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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).

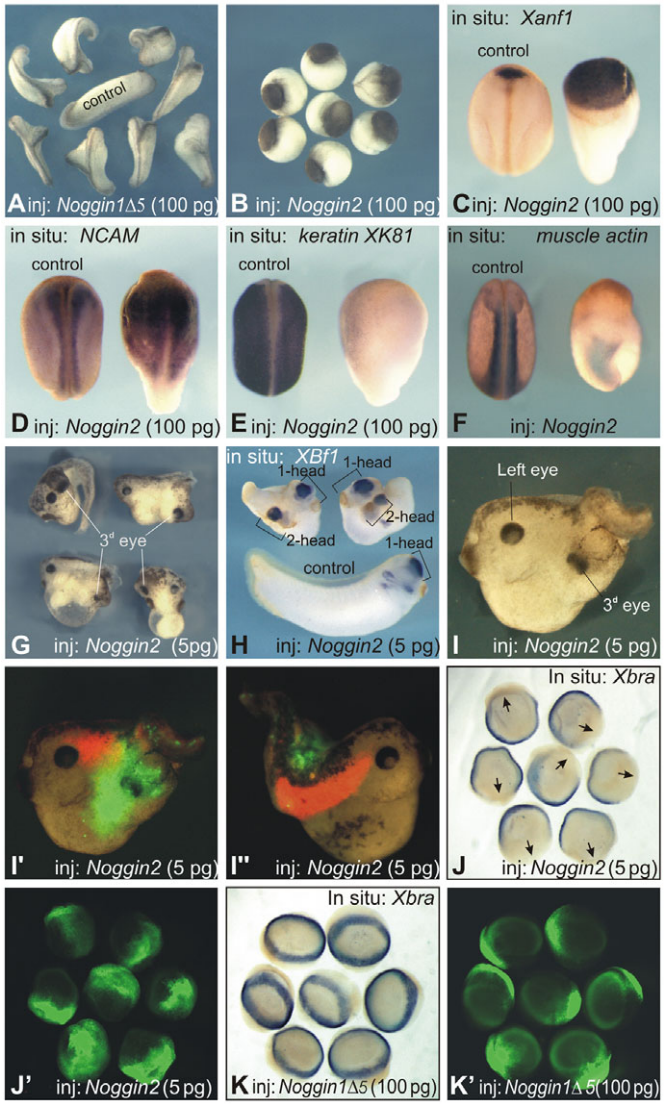

Fig. 1. Wild-type *Noggin2* mRNA elicits effects distinct from those induced by wild-type *Noggin1* mRNA. (**A**,**B**) Ventral injections of *Noggin1Δ5* mRNA induce secondary axes (A), whereas similar injections of *Noggin2* mRNA resulted in mushroom-shaped embryos (B). (**C-F**) Whole-mount in situ 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

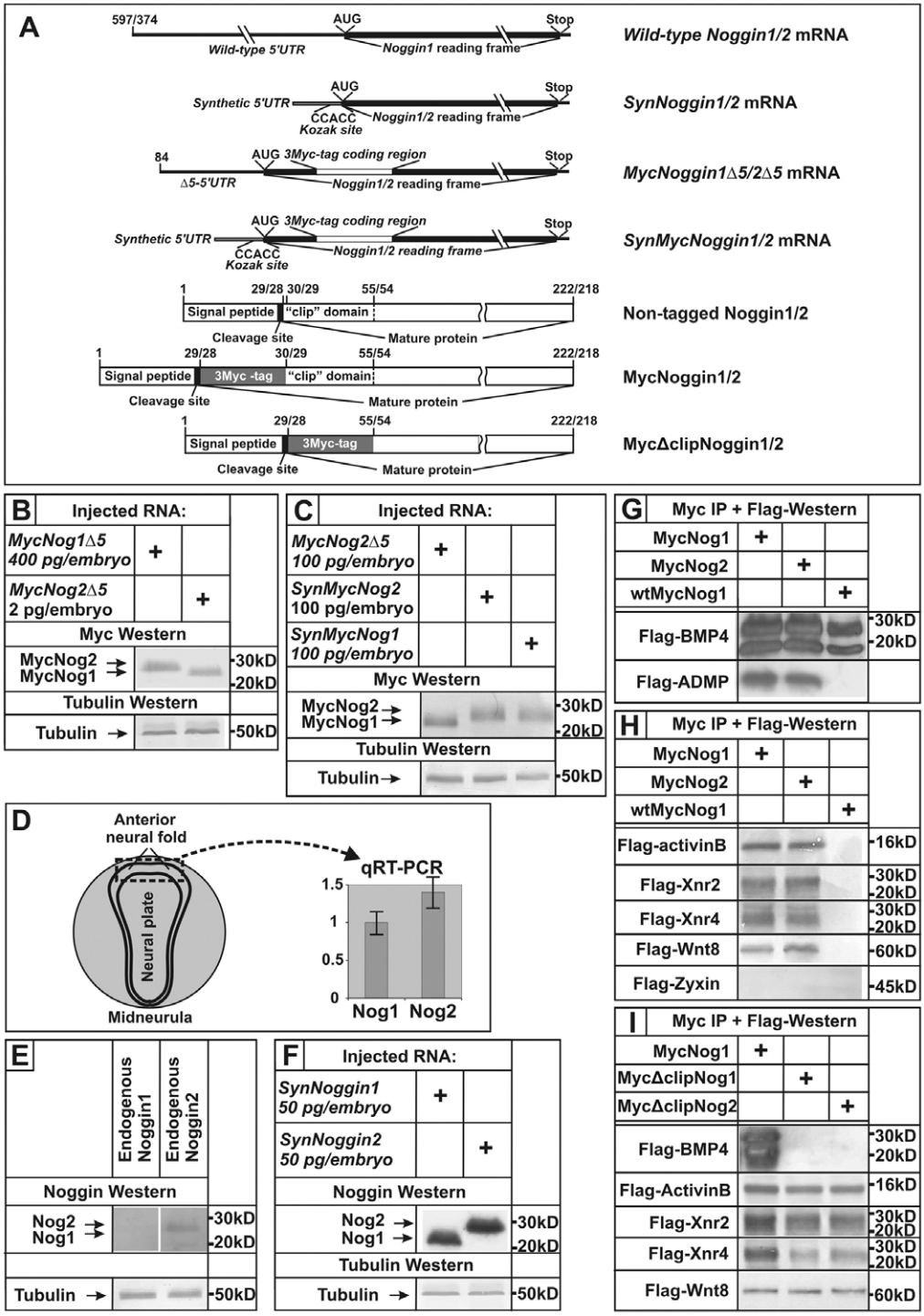

Fig. 2. Noggin1 and Noggin2 can bind TGFβ and Wnt ligands. (**A**) Selected mRNA and proteins used in the present study. (**B**,**C**) Comparison of translation capacities of *MycNoggin1Δ5* and *MycNoggin2Δ5* mRNA (B) or *MycNoggin2Δ5*, *SynMycNoggin1* and *SynMycNoggin2* mRNA (C) injected in two-cell 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.

