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Noggin-mediated Retinal Induction Reveals a Novel Interplay between BMP Inhibition, TGFβ and SHH Signaling

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ABSTRACT

It has long been known that the depletion of Bone Morphogenetic Protein (BMP) is one of the key factors necessary for the development of anterior neuroectodermal structures. However, the precise molecular mechanisms that underlie forebrain regionalization are still not completely understood. Here, we show that Noggin1 is involved in the regionalization of anterior neural structures in a dose-dependent manner. Low doses of Noggin1 expand prosencephalic territories, while higher doses specify diencephalic and retinal regions at the expense of telencephalic areas. A similar dose-dependent mechanism determines the ability of Noggin1 to convert pluripotent cells in prosencephalic or diencephalic/retinal precursors, as shown by transplant experiments and molecular analyses. At a molecular level, the strong inhibition of BMP signaling exerted by high doses of Noggin1 reinforces the Nodal/TGFβ signaling pathway, leading to activation of Gli1 and Gli2 and subsequent activation of Sonic Hedgehog (SHH) signaling. We propose a new role for Noggin1 in determining specific anterior neural structures by the modulation of TGFβ and SHH signaling. STEM CELLS 2015; 00:000–000

INTRODUCTION

The initial phases of neural plate specification and organization require a complex series of signaling events, which are highly conserved in vertebrates. In the classical view of neural induction, in the blastula stage embryo, prospective ectodermal cells acquire an initial neural identity through the release of Fibroblast Growth Factors (FGF) and Bone Morphogenetic Proteins (BMP) antagonists, required for the establishment of a neuroectodermal identity with anterior features. Subsequently, the production of other secreted molecules drives the antero-posterior (FGFs, Wnts and retinoic acid) and dorso-ventral (BMPs and Sonic Hedgehog, SHH) patterning of the neural plate allowing it to acquire a three-dimensional structure and undergo regionalization [1]. Regional differentiation of the neural plate creates the roof plate and floor plate, which be-

come local signaling centers and orchestrate subsequent regionalization and differentiation [2]. The roof plate, under the influence of the surrounding epidermal tissue, produces BMPs and Wnts driving the formation of dorsal neural structures [3]. Midline floor plate cells, instructed by the underlying axial mesendoderm, secrete TGFβ and SHH conferring a ventral identity to the adjacent neural cells [2, 4]. Similarly, antagonizing signals secreted by anterior and posterior organizing centres orchestrate antero-posterior patterning. Following these stimuli, the anterior part of the neural plate gives rise to the forebrain that will subsequently divide to form telencephalon and diencephalon. Finally, the two lateral walls of the diencephalon evaginate, forming the eye vesicles [1].

While many of the factors involved in neural induction and development are well known, the relationships among the different players necessary for specific pat-

terning and differentiation events are still unclear. Noggin1 is one of the first secreted molecules produced by Spemann's Organizer to antagonize BMPs activity, in order to specify neuroectoderm instead of epidermidis in *Xenopus* dorsal ectoderm [5, 6]. The ability of Noggin1 to inhibit BMP and XWnt8 signaling represents one of the mechanisms contributing to the development of the anterior neuroectoderm [7]. Further evidences suggest that Noggin1 could also be involved in retinal differentiation. Noggin1 overexpression in *Xenopus* embryos activates the ectopic expression of the "Eye Field Transcription Factors" (EFTFs, necessary for retinal specification) thus conferring the ability to form an ectopic eye [8]. Moreover, Noggin1 is able to convert pluripotent animal cap cells of *Xenopus* embryos (Animal Cap Embryonic Stem cells, ACES cells) in retinal precursors, which develop in complete and functional eyes after transplantation [9, 10]. The molecular mechanisms by which Noggin1 has this retinal-inducing activity are unknown. Recent evidences from mammalian development show an interaction of BMP inhibition with other signaling cascades, such as Nodal [11, 12] and Sonic Hedgehog (SHH) [11, 13]. We thus decided to test the possible involvement of these signaling pathways in the Noggin1-mediated retinal induction we observe in ACES cells.

Here we show that Noggin1 contributes to the regionalization of the anterior neural plate in a dose-dependent manner, influencing the transcription of anterior neural plate markers, including EFTFs. We suggest a conserved mechanism in the ability of different doses of Noggin1 to elicit a forebrain or a diencephalic/retinal fate both *in vivo* and in ACES cells. Furthermore, our data suggest that Noggin1-dependent diencephalic/retinal induction is mediated by the activation of the SHH cascade, elicited by the reinforcement of Nodal signaling.

MATERIALS AND METHODS

Animals

Experiments were conducted in accordance to the European Community Directive 2010/63/EU and approved by the Italian Ministry of Health and Ethics Committee of the University of Trento (Prot. n.2012-030-31.a, May 6 2013). Adult *Xenopus laevis* were obtained from NASCO (Fort Atkinson, WI, U.S.A.) Animals were reared in a 12/12 hr light/dark cycle at 18°C with food available *ad libitum*. All efforts were made to minimize animal suffering. Embryos were staged according to Nieuwkoop and Faber [14].

In vitro transcription and microinjection in *Xenopus* embryos.

Capped GFP and Noggin1 mRNAs were prepared as in Lan et al. [9]. A volume of 10 nl containing either 400pg GFP mRNA or 400pg GFP mRNA plus 7.5pg or 20pg Noggin1 mRNA was microinjected in the animal pole of

each blastomere in two-cell stage embryos. Microinjected embryos were used either for ACES cells explants at stage 8.5 or collected at neurula stage (stage 15) for whole mount *in situ* hybridization and RT-qPCR experiments.

ACES cells explants, treatments and transplants.

ACES cells were collected at stage 8.5 in 1x MBS as described [9]. To modulate the SHH pathway, ACES cells explants were grown 24 hr at 14°C in 1x MBS (control) or 1x MBS containing either 200nM SAG (sc-212905; Santa Cruz Biotechnology), or 50μM cyclopamine (C4116; Sigma-Aldrich). Embryos used for TGFβ pathway modulation were microinjected with either 7.5pg or 20pg Noggin1 mRNA at two-cell stage and grown at 14°C until stage 8.5 in 1x MBS containing either 25μM SB431542 (S4317; Sigma-Aldrich) or 1ng/ml Nodal (3218-ND-025; R&D, Space Import Export). To modulate both SHH and TGFβ pathways, embryos were injected with 7.5 pg NOG and treated with a combination of 1 ng/ml Nodal plus 50μM cyclopamine. Alternatively, embryos were injected with 20 pg NOG plus 25μM SB431542 plus 200 nM SAG. At stage 8.5, ACES cells were explanted and cultured at 14°C in the same solutions until stage 15. Transplants were performed as described [9]. Images were acquired using a Leica ZM16F Stereomicroscope.

RT-qPCR experiments.

ACES cells at stage 15 were collected and frozen in dry-ice for total RNA extraction. RNA was extracted using Nucleospin RNA XS kit (FC140902L; Macherey-Nagel). RNAs were quantified with NanoDrop Spectrophotometer (Thermo Scientific) and checked for integrity by agarose gel electrophoresis. cDNAs (500pg) were prepared using SuperScript VILO cDNA synthesis Kit (11754250; Life Technologies). RT-qPCRs experiments were performed in a CFX384 thermocycler (Bio-Rad) using 10 ng/reaction of cDNA as template and SYBR Fast Universal Ready Mix Kit (KK4601; Kapa Biosystems). Data were analyzed using Bio-Rad CFX manager software (Bio-Rad). RT-qPCR primers are listed in Supplemental Table 1.

Whole mount *in situ* hybridization.

Whole mount *in situ* hybridization (WM-ISH) was performed as described [15], with the following probes: Emx1 [16], FoxG1 [17], Rx1 [15], XK81 [18], Zic2 [19], BH1 [20]. En2 probe was cloned by RT-PCR using the following primers: T7-En2-For 5'-TAATACGACTCACTATAGGGCATCTCCCTACTGGCTGCTC-3' SP6-En2-Rev 5'-ATTTAGGTGACACTATAGAAGCCCCAGTGTCTCTCTCA-3'. Images were acquired using a Leica ZM16F Stereomicroscope.

Smad4 immunoprecipitation and immunoblot analysis.

ACES cells microinjected with different doses of Noggin1 and subject to the different treatments were solubilized in RIPA buffer and incubated 1 hr in ice. Dynabeads (100 µl; 100.04D, Life Technologies) were added to each suspension and incubated for 20 min at 4°C in order to remove non-specific interactions. Samples were centrifuged (10 min, 10,000xg) and supernatants incubated o/n at 4°C with Smad4-specific antibodies (#9515, Cell Signaling; 1:25 dilution). Samples were then incubated (4 hr, 4°C) with 50 µl Dynabeads. Beads were precipitated and pellets washed three times in RIPA buffer and resuspended in Laemmli Buffer (LB). After resuspension, samples were boiled and centrifuged to eliminate the beads. Total proteins (supernatant) were obtained by centrifugation using methanol:chloroform (4:1) and resuspended in LB. Proteins were quantified using Pierce BCA Protein Assay (#23227; Thermo Scientific). 30 µg proteins were run on 4-12% gradient acrylamide-bisacrylamide gels (#NP0322Box; NuPAGE 4-12%, Life Technologies), transferred to nitrocellulose membranes (Amersham) and probed with specific Smads and phoshorylated-Smads antibodies (#9963; Phospho-Smad antibody sampler kit, Cell Signaling) and GAPDH (sc-32233, Santa Cruz), Images were acquired using ChemiDoc (Bio-Rad) and quantified by Image J free software.

