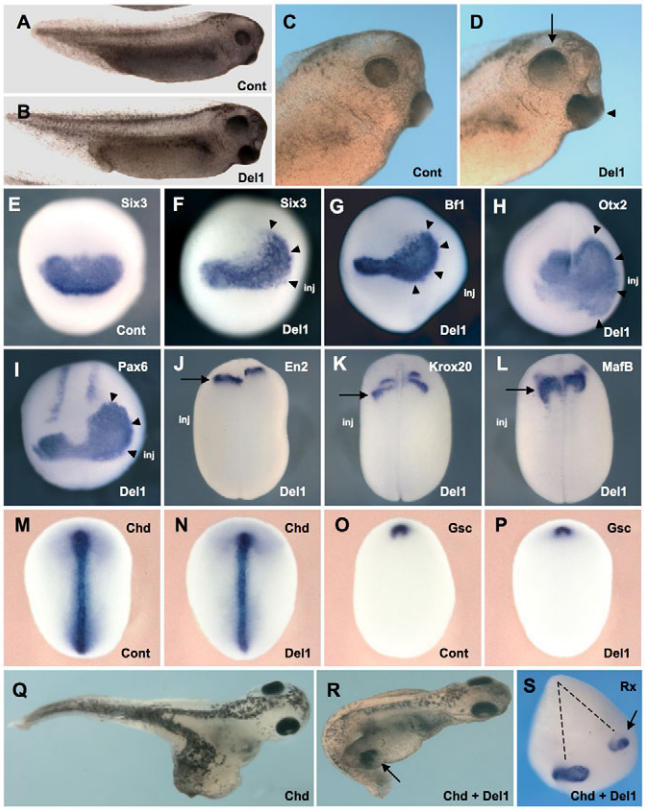

Fig. 1. Del1 expands anterior ectodermal structures of the *Xenopus* embryo. (**A-D**) Uninjected tadpole stage embryo (A,C). Tadpoles after animal-side injection of *Del1* mRNA (200 pg) at the 8-cell stage (B,D). Arrow, eye; arrowhead, cement gland. (**E-P**) Whole-mount in situ hybridization analysis of early neurula (E-I,M-P) or mid neurula (J-L) embryos. *Del1* mRNA (200 pg) was injected into two animal blastomeres on one side of the 8-cell embryo. Arrowheads and arrows indicate expansion and posterior shifts of each marker, respectively. (N,P) *Del1* mRNA (200 pg) was injected into all animal blastomeres of the 8-cell embryo. (**Q-S**) Co-injection of *Del1* and *Chd* mRNAs induced head structures (arrow) in the secondary axis (R). *Chd* (50 pg) and *Del1* (400 pg) mRNAs were injected into one ventro-vegetal blastomere of the 8-cell embryo. Dashed lines indicate the axes of the neurula embryo (S). The ectopic expression of *Rx* in the secondary axis is indicated by an arrow (S).

Overexpression of *Del1* on the left side induced an obvious expansion of the anterior neural markers *Six3* (forebrain; 55%, *n*=40), *Bf1* (telencephalon; 66%, *n*=56), *Otx2* (forebrain/midbrain; 67%, *n*=46) and *Pax6* (forebrain; 57%, *n*=42) on the ipsilateral side (arrowheads in Fig. 1E-I, anterior views). *Del1* overexpression also caused a posterior shift or expansion of the posterior neural markers *En2* (midbrain/hindbrain boundary; 67%, *n*=30), *Krox20* (hindbrain; 54%, *n*=28) and *MafB* (posterior hindbrain; 57%, *n*=30) (arrows in Fig. 1J-L, dorsal views). Under these conditions, in contrast to the 4-cell stage injection in our previous report, we did not observe strong expansion in the expression of mesodermal markers such as *Chordin* (*Chd*; axial mesoderm; 100%, *n*=26) and *Goosecoid* (*Gsc;* anterior mesendoderm; no change in 71% and a minimal expansion in 29%, *n*=28) (Fig. 1M-P, dorsal views).

We next tested the anteriorizing effect of *Del1* in a secondary-axis assay. The ventral injection of BMP inhibitors, such as *Chd*, induces the formation of a secondary axis, which rarely contains the anterior-most head structures, such as the eyes (Glinka et al., 1997; Pera et al., 2001) (Fig. 1Q). Our previous study showed that the ventral injection of *Del1* alone does not induce a secondary axis (Arakawa et al., 2007). Interestingly, the co-injection of *Chd* and *Del1* caused the formation of eye tissues in the secondary axis (33%, *n*=36; Fig. 1R, arrow). The induction of the eye-forming field by *Chd* and *Del1* was confirmed by in situ hybridization with a probe for *Rx* (a forebrain and eye-field marker; 61%, *n*=22; Fig. 1S, anterior view).

These findings show that overexpression of *Del1* in the animal region promotes anteriorization of the neuroectoderm. This anteriorizing effect was presumably overlooked in our previous analysis because the 4-cell stage injection induced dorsalization of the mesoderm, which secondarily caused some degree of anteriorization of the embryo, whereas in the present study the 8-cell stage injection could uncouple the two phenotypes.

The anteriorizing activity of the matrix protein Del1 is mediated by its Discoidin domains

Del1 has two EGF-like domains and two Discoidin domains (Fig. 2A). An integrin-binding RGD motif is present in the first EGF domain. We investigated the relationship between these domains and the activity of Del1 (Fig. 2). Using a V5-tagged construct, we first analyzed the effects of deleting certain domains on the accumulation of Del1 protein in the ECM. RNA-injected animal cap cells were dissociated, cultured on a plastic culture slide, and subjected to immunostaining with an anti-V5 antibody. When the cells were fixed without permeabilization of the plasma membrane (Fig. 2B-F′; Lyn-mCherry, red), a gradient of Del1-V5 and Del1-delN-V5 (lacking the EGF domains) labeling was detected in the pericellular areas of the overexpressing cells (Fig. 2C,E, green), suggesting that these molecules were associated with the pericellular matrix (see also Fig. S1A in the supplementary material). By contrast, almost no Del1-delC-V5 protein was immunodetected around the non-permeabilized cells (Fig. 2D). With permeabilization of the plasma membrane (Fig. 2G-K′), Del1-delC-V5, Del1-V5, and Del1-delN-V5 were detected inside the cells (Fig. 2H-J), indicating that all of these proteins were translated in the cells. Immunoblotting analysis (see Fig. S1B in the supplementary material) showed that Del1-delC-V5 was not detectable in the conditioned medium but was instead detected in the cell lysate, whereas Del1-V5 and Del1-delN-V5 were present in both, suggesting that Del1-delC-V5 was produced by the cells but not secreted. By contrast, the mutation in the RGD motif [*Del1(RGE)-V5*, in which the integrin-binding RGD motif was mutated to non-integrin-binding RGE; Fig. 2A] did not significantly affect the localization of Del1. The Del1(RGE)-V5 protein was detected in the pericellular areas of the non-permeabilized cells (Fig. 2F,F′) and was secreted into the conditioned medium (see Fig. S1B in the supplementary material).