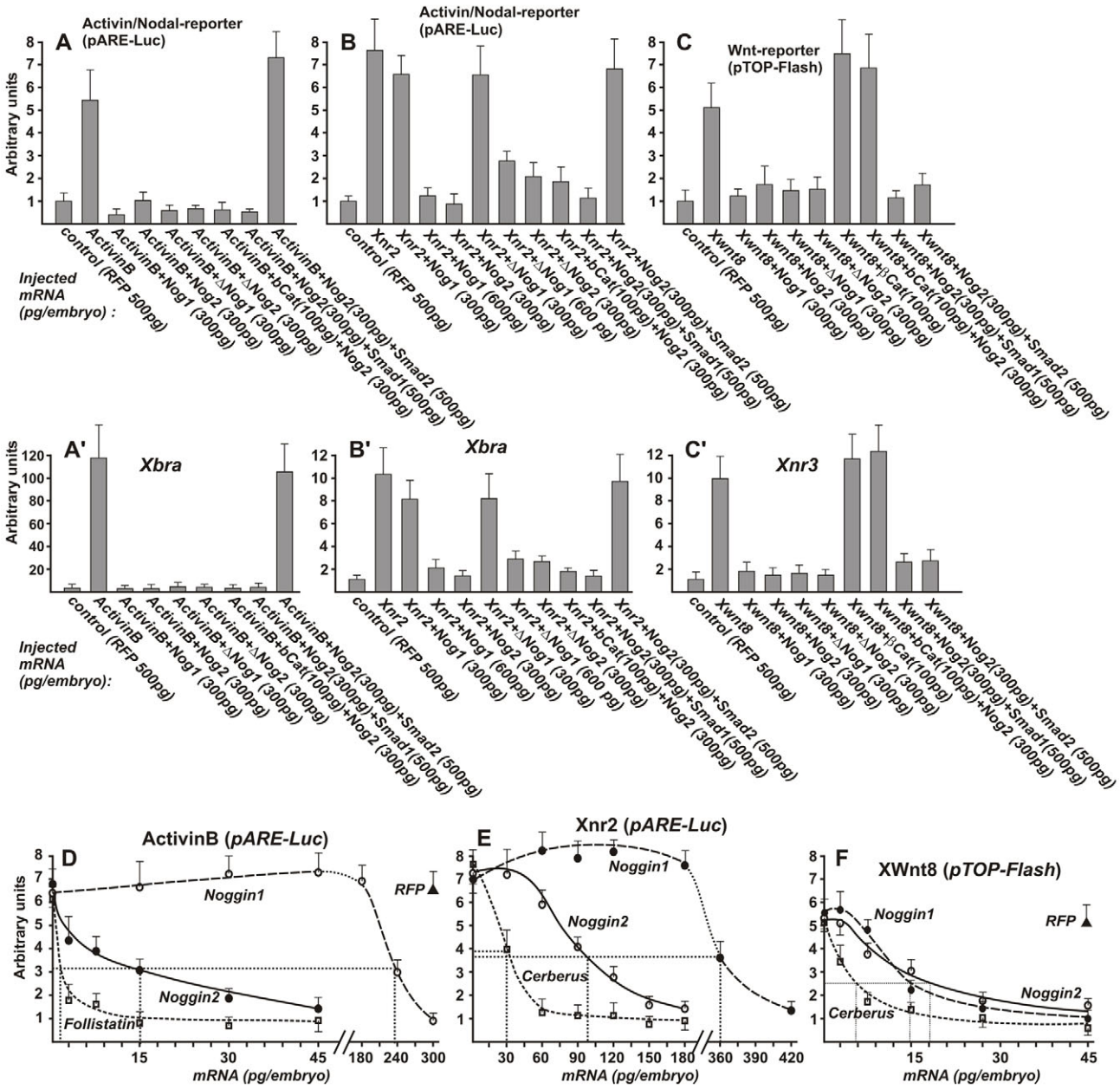

Fig. 3. Noggin proteins inhibit ActivinB, Xnr2 and XWnt8 signaling. (**A-C**) Influence of Noggin proteins translated from *SynNoggin* mRNAs injected into embryos at concentrations indicated below each graph on transcription of luciferase reporters activated by co-injection of *ActivinB* (0.8 pg/embryo), *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

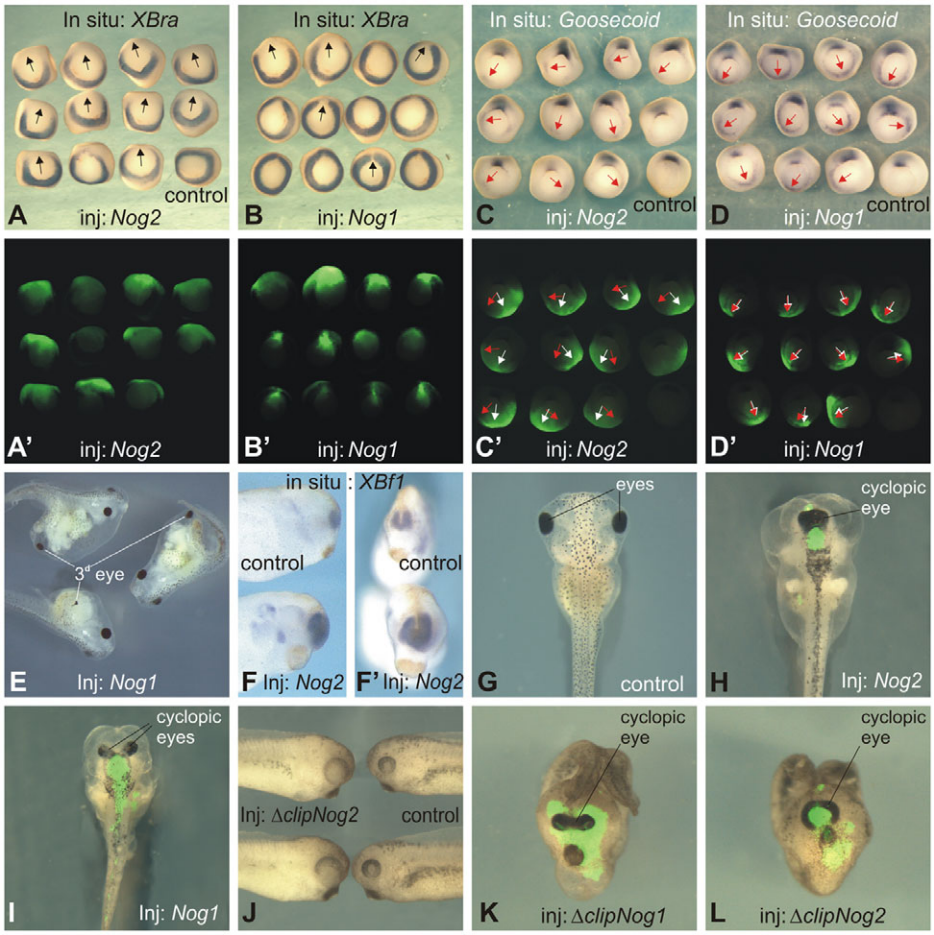

Fig. 4. Noggin proteins translated from mRNA with consensus Kozak (*SynNoggins*) site can influence developmental processes regulated by Nodal/Xnrs and Wnt. (**A**,**A′**) Injection of *Noggin2* mRNA (10 pg/embryo) elicits inhibition of *Xbra* expression in 100% of embryos (black arrows). (**B,B′**) 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

