Int. J. Dev. Biol. 60: 151-157 (2016)
doi: 10.1387/ijdb.150402am

THE INTERNATIONAL JOURNAL OF DEVELOPMENTAL BIOLOGY
www.intjdevbiol.com

Noggin 1 overexpression in retinal progenitors affects bipolar cell generation

ANDREA MESSINA*[,1], SIMONE BRIDI[1,2], ANGELA BOZZA[1,3], YURI BOZZI[3,4], MARIE-LAURE BAUDET[2] and SIMONA CASAROSA*[,1,4]

[1]*Developmental Neurobiology Laboratory, CIBIO, University of Trento, Italy,*
[2]*The Armenise-Harvard Laboratory of Axonal Biology, CIBIO, University of Trento, Italy,*
[3]*Molecular Neuropathology Laboratory, CIBIO, University of Trento and* [4]*CNR Neuroscience Institute, Pisa, Italy*

ABSTRACT Waves of Bone Morphogenetic Proteins (BMPs) and their antagonists are present during initial eye development, but their possible roles in retinogenesis are still unknown. We have recently shown that *noggin 1*, a BMP antagonist, renders pluripotent cells able to differentiate into retinal precursors, and might be involved in the maintenance of retinal structures in the adult vertebrate eye. Here, we report that *noggin 1*, differently from *noggin 2* and *noggin 4*, is expressed during all phases of *Xenopus laevis* retinal development. Gain-of-function experiments by electroporation in the optic vesicle show that overexpression of *noggin 1* significantly decreases the number of bipolar cells in the inner nuclear layer of the retina, without significantly affecting the generation of the other retinal cell types. Our data suggest that BMP signaling could be involved in the differentiation of retinal progenitors into specific retinal subtypes during late phases of vertebrate retinal development.

KEY WORDS: *noggin 1, retinal differentiation, BMP inhibition*

Introduction

The retina is the light-sensitive part of the eye and is involved in the reception and transduction of light signals. The mature vertebrate retina is characterized by a highly organized laminar structure containing specialized neuronal types, such as light-sensitive photoreceptors, bipolar, horizontal and amacrine interneurons, and ganglion cells whose axons form the optic nerve. Each of these cell types carries out specific functions. All retinal cells originate during embryonic development from a common pool of multipotent progenitors (retinal precursor cells, RPCs) located in the inner part of the optic vesicle. Over the years, many studies have characterized the extrinsic and intrinsic factors that orchestrate retinal development and the differentiation of specific retinal neurons (Zagozewski *et al.*, 2015).

The initial phases of eye development are characterized by the activation of a set of transcription factors, collectively defined as Eye Field Transcription Factors (EFTFs). The cooperation of the EFTFs induces the anterior neural plate to generate the eye field, that will subsequently split in two bilateral regions forming the optic vesicles (Zuber *et al.,* 2003). In fact, after neural tube closure, the two dorsolateral walls of the diencephalon produce evaginations that will form the bilateral optic vesicles. The optic vesicles will give rise to the optic cups from which the neural retina (NR) and the retinal pigmented epithelium (RPE) will form. The molecular mechanisms that underlie eye/retina development and the activation of EFTFs are still not fully understood. Inhibitors of Bone Morphogenetic Proteins (BMPs) are secreted by the organizer, a specialized region of the blastula stage embryo showing inductive

Abbreviations used in this paper: A/P axis, antero/posterior axis; AC, amacrine cell; ACES, animal cap embryonic stem cell; ANF, anterior neural fold; ANR, anterior neural ridge; BC, bipolar cell; BMP, bone morphogenetic protein; D/V axis, dorso/ventral axis; Di, diencephalon; EFTF, eye field transcription factor; GC, ganglion cell; GFP, green fluorescent protein; HC, horizontal cell; Hind, hindbrain; INL, inner nuclear layer; IPL, inner plexiform layer; NR, neural retina; NT, notocord; ONL, outer nuclear layer; OPL, outer plexiform layer; OV, optic vesicle; Ph, photoreceptor; PH3+, phospho-histone H3 positive cell; Rhod, rhodopsin; RPC, retinal precursor cell; RPE, retinal pigmented epitelium; RT-qPCR, real-time quantitative PCR; Shh, sonic hedgehog; St, stage; Syn1, synaptophysin 1; Tel, telencephalon; TGFβ, transforming growth factor β; WM-ISH, whole mount *in situ* hybridization; Wnt, Wnt signaling.