We next examined which domains were involved in the anteriorizing activity of Del1 (Fig. 2L-S). *Del1-delN-V5* expanded the area of *Bf1* expression (71%, *n*=24; Fig. 2N). It also induced *Rx* expression in the secondary axis generated by *Chd* (83%, *n*=23; Fig. 2R), indicating that the C-terminal Discoidin domains are sufficient for the anteriorizing activity. Consistent with this idea, Del1(RGE)-V5 retained its anteriorizing activity (*Bf1* expansion in 71%, *n*=38, Fig. 2O; *Rx* expression in the *Chd*-induced secondary axis, 53%, *n*=45, Fig. 2S). These findings demonstrate that the anteriorization activity of Del1 is mediated by the Discoidin domains and is independent of its RGD motif.

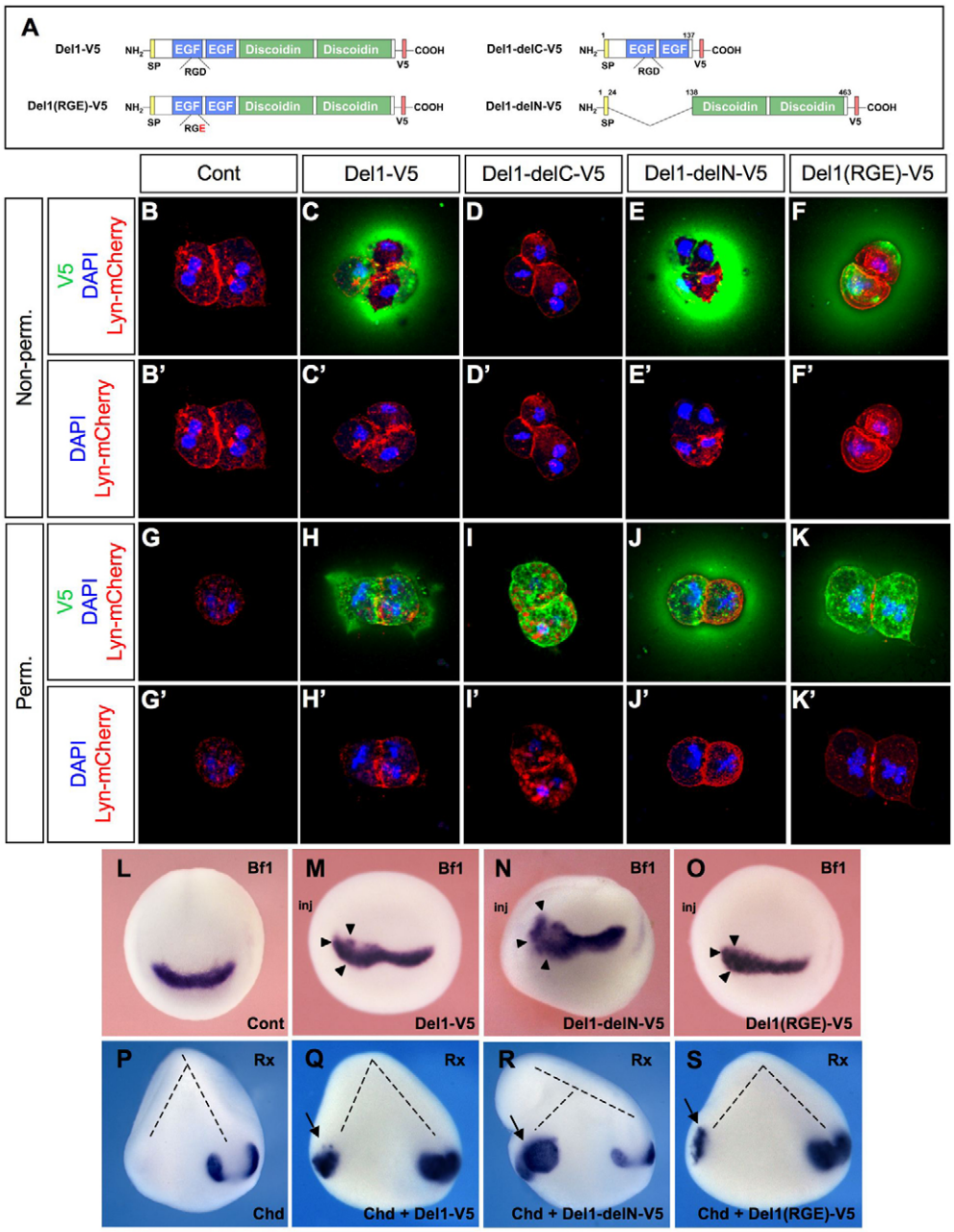

Fig. 2. The pericellular matrix protein Del1 functions as an anteriorizing factor via its Discoidin domains. (**A**) Structure of Del1-V5 and its mutant derivatives. (**B-K′**) *Xenopus* embryos were co-injected with *Lyn-mCherry* (20 pg), *Del1-V5* (400 pg), *Del1-V5(RGE)* (400 pg), *Del1-delC-V5* (400 pg) and *Del1-delN-V5* (400 pg) mRNAs. Cultured cells were fixed with (G-K′) or without (B-F′) permeabilization, and immunostained with an anti-V5 antibody (green). Red, Lyn-mCherry; blue, DAPI. (**L-O**) The expansion of the *Bf1*-positive area (arrowheads) was induced by *Del1-V5* (M; 400 pg; *Bf1* expansion in 84%, *n*=44), *Del1-delN-V5* (N; 200 pg) or *Del1(RGE)-V5* (O; 400 pg) mRNAs. Embryos received injections into two animal blastomeres on one side of the 8-cell embryo. (**P-S**) The ectopic expression of *Rx* in the secondary axis (arrows) was induced by injecting *Chd* alone (P; 50 pg) or with *Del1-V5* (Q; 400 pg; 53%, *n*=45), *Del1-delN-V5* (R; 400 pg) or *Del1(RGE)-V5* (S; 400 pg) mRNAs. Embryos received injections into one ventro-vegetal blastomere of the 8-cell embryo. Dashed lines indicate the axes of the neurula embryo.