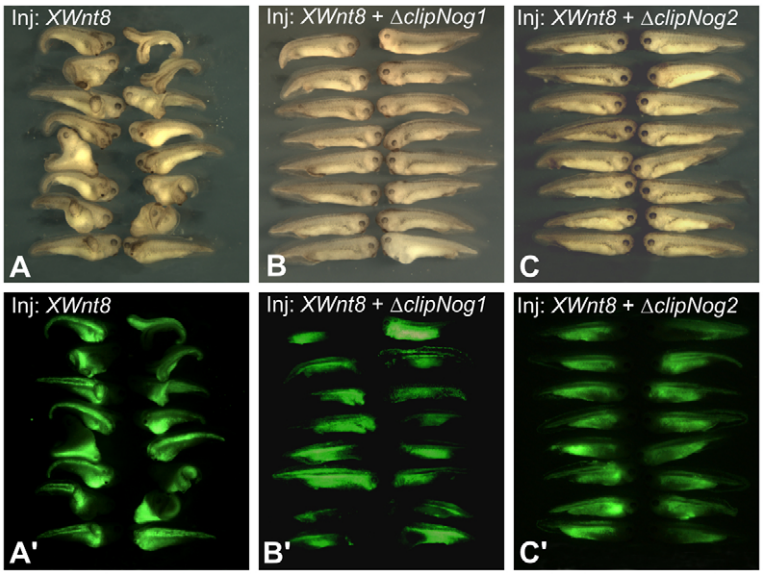

Fig. 5. Δclip-domain mutants of Noggin proteins suppress Wnt signaling. (**A**,**A′**) Ventral injections of *XWnt8* mRNA (3 pg/embryo) elicit development of the head-containing secondary axes due to ectopic induction of the Nieuwkoop center at the blastula stage. (**B-C′**) Co-injection of *ΔclipNoggin1*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

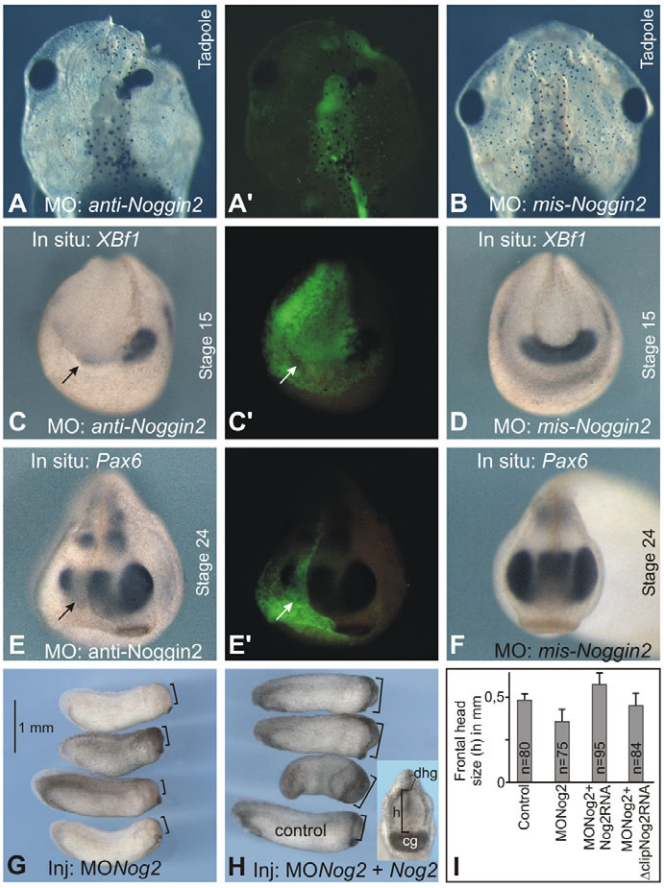

Fig. 6. The effects of inhibition of *Noggin2* mRNA translation by MO. (**A**,**A′**) Head of the tadpole injected with *Noggin2* MO into right side has severe malformations of the right eye, nasal placode and forebrain. Dorsal view, anterior side upwards. (**B**) Head of the control tadpole. (**C**,**C′**) 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).

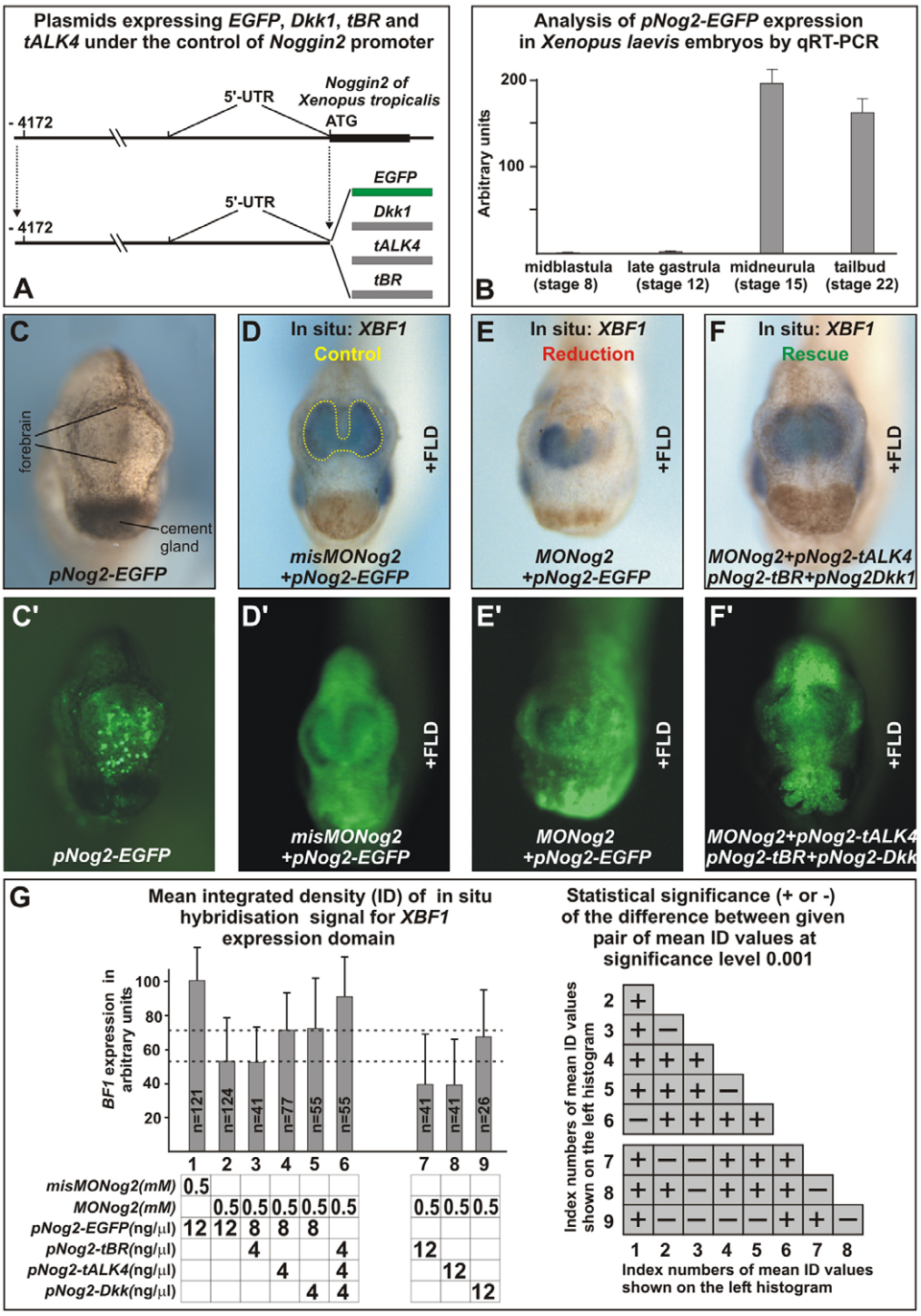

Fig. 7. Rescue of Noggin2 MO morphants by Wnt-, BMP- and Activin/Nodal-specific inhibitors. (**A**) Plasmids expressing *EGFP*, *Dkk1*, *tALK4* and *tBR* under the control of the *Xenopus tropicalis Noggin2* promoter. (**B**) Analysis of *pNog2-EGFP* expression in *Xenopus laevis* embryos by qRT-PCR. 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

