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Novel functions of Noggin proteins: inhibition of Activin/Nodal and Wnt signaling

Andrey V. Bayramov*, Fedor M. Eroshkin*, Natalia Y. Martynova, Galina V. Ermakova, Elena A. Solovieva and Andrey G. Zaraisky‡

SUMMARY

The secreted protein Noggin1 is an embryonic inducer that can sequester TGFβ cytokines of the BMP family with extremely high affinity. Owing to this function, ectopic Noggin1 can induce formation of the headless secondary body axis in *Xenopus* embryos. Here, we show that Noggin1 and its homolog Noggin2 can also bind, albeit less effectively, to ActivinB, Nodal/Xnrs and XWnt8, inactivation of which, together with BMP, is essential for the head induction. In support of this, we show that both Noggin proteins, if ectopically produced in sufficient concentrations in *Xenopus* embryo, can induce a secondary head, including the forebrain. During normal development, however, *Noggin1* mRNA is translated in the presumptive forebrain with low efficiency, which provides the sufficient protein concentration for only its BMP-antagonizing function. By contrast, Noggin2, which is produced in cells of the anterior margin of the neural plate at a higher concentration, also protects the developing forebrain from inhibition by ActivinB and XWnt8 signaling. Thus, besides revealing of novel functions of Noggin proteins, our findings demonstrate that specification of the forebrain requires isolation of its cells from BMP, Activin/Nodal and Wnt signaling not only during gastrulation but also at post-gastrulation stages.

KEY WORDS: Noggin, Activin, Nodal, Wnt, Forebrain, *Xenopus*

INTRODUCTION

The secreted protein Noggin (Noggin1) was first discovered in *Xenopus* as a neural inducer produced by Spemann's organizer (Smith and Harland, 1992). Noggin1 can bind to members of one of two subgroups of the TGFβ cytokines, bone morphogenetic proteins (BMPs), thereby preventing BMP binding to type I and type II serine-threonine kinase receptors and inhibiting signaling mediated by Smad1/5/8 (Groppe et al., 2002; Zimmerman et al., 1996). Because of this function, Noggin1 plays a key role in many processes, including induction of neural tissue and skeletal muscles in early embryogenesis (Smith and Harland, 1992), development of cartilage (Brunet et al., 1998), and differentiation of hair follicles (Botchkarev et al., 1999).

In addition to 'classical' Noggin1, two other Noggin proteins, Noggin2 and Noggin4, have been identified in vertebrates (Furthauer et al., 1999; Fletcher et al., 2004; Eroshkin et al., 2006). Among them, only the biological function of Noggin2, which is specifically expressed in the forebrain rudiment of *Xenopus* and *Danio* embryos, has been studied, and experiments have shown that Noggin2 can duplicate the BMP-antagonizing function of Noggin1 (Furthauer et al., 1999).

Now, we demonstrate that ectopically expressed *Noggin2* can elicit formation of a secondary head in *Xenopus* embryos, an effect requiring simultaneous inhibition of BMP, Nodal and Wnt signaling pathways (Piccolo et al., 1999). To further address these novel functions of Noggin2, we compared the abilities of Noggin1 and Noggin2, translated from synthetic mRNA in early *Xenopus laevis* embryos, to bind to and antagonize several secreted proteins known to be involved in regulation of TGFβ and Wnt signaling. Because preliminary data demonstrated that the translation of *Noggin1* wild-type mRNA was extremely low, special attention was paid to equalize the translational capacities of *Noggin* mRNA in embryos. Surprisingly, we found that, besides BMPs, Noggin1 and Noggin2 can antagonize, albeit less effectively, a set of non-BMP TGFβ ligands, including ActivinB, and *Xenopus* Nodal homologs Xnr2 and Xnr4, which bind to a different set of type I and type II serine-threonine kinase receptors and regulate a specific set of genes through the cytoplasmic effectors Smad2/3 (Shi and Massague, 2003). Accordingly, we demonstrate that, during normal development, Noggin2 suppresses Activin signaling in cells of the forebrain rudiment. The most surprising finding, however, is that both Noggin proteins can antagonize XWnt8 signaling, the inhibition of which is necessary for forebrain development (Kiecker and Niehrs, 2001).

MATERIALS AND METHODS

DNA constructs, luciferase assay and qRT-PCR

Cloning strategies of all constructs are described in Table S1 in the supplementary material. For luciferase assay, *Xenopus* embryos were injected at the two- to four-cell stage by a mixture of one of the luciferase reporter plasmids [GL3-ARE-Luc (Pierreux et al., 2000); TOPflash, Millipore; TCFm-Luc (Hikasa et al., 2010)], the reference pCMV-β-GAL plasmid (50 pg/embryo of each plasmids) and corresponding mRNA. Animal caps (AC) and ventral marginal zone (VMZ) explants were explanted at stage 10, cultured until the stage 11 equivalent in three replicate samples of 10 explants each and processed for luciferase analysis according to the manufacturer's protocol. Similar triplicate samples of explants were subjected to qRT-PCR with primers to *XBra*, *Xnr3* and *ODC* as reference controls (Xanthos et al., 2002).

Immunoprecipitation and antibodies

Xenopus embryos at the two- to four-cell stage were injected with synthetic mRNA (500 pg/blastomere) and in 2 hours transferred to 0.1×MMR for further incubation for 18-20 hours at 15°C. The injected embryos were

Shemyakin-Ovchinnikov Institute of Bioorganic Chemistry, Russian Academy of Sciences, Moscow, 117997, Russia.

*These authors contributed equally to this work
‡Author for correspondence should be addressed (azaraisky@yahoo.com)

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staged according to Nieuwkoop and Faber (Nieuwkoop and Faber, 1967). Embryos at stage 11 were homogenized on ice by pipetting in Dulbecco's phosphate-buffered saline (137 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4 and 8.1 mM Na_2HPO_4) supplemented with 0.1% BSA, 0.1% Triton X-100 and protease inhibitors at a 1:50 dilution (Sigma) (immunoprecipitation buffer, IPB; 10 μl per embryo), and centrifuged at 13,400 ***g*** for 30 minutes at 4°C to remove the yolk. Aliquots of lysates containing standard amount of tagged protein of each type were mixed, adjusted using IPB to 1 ml and incubated for 2 hours at 4°C on a rotating wheel. Lysate mixture (500 μl) was mixed with either anti-Myc antibody (Sigma, clone 9E10, catalog number M 4439) bound to protein G-sepharose beads (Sigma, catalog number P3296) or with 5 μg of antibodies bound to 50 μl of a 1:1 beads suspension, then incubated for 3 hours at 4°C on a rotating wheel and washed five times with IPB. Protein complexes removed from beads were analyzed by blotting as described previously (Martynova et al., 2008). For detection of endogenous Noggin proteins by western blotting, affinity-purified rabbit antibodies raised by PickCell Laboratories (Netherlands) to specific synthetic peptides to Noggin1 (QRRVQQKCAWITIQ) and Noggin2 (LDLSETPYGDRIRMGK) were used as primary antibodies. Goat anti-rabbit F(ab′) fragments of antibody conjugated to alkaline phosphatase (Sigma) was used as a secondary antibody.

Synthetic mRNA, morpholino oligonucleotides, transgenic embryos and in situ hybridization

Synthetic mRNA was prepared by mMessage Machine SP6 Kit (Ambion) after linearization of pCS2-based plasmids with *Not*I or pSP64-based plasmids with *Ase*I. Anti-Noggin morpholinos (0.5 mM final concentration) were: *Noggin1*, 5′-TCACAAGGCACTGGGAAT-GATCCAT; *Noggin2*, 5′-CCTCAGGCAGATTTATCCTCTTCAT; and *misNoggin2*, 5′-CaTCAggCAGAcTcATCCTCaaCAT. Anti-BMP2, -BMP4 and -BMP7 morpholinos were as described previously (Kuroda et al., 2005). All mRNAs and MOs were mixed with Fluorescein Lysine Dextran (FLD) (Invitrogen, 40 kDa, 5 μg/μl) before injection.

Transgenic embryos were generated as described previously (Ermakova et al., 2007). Embryos expressing Kate RFP fluorescent reporter under the control of cardiac actin promoter were obtained from adult transgenic *Xenopus laevis* generated in our laboratory (Shcherbo et al., 2007).

Whole-mount in situ hybridization was performed as described previously (Harland, 1991). To reduce staining variations in different series of experiments in which integrated density of *XBF1* in situ hybridization signal was measured, fresh aliquots of preliminary synthesized and frozen digoxigenin-labeled probe were used in each of these series and staining was performed under constant conditions (by BM Purple for 3 hours at 20°C). After staining, all embryos were bleached in 10% H_2O_2.