Essential role of Del1 in anterior neuroectodermal development

Sagittal sections of neurula stage embryos showed a strong accumulation of *Del1* transcripts in the anterior neural plate (Fig. 3A, bracket), in addition to their diffuse distribution throughout the neural plate and dorsal mesoderm.

We next performed a loss-of-function study of *Del1* in the embryo. The injection of *Del1-MO* (see Fig. S2A,B in the supplementary material) into all the animal blastomeres of the 8-cell embryo caused a head defect (microcephaly) without obvious malformation of the trunk or tail (88%, *n*=24; Fig. 3B,C). In situ hybridization analysis (Fig. 3D-O) showed that *Del1-MO* injection suppressed expression of the forebrain marker *Six3* (83%, *n*=36; Fig. 3E). This suppression was reversed by co-injection of an MO-insensitive *Del1* mRNA (no suppression in 64%, *n*=28; Fig. 3F; see also Fig. S2C,D in the supplementary material). *Del1-MO* also attenuated the expression of other forebrain markers, including *Pax6* (63%, *n*=27) and *Rx* (81%, *n*=42), whereas the expression of these marker genes was restored by MO-insensitive *Del1* mRNA injection (75%, *n*=28 and 74%, *n*=52, respectively) (Fig. 3G-L). By contrast, posterior neural markers, such as *Krox20* and *HoxB9* (spinal cord), were largely unaffected, and the expression of the midbrain/hindbrain marker *En2* was modestly decreased or was shifted anteriorly (81%, *n*=42; Fig. 3N). This effect on *En2* was also reversed by co-injection of MO-insensitive *Del1* mRNA (no effect in 53%, *n*=32; Fig. 3Q). Under these conditions, *Del1-MO* did not substantially change the expression of the mesodermal markers *Xnot* (axial mesoderm) and *Goosecoid* (see Fig. S2E-H in the supplementary material). Taken together with the gain-of-function data (Fig. 1), these findings show that Del1 plays a crucial role in anterior neuroectodermal development in the early *Xenopus* embryo.

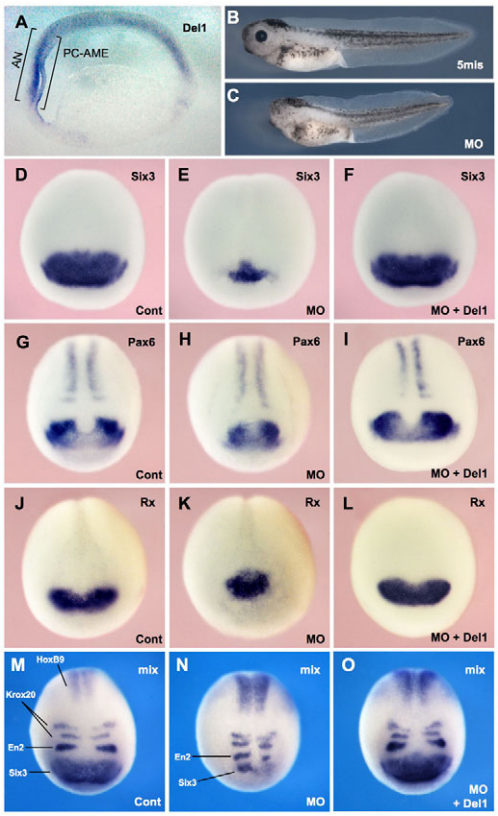

Fig. 3. Del1 is essential for the formation of anterior brain tissues. (**A**) Expression pattern of *Del1* at the early neurula stage (sagittal section; anterior side is left). AN, anterior neuroectoderm; PC-AME, prechordal anterior mesendoderm. (**B**,**C**) Effects of animal cell injection of 5mis-*MO* (12.5 ng) or *Del1-MO* (12.5 ng) at the 8-cell stage. (**D-O**) Effects on expression of neural markers of animal cell injection of *Del1-MO* (25 ng) at the 8-cell stage (E,H,K,N). These effects were rescued by co-injecting MO-insensitive *Del1* mRNA (100 pg) (F,I,L,O). Compare with controls (D,G,J,M). mix, mixed probes (*Six3*, *En2*, *Krox20* and *HoxB9*).