PH3 immunostaining and PH3+ cells count.

Cryostat sections (12 µm) were cut after 1 hour of 4% PFA fixation and OCT inclusion. Immunoistochemistry was perfomed with anti-phosho-histoneH3 (PH3) antibody (06570, Millipore) as described [21]. PH3+ cells were counted on all sections derived from n=5 explants for each condition.

Eye size measurements.

Size of the wild-type and transplanted eyes was evaluated measuring the length of the D/V and A/P axes of the RPE using the ruler function of PhotoshopCS3. Statistical differences were evaluated with respect to D/V and A/P eye of wild-type embryos. We scored as complete those eyes in which the axes were similar or longer than wild-type eyes, and that showed a complete closure of the optic fissure, located ventrally. Those eyes in which one or both axes were shorter than wild-types and/or that had an incomplete closure of the optic fissure were scored as incomplete. The measures of the eye axes are now shown in Supplementary Table 3, and Figure S4 shows representative wild-type, complete and incomplete eyes.

Statistics

Statistical analysis was performed with SigmaPlot 11.0 and Prism 6 (GraphPad) softwares. Values were expressed as mean ± SD and one-way ANOVA or two-tailed Student's t-test were used as appropriate. Level of statistical significance set at $p < 0.05$.

Noggin1 overexpression elicits an anterior neural fate in *Xenopus* embryos and pluripotent cells*.*

Previous studies reported that overexpression of high doses of the neural inducer Noggin1 induce a retinal fate in *Xenopus* ACES cells [9, 10]. This prompted us to investigate whether Noggin1 might play a role not only in retinal development, but more in general in the establishment of regional fates in the nervous system. We thus characterized the ability of different doses of Noggin1 to influence the neural identity of ACES cells of *Xenopus laevis*. In order to verify the amount of Noggin1 present in GFP-only ACES cells (and whole embryos) and after microinjection of the different Noggin1 doses, we performed RT-qPCR experiments that show, at stage 15, a progressive increase of Noggin1 mRNA following the increasing overexpressed doses (Figure S1A). Then, as shown in Figure 1A and B, we analyzed by RT-qPCR the expression of key genes involved in neural tube patterning and regionalization, in ACES cells treated with three different Noggin1 doses. In ACES cells overexpressing 7.5pg of Noggin1 (7.5pg NOG ACES cells; Figure 1A), the transcription of general neural markers such as Sox2 [22] and Pax6 [23, 24, 25] was increased with respect to controls (GFP ACES cells), in accordance with the neural inducing nature of Noggin1. The analysis of patterning genes showed that low Noggin1 doses activate the expression of both anterior (Nog2 and XAnf1) and posterior (Six3, Rx1, Lhx2, BH1) prosencephalic markers with respect to GFP cells [26, 7, 15, 27], while leaving unaltered the expression of genes involved in the development of midbrain (En1, En2; [28]), hindbrain (Hoxb1 and Krox20; [29, 30]) and spinal cord (Hoxb9; [31]). These data suggest a role for low Noggin doses in the induction of a general forebrain identity. For this reason, in all following experiments, the 7.5pg NOG dose was taken as the "reference" dose, against which all other conditions were compared.

We then analyzed the overexpression of an intermediate dose of Noggin1 (12.5pg NOG, Figure 1B), which shows an initial down-regulation of Pax6 and telencephalic genes (Nog2 and FoxG1) and a concomitant up-regulation of ventral diencephalic genes such as Nkx2.1 and Rx1. The analysis of cells overexpressing high Noggin1 doses (20pg NOG ACES cells; Figure 1B) showed, with respect to 7.5pg NOG cells, a significant down-regulation of telencephalic genes (Nog2 and FoxG1), as in 12.5pg NOG overexpressing cells, and a concomitant up-regulation of diencephalic/retinal genes such as Nkx2.1, Six3, Rx1, and Lhx2 but not BH1, expressed in the dorsal diencephalon. The up-regulation of Nkx2.1 and Rx1 is markedly increased with respect to 12.5pg NOG cells. These data strongly suggest the ca-

pability of Noggin1 to modulate the neural identity of *Xenopus* pluripotent cells in a dose-dependent manner, with the highest dose we tested able to specifically establish retinal identity.

In order to elucidate whether Noggin1 could play a similar role *in vivo* during neural patterning, we analyzed by whole mount *in situ* hybridisation (WM-ISH; Figure 2) and by RT-qPCR (Figure S1B) stage 15 *Xenopus* embryos microinjected with GFP, 7.5pg, 12.5pg and 20pg Noggin1. Noggin1 overexpression expanded the anterior neural plate in a dose-dependent manner, as seen by progressive reduction of XK81 expression (epidermal keratin; [18]) accompanied by an enlargement of the Zic2 expression domain, that highlights the neural plate boundary [19]. Thus, Noggin1 overexpression induces an expansion of the anterior neural plate in a dose dependent manner, as previously reported [7]. WM-ISH for patterning genes showed that the injection of 7.5pg Noggin1 induced an expansion of the expression domains of FoxG1 (telencephalon), Emx1 (dorsal telencephalon), Rx1 (diencephalon/retina), BH1 (dorsoposterior diencephalon) and En2 (midbrain), suggesting a role for Noggin1 in the induction of fore- and midbrain structures. The overexpression of 12.5pg NOG leads to a further marked expansion of FoxG1, while only slightly increasing Rx1 expression. Strikingly, the overexpression of 20pg Noggin1 down-regulated the expression of FoxG1, Emx1, BH1 and En2, while increasing Rx1 expression, with respect to all other tested conditions. These results were confirmed by RT-qPCR experiments (Figure S1B), showing that anterior telencephalic (FoxG1, Emx1, Emx2 and Nog2, XAnf1) and mid/hindbrain-spinal cord markers (En2, HoxB1, Krox20 and HoxB9) were repressed in 20pg NOG embryos with respect to all other tested conditions, while diencephalic/retinal markers (Nkx2.1, Six3, Rx1, Lhx2) were significantly over-expressed.

Taken together, these results suggest that Noggin1 contributes with a dose-dependent mechanism to the regionalization of the anterior neural plate. Furthermore, the capability of high Noggin1 doses to induce retinal fates in *Xenopus* pluripotent ACES cells could reflect a role for this molecule in the specification of regional identities *in vivo* during neural development.

The SHH pathway is involved in Noggin1-mediated neural induction of *Xenopus* pluripotent cells.

In order to elucidate the molecular mechanisms through which Noggin1 elicits different regional fates in ACES cells in a dose dependent manner, we analyzed the possible involvement of the SHH and BMP/TGFβ pathways using RT-qPCR. As compared to GFP controls, the overexpression of 7.5pg NOG in ACES cells significantly induced the transcription of genes belonging to the SHH pathway, namely Patched2 (SHH receptor), Smoothened (G protein-coupled receptor), Sufu, Gli1, Gli2, Gli3 (intracellular effectors) and Zic2 (Gli transcrip-

tional cofactor) [32, 33, 34]. Moreover, the transcription of these genes was exponentially induced in 20pg NOG ACES cells with respect to the other two conditions, with the sole exception of Gli3 whose expression remained constant between 7.5pg and 20pg NOG cells (Figure 3A). The analysis of the expression of BMP/TGFβ pathway genes showed a strong down-regulation of the transcription of BMP4, BMP7 and BMP-related Smads (Smad1/5/8) after the microinjection of 7.5pg NOG. This down-regulation is further accentuated by the microinjection of 20pg NOG. Moreover, the transcription of TGFβ genes (Smad2/3, Cripto) and Smad4 was not affected among our three experimental conditions (Figure 3B), in line with Noggin1 ability to inhibit the BMP but not the TGFβ signaling cascade [35]. The only TGFβ-related genes that showed a drastic reduction in its expression between GFP and 7.5pg NOG injected ACES cells is Xnr4, but this reduction was not further accentuated by higher Noggin doses.

We then tested the possible involvement of the SHH pathway in Noggin1-mediated neural induction. In order to verify whether SHH could be a downstream effector of Noggin1 in this process, we treated 20pg NOG ACES cells with cyclopamine, a SHH antagonist [36] from the time of explant (stage 9) to the stage of analysis or transplant (stage 15). We then checked whether ACES cells retained their retina-forming potential after transplantation, as described [9]. For this, we removed the right part of the eye field of stage 15 *Xenopus* embryos, replacing it with 20pg NOG ACES cells, untreated or treated with 50μM cyclopamine. Cyclopamine treatment of 20pg NOG ACES cells almost completely abolished the ability of high Noggin1 doses to rescue eye structures after transplantation: about 80% of treated embryos fail to form an eye (Figure 4A, A' and Tables S2 and S3). Consistent with these data, RT-qPCR experiments showed that cyclopamine treatment of 20pg NOG ACES cells blocked the ability of high Noggin1 doses to up-regulate Rx1, Pax6 and Lhx2 (Figure 4E), thus providing a molecular explanation for the inability of 20pg NOG+cyclopamine treated ACES cells to rescue eye structures after transplantation.

Moreover, the comparison between the RT-qPCR data of 20pg NOG+cyclopamine and 7.5pg NOG showed that cyclopamine seemed to convert high Noggin1-expressing cells in low Noggin1-expressing cells (Figure 4E), supporting our hypothesis of SHH as a downstream effector of Noggin1-mediated retinal induction. To confirm this, we studied whether activation of the SHH pathway in low Noggin1 ACES cells could mimic the phenotype observed in high Noggin1 ACES cells. We thus treated 7.5pg NOG ACES cells with a SHH pathway activator (SAG, Smoothened AGonist) [37] from the time of explant (stage 9) to the stage of analysis or transplant (stage 15) and then performed the previously described assays. Transplants showed that the activation of the SHH pathway by 200nM SAG converted low Noggin1 doses cells in retinal precursors that were able to partially (36%) or completely (40%) rescue eye struc-

tures after transplantation (Figure 4B, B' and Tables S2 and S3). Again, RT-qPCR experiments were consistent with transplantation data, showing that SAG treatment of 7.5pg NOG ACES cells induced Rx1 mRNA to a level similar to that found in 20pg NOG ACES cells (Figure 4E).