RESULTS

Noggin2 induces formation of a secondary head and inhibits mesoderm differentiation

To reveal possible functional differences between Noggin1 and Noggin2, we first compared their abilities to induce secondary body axes in *Xenopus* embryos. Consistent with data of other authors, injection of 20-400 pg/blastomere of full-length *Noggin1* mRNA or its *Δ5* version, which has a significant part of the wild-type 5′UTR deleted (Smith and Harland, 1992), into ventral blastomeres at the four- to eight-cell stage induced headless secondary axes (Fig. 1A).

By contrast, when a similar version of *Noggin2* mRNA was injected, normal development was blocked at the early neurula stage and the mushroom-shaped embryos formed by the tailbud stage (100%, *n*=400) (Fig. 1B). Further analysis revealed a significant anteriorization of these embryos, marked by increased expression of the rostral forebrain marker *Xanf1* (Fig. 1C). Additionally, there was an enormous neuralization of ectoderm (expanded expression of *NCAM*) accompanied by reduced epidermal (*keratin*) and muscle (*muscle actin*) differentiation (Fig. 1D-F). Surprisingly, when the concentration of injected Noggin2 mRNA was reduced to 1-5 pg/embryo, formation of secondary heads with a medially positioned cyclopic eye and forebrain (marked by expression of *XBF1*) was observed in 35% (*n*=94) of embryos (Fig. 1G,H).

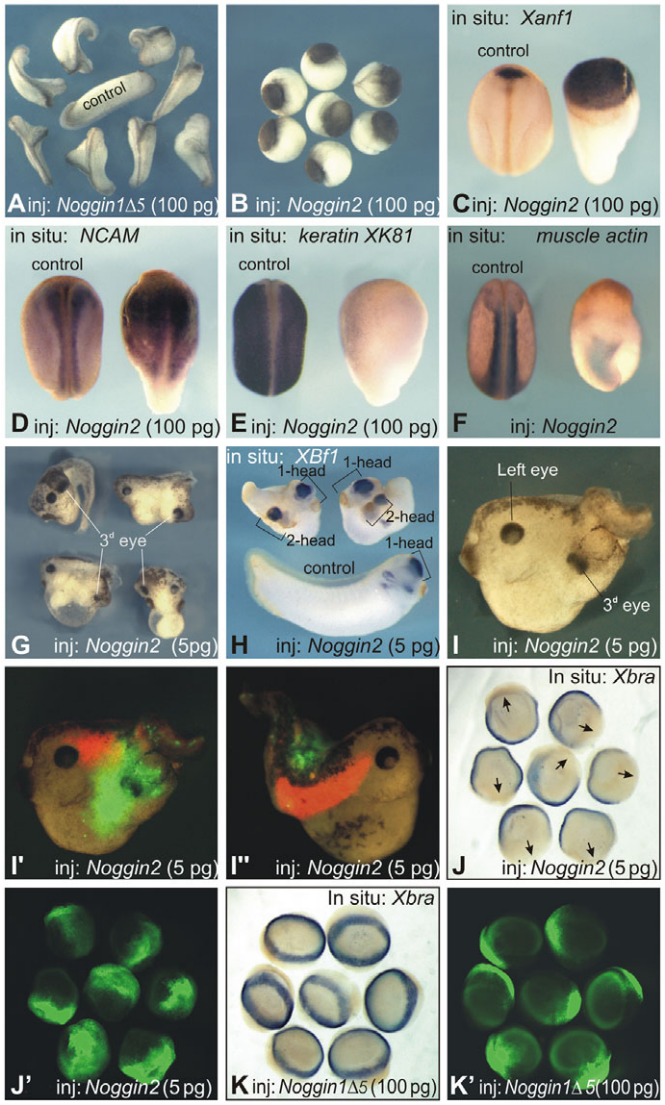

Fig. 1. Wild-type *Noggin2* mRNA elicits effects distinct from those induced by wild-type *Noggin1* mRNA. (**A**,**B**) Ventral injections of *Noggin1Δ5* mRNA induce secondary axes (A), whereas similar injections of *Noggin2* mRNA resulted in mushroom-shaped embryos (B). (**C-F**) Whole-mount in situ hybridization of the control (left in each photo) and the *Noggin2* mRNA-injected (right) embryos demonstrates strong upregulation of the neural (C,D) marker genes, and inhibition of epidermal (E) and muscle (F) marker genes. (**G**) Ventral injections of *Noggin2* mRNA induce formation of secondary heads with cyclopic eyes. (**H**) The forebrain marker *XBF1* is expressed in the secondary heads of embryos injected by *Noggin2* mRNA. (**I-I″**) The embryo of a transgenic line expressing RFP in muscles has reduced muscle differentiation on the left side where a secondary head with cyclopic eye was induced by injection of the mixture of *Noggin2* mRNA and FLD tracer. The same embryo under white light (I) and as an overlay of white light, red and green fluorescent images (I′, left side; I″, right side). (**J-K′**) *Noggin2* (J,J′) but not *Noggin1* mRNA (K-K′) inhibits (arrows) *XBra* expression in blastopore marginal zone. Embryos at stage 10.5 are shown from the vegetal pole.

As was further revealed by transgenic embryos expressing *Kate RFP* in muscles (Shcherbo et al., 2007), ectopic Noggin2 elicited reduction of skeletal muscles on the injected side (Fig. 1I-I″; supplementary material Fig. S1). This indicates that, in contrast to Noggin1, Noggin2 could probably inhibit general mesoderm specification. In support of this, we observed strong inhibition of general mesoderm marker *Xbra* expression in embryos injected with *Noggin2* mRNA, but not with *Noggin1Δ5* mRNA (Fig. 1J-K′).

In normal development, *Noggin2* mRNA is translated with much higher efficiency than *Noggin1* mRNA

The revealed effects of Noggin2 closely resemble those of the head inducer Cerberus, which can simultaneously antagonize BMP, Nodal and Wnt signaling (Piccolo et al., 1999). Therefore, we decided to test by co-immunoprecipitation the ability of Noggin1 and Noggin2 to bind ligands of these three pathways. However, based on the data of Smith and Harland (Smith and Harland, 1992), indicating inhibition of translation by the wild-type 5′UTR of *Noggin1* mRNA, we preliminarily compared the translation of *Noggin1* and *Noggin2* mRNA containing different 5′UTRs (Fig. 2A). As we suspected, *MycNoggin1* mRNA, with either long wild-type 5′UTR or with a part of this sequence consisting of 84 amino acids [*A3* and *Noggin1Δ5* mRNA, respectively, according to Smith and Harland (Smith and Harland, 1992)], was translated at least 200 times less effectively than similar *MycNoggin2Δ5* mRNA (Fig. 2B). By contrast, no significant difference was observed between translation of *SynMycNoggin1* and *SynMycNoggin2* mRNA, in which the wild-type 5′UTR was substituted by a common synthetic 5′UTR containing a consensus Kozak site (Fig. 2A,C). Therefore, we used mRNA with these 5′UTR in the following experiments.

Importantly, the revealed difference in the translation of the wild-type *Noggin1* and *Noggin2* mRNA is not compensated in normal development by their concentrations, as both mRNAs are present in cells of the anterior neural fold in approximately equal concentrations (Fig. 2D). This suggests that the concentration of endogenous Noggin2 may actually be much higher than that of Noggin1.

To test this, we tried to detect endogenous Noggin1 and Noggin2 in explants of the midneurula anterior neural fold with antibodies raised against Noggin1- and Noggin2-specific oligopeptides. Although we were unable to detect any specific signal for Noggin1, a low but clear band was observed with antibodies to Noggin2 (Fig. 2E). Assuming that the antibodies used have demonstrated a similar affinity to exogenous Noggin1 and Noggin2 translated in embryos from synthetic mRNA (Fig. 2F), we concluded that these results agree with the hypothesis of a lower concentration of endogenous Noggin1 compared with Noggin2.

Both Noggin1 and Noggin2 can bind non-BMP TGFβ ligands and Wnt

Using co-immunoprecipitation, the following Flag-tagged ligands known to operate during early embryogenesis were tested for their ability to bind Myc-tagged Noggin1 and Noggin2; ADMP and BMP4 (BMP pathway); ActivinB (Activin βB); two *Xenopus* Nodal homologs, Xnr2 and Xnr4 (Activin/Nodal pathway); and Xwnt8 (canonical Wnt pathway). Each of these proteins was individually translated in *Xenopus* embryos from the injected mRNA and assayed for co-immunoprecipitation with MycNoggin1 and MycNoggin2, which were also translated individually in embryos from *SynMycNoggin1* and *SynMycNoggin2* mRNA (Fig. 2A; supplementary material Fig. S2 for input proteins).