***Address correspondence to:** Andrea Messina. Centre for Integrative Biology - University of Trento, Via Sommarive 9 - 38123 Trento, Italy. Tel.: + 39 0461 282745. Fax: + 39 0461 283937. E-mail: andrea.messina@unitn.it or Simona Casarosa. Centre for Integrative Biology - University of Trento, Via Sommarive 9 - 38123 Trento, Italy. Tel.: + 39 0461 282073. Fax: + 39 0461 283937. E-mail: simona.casarosa@unitn.it

Accepted: 10 March 2016.

ISSN: Online 1696-3547, Print 0214-6282

properties, and stimulate ectodermal cells to acquire neural identity (Smith and Harland, 1992; Wilson and Houart, 2004, Delaune *et al.*, 2005). *noggin 1* is one of the BMP inhibitors that contribute to primary neural induction and mesoderm regionalization in vertebrates (Smith and Harland, 1992; Smith *et al.*, 1993; Zimmerman *et al.,* 1996; Thomsen, 1997). Elegant studies suggested that BMP inhibition by *noggin* overexpression could confer a retina-forming competence to *Xenopus* animal blastomeres normally not fated to become retina (Moore and Moody, 1999). In the last ten years, *noggin 1* was also shown to contribute to the activation of EFTFs in *Xenopus laevis* pluripotent animal cap embryonic stem (ACES) cells (Zuber *et al.,* 2003; Lan *et al.*, 2009; Viczian *et al.*, 2009). The overexpression of high levels of *noggin 1* activates Nodal/TGFβ and Shh pathways in ACES cells eliciting a retinal fate *in vitro*, and these cells are able to induce the formation of a normal eye after transplantation in the eye field region (Lan *et al.*, 2009; Messina *et al.*, 2015). Similar results were obtained treating ACES cells with Noggin protein (Viczian *et al.*, 2009). Moreover, *noggin* shows a conserved expression in the adult retina of different vertebrates (Messina *et al.*, 2014). These studies strongly point to the idea that *noggin 1* may exert a yet unexplored but crucial role in retinal development. However, no *in vivo* data are available that might support this hypothesis. In *Xenopus*, three different *noggin* genes have been isolated: *noggin 1, 2* and *4* (Fletcher *et al.,* 2004; Eroshkin *et al.,* 2006). *Noggin 2* and *4* are expressed at a later developmental stage and in different territories than *noggin 1* (Eroshkin *et al.,* 2006). *Noggin 2* is expressed in the telencephalon and seems to antagonize BMPs, activin/nodal and Wnt signaling (Eroshkin *et al.*, 2006; Bayramov *et al.*, 2011). *Noggin 4* is less characterized than the other two but it seems unable to antagonize BMP during dorso-ventral axis formation in planarians and *Xenopus* (Molina *et al.,* 2011).

Here we performed an analysis of *noggin* expression during early *Xenopus* development, with specific attention to the eye. Our results suggest that *noggin 1* is the most expressed *noggin* gene in the developing *Xenopus* eye. For this reason, we then overexpressed *noggin 1* by *in vivo* electroporation in the developing optic vesicle. Our results indicate that *noggin 1*, but not *noggin 2* nor *noggin 4*, is involved in eye development and that reinforcing *noggin 1* expression in the optic vesicle reduces the number of bipolar cells generated in the electroporated eye, while leaving the other retinal cell types unaffected. We propose that a wave of BMP signaling could play a pivotal role in the maturation of the vertebrate retina and in the differentiation of specific retinal subpopulations during eye development.