Previous studies showed that SHH signaling is implicated in the proliferation of retinal precursors [38, 39]. To rule out the possibility that the effect of SHH observed in our experiments could depend on an effect on ACES cells proliferation, we analyzed the expression of proliferation markers such as cyclinD1 (RT-qPCR; Figure S2A) and phosphohistone H3 (immunohistochemistry; Figure S2B and Table S4) [40]. Neither marker was affected by cyclopamine or SAG, demonstrating that proliferation rate was equivalent in all experimental conditions. These data show a novel role for the SHH signaling pathway in the acquisition of a retinal fate mediated by high Noggin1 doses.

TGFβ pathway is necessary for Noggin1-mediated retinal induction in *Xenopus* pluripotent cells.

TGFβ superfamily members, such as Activin or Nodal-related genes, could be involved in the SHH pathway activation during different developmental processes [41, 11]. We therefore decided to analyze whether the TGFβ signaling pathway was also involved in the Noggin1-mediated retinal induction of ACES cells. We thus treated 20pg NOG ACES cells with a TGFβ specific antagonist, SB-431542 (25μM) [42] and 7.5pg NOG ACES cells with Nodal (1 ng/ml), and verified their retina-forming potential after transplantation. Figure 4C-C' and Tables S2 and S3 show that blocking TGFβ signaling by SB-431542 decreased the capability of 20pg NOG to rescue the formation of complete eye structures to 44% of the treated embryos, with the remaining only forming partial eyes. On the contrary, the treatment of low Noggin1-expressing ACES cells with Nodal enabled them to form a complete or incomplete eye after transplantation (Figure 4D-D') in 74% of embryos (respect to 36% of control untreated embryos; Tables S2 and S3). RT-qPCR of retinal genes confirmed these data (Figure 4F), as SB-431542 treatment of 20pg NOG ACES cells significantly down-regulated Six3, Rx1, Pax6 and Lhx2 expression with respect to untreated 20pg NOG controls, converting the expression profile of high Noggin1-expressing cells in that of low Noggin1-expressing ACES cells. On the contrary, 7.5pg NOG-treated ACES cells exposed to Nodal showed a significant up-regulation of Rx1 expression with respect to untreated controls (Figure 4F). In summary, these data show that activation of the TGFβ pathway is necessary for Noggin1-mediated retinal induction in *Xenopus* pluripotent cells.

We have then analyzed whether the two signaling pathways, SHH and TGFβ, are simultaneously necessary or if the activation of only one could be sufficient to elicit eye formation, even when blocking the other. For this, we have microinjected 7.5pg NOG and then treat-

ed explants, as described above, with 1ng/ml Nodal plus 50μM cyclopamine. We have also injected 20pg NOG and then treated explants with 25μM SB431542 plus 200nM SAG. In neither situation we saw an up-regulation of the EFTFs, signifying that the activation of one single pathway while inhibiting the other is not sufficient to induce a retinal fate (Figure S3A). Thus, both SHH and TGFβ signaling are necessary for Noggin1-mediated retinal induction in *Xenopus* pluripotent cells.

TGFβ signaling activates Gli1 and Gli2 transcription during Noggin1-mediated retinal induction.

The analysis of the expression of genes involved in the SHH pathway (Figure 3A) shows an up-regulation of intracellular effectors but not of SHH itself. Therefore, the activation of the pathway might occur directly by regulating Gli effectors transcription, as already shown [43]. We thus decided to test the effect of our treatments on the transcription of Gli and Zic2 genes, mediators of SHH signaling. As shown by RT-qPCR, Noggin1 increased the expression levels of Gli1, Gli2 and Zic2 in a dose-dependent manner (Figure 5). The induction of Gli1 and Gli2 observed in 7.5pg NOG cells was further increased by the treatment with SAG and Nodal, leading to expression levels similar to those observed in 20pg NOG ACES cells. On the contrary, treatment with cyclopamine did not affect the transcription of Gli1 and Gli2 mRNAs induced by 20pg NOG overexpression. Moreover, SB-431542 treatment down-regulated the expression of Gli1/2 mRNAs. Finally, neither of the "double treatment" combinations is able to activate Gli2 expression to a level similar to that of 20pg NOG (Figure S3B). Zic2 expression was also up-regulated by Noggin1 overexpression in a dose-dependent manner, but none of the treatments affected it. This suggests a direct role for Noggin1 in regulating Zic2 expression, independent of the activation of the SHH and TGFβ pathways.

These results suggest that Gli genes might be the effectors of the SHH pathway in retinal induction mediated by high Noggin1 doses, and that their transcription (and the observed retinal inducing activity) requires an active TGFβ signaling cascade.

Smad4 is the modulator of the TGFβ cascade activation in Noggin1-mediated retinal induction.

To verify the activation state of the BMP signaling cascade in the different experimental conditions, we analyzed the expression of two genes known to be direct transcriptional targets of this pathway, Ventx1 and Tbx3/ET [44, 45]. As expected, both genes showed consistent expression profiles, being dose-dependently down-regulated by the treatment with the different Noggin1 doses. The addition of SB-431542 to 20pg NOG significantly increased their expression levels with respect to both 7.5pg NOG and 20pg NOG (Figure 6C). This could signify that the block of TGFβ

feeds back a signal that enhances the BMP cascade, thus explaining the capability of TGFβ   to increase the retina forming action of low Noggin1 doses.

Since SB-431542 acts by blocking activin/TGFβ receptors, we investigated at which intracellular level the crosstalk between the two pathways could be. As it is widely known, Noggin1 blocks BMP signaling by physically binding to BMPs in the extracellular space [6]. We speculated that the strong block to BMP signaling exerted by high Noggin1 doses could release a greater amount of the intracellular cofactor Smad4 from the BMP-related Smads (Smad 1/5/8), leaving it free to interact with the TGFβ related Smads (Smads 2/3). In fact, Smad4 is the only intracellular effector that is common to both pathways [35]. This could reinforce TGFβ signaling, and in turn activate Gli transcription, as already shown [46]. We thus decided to check the activation of both BMP and TGFβ pathways by analyzing the levels of activated intracellular effectors that co-immunoprecipitated with Smad4. We performed immunoprecipitation experiments using Smad4-specific antibodies followed by immunoblot experiments to test the levels of phosphorylated BMP- and TGFβ-related Smads bound to Smad4, in all the experimental conditions described above. BMP-related Smads were down-regulated in 20pg NOG ACES cells with respect to both GFP and 7.5pg NOG ACES cells, confirming the strong block of BMP signaling in these cells. Moreover, Nodal and SAG treatments did not affect BMP-related Smad phosphorylation in 7.5pg NOG ACES cells, according to their nature of TGFβ and SHH agonist, respectively. On the contrary, SB431542 treatments of 20pg NOG ACES cells showed an increased Smad1/5 phosphorylation with respect to non-treated cells, rendering them more similar to 7.5pg NOG ACES cells (Figure 6B). This suggests that the crosstalk between BMP and TGFβ pathways could be bidirectional. Cyclopamine treatment showed a similar up-regulation of Smad1/5 phosphorylation (Figure 6B), in keeping with the antagonistic activity between BMP and SHH pathways during the development of the floor plate [47]. Figure 6A and B also shows the levels of Smad2 phosphorylation (TGFβ effector). Smad2 phosphorylation decreased in 7.5 pg NOG ACES cells with respect to GFP cells, suggesting that a TGFβ pathway was constitutively active in untreated ACES cells at stage 15, and that this low Noggin1 dose was not sufficient to efficiently block the BMP pathway to produce a strong phosphorylation of TGFβ-related Smads. On the contrary, phosphorylated Smad2 significantly increased in 20pg NOG ACES with respect to 7.5pg NOG cells, showing that high Noggin1 doses reinforce TGFβ signaling, according to our hypothesis. As expected, Nodal treatment of 7.5 NOG cells elicited similar levels of phosphorylated Smad2, in accordance with its TGFβ superfamily membership. SAG treatment also led to an increase of phosphorylated Smad2, comparable to 20pg NOG cells, supporting the idea that the SHH pathway could contribute to Noggin1-mediated retinal induction. On the contrary, SB-431542 was able to block the phosphorylation of Smad2 in 20pg NOG cells, and to repress their eye rescuing abilities after transplantation (Figure 6B).

These data confirm our hypothesis of a regulatory crosstalk between BMP and TGFβ signaling, dependent on the quantity of Smad4 available to participate to either one of the pathways.

Despite many of the factors involved in neural development are known, their molecular interactions still remain somewhat elusive. Understanding the interplay among the signaling pathways that drive nervous system regionalization would be necessary, for example, for efficient generation of specific neuronal types in culture, allowing advances in regenerative medicine approaches. Our data demonstrate novel interactions among signaling pathways in retinal induction of *Xenopus* pluripotent cells. Retinal induction mediated by high Noggin1 doses requires active TGFβ and SHH pathways. TGFβ signaling activates Gli mRNA expression, in turn triggering SHH-dependent transcription. Finally, we show that BMP inhibition exerted by high Noggin1 doses enhances TGFβ signaling by releasing the Smad4 cofactor, leaving it free to interact with TGFβ-related Smads.