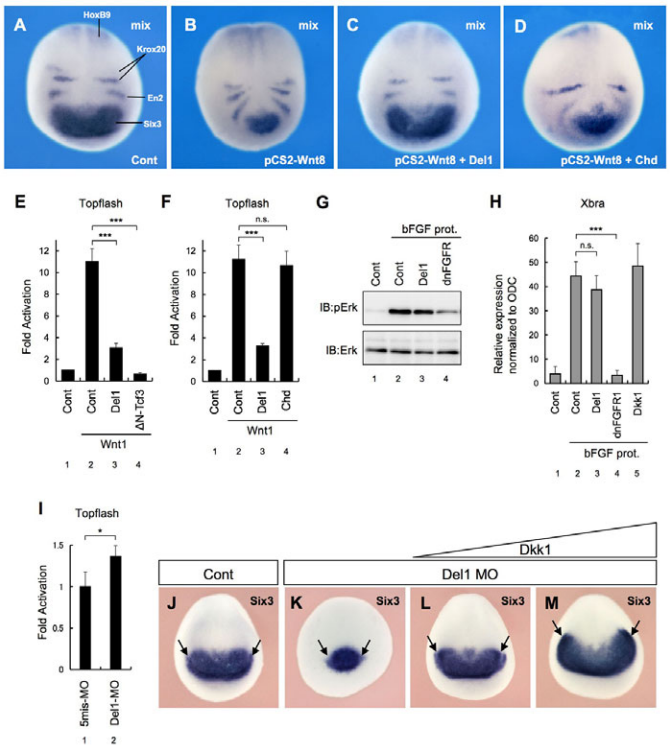

Fig. 4. Del1 antagonizes Wnt signaling in vitro and in vivo. (**A-D**) Co-injection of *Del1* (400 pg, C) rescued posteriorization caused by injecting *pCS2-Wnt8* (10 pg, B), whereas co-injection of *Chd* did not (100 pg, D); compare with the control (A). *Xenopus* embryos received injections into all the animal blastomeres at the 8-cell stage mix, mixed probes (*Six3*, *En2*, *Krox20* and *HoxB9*). (**E**,**F**) Animal caps were injected with *Wnt1* (10 pg), *Del1* (500 pg), Δ*N-Tcf3* (10 pg), or *Chd* (100 pg) mRNAs, then analyzed for luciferase activity. (**G**,**H**) Animal caps were injected with *Del1* (500 pg), dominant-negative *FGF receptor* (*dnFGFR*; 100 pg), or *Dkk1* (100 pg) mRNAs. (G) Explants were cultured with 50 ng/ml bFGF protein for 30 minutes and analyzed by immunoblotting. (H) Explants were cultured with 500 ng/ml bFGF protein for 3 hours and *Xbra* expression was analyzed by qPCR. (**I**) *Del1-MO* (25 ng) or 5mis-*MO* (25 ng) and reporter plasmids were animally injected into 8-cell stage embryos, and the anterior quarter of embryos was excised at stage 13 and subjected to the dual luciferase assay. *, $P<0.05$; ***, $P<0.001$. n.s., not significant. Error bars represent s.d. (**J-M**) Suppression of *Six3* expression by injecting 25 ng of *Del1-MO* (K; suppression in 60%, $n=30$) was reversed by co-injecting 5 pg (L; equivalent to normal in 96%, $n=27$) or 25 pg (M; equivalent to normal or expansion in 100%, $n=24$) *Dkk1* mRNA. Embryos received injections into all the animal blastomeres at the 8-cell stage and were harvested at the mid-neurula stage. Arrows indicate expression borders of *Six3*.

Next, we examined whether *Del1* is directly involved in the AP patterning of neural tissues. Consistent with previous studies (Onai et al., 2004; Sasai et al., 1995), qPCR analysis showed that *Chd*-injected neuralized animal cap explants strongly expressed the anterior neural marker *Six3*, whereas they showed little expression of the posterior neural markers *En2* and *Krox20* (see Fig. S3A in the supplementary material, lanes 2, 5 and 8). The co-injection of *Del1-MO* induced the expression of *En2* and *Krox20* and moderately attenuated that of *Six3* (lanes 3, 6 and 9), whereas the expression of the general neural marker *NCAM* was largely unaffected (lanes 11 and 12). These findings indicate that Del1 is directly required for protecting the neuroectoderm from caudalization.

We next performed overexpression analysis of Del1 in neuralized animal caps that were caudalized by Wnt signaling. The co-injection of *Wnt1*, even at a low dose, suppressed anterior neural markers and induced posterior genes (see Fig. S3B in the supplementary material, lanes 2, 5 and 8). This posteriorizing effect of *Wnt1* was reversed by *Del1* co-injection, which increased anterior marker and suppressed posterior marker expression (lanes 3, 6 and 9). The co-injection of *Wnt1* at a moderate dose preferentially induced *Krox20* but not *En2* expression (see Fig. S3C in the supplementary material), presumably reflecting an even stronger caudalizing effect of Wnt. In this case, *Del1* co-injection induced both *Six3* and *En2* and suppressed *Krox20* expression, showing that Del1 shifts the regional identity of neural tissues in an anterior direction.

These findings suggest that Del1 plays a crucial role in the anterior specification of the neuroectoderm.

Del1 interferes with canonical Wnt signaling

Since *Del1* reversed the Wnt-induced posteriorization, we next examined the effect of Del1 on Wnt signaling in vivo (Fig. 4A-D). As previously reported (Christian and Moon, 1993), Wnt plasmid injection suppressed the expression of the anterior neuroectodermal markers in the embryo (Fig. 4B; *Six3*

suppression in 95%, *n*=40). This Wnt-induced posteriorization was reversed by the co-injection of *Del1* (Fig. 4C; no *Six3* suppression in 87%, *n*=38). Importantly, such a reversal did not occur upon co-injection of the BMP inhibitor *Chd* (Fig, 4D; *Six3* suppression in 95%, *n*=43), showing that attenuation of the BMP signal by Del1 cannot by itself explain the reversal. In the *Topflash-luciferase* reporter assay using animal cap cells, *Wnt1* injection substantially increased the luciferase activity (Fig. 4E, lane 2), and this increase was strongly suppressed by co-injecting *Del1* (Fig. 4E, lane 3; in lane 4, Δ*N-Tcf3* was used as a positive control) (Molenaar et al., 1996). Again, such a suppression did not occur upon co-injection with *Chd* (Fig. 4F, lane 4). These data show that *Del1* causes attenuation of canonical Wnt signaling and that this is unlikely to occur via the suppression of BMP signaling, suggesting a direct anti-Wnt activity for Del1.

Previous studies have shown that, in addition to Wnt signals, FGF and Nodal play an important role in early AP patterning in *Xenopus* (Kengaku and Okamoto, 1995; Piccolo et al., 1999). FGF signals also posteriorized the AP identity of *Chd*-induced neural tissues (see Fig. S4A, lane 4, in the supplementary material), and *Del1* co-injection reversed the posteriorized phenotypes caused by bFGF in the neuralized animal cap (see Fig. S4A, lane 5, in the supplementary material). However, *Del1* injection did not inhibit the phosphorylation of Erk (Fig. 4G, lane 3; in lane 4, *dnFGFR* was used as a positive control) (Amaya et al., 1991) or substantially suppress the induction of *Xbra* expression in the animal cap treated with bFGF (Fig. 4H, lane 3). This discrepancy may be explained, at least in part, by a previous report that the posteriorizing activity of FGF can be reversed by the Wnt inhibitor Dkk1 (Kazanskaya et al., 2000), indicating the requirement for Wnt signaling downstream of FGF.

Del1 injection did not substantially affect Nodal/Activin signaling either. *Del1* injection neither affected the *activin response element* (*ARE*)-*luciferase* activity (Sasai et al., 2008) nor strongly interfered with the Activin-induced induction of *Mix.2* expression in the animal cap (see Fig. S4B,C in the supplementary material).

These observations indicate that the anteriorizing activity of Del1 is relevant to the attenuation of Wnt signaling. Consistent with this idea, injection of *Del1-MO* elevated the *Topflash-luciferase* activity in the anterior region of the embryo as compared with the level in the control injected with *5mis-MO* (Fig. 4I). In addition, the decrease in *Six3* expression upon *Del1-MO* injection was reversed by co-injecting the Wnt inhibitor *Dkk1* in a dose-dependent manner (Fig. 4J-M).