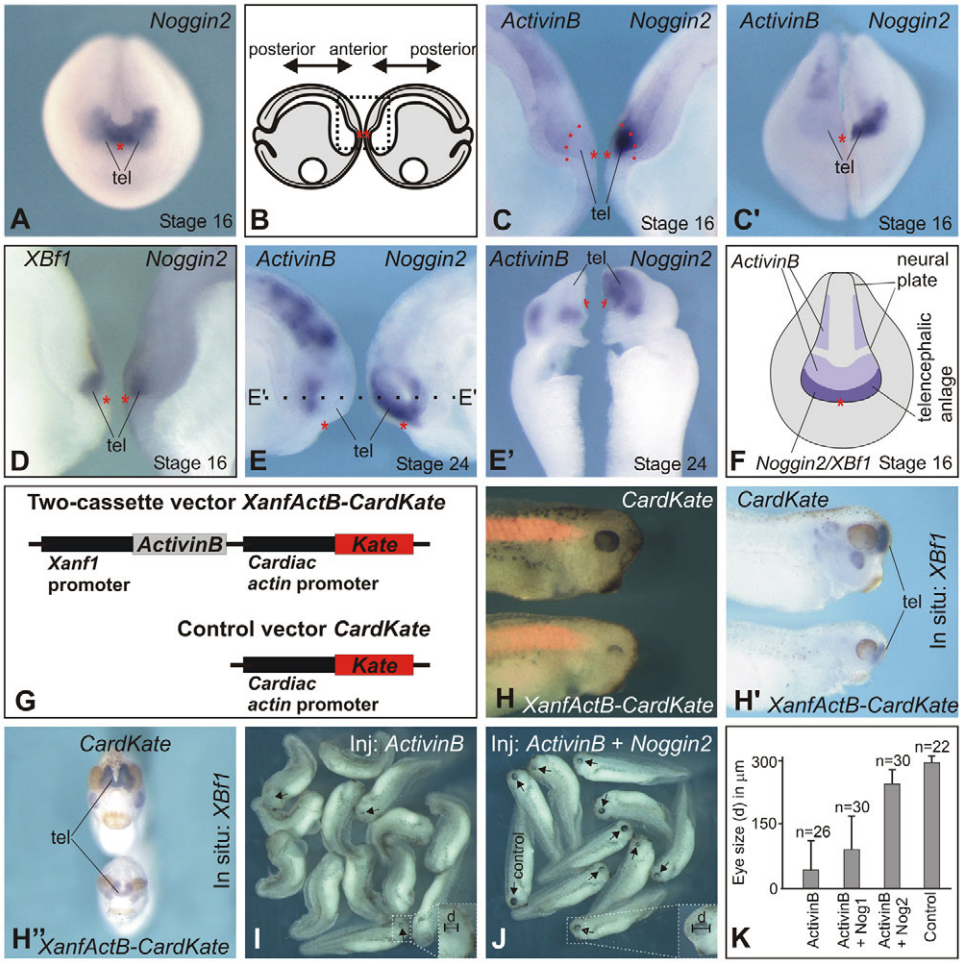

Fig. 8. The inhibition of Activin signaling at the anterior margin of the neural plate is essential for the forebrain development. (A-F) Expression patterns of *ActivinB* and *Noggin2* in the forebrain rudiment revealed by the whole-mount in situ hybridization. (**A**) At the midneurula stage, *Noggin2* 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

Supplementary material available online at
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Table S1. Cloning strategies of constructs