Previous studies suggested a yet uncovered role for Noggin1 in inducing retinal identity in pluripotent cells. In the anterior neural plate, retinal identity is conferred by a set of transcription factors (EFTFs), necessary and sufficient for eye development [8]. In *Xenopus*, EFTFs overexpression confers retinal identity to pluripotent ACES cells [10]. We showed that high Noggin1 doses were able to activate EFTF expression in ACES cells, converting them into retinal precursors able to generate a functional eye after transplantation [9]. Here, we have analyzed the molecular mechanisms underlying the retinal-inducing capability of Noggin1. We show that Noggin1 effects rely on the activation of the SHH pathway, driven by Nodal signaling. Nodal can cooperate with Noggin1 to activate the transcription of Gli2 mRNA in *Xenopus* pluripotent cells. Therefore, we suggest a new mode of action for Noggin1 in the specification of the anterior neural plate and eye.

Noggin1 regionalizes the forebrain in a dose-dependent manner.

In mammals, Noggin has been implicated in holoprosencephaly, a craniofacial/neural developmental anomaly characterized by forebrain defects [12, 48]. Unlike mammals, *Xenopus* has three different Noggin genes (Noggin1, Noggin2, Noggin4; [49]) and the overexpression of both Noggin1 and 2 is able to form a complete secondary axis [50, 7]. In addition to this common ability, recent data suggest that different Noggin proteins might have different roles during development. Nog-

gin2 is able to bind not only to BMPs, but also to XWnt8 and, with lower affinity, to other TGFβ factors, contributing to the formation of forebrain structures [7]. Noggin4 seems to play a different role, as it is not able to induce neural tissue in *Xenopus* ectoderm [51]. Here, we suggest that Noggin1 drives forebrain regionalization in a dose-dependent manner. Both RT-qPCR and WM-ISH data show that low Noggin1 doses (7.5pg NOG) convert ACES cells in forebrain precursors (Figures 1A, 2 and S1B). Therefore, the ability of Noggin1 to interfere with the regionalization of anterior neural plate and to expand forebrain boundaries might represent the mechanism through which Noggin1 establishes an anterior neural identity in ACES cells. On the contrary, the microinjection of high Noggin1 doses (20pg NOG) abrogates telencephalic fate promoting only the up-regulation of specific diencephalic/retinal markers in both ACES cells and whole embryos (Figure 1B, 2 and S1B), supporting our previous data [9]. Our results suggest a new role for Noggin1 in the regionalization of the *Xenopus* anterior neural plate, thus supporting the idea of different roles for different Noggin proteins in lower vertebrates [7, 51]. Noggin1 is also expressed in adult vertebrate photoreceptors, suggesting a role in retinal function [21].

The SHH pathway mediates high Noggin1-dependent retinal induction in ACES cells.

The SHH pathway plays important roles in vertebrate organogenesis, such as dorsoventral brain patterning and retinal development [52]. Published data show an involvement of the SHH cascade in different aspects of retinal development, such as optic cup formation, optic stalk development, final retinal lamination and proliferation and multipotency of retinal progenitors [38, 39, 53]. Here we propose a new role for the SHH pathway, that is a cooperation with high levels of Noggin1 in confering a retinal identity to pluripotent cells. RT-qPCR experiments showed that overexpression of Noggin1 mRNA in ACES cells is able to up-regulate genes belonging to the SHH pathway in a dose-dependent manner (Figure 3A). We performed functional experiments in which blocking SHH signaling using cyclopamine abrogated the ability of high Noggin1 doses to induce EFTFs transcription and to rescue eye formation after transplantation (Figure 4A, A', E). On the contrary, the treatment of low Noggin1 overexpressing ACES cells with SAG, a SHH agonist, activated some EFTFs expression and rescued eye formation in transplanted embryos (Figure 4B, B', E), in accordance with our previous observations that SHH overexpression ectopically activates Rx1 expression [22]. Proliferation of retinal progenitors is under the control of SHH pathway, but we see no difference in the levels of proliferation markers in our explants (Figure S2 and Table S4). The ability of Noggin to induce the SHH pathway, and vice versa, was previously described in the dermal papilla [13], but not in neural tissue. The data we present here are the first

evidences of a similar crosstalk in Noggin1-mediated retinal induction.

Nodal is necessary to activate the SHH pathway in Noggin1-dependent retinal induction.

Forebrain specification requires the release of different secreted molecules implicated in neural identity acquisition (FGFs and BMPs antagonists), antero-posterior (FGFs, Wnts and retinoic acid), and dorso-ventral (BMPs and SHH) neural tube patterning. The interactions among these signaling cascades establish specific signaling centers. Ventral midline cells release high levels of Nodal and SHH allowing ventral forebrain structures to subdivide into ventral telencephalon, ventral diencephalon and optic vesicles [1, 2]. In lower vertebrates, evidences suggest an initial role for Nodal in the induction and a subsequent role for SHH in the patterning of the anterior neural plate [2]. Here we suggest that similar interactions among the same molecular pathways contribute to drive high Noggin1-expressing ACES cells toward a retinal precursors identity. Blocking TGFβ type I receptors by SB431542 inhibits EFTFs transcription in 20pg NOG ACES cells and abolishes the ability of these cells to rescue eye development after transplantation (Figure 4C, C', F). This is in line with zebrafish data showing a misexpression of Rx genes in the forebrain of *cyclops* mutants in which Nodal-related2 signaling is abolished [54]. On the contrary, Nodal treatment of 7.5pg NOG ACES cells enables them to become retinal precursors, by activating Rx1 expression and converting them in retinal cells (Figure 4D, D', F).

Many studies suggest that Nodal genes can influence the activation of the SHH pathway. In zebrafish, the signaling cascade activated by *cyclops*, a Nodal-related gene, can directly regulate *shh* expression during ventral forebrain patterning [4]. In *Xenopus* embryos, a synergistic action of Noggin1 and Nodal-related genes induces *shh* expression in notochord and spinal cord [11]. In mammals, BMP antagonism protects Nodal signaling and promotes forebrain patterning maintaining *shh* expression [12]. Albeit we see no activation of *shh* expression in 20pg NOG ACES cells, we observe a dramatic mRNA induction of Gli1/2 and Zic2, are SHH intracellular effectors (Figures 3B and 5). We show an involvement of Nodal signaling in the modulation of Gli1/2 transcription, while Zic2 expression is modulated by Noggin1 expression levels independently of Nodal signaling.

Activation of the SHH pathway by SAG treatment in 7.5pg NOG ACES cells leads to an up-regulation of Gli2 expression. This is in accordance with data demonstrating a positive feedback of activated Gli2 protein on its transcription [55]. On the contrary, no relevant differences in the transcription of Gli1/2 or Zic2 mRNAs were detected after treatment of 20pg NOG ACES cells with cyclopamine (Figure 5). As cyclopamine acts by direct binding and block of the Smoothened receptor [36], it may inhibit Gli protein activation, but not necessarily their transcription, as no data is available on negative

feedback regulation on Gli transcription by SHH signaling inhibition. This suggests that the reinforcement of the SHH cascade observed in Noggin1-mediated retinal induction could be due to a direct effect of Nodal signaling on Gli1/2 transcription. An indirect evidence of a possible involvement of Gli2 in retina formation is shown in humans, by the association of GLI2 mutations with anophthalmia [56, 57]. The activation of Nodal/TGFβ-specific receptors is characterized by an increase of Smad2/3 phosphorylation inside the cell. The phosphorylated Smad2/3 complex then interacts with Smad4 and translocates inside the nucleus activating the transcription of Nodal/TGFβ-related targets [35]. In zebrafish embryos, it has been shown that Smad2 influences the expression of SHH [4]. Moreover, in human cell cultures, the activated Smad2/3 complex is able to directly bind the Gli2 promoter, regulating Gli2 transcription and thus activating the SHH pathway [46]. We hypothesize a similar mechanism of SHH cascade activation by the phosphorylated Smad2/3 complex in *Xenopus* ACES cells. We have performed Smad4 immunoprecipitation experiments, followed by immunoblots for Nodal/TGFβ-related (Smad2) or BMP-related (Smad1/5) phosphorylated Smads (Figure 6A and B). In 20pg NOG ACES cells there is an increase in the phosphorylation of Smad2 with respect to 7.5pg NOG ACES cells. The high levels of Noggin1 found in 20pg NOG explants bind BMPs in the extracellular space, lowering the levels of phosphorylated Smad 1/5 and increasing the possibility of the Smad4 cofactor to interact with phosphorylated Smad2, as shown by the increased levels of Smad4/phospho-Smad2 complex after immunoprecipitation. This interaction is made possible by a basal level of active TGFβ signaling, as shown by the presence of phospho-Smad2 even in GFP and 7.5 NOG ACES cells (Figure 6A and B). As more Smad4 is released from Smad1/5/8 by the increasing BMP inhibition, it becomes available to interact with phospho-Smad2, thus increasing TGFβ signaling. This could explain the observed Gli1/2 transcriptional activation and the subsequent SHH cascade activation.

Consistent with this, both Nodal and SAG treatments of 7.5pg ACES cells increase Smad2 phosphorylation, bringing it to levels comparable to those of 20pg NOG ACES cells. On the contrary, these treatments do not affect Smad1/5 phosphorylation (Figure 6A and B). This supports the hypothesized role for active Smad2 in activating Gli2 transcription thus determining the observed SHH-mediated retinal induction. SB431542 treatment of 20pg NOG ACES cells blocks phosphorylation of Smad2, as expected, while increasing Smad1/5 phosphorylation. SB431542 treatment causes 20pg NOG ACES cells to behave like 7.5pg NOG ACES cells, blocking Gli1/2 transcription and eye formation in transplant experiments. This result supports a role for TGFβ in the transcription of Gli2 mRNAs as previously described [46].