Del1 inhibits the intracellular Wnt pathway downstream of the nuclear accumulation of β-catenin

In *Topflash* assays using animal cap cells, Del1 suppressed the luciferase activity induced by the injection of *Dishevelled* (*Dsh*) (Fig. 5A), dominant-negative *GSK3*β (Fig. 5B) (Pierce and Kimelman, 1995) and β-*catenin* (Fig. 5C), suggesting that Del1 functions antagonistically to the canonical Wnt pathway downstream of these intracellular mediators.

We next analyzed the nuclear localization of β-catenin (Fig. 5D-H), a key regulatory step for the transduction of Wnt signaling (Moon et al., 2004). Immunostaining showed that the Wnt1-induced nuclear level of β-catenin (Fig. 5E) was not substantially reduced by co-injection of *Del1* (Fig. 5F), in contrast to the clear reduction caused by co-injection of *Dkk1*, an upstream antagonist of Wnt signaling (Fig. 5G). These observations support the idea that Del1 acts downstream of the nuclear localization of β-catenin in the canonical Wnt pathway.

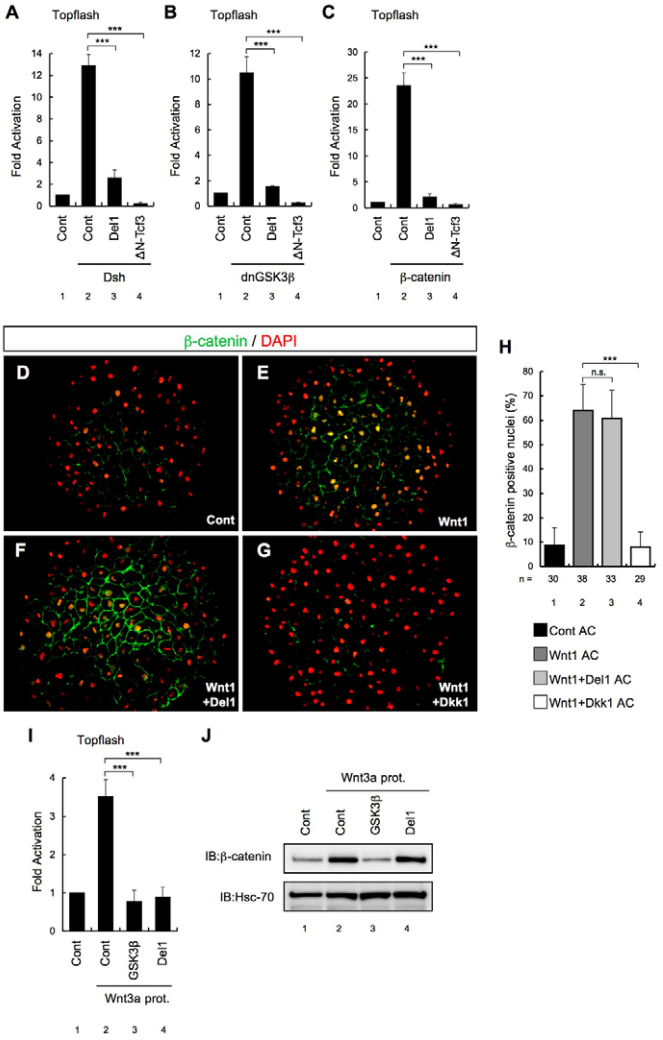

Fig. 5. Del1 interferes with the intracellular Wnt pathway downstream of the nuclear accumulation of β-catenin. (**A-C**) Co-injection of *Del1* (500 pg) antagonized the upregulation of *Topflash* activity caused by injecting *Dsh* (100 pg, A), dominant-negative *GSK3*β (*dnGSK3*β; 200 pg, B) or β-*catenin* (10 pg, C). Δ*N-Tcf3* (10 pg) was used as a positive control. (**D-H**) Cryosections (D-G) were prepared from animal caps injected with *Wnt1* alone (10 pg, E) or together with *Del1* (500 pg, F) or *Dkk1* (100 pg, G) mRNAs, then immunostained with an anti-β-catenin antibody (green). Red, DAPI. (H) The percentage of β-catenin-positive nuclei in each cryosection. (**I**,**J**) Dissociated animal cap cells were prepared from *Xenopus* embryos injected with *GSK3*β (200 pg) or *Del1* (500 pg) mRNAs, then cultured with or without 25 ng/ml Wnt3a protein for 3 hours and analyzed for luciferase activity (I) or by immunoblotting (J). ***, *P*<0.001. n.s., not significant. Error bars represent s.d.

In dissociated animal cap cells, the addition of recombinant Wnt3a protein increased *Topflash-luciferase* activity (Fig. 5I, lane 2) and the level of β-catenin protein (Fig. 5J, lane 2), which is indicative of a reduction in GSK3β-mediated degradation. *GSK3*β injection inhibited the increases in β-catenin and luciferase levels (Fig. 5I,J, lane 3). By contrast, although *Del1* injection also suppressed the Wnt3a-induced luciferase activity, it did not reduce the level of β-catenin (Fig. 5I,J, lane 4). These findings support the idea that Del1 function is not mediated by the conventional GSK3β-regulated β-catenin degradation.

The Ror2 pathway is essential for mediating the anteriorizing activity of Del1 in the neuroectoderm

The evidence above suggests that Del1 interferes with the canonical Wnt pathway downstream of the nuclear accumulation of β-catenin. Previous studies have shown that, like Del1, the non-canonical Wnt signal can inhibit the pathway downstream of β-catenin via the Ror2 (Mikels and Nusse, 2006) or NLK (Ishitani et al., 2003) pathway. Therefore, we examined the involvement of Ror2 and NLK in the anteriorizing activity of Del1 (Fig. 6A-F).

The expansion of *Bf1* expression by overexpression of *Del1* mRNA (Fig. 6B; 71%, *n*=21) was strongly suppressed by co-injection of *Ror2-MO* (Fig. 6D; no expansion in 100%, *n*=23), although *Ror2-MO* injection alone had only a marginal phenotypic effect at this dose (Fig. 6C). By contrast, the injection of *NLK-MO* (Satoh et al., 2007) did not effectively suppress the *Del1*-induced *Bf1* upregulation (Fig. 6F; *Bf1* expansion in 44%, *n*=25), even at the dose at which the *NLK-MO* injection alone showed an inhibitory effect on *Bf1* expression (Fig. 6E). These results raised the possibility that the anteriorizing activity of Del1 preferentially depends on the Ror2 pathway.