The results revealed that, in addition to BMP4, both Noggin proteins were precipitated, albeit less effectively, with ADMP, all non-BMP TGFβ ligands and XWnt8 (Fig. 2G,H). At the same time, no interactions were detected with the control cysteine-rich protein 3×-Flag-tagged Zyxin (Fig. 2H). Interestingly, when the low translation version of *MycNoggin1* mRNA containing the wild-type *Δ5* 5′UTR was used, a signal above background was detected only for BMP4 (Fig. 2G,H). This result confirms that, despite its potential ability to bind non-BMP TGFβ ligands and Wnt, Noggin1, owing to its low concentration in embryos, can inhibit only BMP ligands in normal development, which have a much higher affinity to Noggin1 than to other TGFβ proteins or to Wnt. This is consistent with the generally accepted theory that Noggin1 operates in embryos primarily as a BMP inhibitor.

It has previously been shown that the N-terminal clip domain of Noggin1 plays a crucial role in its binding to BMP (Groppe et al., 2002). To determine whether the same domain is responsible for binding of Noggin1 and Noggin2 to non-BMP TGFβ ligands and XWnt8, we tested the interaction of these ligands with Myc-tagged ΔclipNoggin1 and ΔclipNoggin2 mutants lacking the 28 N-terminal amino acid residues that form the clip domain (Fig. 2A).

The deletion of clip-domain sharply reduced binding of Noggin proteins to BMP4 to background levels (Fig. 2I). Surprisingly, both ΔclipNoggin1 and ΔclipNoggin2 could bind all non-BMP TGFβ ligands and XWnt8 (Fig. 2I). These data indicate that other regions, but not the clip-domain, of Noggin molecules are responsible for their binding to these ligands.

Noggin1 and Noggin2 can inhibit Activin/Nodal and Wnt signaling

To verify whether Noggin1, Noggin2 and their clip-domain deletion mutants can, in principle, antagonize ActivinB, Xnr2 or XWnt8, we first tested the ability of non-tagged variants of Noggin proteins produced in a large excess over the non-tagged ligands to inhibit expression of luciferase reporters driven by specific promoter elements for the Smad2 and β-catenin pathways.

Under these conditions, we observed inhibition of the pARE-Luc (Smad2 pathway) and pTOPflash (β-catenin pathway) reporters activated by ActivinB/Xnr2 and XWnt8, respectively, in the animal cap and ventral marginal zone explants of embryos co-injected with either full-length *Noggin* or *Δclip* mutant mRNA (Fig. 3A-C). Although the large excess of both Noggin proteins, as well as their deletion mutants, were almost equally as effective at inhibiting ActivinB and XWnt8 signaling, Noggin2 and ΔclipNoggin2 inhibited Xnr2 signaling to a greater degree (Fig. 3B). Lower inhibitory effects were detected when Myc-tagged versions of the Noggin proteins were tested (not shown).

The ability of Noggin proteins and their deletion mutants to antagonize ActivinB, Xnr2 and XWnt8 was also confirmed by analysis of the expression of direct endogenous targets of the Smad2 and β-catenin pathways (*Xbra* and *Xnr3*, respectively) in embryonic explants (Fig. 3A′-C′).

Importantly, full-length Noggin proteins, as well as their Δclip mutants, were unable to inhibit ActivinB, Xnr2 or Wnt signaling pathways when these pathways were activated intracellularly by overexpression of the corresponding effectors of these pathways, Smad2 and β-catenin (Fig. 3A-C′). These results confirm an extracellular mode of Noggin proteins activity. By contrast, overexpression of TGFβ effectors Smad2 or Smad1 was unable to prevent inhibition of XWnt8 signaling by Noggin proteins. Reciprocally, no blocking of the inhibitory influence of Noggin proteins upon Activin or Xnr2 signaling was observed when they

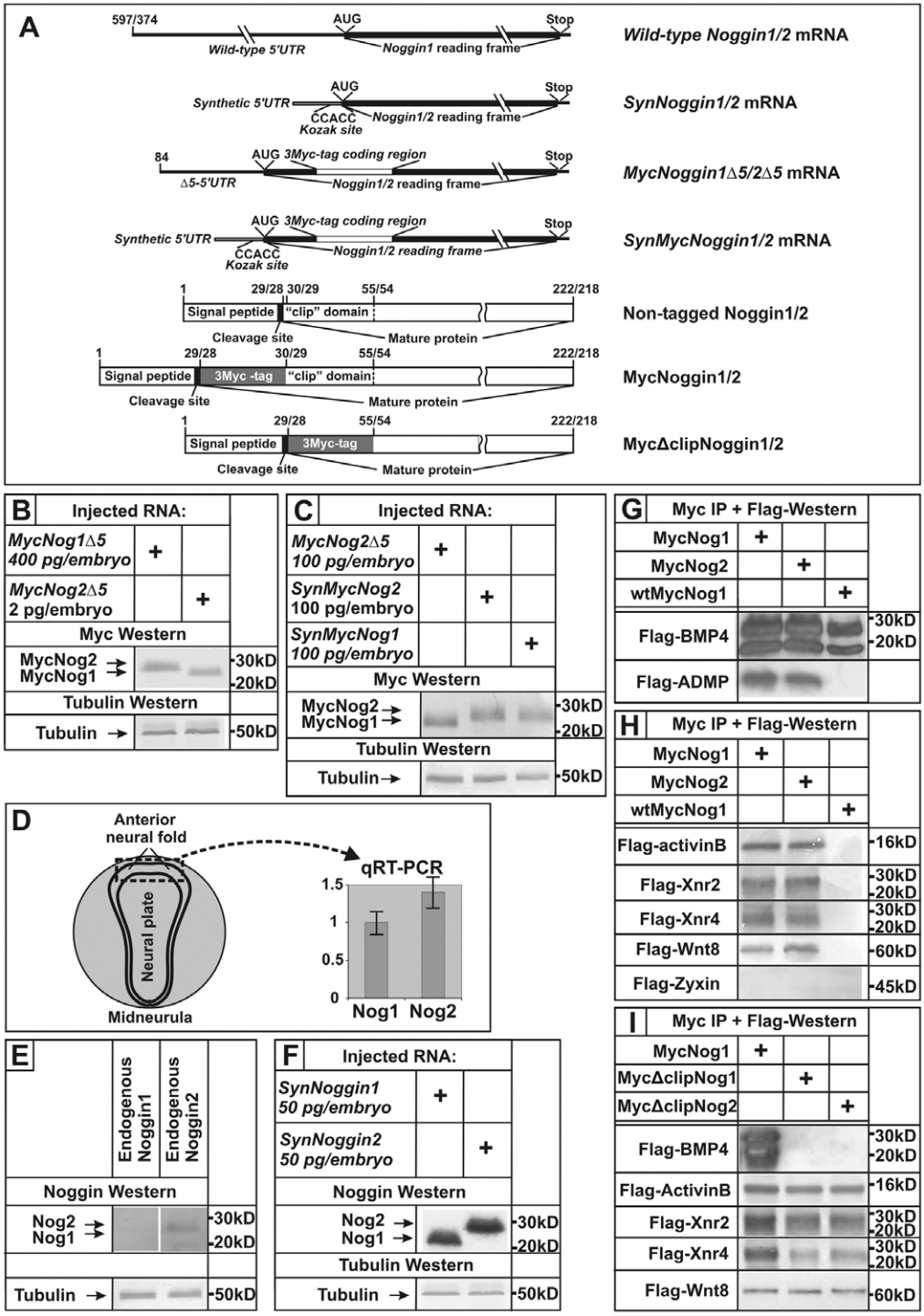

Fig. 2. Noggin1 and Noggin2 can bind TGFβ and Wnt ligands. (**A**) Selected mRNA and proteins used in the present study. (**B**,**C**) Comparison of translation capacities of *MycNoggin1Δ5* and *MycNoggin2Δ5* mRNA (B) or *MycNoggin2Δ5*, *SynMycNoggin1* and *SynMycNoggin2* mRNA (C) injected in two-cell embryos at the indicated concentrations. Five embryos of each type were collected at stage 10 in 50 μl of lysis buffer and Noggin proteins were revealed by western blotting with anti-Myc antibody either in 1/5 (B) or in 1/125 (C) aliquots of this volume. Here and below, α-tubulin was detected with anti-tubulin antibodies (DM1A, Sigma, final dilution 1:10,000) as a loading control. (**D**) qRT-PCR analysis of endogenous *Noggin1* and *Noggin2* mRNA in the anterior neural fold explants of stage 15 embryos. (**E**,**F**) Only endogenous Noggin2 (lane 2), but not Noggin1 (lane 1), was detected in the anterior neural fold explants of stage 15 embryos by antibodies specific to Noggin1 and to Noggin2 (E), despite these antibodies demonstrating similar affinities to exogenous Noggin1 (lane 3) and Noggin2 (lane 4) translated from injected *SynNoggin1* and *SynNoggin2* mRNA (F). In the last case, a mixture of antibodies to both Noggin proteins was used. (**G**,**H**) Both Noggin1 and Noggin2 (Ng1 and Ng2) translated from *SynMycNoggin*1 and *SynMycNoggin2* mRNA co-precipitate with Flag-tagged BMP4, ADMP, Activin, Xnr2, Xnr4 and XWnt8. In case of Noggin1 translated from *MycNoggin1Δ5*mRNA (wtNg1), only precipitation with BMP was detected. No precipitation of Noggin proteins was revealed with Flag-tagged Zyxin (negative control). (**I**) Deletion of the clip-domain sharply reduce ability of Noggin proteins (ΔNg1, ΔNg2) to bind BMP4 but much more poorly influences the binding of Noggin to all non-BMP TGFβ ligands tested and to XWnt8.