Finally, cyclopamine treatment of 20pg NOG ACES cells restores Smad1/5 phosphorylation to levels com-parable to those found in 7.5pg NOG ACES cells and increases Smad2 phoshorylation to levels higher than those found in 20pg NOG samples, destabilizing Gli2 transcription and blocking eye formation in transplant experiments. The non-competence of cyclopamine-treated 20pg NOG ACES cells to become retinal precursors could be explained by the transcriptional antagonism between the SHH and BMP pathways, as in dorsoventral neural tube patterning [58, 1]. As the SHH pathway is blocked by cyclopamine, ACES cells may start to produce BMP, as shown by the increase of phosphorylated Smad1/5 levels, and they could be converted in more dorsal precursors as previously described [58].

Taken together, these results suggest that high levels of Noggin1 could stabilize the Nodal signaling cascade activating the phosphorylation of Smad2. This, in turn, could increase Gli1/2 transcription, triggering SHH pathway and establishing a retinal identity in high Noggin1-expressing ACES cells.

In Figure 7 we propose a model depicting how the crosstalk among signaling pathways, elicited by the overexpression of different Noggin1 doses, is able to confer specific forebrain or diencephalic/retinal identities to ACES cells.

CONCLUSIONS

In conclusion, Noggin1 contributes to the regionalization of the anterior neural plate, influencing the transcription of anterior neural plate markers with a dose dependent mechanism, thus eliciting a general forebrain or a diencephalic/retinal identity. The observed Noggin1-mediated retinal induction is, at least partially, due to the involvement of Nodal and SHH signaling during neural precursors specification. Moreover, our results point to a role for a graded expression of TGFβs in anterior neural plate and forebrain patterning, as already suggested in zebrafish and mammals [4, 12]. This clearly shows that the interactions among different signaling pathways at the basis of brain regionalization still reserve us much to be discovered. For this purpose, pluripotent stem cells are a challenging and powerful tool. Their *in vitro* differentiation provides us with a simpler yet quite faithful model to identify novel interactions and molecular mechanisms responsible for neural tissue specification and regionalization [59, 60, 61, 62].

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AUTHOR CONTRIBUTIONS

A.M.: conception/design, data collection/assembly, data analysis/interpretation, manuscript writing, final approval of manuscript; L.L.: data collection/assembly; T.I., A.B.: data analysis/interpretation; M.A., R.V.: contribution of reagents, data analysis/interpretation, manuscript revision; F.C.: contribution of reagents, data analysis/interpretation, manuscript revision, financial support; Y.B.: data analysis/interpretation, manuscript writing; financial support; S.C.: conception/design, data collection/assembly, data analysis/interpretation, manuscript writing, final approval of manuscript; financial support.

DISCLOSURE OF POTENTIAL CONFLICT OF INTEREST

The Authors declare no competing commercial interests in relation to the present work.

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Figure 1. Expression of patterning genes in ACES cells.
(A, B) RT-qPCR experiments characterizing the antero-posterior identity of 7.5pg NOG ACES cells (stage 15), as compared to GFP-injected ACES cells (A) and that of 12.5pg NOG ACES cells and 20pg NOG ACES cells (stage 15), as compared to 7.5pg NOG ACES cells (B). Values (mean ± s.d. from three independent experiments) are normalized to GFP-injected ACES cells (A) or 7.5pg NOG ACES cells (B). Statistical significance: p≤0.05 (*; ▪); p≤0.005 (**; ▪▪); p≤0.0005 (***; ▪▪▪). See also Figure S1.
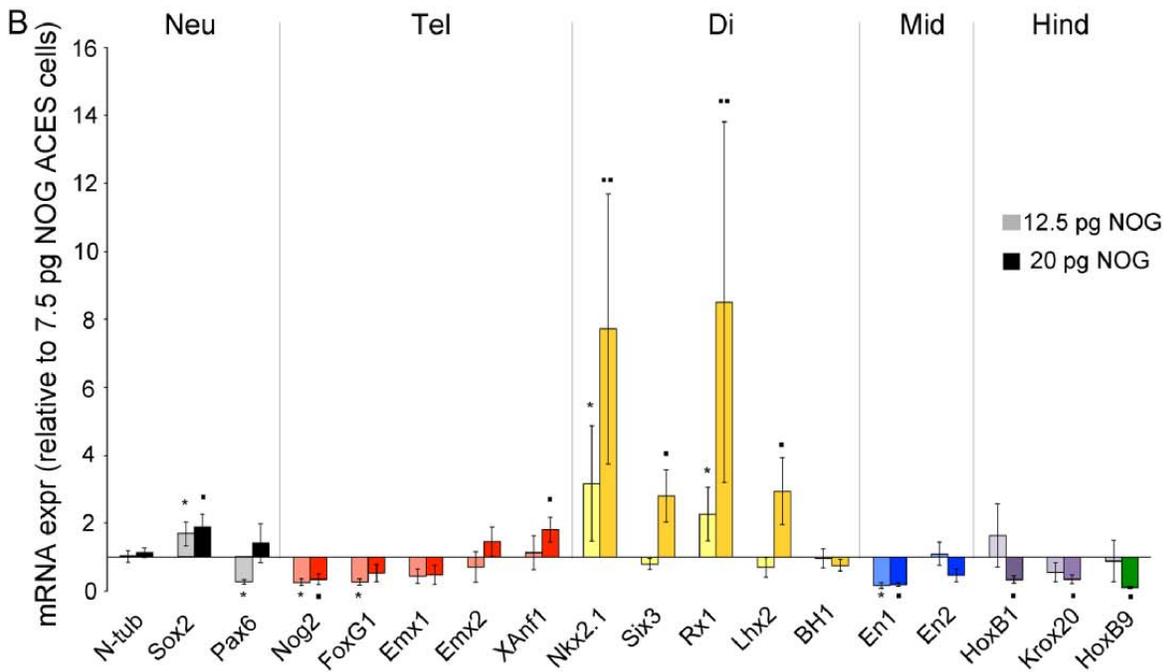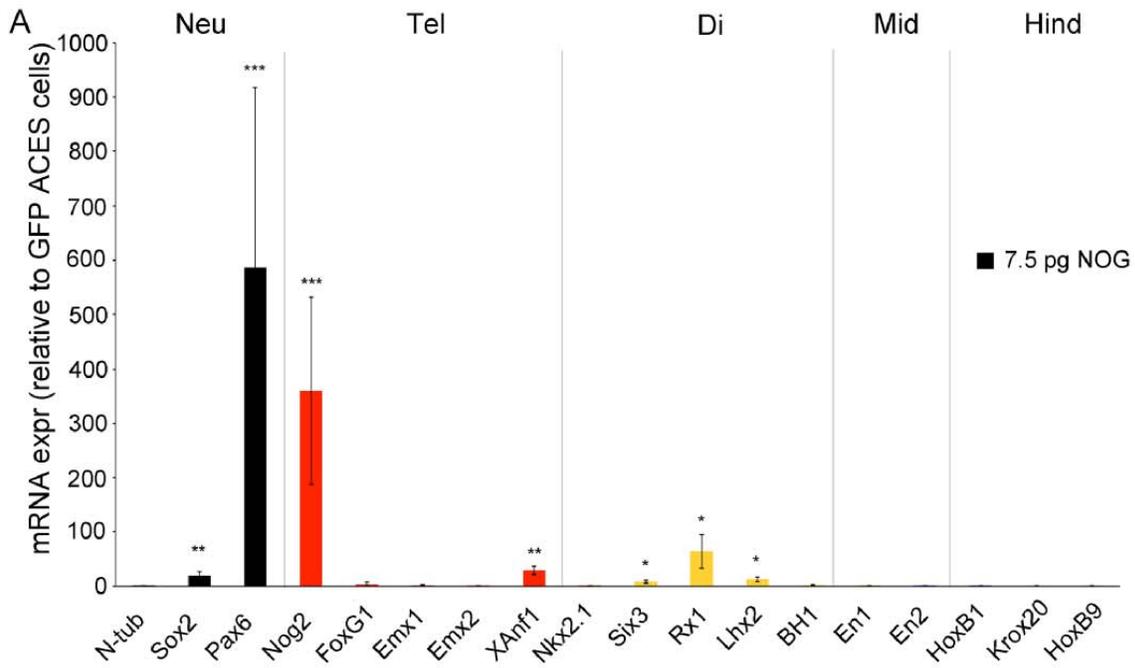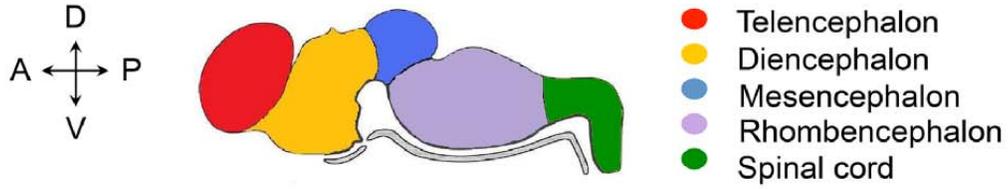
Figure 2. Patterning genes expression in stage 15 *Xenopus* embryos.
Effect of GFP, 7.5pg NOG, 12.5pg NOG and 20pg NOG microinjection in whole embryos at stage 15 by whole mount *in situ* hybridisation. Pictures show staining for XK81 (epidermidis), Zic2 (neural plate boundary), FoxG1 (telencephalon), Emx1 (dorsal telencephalon), Rx1 (diencephalon), BH1 (dorso- posterior diencephalon), En2 (midbrain). See also Figure S1.