DNA constructs	PCR primers and cloning strategy used to prepare expression plasmids
pNog2-EGFP	1st step. Obtaining of 4172 bp DNA fragment including 5'UTR and 5'non-coding region of *Noggin2* gene by two rounds of nested PCR from *Xenopus tropicalis* genomic DNA with the following two pairs of primers: 5'-GGTTAATAAGGGCTTGCTGAAC and 5'- CTCAGGCAGATTTATCCTCTTC (26 cycles); 5'-TAACGTCGACATAGCTGCCGATCAGTAGGTC and 5'- ATCCACCGGTGATGTTCAACCCCCTTCAATG (15 cycles). 2nd step. Cloning into SalI/AgeI sites of pEGFP plasmid (Clontech). Checking by sequencing.
pNog2-tALK4	1st step. Obtaining of *tALK4* cDNA fragment from gastrula first-strand total cDNA with primers: 5'-ATATACCGGTGCCACCATGGCGGAGCTACCGGCCTT and 5'- AATGCGGCCGCTCAGATAGTTCTCGCCACAGT. 2nd step. Cloning instead of *EGFP* into AgeI/NotI sites of *pNog2-EGFP*. Checking by sequencing.
pNog2-tBR	1st step. Obtaining of *tBR* cDNA fragment from *ptBR* with primers: 5'-AATAACCGGTGCCACCATGAGAGAACGACTTTTCATTG and 5'- AATGCGGCCGCTTATTTGTAAATCCATATGATAAGA . 2nd step. Cloning instead of *EGFP* into AgeI/NotI sites of *pNog2-EGFP*. Checking by sequencing.
pNog2-Dkk	1st step. Obtaining of *Dkk1* cDNA from gastrula first-strand total cDNA with primers: 5'- AATAACCGGTGCCACCATGGGCAGCAACATGTT and 5'- AATGCGGCCGCTTAGTGTCTTTGGCAAGTGTGA. 2nd step. Cloning instead of *EGFP* into AgeI/NotI sites of *pNog2-EGFP*. Checking by sequencing.
pXanfActB-CardKate	To construct double-cassette vector *pXanfActB-CardKate*, *Otx2* cDNA in *pXanf1-Otx2-CardKate* (Ermakova et al., 2007) was swapped by AgeI and NotI with *ActivinβB* cDNA (see below)
Synthetic mRNA	PCR primers and cloning strategy used to prepare DNA templates for generation of synthetic mRNA
Noggin1Δ5	1st step. PCR from *pNogginΔ5* with forward primer 'Ng1 Δ5': 5'- ATAACCGGTGAATTCCTCCTCTGATGCAT and reverse primer 'Ng1 stop': 5'- ATTCTCGAGTCTCAGCATGAGCATTTGCA. Here and below restriction sites are underlined, start and stop codons are framed. 2nd step. Cloning by AgeI (blunted) and XhoI into BamHI (blunted) and XhoI of pCS2 plasmid; checking by sequencing. Final construct: *pCS2-Noggin1Δ5*.
Noggin2Δ5	1st step. PCR from *pBluescriptNoggin2* with forward primer 'Ng2 Δ5': 5'- ATAACCGGTAATCTAACGATCTGTAACTATTG and reverse primer 'Ng2 stop': 5'- ATTCTCGAGTTAGCATGAACACTTACACTCTG. 2nd step. Cloning by AgeI (blunted) and XhoI into BamHI (blunted) and XhoI of pCS2 plasmid; checking by sequencing. Final construct: *pCS2-Noggin2Δ5.*
SynNoggin1	1st step. PCR from *pNogginΔ5* with forward primer 'Ng1 synt 5'(Kozak site is in italics): 5'-AATTACCGGT*CGCCACC*ATGGATCATTCCCAGTGCC and 'Ng1 stop'. 2nd step. Cloning by AgeI (blunted) and XhoI into BamHI (blunted) and XhoI of pCS2 plasmid; checking by sequencing. Final construct: *pCS2-SynNoggin1*.
SynNoggin2	1st step. PCR from *pBluescrptNoggin2* with forward primer 'Ng2 synt 5'(Kozak site is in italics): 5'-AATTACCGGT*CGCCACC*ATGAAGAGGATAAATCTGC and 'Ng2 stop'. 2nd step. Cloning by AgeI (blunted) and XhoI into BamHI (blunted) and XhoI of pCS2 plasmid; checking by sequencing. Final construct: *pCS2-SynNoggin2*.
ΔclipNoggin1	1st step. Obtaining of 5' fragment of *ΔclipNoggin1* cDNA. PCR from pNogginΔ5 with 'Ng1 synt 5' and 5'-CTTCTCCTTGGGATAATGTTGGCAACCCCCTT. 2nd step. Obtaining of 3' fragment of Δclip *Noggin1* cDNA. PCR from pNogginΔ5 with 5'-TTGCCAACATTATCCCAAGGAGAAGGATCTTA and 'Ng1 stop'. 3d step. Two overlapping cDNA fragments obtained at the previous two steps were purified from non-incorporated primers, mixed, denatured, annealed and subjected to PCR with 'Ng1 synt 5' and 'Ng1 stop' primers. 4th step. Cloning by AgeI (blunted) and XhoI into BamHI (blunted) and XhoI of pCS2 plasmid; checking by sequencing. Final construct: *pCS2-ΔclipNoggin1*.
ΔclipNoggin2	1st step. Obtaining of 5' fragment of *ΔclipNoggin2* cDNA. PCR from *pBluescrptNoggin2* with 'Ng2 synt 5' and –CTGCTCCTTTGGATAAGGCTGACAGCACCCCT. 2nd step. Obtaining of 3' fragment of *ΔclipNoggin2* cDNA. PCR from *pBluescriptNoggin2* with 5'-CTGTCAGCCTTATCCAAAGGAGCAGGATCTGG and 'Ng2 stop'. 3d step. Two overlapping cDNA fragments obtained at the previous two steps were purified from non-incorporated primers, mixed, denatured, annealed and subjected to PCR with 'Ng2 synt 5' and 'Ng2 stop' primers. 4th step. Cloning by AgeI (blunted) and XhoI into BamHI (blunted) and XhoI of pCS2 plasmid; checking by sequencing. Final construct: *pCS2-ΔclipNoggin2*.
misMONoggin2	1st step. PCR from *pBluescriptNoggin2* with 'Ng2 stop' and the mixture (ratio of 1:10 pM, respectively) of 'Ng1 synt 5' and Noggin1/Noggin2 adaptor primer 5'-GATCATTCCCAGTGCCTTGTGA*CTTTTTTGCTTTGCTTGTG* (*Noggin1* sequence is underlined, *Noggin2* sequence is in italic). 2nd step. Cloning by AgeI (blunted) and XhoI into BamHI (blunted) and XhoI of pCS2 plasmid; checking by sequencing. Final construct: *pCS2-misMONoggin2.*
misMOΔclipNoggin2	The same PCR strategy was utilized as was used to generate *pCS2-misMONoggin2* except *pCS2-ΔclipNoggin2* was taken as a template for PCR. Final construct: *pCS2-misMOΔclip Noggin2.*
MycNoggin1Δ5	1st step. Obtaining of 5' fragment of *MycNoggin1Δ5* cDNA. PCR from *pNogginΔ5* with 'Ng1 Δ5' and a mixture of two reverse primers taken in ratio of 1:10 pM respectively: 5'-CAGATCCTCTTCAGAGATGAGTTTCTGCTCATAATGTTGGCAACCCCCTTG ('Ng1 Myc rev') and 5'-GAGGTCTTCCTCCGATATCAGCTTCTGTTCCAGATCCTCTTCAGAGATG ('Myc rev'). here and below Myc-tag coding sequences are underlined by dotted line. 2nd step. Obtaining of 3' fragment of *MycNoggin1Δ5* cDNA. PCR from *pNogginΔ5* with 'Ng1 stop' and a mixture of two forward primers taken in ratio of 1:10 pM respectively (Myc-tag coding sequences are underlined): 5'-GAGCAGAAACTCATCTCTGAAGAGG ATCTGCTGCACATCAGACCGG CT ('Ng1 Myc forw') and