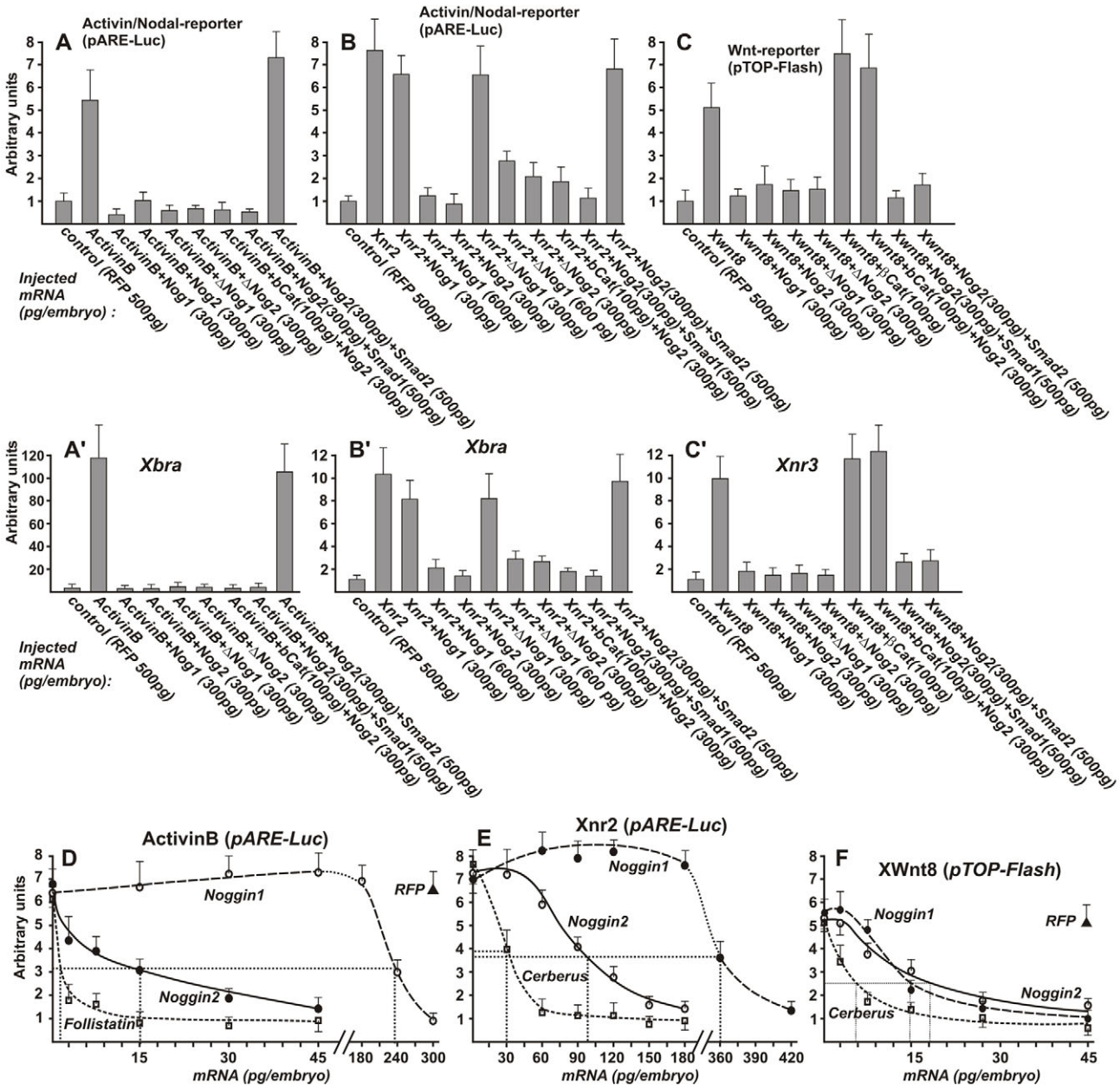

Fig. 3. Noggin proteins inhibit ActivinB, Xnr2 and XWnt8 signaling. (**A-C**) Influence of Noggin proteins translated from *SynNoggin* mRNAs injected into embryos at concentrations indicated below each graph on transcription of luciferase reporters activated by co-injection of *ActivinB* (0.8 pg/embryo), *Xnr2* (10 pg/embryo), *XWnt8* (5 pg/embryo), *Smad1*, *Smad2* or β-*catenin* mRNA. (**A′-C′**) qRT-PCR analysis of ActivinB, Xnr2 and XWnt8 target gene expression in siblings of samples analyzed in A-C. (**D-F**) Comparison of inhibitory activities of Noggin proteins, Cerberus and Follistatin. Two-cell embryos were injected with *Activin* (0.8 pg/blastomere), *Xnr2* (10 pg/embryo) or *XWnt8* mRNA (5 pg/embryo) mixed with increasing concentrations of *Noggin1*, *Noggin2, Cerberus* and *Follistatin* mRNA. Concentration of mRNA necessary to decrease halve the reporter signal activated by corresponding ligand is indicated by broken vertical lines. Injections of *RFP* mRNA at the indicated highest concentrations were used as negative controls. All graphs represent means of triplicate experiments. Bars indicate s.d.

were co-expressed with the BMP and Wnt effectors Smad1 and β-catenin, respectively (Fig. 3A-C′). These data prove that the inhibitory effects exerted by Noggin proteins on expression of ARE and TOPflash reporters were caused by interactions of Noggin proteins with true ligands of Wnt or Activin/Nodal signaling pathways and were not the result of possible crossregulation of these pathways.

Finally, we wanted to compare the efficiency of Noggin1 and Noggin2 as inhibitors of ActivinB, Xnr2 and XWnt8 with Cerberus (an inhibitor of Nodal and Wnt) and Follistatin (an inhibitor of Activin). To achieve this, we determined the mRNA concentrations that would be necessary to inject into embryos in order to decrease the reporter signal by half. We first confirmed that equal amounts of *SynMycNoggin1*, *SynMycNoggin2*, *SynMycCerberus* and *SynMycFollistatin* mRNA with common Kozak site and other regions of 5′- and 3′UTRs produced approximately equal amounts of Myc-tagged proteins (supplementary material Fig. S3A, Table S1). Then we established that Noggin2 inhibits ActivinB ~10 times less efficiently than Follistatin, whereas its ability to inhibit Xnr2 and