	GFP	7.5 pg NOG	12.5 pg NOG	20 pg NOG

Epidermidis

XK81

Neural Plate

Zic2

Telencephalon

FoxG1

Dorsal Telenc.

Emx1

Diencephalon

Dorsal Dienc.

Midbrain

500 uM
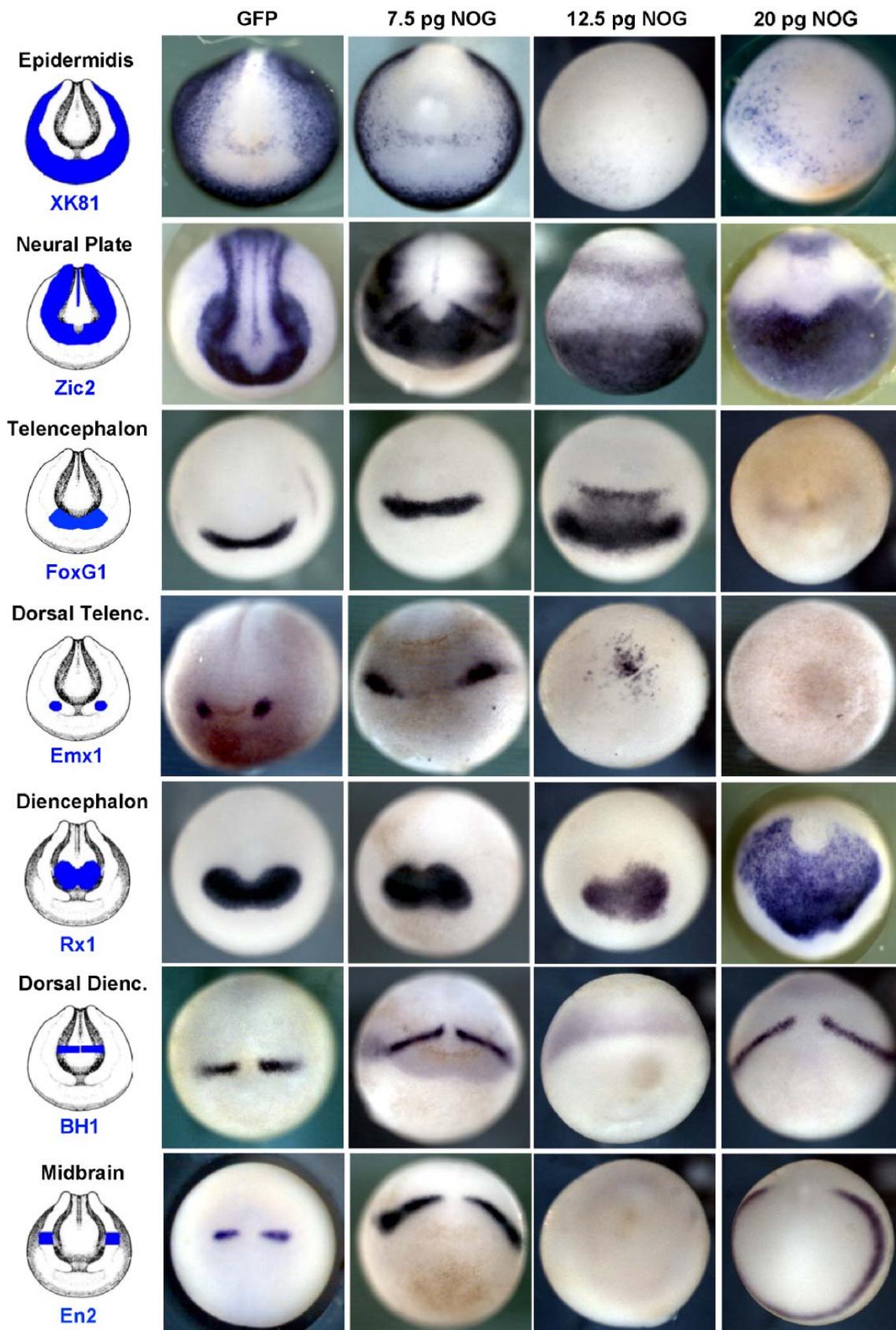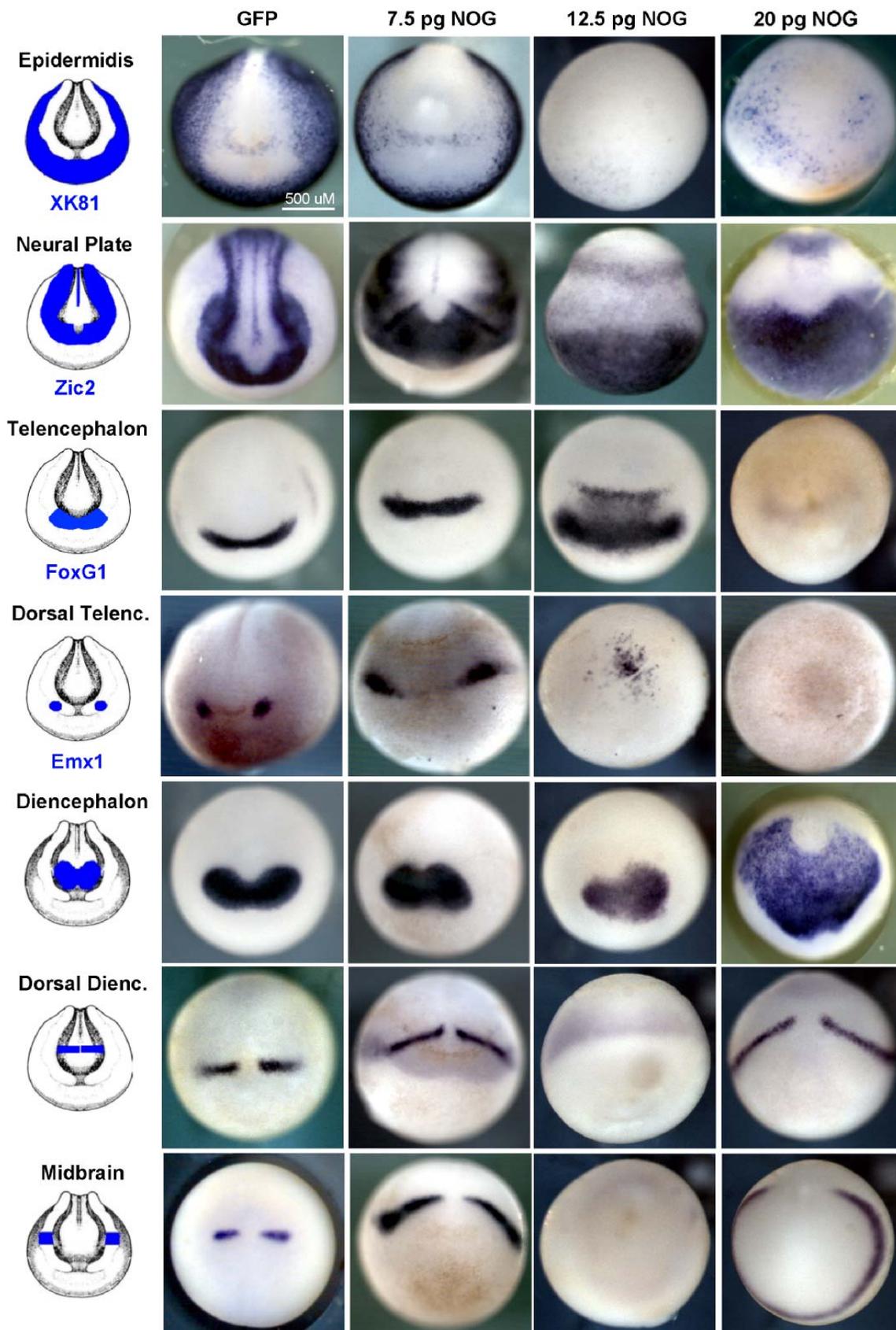
Figure 3. SHH and BMP/TGFβ pathways gene expression in ACES cells.
RT-qPCR on (A) SHH and (B) BMP/TGFβ pathway genes in GFP, 7.5pg NOG and 20pg NOG ACES cells (stage 15). GFP (*) and 20pg NOG (▪) values (mean ± s.d. from three independent experiments) are normalized to 7.5pg NOG values. Statistical significance: p≤0.05 (*; ▪); p≤0.005 (**; ▪▪); p≤0.0005 (***; ▪▪▪).
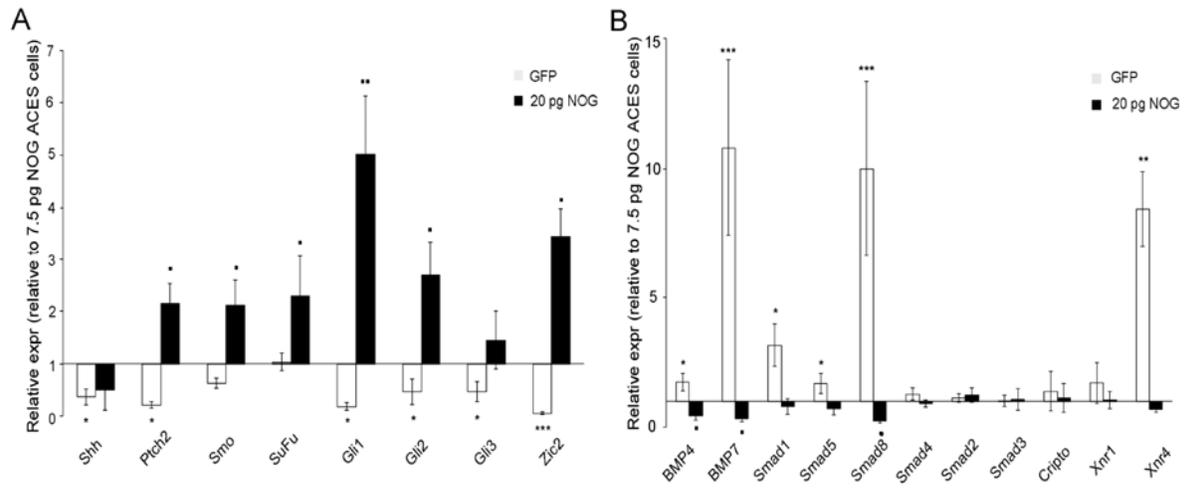
Figure 4. Functional validation experiments on SHH and TGF pathway in ACES cells.
(A-D') Transplantation experiments of ACES cells treated with 20pg NOG (A, C), 7.5pg NOG (B, D), 20pg NOG+cyclopamine (A'). 7.5pg NOG+SAG (B'), 20pg NOG+SB431542 (C'), 7.5pg NOG+ Nodal (D').