Results

Noggin *expression in the developing* Xenopus *retina*

We performed RT-qPCR experiments in order to analyse *noggin* genes expression between blastula stage (stage 9) and mature larval stage (stage 42) in which the retina is fully formed and functional. As expected, *noggin 1* is detected at high levels in blastula stage embryos, and its expression remains the highest detected also at neurula stage (stage 15, in which the neural plate is formed the and the eye field starts to develop), as shown in Fig. 1A and B. We then analysed the expression levels of the *noggin* genes in dissected optic vesicles (stage 25, Fig. 1C) and eyes (stage 42, Fig. 1D). Our results show that *noggin 1* is the most abundantly expressed *noggin* gene in eye structures at both stages analysed. *Noggin 2* was present at lower levels with respect to *noggin 1* in all analysed stages (Fig. 1A-D). In fact low levels of *noggin 2* transcripts were detected at blastula and neurula stages but also in the forming optic vesicle (stage 25) and in the mature eye (stage 42). On the contrary, *noggin 4* mRNA was the least abundant *noggin* gene, showing expression levels at the limit of detection in all analysed stages (Fig. 1A-D). These data only partially overlap with our analysis on adult retinae, in which we described a prominent expression of *noggin 1* and low but comparable levels of *noggin 2* and *noggin 4* (Messina *et al.*, 2014).

We performed whole mount *in situ* hybridization experiments (WM-ISH) in order to localize *noggin* genes expression during *Xenopus* optic vesicle and eye development, using probes specific for *noggin 1*, *2* and *4*. Expression patterns of *noggin* genes were previously published, but they did not describe any detail of their possible localization in the developing eye (Fletcher *et al.*, 2004; Eroshkin *et al.*, 2006, Molina *et al.*, 2011). At early neurula, *noggin 1* was expressed along the midline and on the borders of the anterior neural plate with the most intense expression in the region that will give rise to the dorso-lateral forebrain (Fig. 2A). At late neurula (stage 18), *noggin 1* was strongly detected in the lateral walls of the diencephalon, from which the optic vesicles will form, and more weakly in the anterior telencephalon (Fig. 2B). At stage 21, a consistent expression of *noggin 1* was present in the diencephalon and at low levels in the optic vesicles, telencephalon, hindbrain and spinal cord (Fig. 1C). At stage 25, *noggin 1* showed

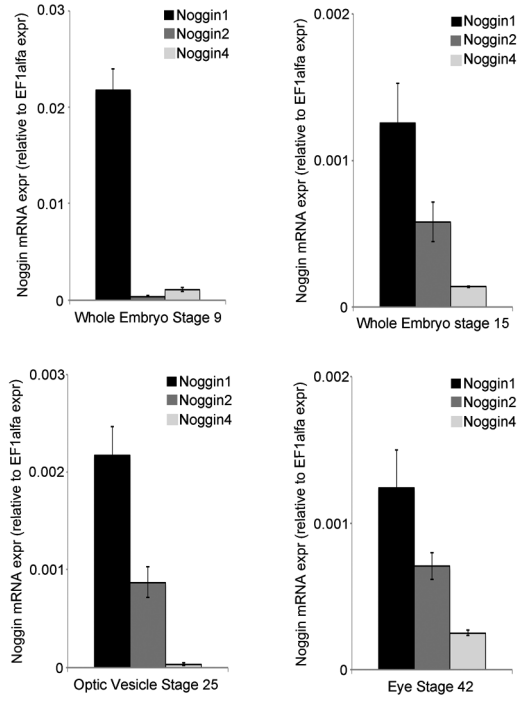

Fig. 1. *Noggin 1, noggin 2* and *noggin 4* expression during different *Xenopus* developmental stages by RT-qPCR. *At all stages analysed* noggin 1 *mRNA is more expressed than* noggin 2 *and* 4. Noggin 2 *mRNA increases in parallel to forebrain development.* Noggin 4 *is at the limit of detection in all samples analysed. Values (mean ± s.d. from three independent experiments) are normalized to EF1α expression at the same stage.*

multiple expression domains, such as the olfactory epithelium, diencephalon, mesencephalon, the anterior part of the hindbrain and the border of the optic vesicles (Fig. 2D).