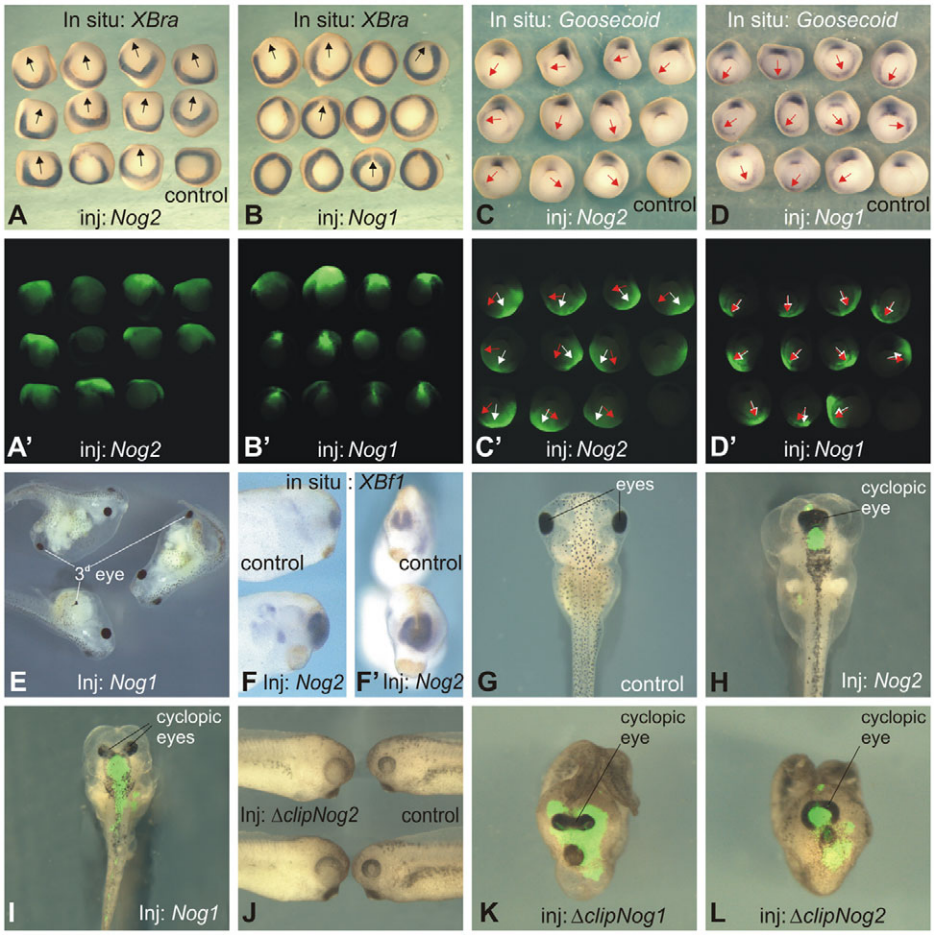

Fig. 4. Noggin proteins translated from mRNA with consensus Kozak (*SynNoggins*) site can influence developmental processes regulated by Nodal/Xnrs and Wnt. (**A**,**A′**) Injection of *Noggin2* mRNA (10 pg/embryo) elicits inhibition of *Xbra* expression in 100% of embryos (black arrows). (**B,B′**) Injections of *Noggin1* mRNA (10 pg/embryo) resulted in inhibition of *Xbra* expression in 40% of embryos (black arrows). (**C**,**C′**) Injections of *Noggin2* mRNA (10 pg/embryo) do not induce *goosecoid* expression in areas exactly corresponding to the injection sites. A very weak *goosecoid* expression can be seen just at the periphery of the injected areas, where the concentration of Noggin2 presumably drops down to the level insufficient for effective inhibition of Xnrs but still enough to antagonize BMP (see deviation between red and white arrows indicating maximums of *goosecoid* expression and FLD tracer signal, respectively). (**D**,**D′**) Injections of *Noggin1* mRNA (10 pg/embryo) induce a weak *goosecoid* expression. Areas of ectopic *goosecoid* expression exactly correspond to the injection sites (red and white arrows coincide). (**E**) Ventral injections of *SynNoggin1* mRNA (5 pg/embryo) induce development of the head-containing secondary axes with cyclopic eyes, the result indicating simultaneous inhibition of BMP, Nodal/Xnrs and Wnt signaling. (**F**,**F′**) Consistent with the ability of Noggin2 to inhibit Wnt, dorsal injections of *Noggin2* mRNA (5 pg/embryo) elicit enlargement of the forebrain rudiment marked by *XBF1* expression. (**G**) The control 5-day old tadpole as it is seen from the dorsal side. (**H**,**I**) Dorsal injections of *Noggin2* or *Noggin1* mRNA (5 pg/embryo) elicit cyclopic phenotype. (**J**) Injections of *ΔclipNoggin2* mRNA elicit enlargement of eyes, an effect consistent with the ability of this mutant to inhibit Wnt signaling. (**K**,**L**) Dorsal injections of *ΔclipNoggin2* or *ΔclipNoggin1* mRNA cyclopic phenotype. Anterior view.

XWnt8 was only three times less than that of Cerberus (Fig. 3D-F). At the same time, Noggin1 was as effective as Noggin2 in antagonizing XWnt8, but had ~15 and four times lower efficiency for the inhibition of ActivinB and Xnr2, respectively (Fig. 3D,E). Remarkably, no difference was revealed in abilities of Noggin proteins to inhibit the BMP-activated TCFm-Luc reporter controlled by Vent2 promoter deprived of TCF-binding site (Hikasa et al., 2010) (supplementary material Fig. S3B).

Noggin proteins can influence developmental processes regulated by Nodal/Xnrs and Wnt

The ability of Noggin proteins to bind and antagonize non-BMP TGFβ proteins and Wnt8 predicts that Noggin proteins translated at sufficient concentrations may influence developmental processes regulated by Smad2 and β-catenin pathways.

To study the effects of Noggin proteins on Smad2-dependent signaling, we analyzed expression of two genetic targets of this pathway, *XBra* and *goosecoid*, by in situ hybridization. As was demonstrated by other authors, 'pure' antagonists of BMP signaling, such as Noggin1 translated from the wild-type mRNA, can induce *goosecoid* expression in the ventral part of marginal zone but cannot inhibit *XBra* (Eimon and Harland, 1999). By contrast, factors able to antagonize both Smad1 and Smad2 signaling pathways, such as Cerberus, can inhibit *XBra* expression but cannot induce *goosecoid* (Bouwmeester et al., 1996; Eimon and Harland, 1999).

Strong inhibition of *XBra* (100%, *n*=65) and no induction of *goosecoid* was observed in embryos injected with *SynNoggin2* mRNA, a result consistent with the ability of Noggin2 to bind Xnrs and to antagonize Smad2 signaling (Fig. 4A-A′,C-C′). At the same time, injection of *SynNoggin1* mRNA resulted in a lower

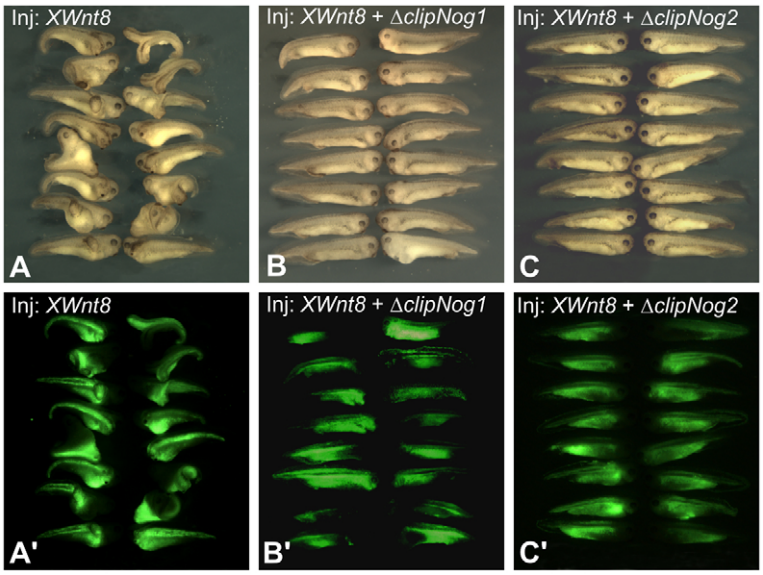

Fig. 5. Δclip-domain mutants of Noggin proteins suppress Wnt signaling. (**A**,**A′**) Ventral injections of *XWnt8* mRNA (3 pg/embryo) elicit development of the head-containing secondary axes due to ectopic induction of the Nieuwkoop center at the blastula stage. (**B-C′**) Co-injection of *ΔclipNoggin1* or *ΔclipNoggin2* mRNA (50 pg/embryo) with *XWnt8* resulted in suppression of secondary axes.

percentage of embryos with inhibited *XBra* expression (40%, *n*=70). Furthermore, a very weak ectopic expression of *goosecoid* was observed in the injected areas (Fig. 4B-B′,D-D′). The latter result can be explained by a lower ability of Noggin1 to antagonize Xnrs and, thus, Smad2 signaling. Consistent with results of binding experiments, Δclip mutants of both Noggin proteins also inhibited endogenous expression of *XBra* (supplementary material Fig. S4).

We demonstrated that injections of even 400 pg/blastomere of *Noggin1Δ5* mRNA, which resulted in a low concentration of Noggin1 protein, led exclusively to the formation of headless secondary axes, which indicates inhibition of BMP signaling only. To test whether higher concentrations of Noggin1 can induce secondary heads, which would be consistent with the ability of Noggin1 to antagonize Nodal and Wnt signaling, we injected 3-5 pg/blastomere of highly translated *SynNoggin1* mRNA into four- to eight-cell stage embryos. Indeed, formation of secondary axes with forebrains and cyclopic eyes were observed (Fig. 4E). However, the percentage of these axes was lower than that of axes induced by Noggin2 (15%, *n*=126 versus 35%, *n*=120, respectively). Moreover, all the head-containing axes induced by Noggin1 had somites. By contrast, no somites were revealed in axes induced by Noggin2. This difference can be explained by stronger inhibition of Nodal/Xnr signaling by Noggin2, which thereby resembles Cerberus, a protein that induces exclusively the head but not the trunk part of the body axis owing to its ability to inhibit Nodal and Wnt signaling.

When 2-5 pg/blastomere of highly translated mRNA encoding Noggin proteins or their Δclip mutants was injected into animal dorsal blastomeres, an enlargement of the forebrain accompanied by cyclopia was observed (Fig. 4F-L). Remarkably, Noggin2 and its Δclip mutant manifested themselves as more potent inducers of these effects. Obviously, such a difference cannot be explained by different influences of Noggin1 and Noggin2 on BMP and Wnt signaling, as both Noggin proteins inhibit these signaling cascades with approximately equal efficiency; moreover, ΔclipNoggin proteins cannot bind to BMP at all. More likely this difference was the result of stronger inhibition by Noggin2 of Nodal/Xnr signaling, which is known to be crucial for the splitting of the originally common eye field into two separate anlages (Schier et al., 1996).