Ror2 expression was widely observed in the embryonic ectoderm during the early and mid-gastrula stages (later, its expression becomes stronger in the caudal neural tissue) (Hikasa et al., 2002) and also in neuralized animal cap explants (see Fig. S5A-E in the supplementary material). We performed an animal cap assay to examine whether the Ror2 pathway was directly involved in the Del1-induced anteriorization of the neuroectodermal tissue.

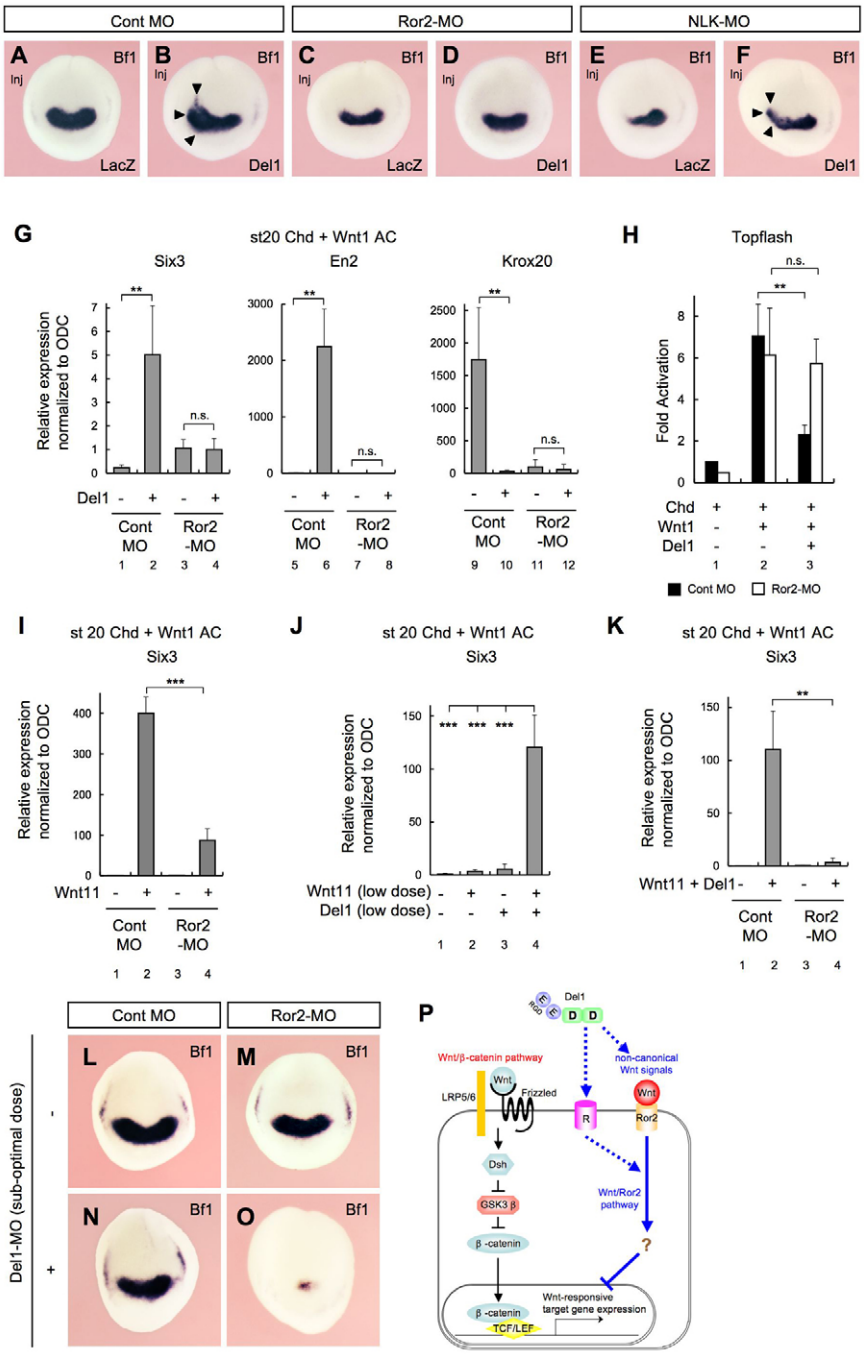

Fig. 6. The Ror2 pathway is essential for Del1 function. (**A-F**) Combinations of *Del1* (400 pg), *lacZ* (400 pg), control-*MO* (4 ng), *Ror2-MO* (4 ng) and *NLK-MO* (4 ng) as shown were injected into the two unilateral animal blastomeres at the 8-cell stage and harvested at the neurula stage. Arrowheads indicate expansion of *Bf1*. *Ror2-MO* injection at a dose higher than 4 ng frequently caused spina bifida or exogastrulation in vivo, presumably because of convergent-extension defects. (**G**) Animal caps were injected with combinations of *Chd* (100 pg), *Wnt1* (5 pg), *Del1* (500 pg), control-*MO* (8 ng) and *Ror2-MO* (8 ng) as shown, cultured until the siblings reached stage 20, and analyzed by qPCR. (**H**) Animal caps were injected with *Chd* (100 pg), *Wnt1* (10 pg), *Del1* (500 pg), control-*MO* (8 ng) or *Ror2-MO* (8 ng) and analyzed for luciferase activity. (**I**) Animal caps were injected with combinations of *Chd* (100 pg), *Wnt1* (5 pg), *Wnt11* (200 pg), control-*MO* (8 ng) and *Ror2-MO* (8 ng) as shown, cultured until the siblings reached stage 20, and analyzed by qPCR. (**J**,**K**) Animal caps were injected with combinations of *Chd* (100 pg), *Wnt1* (5 pg), *Wnt11* (10 pg), *Del1* (200 pg), control-*MO* (8 ng) and *Ror2-MO* (8 ng) as shown, cultured until the siblings reached stage 20, and analyzed by qPCR. **, *P*<0.01; ***, *P*<0.001. n.s., not significant. Error bars represent s.d. (**L-O**) Animal cell injection of *Del1-MO* (10 ng) and *Ror2-MO* (4 ng) coordinately suppressed the expression of *Bf1* at the neurula stage. The frequency of strong *Bf1* suppression was: 0%, *n*=29 (L); 0%, *n*=47 (M); 9%, *n*=43 (N); 80%, *n*=35 (O). (**P**) Working hypothesis for Del1 function in the modulation of the canonical Wnt pathway (see text for details). E, EGF-like domain; D, Discoidin domain; R, presumptive Del1 receptor; LRP5/6, LDL receptor-related protein 5/6. Question marks indicate presumptive intracellular modulator(s) of the Wnt/Ror pathway.