	5′-GAACAGAAGCTGATATCGGAGGAAGACCTCGAGCAGAAACTCATCTCTG (‘Myc forw’). 3[d] step. Obtaining of cDNA encoding full *Noggin∆5* with three copies of Myc-tag epitop behind signal peptide cleavage site (see Fig. 2A). Two overlapping cDNA fragments obtained at the previous two steps were purified from non-incorporated primers, mixed, denatured, annealed and subjected to PCR with ‘Ng1 ∆5’ and ‘Ng1 stop’ primers. 4[th] step. Cloning by AgeI (blunted) and XhoI into BamHI (blunted) and XhoI of pCS2 plasmid; checking by sequencing. Final construct: *pCS2-MycNoggin1∆5*.
MycNoggin2∆5	1[st] step. Obtaining of 5′ fragment of *MycNoggin2∆5* cDNA. PCR from *pBluescriptNoggin2* with ‘Ng2 ∆5’ and a mixture of two reverse primers taken in ratio of 1:10 pM respectively: 5′-CAGATCCTCTTCAGAGATGAGTTTCTGCTCATAAGGCTGACAGCACCCCTGA (‘Ng2 Myc rev’) and ‘Myc rev’. 2[nd] step. Obtaining of 3′ fragment of *MycNoggin2∆5* cDNA. PCR with ‘Ng1 stop’ and a mix of two forward primers taken in ratio of 1:10 pM respectively: 5′-AGCAGAAACTCATCTCTGAAGAGGATCTGCTCAGGCTTAGACCCTCT (‘Ng2 Myc forw’) and ‘Myc forw’. 3[d] step. Obtaining of cDNA encoding full *Noggin2∆5* with three copies of Myc-tag epitop behind signal peptide cleavage site (see Fig. 2A). Two overlapping cDNA fragments obtained at the previous two steps were purified from non-incorporated primers, mixed, denatured, annealed and subjected to PCR with ‘Ng2 ∆5’ and ‘Ng2 stop’ primers. 4[th] step. Cloning by AgeI (blunted) and XhoI into BamHI (blunted) and XhoI of pCS2 plasmid; checking by sequencing. Final construct: *pCS2-MycNoggin2∆5*.
wtMycNoggin1	The same PCR-based strategy was utilized as was used for preparing *MycNoggin1∆5* cDNA (see above), except *pNoggin A3* plasmid and different forward primer was used for obtaining of 5′ fragment of *wtMycNoggin1* cDNA: (Ng1 wt5′) 5′- ATAACCGGTTAATAAATCCTAAGTAGCCAGA. Final construct: *pCS2-wtMyc Noggin1*.
wtMycNoggin2	The same PCR-based strategy was utilized as was used for preparing *MycNoggin1∆5* cDNA (see above), except *pBluescript-wtNoggin2* and different forward primer was used for obtaining of 5′ fragment of *wtMycNoggin2* cDNA: (Ng2 wt5′) 5′- ATAACCGGTTGATTCTGCCTTACTTACTGACACA. Final construct: *pCS2-wtMyc Noggin2*.
SynMycNoggin1	1[st] step. PCR from *pCS2-MycNoggin1∆5* with primers ‘Ng1 synt 5′ and ‘Ng1 stop’. 2[nd] step. Cloning by AgeI (blunted) and XhoI into BamHI (blunted) and XhoI of pCS2 plasmid; checking by sequencing. Final construct: *pCS2- SynMycNoggin1*.
SynMycNoggin2	1[st] step. PCR from *pCS2-Myc Noggin2∆5* with primers ‘Ng2 synt 5′ and ‘Ng2 stop’. 2[nd] step. Cloning by AgeI (blunted) and XhoI into BamHI (blunted) and XhoI of pCS2 plasmid; checking by sequencing. Final construct: *pCS2- SynMycNoggin2*.
Myc∆clipNoggin 1	The same PCR strategy was utilized as was used to generate *MycNoggin1∆5*, except ‘Ng1 synt 5′ primer was taken instead of ‘Ng1 ∆5’ and 5′-GAGCAGAAACTCATCTCTGAAGAGGATCTGCCCAAGGAGAAGGATCTTA (‘∆-clip-Ng1 forw’) was taken instead of ‘Ng1 Myc forw’. Final construct: *pCS2-Myc∆clip Noggin1*.
Myc∆clipNoggin 2	The same PCR strategy was utilized as was used to generate *MycNoggin2∆5*, except ‘Ng2 synt 5′ primer was taken instead of ‘Ng2 ∆5’ and 5′-GAGCAGAAACTCATCTCTGAAGAGGATCTGCCCAAGGAGAAGGATCTTA (‘∆-clip-Ng1 forw’) instead of ‘Ng2 Myc forw’. Final construct: *pCS2-Myc∆clip Noggin2*.
BMP4	1[st] step. Obtaining of cDNA encoding full BMP4. PCR from *Xenopus laevis* gastrula first strand cDNA with primers full BMP4 forward: 5′-AATTGGATCCGCCACCATGATTCCTGGTAACCGAA and stop BMP4 reverse: 5′-AATCTCGAGTCAACGGCACCCACACCCTTCCA. 2[nd] step. The obtained cDNA fragments was cloned into pCS2 plasmid either by BamHI and XhoI and correct clone was selected by sequencing. Final construct: pCS2-BMP4.
Xnr2	1[st] step. Obtaining of cDNA encoding full Xnr2. PCR from *Xenopus laevis* gastrula first strand cDNA with primers full Xnr2 forward: 5′-AATTGAATTCGCCACCATGGCAAGCCTAGGAGTCATC and stop Xnr2 reverse: 5′-AATCTCGAGTCAGTTACATCCACACTCATCCA. 2[nd] step. Cloning of the obtained cDNA fragments was cloned into pCS2 plasmid either by EcoRI and XhoI and correct clone was selected by sequencing. Final construct: pCS2-Xnr2.
Xnr4	1[st] step. Obtaining of cDNA encoding full Xnr4. PCR from *Xenopus laevis* gastrula first strand cDNA with primers full Xnr4 forward: 5′-AATTGGATCCGCCACCATGCATCTATACTTTTACTGTCT and stop Xnr4 reverse: 5′-AATCTCGAGTCACTGGCAGCCACACTCTTC. 2[nd] step. The obtained cDNA fragments was cloned into pCS2 plasmid either by BamHI and XhoI and correct clone was selected by sequencing. Final construct: pCS2-Xnr4.
FlagActivinB	1[st] step. Obtaining of double-stranded cDNA fragment encoding three Flag epitops. Annealing 5′-TAAGTCGACTACAAAGACGATGATGACAAAGATTACAAGGATGACGACG and 5′-TAACTCGAGTTTGTCATCATCGTCTTTGTAGTCCTTATC*GTCGTCATCC* (complementary sequences are in italics), filing of nested ends by Klenow fragment of DNA polymerase, restriction by SalI and XhoI. 2[nd] step. Cloning of the obtained cDNA in XhoI site of *pSP64-ActivinB*. The resulting construct had a sequence encoding for three Flag-tag epitops located posterior to the ActivinβB pre-proregion cleavage site (cleavage site is framed, Flag equences are underlined):…RGLDYKDDDDKDYKDDDDKDYKDDDDKLECDG…Importantly, in the resulting plasmid (*pSP64-Flag- ActivinB*), the region encoding for mature ActivinβB was flanked from 3′-end by XhoI site which allowed us to generate Flag- tagged chimeric constructs, composed of the pre-proregion of ActivinβB and the mature region of any desired TGF-beta factor. Final construct: *pSP64-FlagActivinB*.
FlagADMP	1[st] step. Obtaining of cDNA fragment encoding for mature part of ADMP. PCR from Xenopus laevis gastrula first strand cDNA with primers mature ADMP forward: 5′-ATTCTCGAGTCAGTAGAAGAAGATGGACAA and stop ADMP reverse: 5′-ATAGAATTCTTAGTGGCACCCGCAGCT. 2[nd] step. The obtained cDNA fragment was cloned into *pSP64-FlagActivinB* plasmid by XhoI and EcoRI instead of the