To test the ability of Noggin to antagonize processes regulated by Wnt signaling, we determined whether their Δclip mutants could suppress, like Cerberus and Dkk (Glinka et al., 1997; Glinka et al., 1998), the formation of secondary body axes developing as a result of induction of an additional Nieuwkoop center by ectopic Wnt8 acting at the mid-blastula stage. Indeed, when we co-injected *XWnt8* mRNA with *ΔclipNoggin1* or *ΔclipNoggin2* mRNA, a sharp reduction in the number of embryos with a double axis, when compared with *XWnt8* mRNA-injected embryos, was observed (Fig. 5, compare A with B,C).Thus, this test confirms the ability of Noggin proteins to suppress Wnt signaling.

Noggin2 activity in the anterior neural plate is essential for the forebrain development

To test whether Noggin proteins are necessary for the forebrain development, we arranged loss-of-function experiments by injecting eight-cell embryos with specific antisense morpholino oligonucleotides (MO) (for MO effectiveness, see supplementary material Fig. S5A). No significant abnormalities were detected when *Noggin1* MO or the control *misNoggin2* MO (with seven mismatches) were injected. By contrast, tadpoles injected with a *Noggin2* MO had reduced telencephalons, eyes and nasal placodes (90%, *n*=116) (Fig. 6A,B). Consistently, these embryos had a reduced expression of telencephalic (*XBF1*) and eye (*Pax6*) markers (Fig. 6C-F).

To test further the specificity of *Noggin2 MO* effects, we co-injected them with either full-length *Noggin2* or *ΔclipNoggin2* mRNA, both of which lack the MO target site. When *Noggin2* MO was injected alone, an obvious reduction in the size of the head at stage 26 was observed (Fig. 6G,I). By contrast, co-injection of full-length *Noggin2* mRNA elicited statistically significant ($P<0.001$) rescue of this phenotype (Fig. 6H,I). Lower but still statistically significant ($P<0.001$) rescue effect was detected in case of *ΔclipNoggin2* co-injection (Fig. 6I; supplementary material Fig. S5B). By contrast, no rescue was observed if *Noggin2* MO was co-injected with the mixture of *BMP2*, *BMP4* and *BMP7* MOs (supplementary material Fig. S5C-G). This result indicates that Noggin2 in the anterior neural plate is essential for the inhibition of other pathways in addition to BMP signaling.

To determine the role of each specific type of Noggin2 inhibitory activity during normal forebrain development, we sought to rescue Noggin2 morphants by targeting the following inhibitors of Wnt, Activin/Nodal and BMP signaling to cells normally expressing Noggin2: Dkk1, and truncated type I receptors tALK4 and tBR (Chang et al., 1997; Glinka et al., 1998; Graff et al., 1994; Kondo et

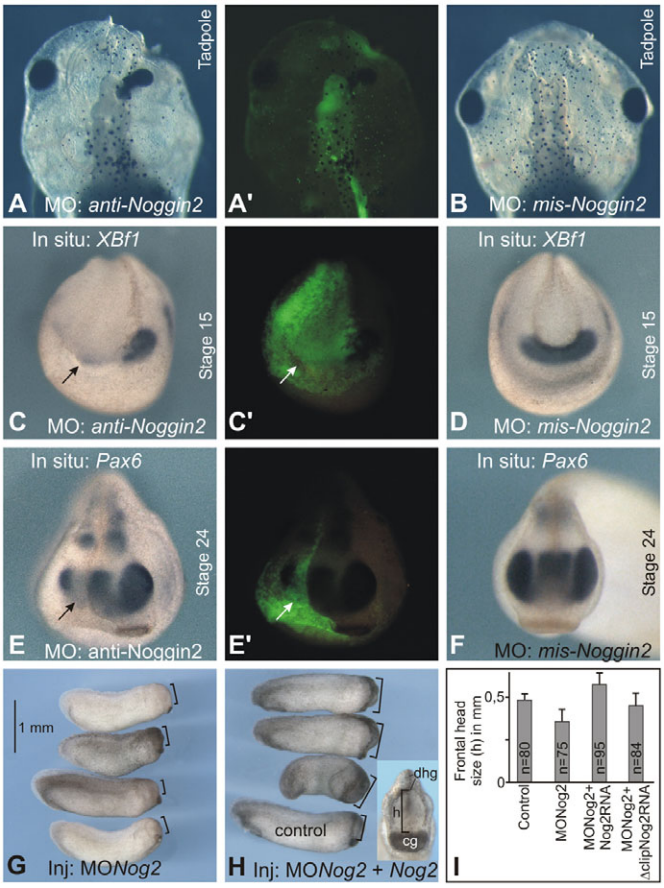

Fig. 6. The effects of inhibition of *Noggin2* mRNA translation by MO. (**A**,**A′**) Head of the tadpole injected with *Noggin2* MO into right side has severe malformations of the right eye, nasal placode and forebrain. Dorsal view, anterior side upwards. (**B**) Head of the control tadpole. (**C**,**C′**) The embryo injected with *Noggin2* MO into the right side has a reduced expression of the forebrain marker *XBF1* (arrows) in the injected cells at midneurula stage. Anterior view, dorsal side upwards. (**D**) Normal expression pattern of *XBF1* in embryo injected with *mis-Noggin2* MO. (**E**,**E′**) The embryo injected with *Noggin2* MO on the right side has a reduced expression of *Pax6* (arrows) in the injected cells at the tailbud stage. Anterior view, dorsal side upwards. (**F**) Normal expression pattern of *Pax6* in the embryo injected with the *misNoggin2* MO. (**G**,**H**) Embryos injected with *Noggin2* MO and rescued by co-injection of *Noggin2* mRNA. Brackets indicate the distance (h) from the beginning of the dorsal hatching gland (dhg) to the dorsal margin of the cement gland (cg) (see inset photo in H for details). Anterior view, dorsal towards the top. (**I**) Mean values of 'h' in embryos injected with the indicated MO and mRNAs. Error bars indicate s.d., *n*, number of embryos. Statistical significance of the difference between mean values was confirmed by independent two-sample Student's *t*-test for unequal sample sizes, unequal variance.

al., 1996). To achieve this, we co-injected embryos at the eight-cell stage in the animal corners of both dorsal blastomeres (presumptive forebrain) with *Noggin2* MO and different combinations of plasmids expressing aforementioned inhibitors under the control of a 4172 bp fragment of the *Noggin2* promoter. (Fig. 7A). Our preliminary study confirmed the validity of using this promoter to mimic the endogenous expression pattern of *Noggin2* (Fig. 7B). As a sensitive readout of the forebrain development, we measured in subsequent experiments the integrated density of in situ hybridization signal within the expression domain of the telencephalic marker *XBF1* at stage 26 (Fig. 7D-F).

When tALK4- or Dkk1-expressing plasmids were co-injected (4 ng/μl) with Noggin2 MO, statistically highly significant partial rescue effects ($P<0.001$) were observed in both cases (Fig. 7G; supplementary material Table S2). At the same time, no rescue was detected in case of tBR-expressing plasmid when co-injected with Noggin2 MO (Fig. 7G). Remarkably, higher amounts of injected plasmids (12 ng/μl) either yielded no further increase of rescue effect (Dkk1) or caused a decrease of *XBF1* expression domain in comparison with injections of MO alone (tALK4 and tBR) (Fig. 7G). By contrast, almost complete rescue effect was detected when plasmids expressing all three inhibitors were injected together (*$P<0.001$) (Fig. 7G).

The inhibition of Activin signaling at the anterior margin of the neural plate is essential for the forebrain development

A crucial role for the inhibition of BMP and Wnt signaling in the rostral part of neural anlage at post-gastrulation stages for the forebrain development has already been demonstrated (Kiecker and Niehrs, 2001; Lagutin et al., 2003; Onai et al., 2004). By contrast, the importance of inhibition of Activin/Nodal signaling, at these stages, as far as we know, has not been addressed previously. To our knowledge, the most probable regulator of Smad2 signaling expressed during neurulation near the anterior margin of the neural plate (Dohrmann et al., 1993) (and thus capable of being a target for Noggin2) is ActivinB.

To test this, we first of all compared the expression patterns of *ActivinB* and *Noggin2* in embryos split bilaterally before in situ hybridization. *ActivinB* is expressed during gastrulation at a very low level but starts to increase progressively in parallel with the expression of *Noggin2* at the beginning of neurulation. Importantly, these two genes are expressed in complementary patterns, a fact consistent with a possible role for Noggin2 in the inhibition of Activin signaling (Fig. 8A-F). Thus, expression of *Noggin2* occurs in cells of the internal layer of the anterior neural ridge, in a region corresponding to the presumptive telencephalon, which is marked by expression of *XBF1*, whereas *ActivinB* is expressed just posterior to *Noggin2* (Fig. 8C,D). Similar mutually exclusive expression of *Noggin2* and *ActivinB* is also observed in the brain rudiment after neurulation (Fig. 8E,E′).