In *Chd*/*Wnt1*-injected animal caps, co-injection of *Del1* induced the expression of *Six3* and *En2* (Fig. 6G, lanes 2 and 6) at the cost of expression of *Krox20* (lane 10). Interestingly, this *Del1*-induced anteriorization in the explants was strongly suppressed by *Ror2-MO* co-injection (Fig. 6G, lanes 4 and 8), and the reduction of *Krox20* expression by *Del1* became marginal in the Ror2-depleted animal caps (compare lanes 11 and 12 with 9 and 10). In the *Topflash-luciferase* assay using animal caps, suppression of the Wnt-induced luciferase activity by *Del1* (Fig. 6H, black bars in lanes 2 and 3) was diminished when *Ror2-MO* was co-injected (white bars in lanes 2 and 3), indicating that Del1 inhibited the canonical Wnt signaling in a Ror2-dependent manner.

Previous studies using cultured cells have shown that non-canonical Wnt ligands, such as Wnt5a and Wnt11, bind directly to the Ror2 receptor and inhibit the canonical Wnt pathway downstream of β-catenin (Hikasa et al., 2002; Maye et al., 2004; Mikels and Nusse, 2006). Consistent with this idea, we observed in the animal cap assay that overexpression of the non-canonical ligands Wnt11 or Wnt5a almost completely suppressed the luciferase activity induced by *Wnt3a* or *Δβ-catenin* [which encodes a degradation-resistant form of β-catenin (Yost et al., 1996)] (see Fig. S6A and Fig. S7A in the supplementary material). By contrast, this suppression was only partial (~50%) when Ror2 was knocked down by MO (see Fig. S6B in the supplementary material, compare lanes 5 and 6 with 2 and 3), suggesting that Ror2 also mediates a substantial portion of the Wnt11- or Wnt5a-induced suppression of the canonical signal in the embryonic *Xenopus* cells. The injection of *Wnt11* or *Wnt5a* induced expression of anterior neural markers, such as *Six3*, at the cost of posterior gene expression in *Chd*/*Wnt1*-injected animal caps (see Fig. S6C and Fig. S7B in the supplementary material). This induction of *Six3* by *Wnt11* or *Wnt5a* was reversed by *Ror2-MO* co-injection (Fig. 6I; see Fig. S7C in the supplementary material, lanes 2 and 4). These results suggested that activation of the Ror2-mediated non-canonical pathway substantially inhibited canonical Wnt signaling and promoted anteriorization in the neuralized animal cap.

Finally, given that the anteriorizing activities of both Del1 and Wnt11 or Wnt5a depend on the Ror2 pathway, we examined whether the *Del1* signal and the *Wnt11*-induced non-canonical signal exhibited any functional interaction. We injected small doses of *Del1* and *Wnt11* (Fig. 6J) or *Wnt5a* (see Fig. S7D in the supplementary material) mRNAs into the *Chd*/*Wnt1*-injected animal cap. When injected individually, *Del1* or *Wnt11* showed only a moderate effect on *Six3* expression (Fig. 6J, lanes 2 and 3). By contrast, when injected together, they synergistically elevated *Six3* expression (lane 4). Importantly, this strong upregulation of the anterior marker was suppressed by co-injection of *Ror2-MO* (Fig. 6K, compare lanes 2 and 4), showing that this phenomenon is Ror2 dependent. Similarly, in the *Topflash* assay (see Fig. S6D in the supplementary material), a sub-effective dose of *Wnt11* (lane 3) enhanced the *Del1*-induced attenuation of luciferase activity (lanes 4 and 5). Then, we tested whether the functional interaction of Del1 and Ror2 was also observed in vivo (Fig. 6L-O; see also Fig. S8 in the supplementary material). The single injection of a sub-optimal dose of *Ror2-MO* or *Del1-MO* only marginally suppressed *Bf1* expression (Fig. 6M,N). By contrast, the injection of both *Ror2-MO* and *Del1-MO* strongly inhibited *Bf1* expression (Fig. 6O), suggesting that Ror2 and Del1 work together in neural anteriorization in the embryo.

Collectively, these findings indicate that Del1 promotes anterior neuroectoderm development via inhibition of the canonical Wnt signaling pathway in a Ror2-dependent manner.

DISCUSSION

Essential role of the matrix protein Del1 in AP patterning of the *Xenopus* embryo

Several soluble inhibitors of Wnt signals have been identified, including Cerberus, Frzb, Dkk1, WIF1 and Crescent. These factors inhibit the cellular reception of Wnt signals by binding to the Wnt ligands or their receptor complexes (reviewed by Kawano and Kypta, 2003). Compared with these factors, Del1 is unique in two aspects. First, Del1 interferes with the Wnt intracellular pathway, presumably via a non-integrin receptor (as discussed below). Second, Del1 is a matrix protein that accumulates pericellularly. In this sense, Del1 is not a typical diffusible inducer that emanates from the head organizer (e.g. Cerberus from the prechordal plate), but rather acts as a local modulator of the signaling microenvironment within the anterior neuroectoderm. A reasonable interpretation is that the anterior neuroectoderm protects itself from the posteriorizing Wnt signal by accumulating Del1 pericellularly and shifting its microenvironment in an 'anti-Wnt' direction. Previous studies have also identified several Wnt-modulating matrix proteins, such as Syndecan-4 (Muñoz et al., 2006), Glypican-4 (Ohkawara et al., 2003) and R-spondin (Wei et al., 2007; Kim et al., 2008). Thus, it appears that the fine-tuning of local Wnt responsiveness is orchestrated by an intricate network of positive and negative Wnt-modulating matrices.

Del1 attenuates both Wnt and BMP signals via its Discoidin domains

As our previous report indicated that the anti-BMP activity of Del1 is also independent of the RGD motif, it was important to rule out the possibility that the anti-Wnt activity of Del1 is secondary to its anti-BMP function, as this direction of interaction was previously suggested in another context (Nishita et al., 2000). The following observations in the present study indicate that such a secondary effect is unlikely. First, *Del1* has a qualitatively distinct activity, i.e. eye induction, in the secondary axis that is induced by BMP inhibition. Second, similarly, *Del1* promotes an obvious anteriorization in the neural tissue induced from animal caps by Chd, suggesting that Del1 plays more roles than the BMP antagonist Chd does. Third, Wnt-induced posteriorization of the embryo is not reversed by co-injecting *Chd*. Furthermore, *Del1* strongly inhibits Wnt signaling in the animal cap, whereas *Chd* affects the *Topflash* activity only marginally. In addition, a *BRE-luciferase* assay (see Fig. S9 in the supplementary material) showed that inhibition of the BMP signal by Del1, unlike the inhibition of the canonical Wnt signal (as discussed below), is largely independent of the Ror2 pathway.