	fragment encoding mature ActivinB and checked by sequencing. Final construct: *pSP64-FlagADMP*.
FlagBMP4	1st step. Obtaining of cDNA fragment encoding for mature part of BMP4. PCR from *pCS2-BMP4* with primers mature BMP4 forward: 5′-AATCTCGAGCAGAGACCCCGTAAAAAAAAC and stop BMP4 reverse. 2nd step. The obtained cDNA fragment was cloned into *pSP64-FlagActivinB* plasmid by XhoI and EcoRI (blunted) instead of the fragment encoding mature ActivinB and checked by sequencing. Final construct: *pSP64-FlagBMP4*.
FlagXnr2	1st step. Obtaining of cDNA fragment encoding for mature part of Xnr2. PCR from *pCS2-Xnr2* with primers mature Xnr2 forward: 5′-TAACTCGAGATTGTCATGAACACCATCCCTTC and stop Xnr2 reverse. 2nd step. The obtained cDNA fragment was cloned into *pSP64-FlagActivinB* plasmid by XhoI and EcoRI (blunted) instead of the fragment encoding mature ActivinB and checked by sequencing. Final construct: *pSP64-FlagXnr2*.
FlagXnr4	1st step. Obtaining of cDNA fragment encoding for mature part of BMP4. PCR from *pCS2-Xnr4* with primers mature Xnr4 forward: 5′- ATACTCGAGTTTAAGGAACATGTTATGGGT and stop Xnr4 reverse. 2nd step. The obtained cDNA fragment was cloned into *pSP64-FlagActivinB* plasmid by XhoI and EcoRI (blunted) instead of the fragment encoding mature ActivinB and checked by sequencing. Final construct: *pSP64-FlagXnr2*.
XWnt8	1st step. Obtaining of cDNA fragment encoding for mature part of BMP4. PCR from *pCSKA-XWnt8* first strand cDNA with primers XWnt8 forward: 5′-AATTGGATCCGCCACCATGCAAAACACCACTTTGTTCATC and XWnt8 reverse: 5′-ATGCATGCTCGAGTCATCTCCGGTGGCCTCT. 2nd step. The obtained cDNA fragment was cloned into pCS2 plasmid by BamHI and XhoI and checked by sequencing. Final construct: *pSP64-Xwnt8*.
Xwnt8-Flag	1st step. Obtaining of *XWnt8* cDNA deprived of 3′-terminal stop-codon by PCR from *pCSKA-XWnt8* with primers XWnt8 forward and XWnt8 reverse: 5′-ATGCATGCTCATGATTCTCCGGTGGCCTCT. 2nd step. The obtained cDNA fragment was cloned by EcoRV/BamHI (blunted) and NcoI/PagI sites into pCS4- 3Flag plasmid (gift from Dr Asashima) and checked by sequencing. Final construct: *pSP64-Xwnt8-Flag*.
SynNog2-Cer	Preparing of chimeric cDNA encoding for signaling peptide of Noggin2 and mature part of Cerberus. 1st step. Obtaining of cDNA fragment encoding Noggn2 signal peptide by PCR from *pCS2-SynNoggin2* with primers ‘Ng2 synt 5′ and 5′-ATAAGGCTGACAGCACCCCT. 2nd step. Obtaining of cDNA fragment encoding mature Cerberus by PCR from *pSP35-Cer* with primers 5′-AGGGGTGCTGTCAGCCTTATCACTCAGAAGGACGAGAAAG and 5′-AATTCTCGAGTTAATGGTGCAGGGTAGTAGATGTAT (‘Cer-stop’). 3d step. Two overlapping cDNA fragments obtained at previous two steps were purified from non-incorporated primers, mixed, denatured, annealed and subjected to PCR with ‘Ng2 synt 5′ and ‘Cer-stop’ primers. 4th step. Cloning by AgeI (blunted) and XhoI into BamHI (blunted) and XhoI sites of pCS2 plasmid. Checking by sequencing. Final construct: *pCS2-SynNog2-Cer*.
SynMycNog2-Cer	Preparing of chimeric cDNA encoding for signaling peptide of Noggin2 and mature part of Cerberus. 1st step. Obtaining of cDNA fragment encoding Noggn2 signal peptide by PCR from *pCS2-SynMycNoggin2* with primers ‘Ng2 synt 5′ and 5′-CAGATCCTCTTCAGAGATGAGTTTCTGCTCTAGGTCT. 2nd step. Obtaining of cDNA fragment encoding mature Cerberus by PCR from *pSP35-Cer* with primers 5′-TCATCTCTGAAGAGGATCTGCACTCAGAAGGACGAGAAAG and ‘Cer-stop’. 3d step. Two overlapping cDNA fragments obtained at previous two steps were purified from non-incorporated primers, mixed, denatured, annealed and subjected to PCR with ‘Ng2 synt 5′ and ‘Cer-stop’ primers. 4th step. Cloning by AgeI (blunted) and XhoI into BamHI (blunted) and XhoI sites of pCS2 plasmid. Checking by sequencing. Final construct: *pCS2-SynMucNog2-Cer*.
SynNog2-Fol	Preparing of chimeric cDNA encoding for signaling peptide of Noggin2 and mature part of Follistatin. 1st step. Obtaining of cDNA fragment encoding Noggn2 signal peptide by PCR from *pCS2-SynNoggin2* with primers ‘Ng2 synt 5′ and 5′-ATAAGGCTGACAGCACCCCT. 2nd step. Obtaining of cDNA fragment encoding mature Follistatin by PCR from *p64TNE-XFS319* with primers 5′-AGGGGTGCTGTCAGCCTTATAATTGCTGGCTGCAGCAGTC and 5′-ATTCTCGAGTCACTTACAGTTGCAAGAT (‘Fol-stop’). 3d step. Two overlapping cDNA fragments obtained at previous two steps were purified from non-incorporated primers, mixed, denatured, annealed and subjected to PCR with ‘Ng2 synt 5′ and ‘Fol-stop’ primers. 4th step. Cloning by AgeI (blunted) and XhoI into BamHI (blunted) and XhoI sites of pCS2 plasmid. Checking by sequencing. Final construct: *pCS2-SynNog2-Fol*.
SynMycNog2-Fol	Preparing of chimeric cDNA encoding for signaling peptide of Noggin2 and mature part of Follistatin. 1st step. Obtaining of cDNA fragment encoding Noggn2 signal peptide by PCR from pCS2-SynMycNoggin2 with primers ‘Ng2 synt 5′ and 5′-CAGATCCTCTTCAGAGATGAGTTTCTGCTCTAGGTCT. 2nd step. Obtaining of cDNA fragment encoding mature Follistatin by PCR from *p64TNE-XFS319* with primers 5′-TCATCTCTGAAGAGGATCTGAATTGCTGGCTGCAGCAGTC and ‘Fol-stop’. 3d step. Two overlapping cDNA fragments obtained at previous two steps were purified from non-incorporated primers, mixed, denatured, annealed and subjected to PCR with ‘Ng2 synt 5′ and ‘Fol-stop’ primers. 4th step. Cloning by AgeI (blunted) and XhoI into BamHI (blunted) and XhoI sites of pCS2 plasmid. Checking by sequencing. Final construct: *pCS2-SynMycNog2-Fol*.

Table S2. Rescue of Noggin2 MO morphants by Wnt-, BMP- and Activin/Nodal-specific inhibitors