(E, F) RT-qPCR experiments analyzing anterior neural and retinal gene expression after SHH (E) and TGFβ (F) pathway modulation. Values (mean ± s.d. from three independent experiments) are normalized to 20pg NOG values. Statistical significance was calculated with respect to 20pg NOG (*) or 7.5pg NOG (▪). p≤0.05 (*; ▪); p≤0.005 (**; ▪▪); p≤0.0005 (***; ▪▪▪). See also Figure S2, Table S1 and Table S2.
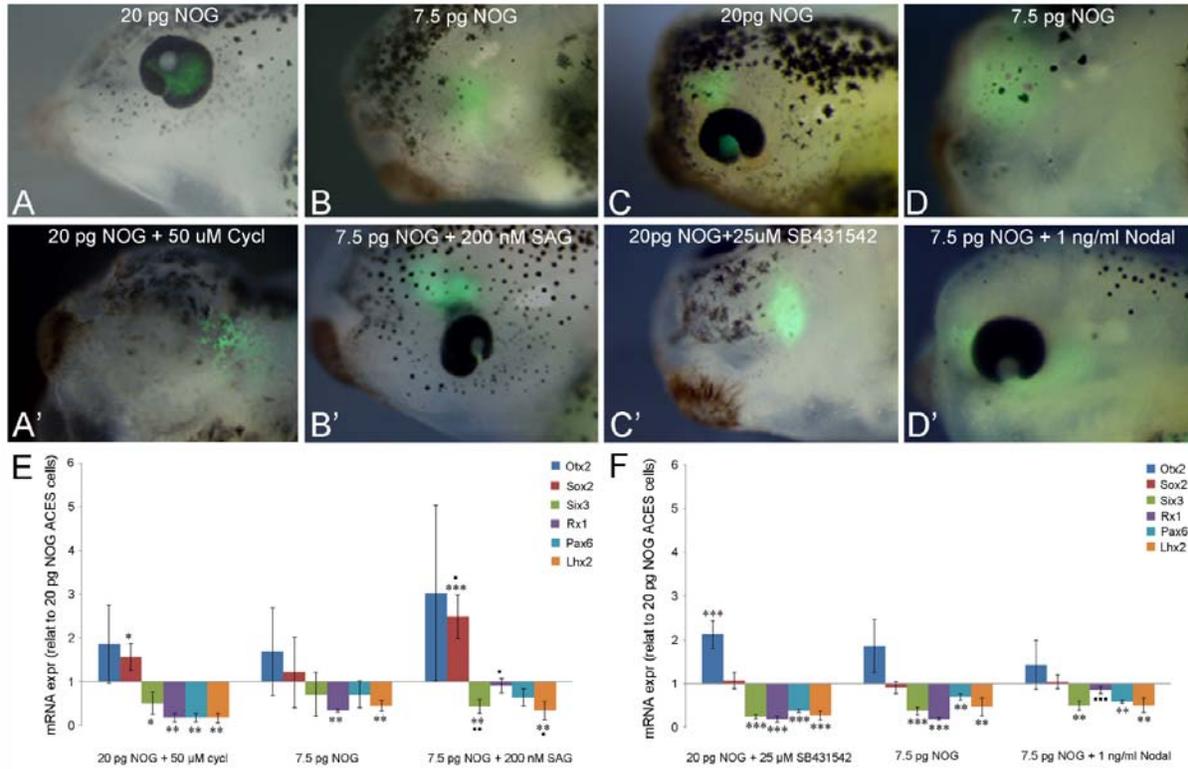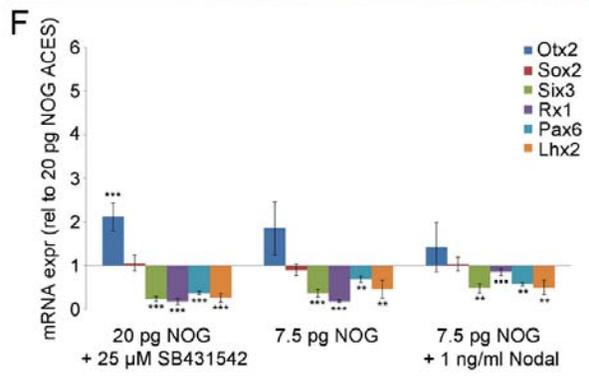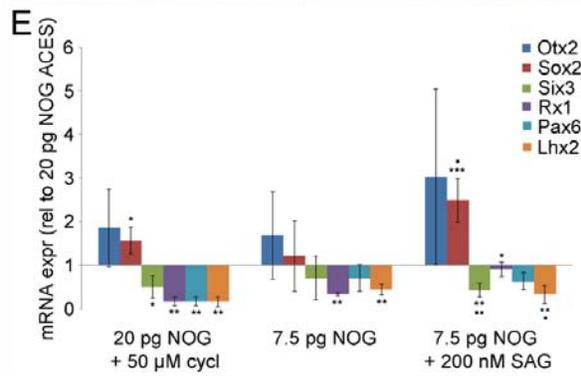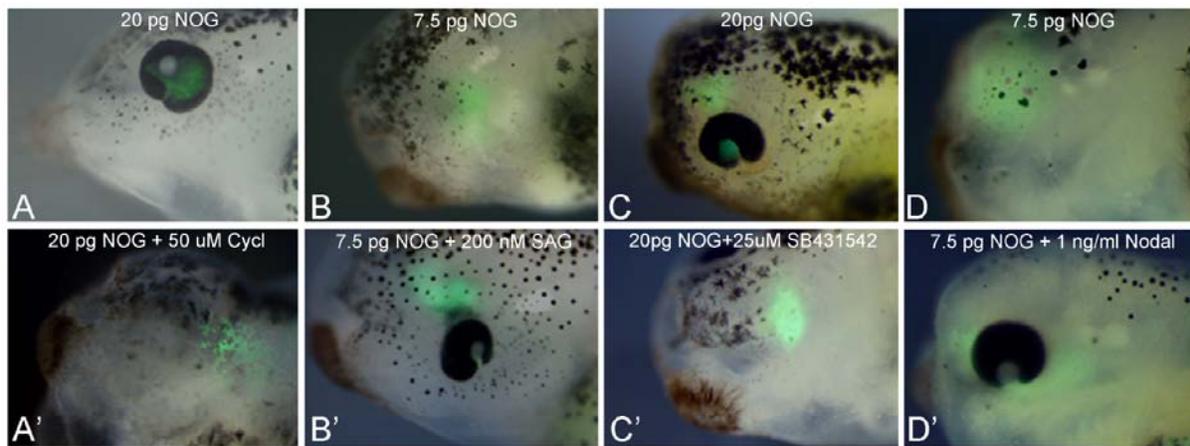
Figure 5. Effect of all experimental conditions on Zic2 and Gli mRNA expression.
Values (mean ± s.d. from three independent experiments) are normalized to 7.5pg NOG values; p≤0.05 (*); p≤0.005 (**); p≤0.0005 (***).
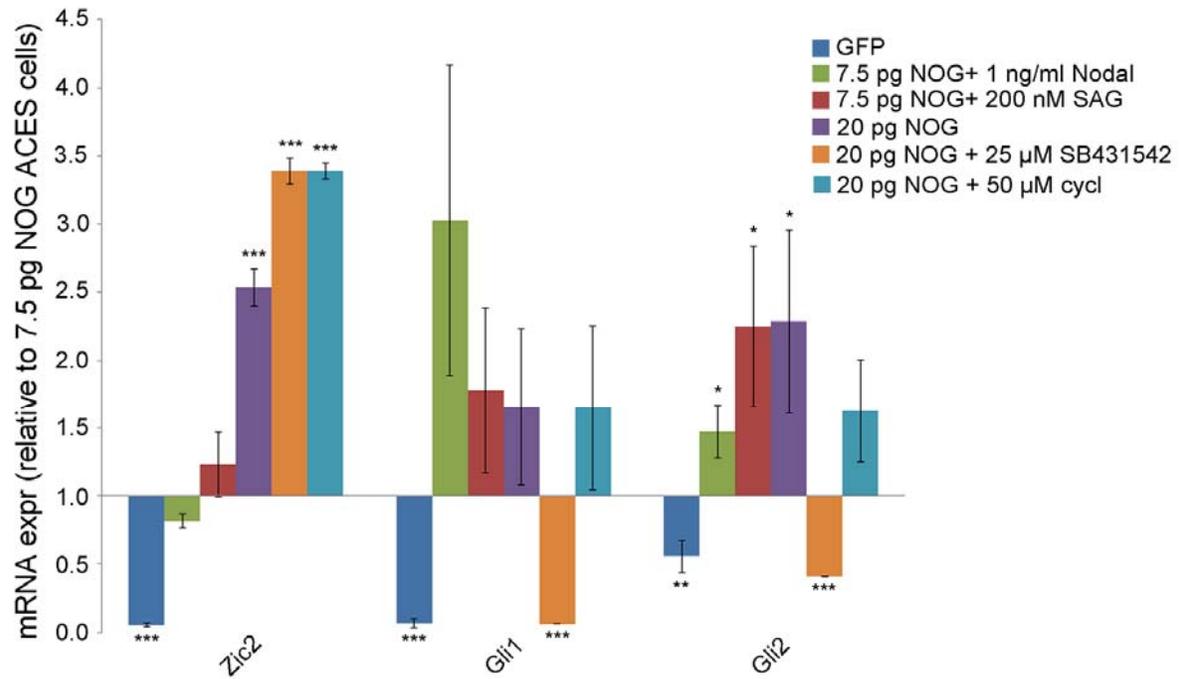
Casarosa et al
Figure 5