To test whether a reduced level of *ActivinB* in the *Noggin2* expression territory is crucial for telencephalic development, we artificially expanded *ActivinB* expression rostrally in transgenic embryos bearing the double-cassette vector with *ActivinB* cDNA under the control of the homeobox gene *Xanf1* promoter and *Kate RFP* cDNA driven by cardiac actin promoter (Fig. 8G; see supplementary material Fig. S6A-C for normal expression patterns of *ActivinB*, *Noggin2* and *Xanf1*). As a result, a reduction of the forebrain, including eyes, was observed in all embryos bearing the double-cassette vector (70%, $n=29$ in two experiments) but not in the control, carrying a single-cassette *CardKate* vector (0%, $n=26$ in two experiments) (Fig. 8H-H″).

In addition, similar reduction of eyes, reflecting overall reduction of the forebrain, were revealed when 0.1 pg/blastomere of ActivinB mRNA was injected into a pair of animal dorsal blastomeres in 16- to 32-cell embryos (higher concentrations of *ActivinB* mRNA elicits blocking of normal development) (Fig. 8I,K). At the same time, partial rescue of eyes was observed when 3 pg/blastomere *SynNoggin2* or *SynΔclipNoggin2* was co-injected with *ActivinB* mRNA. A much lesser rescue effect was detected when *SynNoggin1*or *SynΔclipNoggin1* mRNA was co-injected (Fig. 8J,K; supplementary material Fig. S6D,F-I).

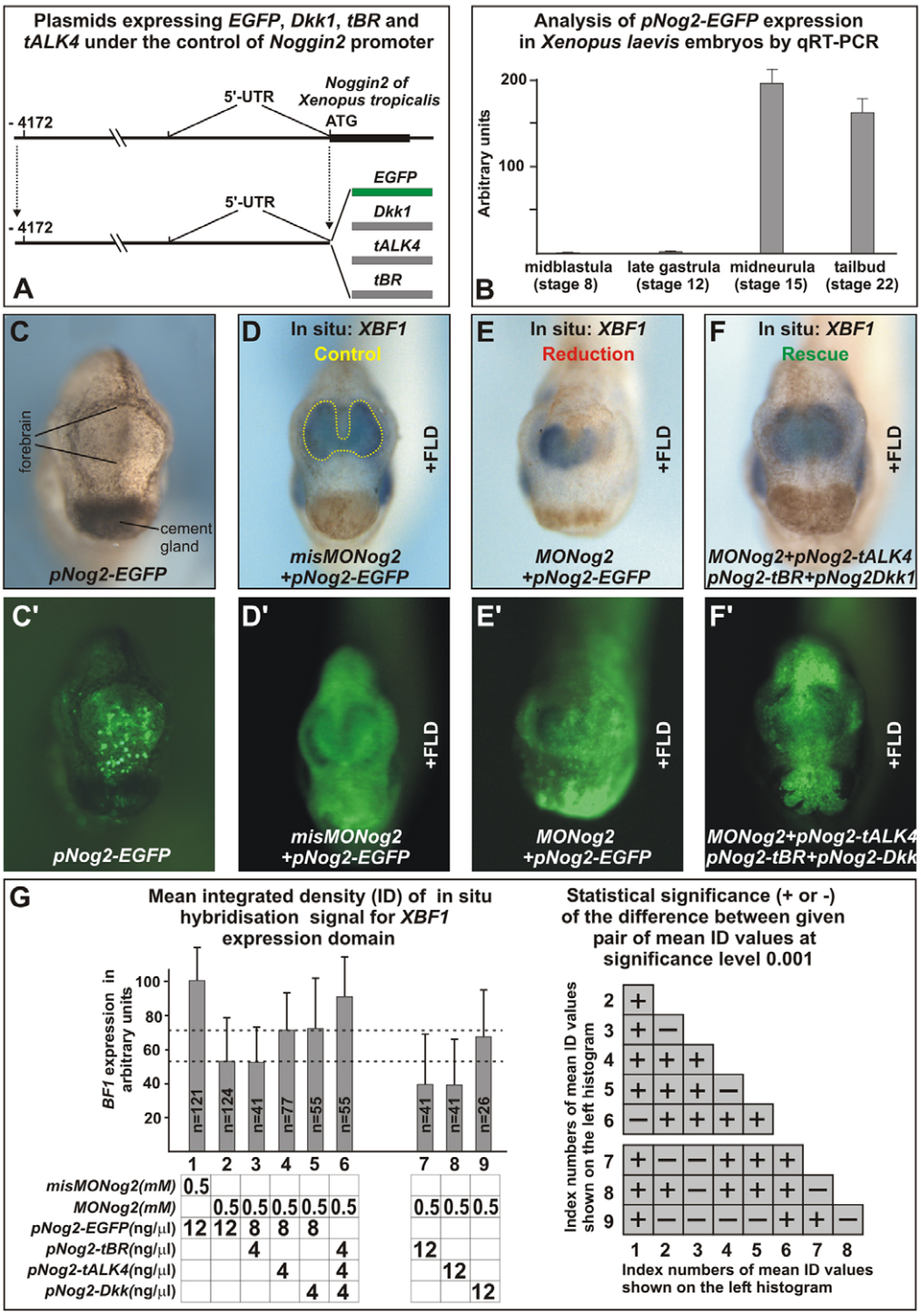

Fig. 7. Rescue of Noggin2 MO morphants by Wnt-, BMP- and Activin/Nodal-specific inhibitors. (**A**) Plasmids expressing *EGFP*, *Dkk1*, *tALK4* and *tBR* under the control of the *Xenopus tropicalis Noggin2* promoter. (**B**) Analysis of *pNog2-EGFP* expression in *Xenopus laevis* embryos by qRT-PCR. Each of the dorsal animal blastomeres of eight-cell embryos was injected in the animal corners with 2 nl of *pNog2-EGFP* (4 ng/µl). Embryos were collected at the indicated stages in batches of 10 in triplicate and qRT-PCR was performed on RNAase-free DNAase pre-treated extracted RNA using primers specific to *EGFP* and *ODC* as references. (**C**,**C′**) A typical stage 26 embryo expressing injected *pNog2-EGFP* in the forebrain. Anterior view, dorsal side upwards. (**D-F′**) Expression domains of *XBF1* (framed by yellow line on D) in forebrains of embryos injected by the indicated mixtures of MO, plasmids and FLD. The integrated density of *XBF1* in situ hybridization signal was measured using the ImageJ program. (**G**) Statistical analysis of integrated density of in situ hybridization signal within *XBF1* expression domain of stage 26 embryos injected with the indicated mixtures of MO and plasmids. The analysis was performed using independent two-sample Student's *t*-test for unequal sample sizes, unequal variance. Broken lines separate values that are most significantly different from one another. Errors bars represent s.d. See supplementary material Table S2 for original data.

DISCUSSION

Non-BMP TGFβ ligands and Wnts are targets of Noggin proteins

We demonstrate here that Noggin1 and Noggin2 can antagonize, besides BMP, several non-BMP TGFβ ligands and XWnt8. At the molecular level, the inhibition of Activin/Nodal and Wnt signaling is proven most evidently by abilities of Noggin proteins and their Δclip mutants deprived of BMP antagonizing activity to downregulate pathway-specific luciferase reporters and to suppress expression of the endogenous genetic markers induced by Activin/Xnr2 and XWnt8.

At functional level, Noggin proteins and their Δclip mutants also satisfy criteria necessary for Wnt and Nodal/Xnr inhibitors, including the ability to inhibit mesoderm specification, to induce the cyclopic phenotype and to suppress Wnt effects.