	misMONog2 +12 ng/µl pNog2-EGFP (control)	MONog2 +12 ng/µl pNog2-EGFP	MONog2 +4 ng/µl pNog2-tALK4 +8 ng/µl pNog2-EGFP	MONog2 +12 ng/µl pNog2-tALK4	MONog2 +4 ng/µl pNog2-tBR +8 ng/µl pNog2-EGFP	MONog2 +12 ng/µl pNog2-tBR	MONog2 +4 ng/µl pNog2-Dkk1 +8 ng/µl pNog2-EGFP	MONog2 +12 ng/µl pNog2-Dkk1	MONog2 +4 ng/µl pNog2-tALK4 +4 ng/µl pNog2-tBR +4 ng/µl pNog2-Dkk1
1	65,027	18,466	51,276	0,469	17,151	44,633	56,892	26,834	44,829
2	34,832	45,217	46,939	1,335	42,062	25,158	45,017	53,418	47,522
3	65,525	11,118	62,906	29,124	0,001	21,345	78,737	68,715	61,948
4	58,845	29,020	6,768	6,400	3,722	6,883	50,533	85,853	65,611
5	58,775	45,360	20,873	31,445	46,888	42,871	52,530	54,498	71,674
6	60,002	59,561	48,942	0,460	43,562	0,020	66,430	30,460	44,508
7	62,793	40,557	45,586	7,815	29,940	11,124	63,631	26,999	60,752
8	60,369	48,715	37,657	0,657	31,107	15,362	12,629	50,718	50,820
9	72,912	19,776	49,257	3,552	46,155	32,034	49,773	21,121	68,236
10	64,355	69,390	50,672	17,063	27,445	18,025	50,566	38,251	63,730
11	48,519	36,176	45,721	6,184	48,066	45,376	22,229	58,500	37,169
12	65,840	40,275	46,983	19,630	39,331	35,489	29,428	55,239	66,965
13	60,395	44,058	61,656	5,545	55,884	44,081	80,910	26,917	48,671
14	75,498	51,503	58,961	26,785	16,188	0,056	0,770	49,792	64,688
15	60,854	51,988	31,526	62,682	35,975	18,897	35,965	50,510	60,003
16	50,728	41,447	58,215	35,610	34,750	59,237	59,434	34,525	69,080
17	68,022	47,541	52,094	18,986	28,170	50,464	43,779	54,319	65,704
18	65,889	18,320	36,770	32,710	29,428	6,755	44,589	56,182	60,421
19	74,452	27,054	44,725	6,766	33,651	29,597	66,267	27,039	62,391
20	82,832	61,305	30,922	53,088	28,980	55,016	62,232	21,461	58,668
21	60,413	34,690	59,141	30,847	44,572	31,009	22,984	30,653	64,576
22	79,620	11,112	41,996	4,994	15,847	15,340	54,008	26,639	40,552
23	69,589	53,238	58,011	12,649	22,355	0,121	47,078	60,056	46,597
24	13,024	54,110	63,153	28,891	14,270	38,261	39,004	17,421	51,047
25	74,064	1,681	52	41,317	53,238	76,198	32,876	23,325	46,837
26	56,728	18,672	17,797	29,807	27,730	14,518	52,490	30,528	8,168
27	83,949	25,717	42,812	38,722	43,765	0,013	41,135		72,847
28	58,875	26,193	42,827	20,181	42,829	9,574	67,345		83,698
29	47,798	27,290	32,599	13,103	22,010	27,401	76,164		61,323
30	59,661	29,803	37,725	29,766	33,083	39,538	36,494		49,891
31	70,296	30,659	30,377	34,100	42,377	44,466	54,863		42,047
32	71,781	31,223	42,03	48,012	32,322	13,541	38,307		44,872
33	73,120	36,296	66,278	29,044	7,331	5,802	5,937		84,344
34	16,730	39,272	38,074	29,627	38,489	0,002	41,638		58,345

35	72,822	39,470	37,537	27,753	32,567	18,156	42,744	35,401
36	68,139	39,725	40,153	40,927	42,262	11,137	23,163	43,095
37	54,581	42,403	33,378	26,872	49,840	28,540	44,514	53,711
38	58,121	43,195	39,417	12,595	31,618	25,975	35,814	76,933
39	58,181	51,311	45,425	0,216	42,010	19,354	40,634	83,028
40	60,073	56,424	43,707	62,664	43,022	22,592	54,137	45,035
41	67,999	57,708	44,968	37,652	32,400	8,565	0,402	52,277
42	54,339	67,409	63,141			10,768	42,100	47,050
43	56,480	3,183	43,746			15,957	53,665	63,225
44	61,619	36,000	40,506			10,360	49,973	69,217
45	64,586	35,082	64,047			69,734	0,051	45,232
46	56,109	28,506	32,886			16,491	29,711	62,568
47	75,318	2,734	47,719			7,577	66,308	24,518
48	52,103	38,635	42,428			23,653	45,687	55,823
49	77,502	46,918	10,251			18,014	46,263	73,827
50	63,058	0,775	37,867			36,817	55,820	48,960
51	55,567	47,637	35,081			37,153	45,606	71,032
52	66,610	49,460	50,431			7,532	42,851	54,389
53	53,867	49,720	40,501				70,679	47,204
54	51,437	61,996	53,52				57,559	49,716
55	65,312	20,230	52,541				25,155	80,095
56	70,740	15,836	48,775					
57	48,217	38,242	18,157					
58	58,268	0,068	38,04					
59	65,264	26,268	51,181					
60	48,887	31,732	48,387					
61	61,216	0,919	43,896					
62	66,240	15,928	40,561					
63	45,598	33,877	24,745					
64	69,509	59,306	14,63					
65	93,091	0,541	27,696					
66	59,542	9,136	21,294					
67	60,517	4,276	39,524					
68	71,822	16,411	68,043					
69	64,274	17,824	49,478					
70	67,137	33,616	44,251					
71	53,646	45,420	44,21					
72	62,328	45,760	48,204					
73	75,883	26,202	71,378					

74	70,338	28,096	43,206
75	68,865	48,563	42,18
76	68,557	28,488	76,428
77	67,372	47,711	33,966
78	67,127	26,557	
79	65,744	30,373	
80	65,571	34,927	
81	65,375	25,396	
82	64,829	24,100	
83	61,133	43,964	
84	60,768	27,454	
85	57,998	44,971	
86	56,956	22,152	
87	54,117	42,108	
88	53,566	38,915	
89	51,875	33,208	
90	49,819	36,842	
100	48,652	23,845	
101	50,683	31,158	
102	77,589	10,862	
103	51,687	0,018	
104	63,411	29,426	
105	64,552	37,932	
106	45,910	36,187	
107	43,992	6,714	
108	65,040	24,739	
109	82,276	30,469	
110	83,382	26,549	
111	58,617	8,79	
112	34,942	55,674	
113	51,522	22,083	
114	63,192	38,312	
115	38,038	37,418	
116	67,590	51,857	
117	80,815	31,811	
118	82,619	25,885	
119	35,145	27,717	
120	81,160	42,72	
121	75,965	40,681	

122	38,627
123	20,302
124	20,212

Integrated density (ID) of in situ hybridization signal within *XBF1* expression domains of stage 26 *Xenopus laevis* embryos measured in arbitrary units by ImageJ image processing program (http://rsb.info.nih.gov/ij/index.html) and photographed at a constant light and exposition conditions by Leica M205 fluorescent stereomicroscope. For each type of injections, the data were collected from three to five independent experiments. Each series of experiments (experiments carried out in 1 day on the eggs obtained from the same pair of frogs) included control embryos, the antiNoggin2 MO-injected embryos and embryos injected by the MO mixed with different combinations of plasmids (from two to five combinations in different series of experiments). No significant difference (exciding 1%) between mean IDs of control embryos obtained in the same day from the same pair of frogs but processed for in situ hybridization in different vials were detected. Nevertheless, in each series of experiments, about 10 control embryos non-labeled by FLD were processed in the same vial with embryos injected by antiNoggin2 MO for to control possible difference between the in situ hybridization conditions. However, in neither of case was a significant difference revealed between these and the control embryos (labeled by FLD), which was processed separately. On the other hand, up to 15% difference in mean IDs was detected between control embryos from different series of experiments (obtained in different days from different pairs of frogs). Therefore, to normalize the results of different series of experiments, the data obtained in different days were normalized using the following procedure: (1) mean values of the *XBF1* expression area in control embryos were calculated for each series of experiments; (2) one of these control mean values was chosen (arbitrary) as a standard and the correction coefficients in respect to this standard were calculated for each of all the other control mean values; (3) IDs of all individual embryos in all series of experiments were normalized by using the corresponding correction coefficients.