Figure 6. Activation of BMP and TGFβ pathways.
(A) Representative immunoblots after Smad4 immunoprecipitation.
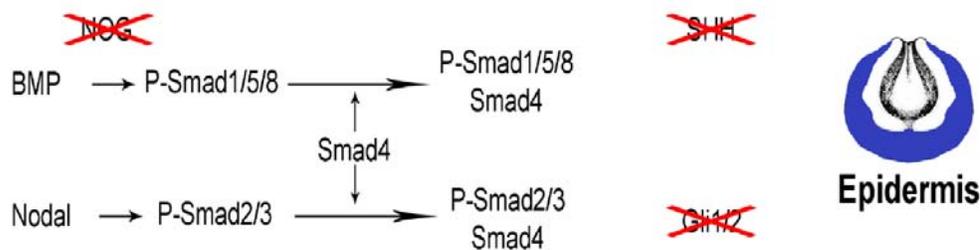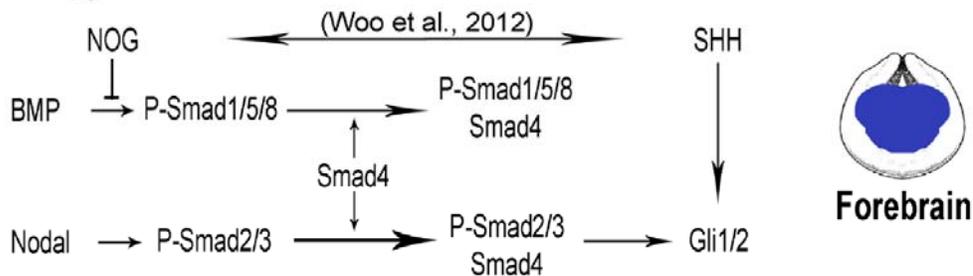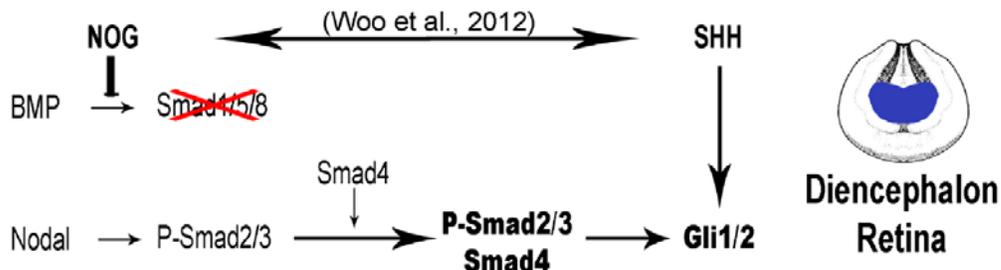(B) Smad4 immunoprecipitation followed by immunoblot with anti P-Smad1/5, Smad1, Smad5, P-Smad2, Smad2 and GAPDH antibodies. Histograms represent densitometric quantification of P-Smads/Smad. GAPDH was performed on IP-Smad4 and supernatant (positive control). (C) RT-qPCR experiments analyzing the effect of all experimental conditions on the expression of BMP-related targets. In (B) and (C), values (mean ± s.d. from three independent experiments) are normalized to 7.5pg NOG values; p≤0.05 (*); p≤0.005 (**); p≤0.0005 (***).
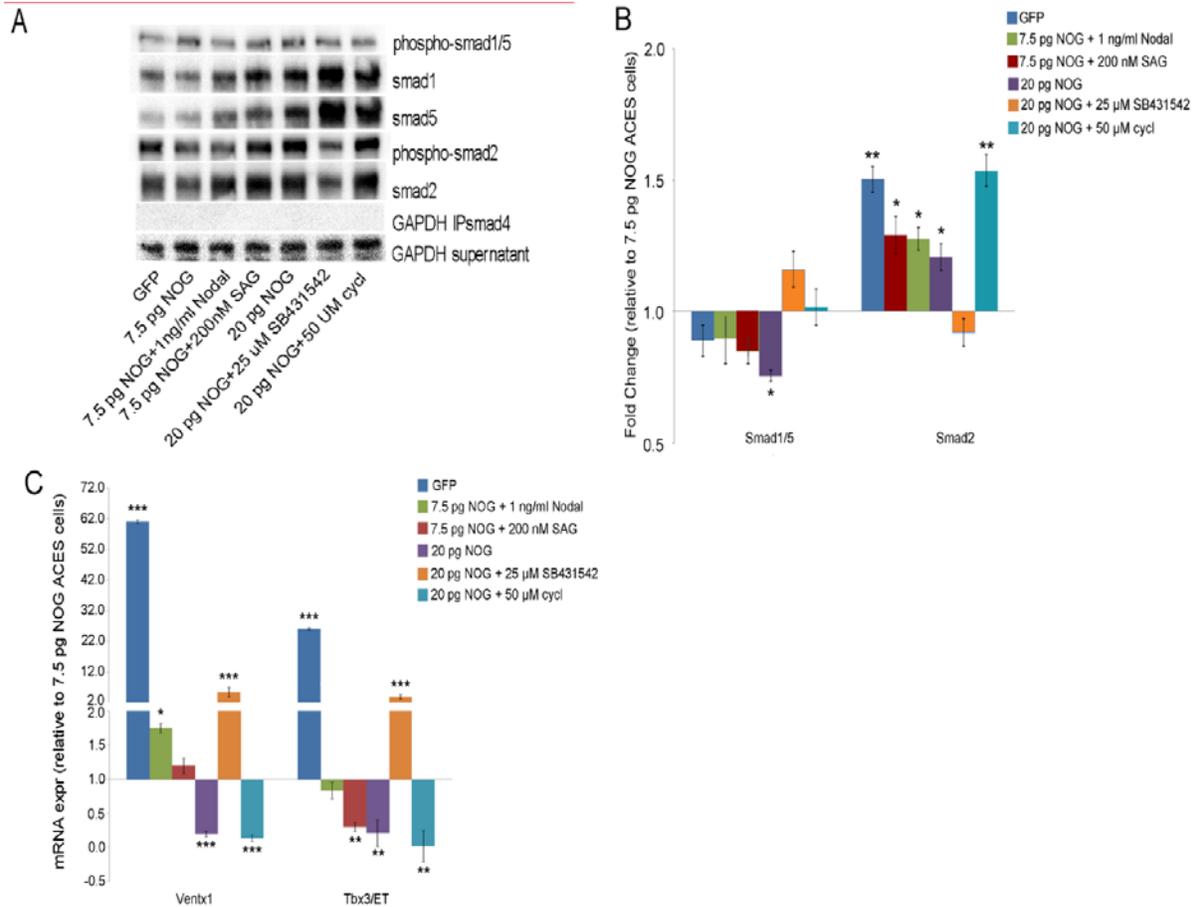
Figure 7. Model of neural identity acquisition mediated by different Noggin doses.
(A) In GFP ACES cells, BMP and Nodal pathways are constitutively active. GFP explants show a basal phosphorylation level of BMP-related Smad1/5/8 (P-Smad1/5/8) and Nodal-related Smad2/3 (P-Smad2/3). P-Smad1/5/8 and P-Smad2/4 interact with their common cofactor Smad4 thus activating the transcription of specific target genes. This will result in the induction of an epidermal fate in ACES cells. No expression of Noggin1, Shh nor Gli1/2 is detected in GFP ACES cells.
(B) In 7.5 pg NOG ACES cells, the overexpression of low doses of Noggin1 reduces the levels of P-Smad1/5/8. Smad4 continues to interact with P-Smad1/5/8, but also reinforces the P-Smad2/3 complex that activates the transcription of Gli1/2. These molecular interactions convert ACES cells into forebrain cells.
(C) In 20 pg NOG ACES cells, the higher levels of Noggin1 completely block Smad1/5/8 phosphorylation, further reinforcing Nodal signalling through the interaction of P-Smad2/3 with Smad4, now completely free from the BMP cascade. This in turn increases Gli1/2 transcription. These molecular cascades reinforce SHH signalling by two different processes: the crosstalk between NOG and SHH (Woo et al., 2012), and Gli1/2 transcription mediated by P-Smad2/3 and Smad4 activity. This cooperation is able to confer diencephalic/retinal identity to 20 pg NOG ACES cells.