From a functional viewpoint, whatever the molecular mechanism might be, the double inhibition of Wnt and BMP signals by the same protein (i.e. the multi-functional Del1) is an efficient way to promote head development (Niehrs, 2004), as has been shown for the secreted head organizer factor Cerberus (Piccolo et al., 1999). It is likely that Del1, a matrix protein, acts more locally to consolidate the forebrain fate.

Ror2-dependent mechanism of Del1 signaling

The present study showed that Del1 inhibits canonical Wnt signaling downstream of the nuclear accumulation of β-catenin. In addition, we demonstrated that this anti-canonical Wnt activity of Del1 is dependent on the Ror2 pathway, which is activated by the non-canonical Wnt ligands. This synergistic interaction between Del1 and the Ror2 pathway is essential for anterior neural development in vivo (Fig. 6L-O). Fig. 6P illustrates our working

hypothesis deduced from the results of the present study. Del1 attenuates canonical Wnt signaling by evoking a Ror2-sensitive intracellular cascade that interferes with β-catenin/Tcf-dependent activation of target genes (Mikels and Nusse, 2006). It is an important future challenge to elucidate the detailed molecular cascade involved in Del1 signaling. It has been reported that Wnt5a/Ror2 signaling induces *XPAPC* expression by activating the Cdc42-JNK-ATF2 pathway (Schambony and Wedlich, 2007). However, in our preliminary study, unlike Wnt5a-induced *XPAPC* expression, which is suppressed by dominant-negative Cdc42 (dnCdc42) (Choi and Han, 2002), the anti-canonical Wnt activity of Del1 or Wnt5a was not affected by dnCdc42 (see Fig. S10 in the supplementary material), suggesting that the anti-Wnt activity of Del1/Wnt5a is mediated by other signaling pathways.

In addition, it remains to be determined whether Del1 signaling merges with the Ror2 signaling pathway at the Ror2 receptor level or acts in the intracellular pathway downstream of Ror2 (Fig. 6P, dotted arrows). We have so far failed to detect any significant binding of Del1 to Ror2-Fc protein (the extracellular domain of Ror2 fused with the human IgG Fc domain in the C-terminal region) or any facilitated binding of Wnt11 to Ror2-Fc protein in the presence of Del1. The finding that the anti-BMP activity of Del1 is independent of Ror2 suggests the presence of a non-Ror2/non-integrin receptor (R in Fig. 6P) system for Del1, at least for its anti-BMP activity. One technical hindrance for Ror2 signaling analysis at present is the lack of a method to activate Ror2 in a ligand-independent manner. For instance, *Ror2* overexpression does not cause the anteriorization of the Chd/Wnt1-injected animal cap (see Fig. S5F in the supplementary material). It is inferred that endogenous *Ror2* expression might already be close to the threshold level, as indicated by the fact that the optimal amount of *Ror2* required to reverse the *Ror2-MO* phenotype is fairly low (see Fig. S5G in the supplementary material).

Another intriguing question is whether Del1 also affects the Ror2-mediated planar cell polarity (PCP) pathway (Hikasa et al., 2002), which is a typical non-canonical Wnt cascade, in the opposite direction. Activin-treated animal caps differentiate mainly into dorsal mesodermal tissues (Osada and Wright, 1999) and exhibit a massive convergent-extension movement that depends on the PCP pathway. In our preliminary study using Activin-treated animal caps, *Del1* injection inhibited the extension of the mesodermalized animal cap. However, it was not easy to judge whether this is a direct consequence of the effects of Del1 on the PCP pathway or a secondary influence of its anteriorization effect on the dorsal mesoderm (see Fig. S11 in the supplementary material). *Del1* injection increased the expression of the anterior dorsal marker *Goosecoid*, but suppressed the trunk mesodermal markers *MyoD*, *alpha-actin* and *Xbra*. By contrast, *Del1* did not substantially affect the expression of the dorsal mesodermal marker *Chd*, which is expressed in both the anterior and posterior axial regions (Sasai et al., 1994), suggesting that Del1 promotes anteriorization not only of the ectodermal tissue but also of the dorsal mesodermal tissue. Because the convergent-extension movement is observed predominantly in the trunk mesoderm but not strongly in the head mesoderm (Wallingford et al., 2002), the relationship between the suppression of the convergent-extension movement and the promotion of anteriorization is not readily separable in this experimental system and will require careful future investigation.

An additional remaining question is why the overexpression of the anti-Wnt factor Del1 (even when its mRNA is injected radially at the 4-cell stage) does not inhibit the formation of the dorsal axis (Arakawa et al., 2007), which is known to depend on intracellular β-catenin signaling (Heasman, 2006; Weaver and Kimelman, 2004). Consistent with this finding, *Del1* did not suppress the induction of a secondary axis by *Wnt8* mRNA injection, which mimics the maternal effect (see Fig. S12 in the supplementary material). The axis-inducing β-catenin signal is known to reflect maternal Wnt activity. In contrast to early/maternal Wnt signaling, late/zygotic Wnt signaling that occurs after the MBT (particularly after the late blastula stage) plays a major role in the posterior and ventral specification of embryonic tissues (Niehrs, 2004). In the neural tissue, late/zygotic Wnt signaling has a strong patterning activity that promotes posterior differentiation at the expense of the development of anterior tissues (Kiecker and Niehrs, 2001) (Fig. 3A,B). The gain- and loss-of-function *Del1* phenotypes shown in this report are consistent with the idea that Del1 antagonizes Wnt signaling preferentially in its late/zygotic posteriorization function. The dependence of Del1 activity on Ror2 explains, at least in part, its late Wnt-specific phenotype, as *Ror2* expression is zygote specific (Hikasa et al., 2002) and no obvious maternal effects, such as axial phenotypes, have been reported for *Ror2-MO* injection (Schambony and Wedlich, 2007) (our unpublished observations).

In conclusion, the matrix-associated protein Del1 acts as an extracellular modulator that controls the zygotic canonical Wnt pathway in a close relationship with Ror2-dependent non-canonical Wnt regulation.

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Competing interests statement
The authors declare no competing financial interests.

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