The abilities of Noggin proteins and their clip-domain deletion mutants to inhibit Activin/Nodal and Wnt signaling are consistent with their binding to corresponding ligands in co-immunoprecipitation assay. Despite the immunoprecipitation signals observed with these ligands being much weaker than in case of Noggin proteins precipitated with BMP4, the interactions were seemingly specific for two reasons. First, all ligands taken for

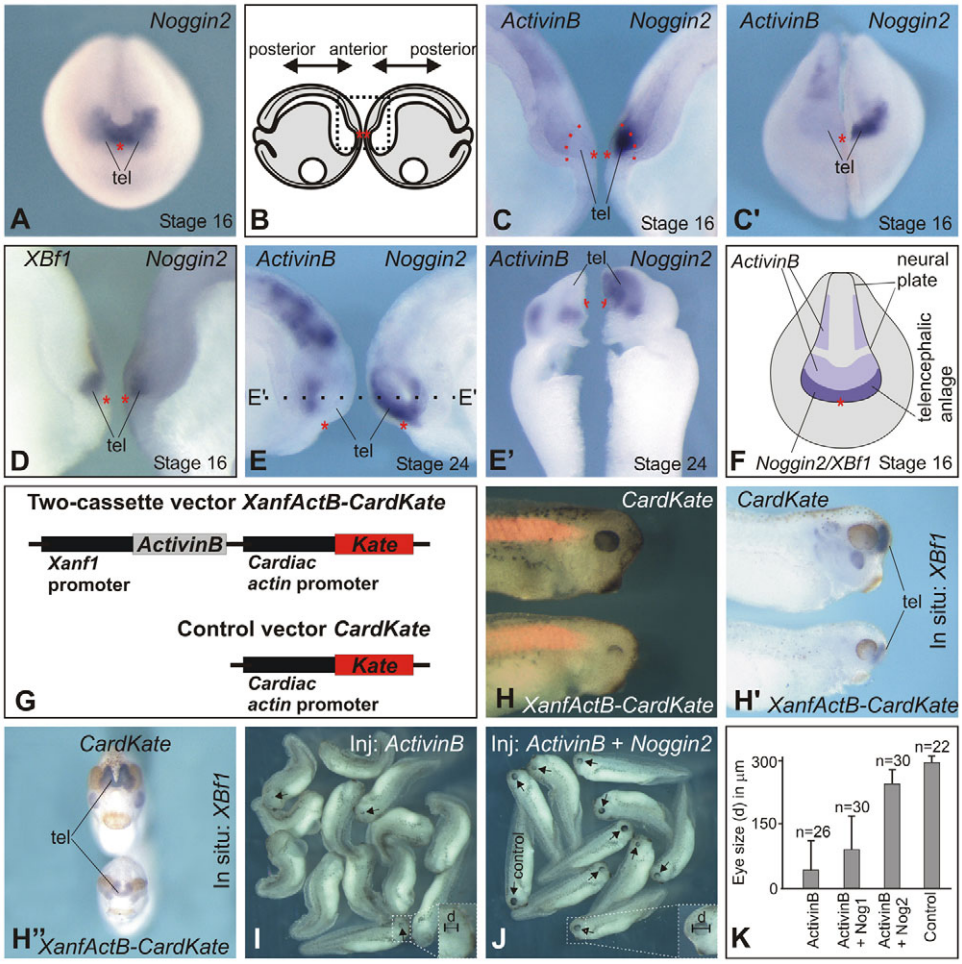

Fig. 8. The inhibition of Activin signaling at the anterior margin of the neural plate is essential for the forebrain development. (A-F) Expression patterns of *ActivinB* and *Noggin2* in the forebrain rudiment revealed by the whole-mount in situ hybridization. (**A**) At the midneurula stage, *Noggin2* is specifically expressed in cells of the anterior neural fold. Anterior view of the whole embryo, dorsal side upwards. Red asterisk marks the median of anterior neural fold. (**B**) The area of embryonic halves (outlined) shown in C-E. (**C**) Expression of *ActivinB* and *Noggin2* on halves of the same midneurula embryo. *ActivinB* is not expressed in the triangular region of the neurectoderm, between the broken red line and asterisk, in which *Noggin2* is expressed. (**C′**) The same hemi-section as in C, but combined together to form the whole embryo and shown from the anterior side. (**D**) Expression of the telencephalic marker *XBF1* and *Noggin2* on halves of the same midneurula embryo. *XBF1* and *Noggin2* are expressed in the same cells at the anterior margin of neural plate. (**E**) Expression of *ActivinB* and *Noggin2* on bilateral halves of the same tailbud embryo. *ActivinB* is not expressed in the telencephalon rudiment (tel) in which *Noggin2* is expressed. (**E′**) Expression of *ActivinB* and *Noggin2* on halves shown in E but further sectioned along the broken line indicated in E; dorsal view. (**F**) The expression patterns of *ActivinB*, *XBF1* and *Noggin2* in the midneurula embryo; anterior view, dorsal side upwards. (G-K) Inhibition of ActivinB by Noggin2 is necessary for normal development of the forebrain. (**G**) The double cassette and control vectors used for targeting of *ActivinB* expression to the forebrain rudiment under the control of *Xanf1* promoter. (**H-H″**) In contrast to the transgenic embryo bearing control vector *CardKate* (upper row), the embryo containing double-cassette vector *XanfActB-CardKate* (bottom row) has reduced eyes and telencephalon (marked by *XBF1* expression). H and H′, side views; H″, anterior view of the same pair of embryos. (**I**,**J**) Examples of embryos injected into the presumptive head region by *ActivinB* mRNA (0.5 pg/embryo) have smaller eyes (arrows) reflecting the smaller forebrains. Co-injection with *ActivinB* mRNA of *Noggin2* mRNA (3 pg/embryo) resulted in partial rescuing of normal phenotype. See control embryos in supplementary material Fig. S6E. (**K**) Average eye sizes (d) of embryos injected with indicated mRNAs. Statistical significance of the difference between mean values was confirmed by Student's *t*-test. Error bars show s.d.

immunoprecipitation were produced separately from Noggin proteins in different batches of embryos, and thus could not form any artifact clusters with Noggin proteins before immunoprecipitation. Second, no immunoprecipitation was detected in similarly arranged control experiments for BMP4 and delta-clip mutants of Noggin proteins or for some non-BMP ligands and for the cysteine-rich protein Zyxin.

Given that the removal of N-terminal clip-domain of Noggin1 and Noggin2 did not suppress binding of Noggin proteins to non-BMP ligands, these interactions probably occur by a mechanism other than Noggin binding to BMP. Moreover, one may speculate that, in contrast to Noggin/BMP binding, this mechanism could be based on a principle other than a simple competition of Noggin proteins with corresponding receptors for ligand binding. First, in the case of a competitive mechanism, the efficiency of binding to a ligand should be high enough to compete with the receptor, which is seemingly not the case for Noggin proteins binding to non-BMP ligands. Second, with the competitive mechanism, the efficiency of inhibition should be directly related to the efficiency of binding. However, although no difference was observed between affinities

of Noggin1 and Noggin2 for ActivinB and Xnr proteins, a significant difference was revealed between their capacities to inhibit signaling activities of these ligands – with Noggin2 demonstrating higher inhibition. Although a competitive mechanism could not explain this result, it could be explained by a non-competitive mechanism that allows no direct link between binding and inhibition.

Of note, in comparison with the inhibition of Activin/Nodal and Wnt signaling by Follistatin and Cerberus, the inhibitory effects of Noggin proteins on non-BMP TGFβ and Wnt signaling are less effective and could not be detected in experiments with wild-type Noggin1 mRNA, which contains a short 5′UTR motif that strongly inhibits translation. This structural peculiarity of wild-type *Noggin1* mRNA, which allowed the detection only of the highly effective anti-BMP activity of Noggin1, could explain why its ability to antagonize non-BMP TGFβ and Wnt ligands has not been previously reported.

Inhibition of Activin and Wnt signaling by Noggin2 is necessary for the rostral forebrain development

We demonstrate here for the first time that inhibition of three signaling pathways by Noggin2, Activin/Nodal, BMP and Wnt in cells of the anterior margin of the neural plate is essential for the normal forebrain development.

The importance of inhibition of Wnt and BMP signaling in the rostral part of neural anlage at post-gastrulation stages, i.e. only at the place and time where and when *Noggin2* is expressed, has been previously demonstrated by gain- and loss-of-function experiments (Kiecker and Niehrs, 2001; Lagutin et al., 2003; Onai et al., 2004). Now, we demonstrate that the truncated BMP receptor alone is unable to rescue the effects of Noggin2 MO. This indicates that inhibition of BMP signaling in the anterior margin of the neural plate during neurulation is not sufficient for the forebrain specification without simultaneous inhibition of Activin and Wnt.

Although it is essential for mesoderm induction at pre-gastrula stages, Activin/Nodal signaling must be inhibited, together with BMP and Wnt signaling, in the anterior region of embryo during gastrulation to allow development of the head structures, including the telencephalon and eyes (Niehrs, 1999; Piccolo et al., 1999). Our present finding demonstrates that protection of the presumptive rostral forebrain from ActivinB, a protein that is found adjacent to the anterior margin of the neural plate, is crucial beyond the end of gastrulation; Noggin2 is the agent responsible for this protection.

As there is no Noggin2 in mammals, the question arises of what mammalian protein(s) could replace physiological functions of Noggin2. One could suppose that this might be Noggin1, the only Noggin protein present in mammals. However, no forebrain abnormalities similar to those observed in the present work have been reported in *Noggin1* knockout mice (McMahon et al., 1998). Therefore, the function of Noggin2 in mammals could be executed by some other inhibitors of Activin/Nodal and Wnt signaling. Alternatively, some deep changes in the mechanism of forebrain development could have taken place in mammalian ancestor that allowed the omission of inhibition of these signals after gastrulation. Further study will be necessary to distinguish between these alternatives.

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Competing interests statement

The authors declare no competing financial interests.

Supplementary material

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