Noggin 2 was expressed in the anterior neural ridge at stage 15 and remained localized in the most anterior portion of the forebrain (stage 18) and telencephalon (stages 21 and 25) (Figure 2E-H), as previously shown in both *Xenopus tropicalis* and *laevis* (Fletcher *et al*., 2004; Eroshkin *et al*., 2006).

Noggin 4 expression was at the limit of detection for WM-ISH between stage 15 and 18, after which a faint expression seemed to be localized in the anterior neural folds (Fig. 2J, K, L). *Noggin 4* expression levels remained low until stage 25 (Fig. 2M). At stage 25 however, *noggin 4* became weakly detectable in the head epidermis and in the dorsal part of the neural tube including the forebrain and the eye (Fig. 2M).

We also analysed *noggin* genes expression during later eye development, but low expression levels did not allow for *in situ hybridization* experiments on sections, while WM-ISH processed embryos displayed a strong background that masked the specific signal for all tested probes (data not shown). For these reasons, we reported noggin protein expression in stage 42 mature retinae, using an antibody that recognizes all noggin proteins. We observed a prominent expression of noggin in the outer nuclear layer (ONL; Fig. 2 M,O,P), which colocalized with rhodopsin (Fig. 2 O,P,Q), a specific photoreceptor marker. Noggin immunoreactivity was also detected in the outer and inner plexiform layers (OPL and IPL, respectively) (Fig. 2 Q,S,T) as shown by the colocalisation with synaptophisin 1 (Fig. 2 S,T), which stains synapses localized in these two layers. This data is in line with our previous results, showing a similar distribution in the adult *Xenopus* retina (Messina *et al*., 2014). Taken together, mRNA and protein expression data just described suggest that, at stage 42, noggin protein immunoreactivity could be prevalently due to *noggin 1* and *2* expression, rather than to *noggin 4*. They also suggest that *Xenopus noggin 1* could be involved in some, if not all, phases of optic vesicle and eye development until final retina maturation, when *noggin 2* also starts to be expressed.

Overexpression of noggin 1 *in the optic vesicle*

Previous studies have analysed *noggin 1* role starting from the initial phases of *Xenopus* development (Smith and Harland, 1992; Bayramov *et al*., 2011) or in *ex-vivo* experiments using animal cap explants (Zuber *et al*., 2003; Lan *et al*., 2009; Viczian *et al*; 2009; Wong *et al*., 2015; Messina *et al*., 2015). No data are reported for a possible role of *noggin 1* in later stages of eye development *in vivo*, however.

To address this issue, we performed electroporation experiments in order to specifically overexpress *noggin 1* in the developing *Xenopus* optic vesicle and retina. Electroporation experiments were carried out at stage 26 (Falk *et al*., 2007; Baudet *et al*., 2011). At this stage, the optic vesicles have detached from the lateral walls of the diencephalon, to which they remain attached by the optic stalk. This allows diffusion of molecules and their subsequent cellular transfer by electroporation specifically in one of the optic vesicles, leaving the contralateral one unaltered. We electroporated pCS2+-Noggin1 plasmid DNA (together with pCS2+-GFP) and analysed eye development at stage 35 and at stage 42, verifying if *noggin 1* overexpression had an effect on eye size or on retinal precursors proliferation/differentiation. Fig. 3 (A-F) shows that electroporation specifically targeted the eye and not other regions of the head or brain. Our data showed that

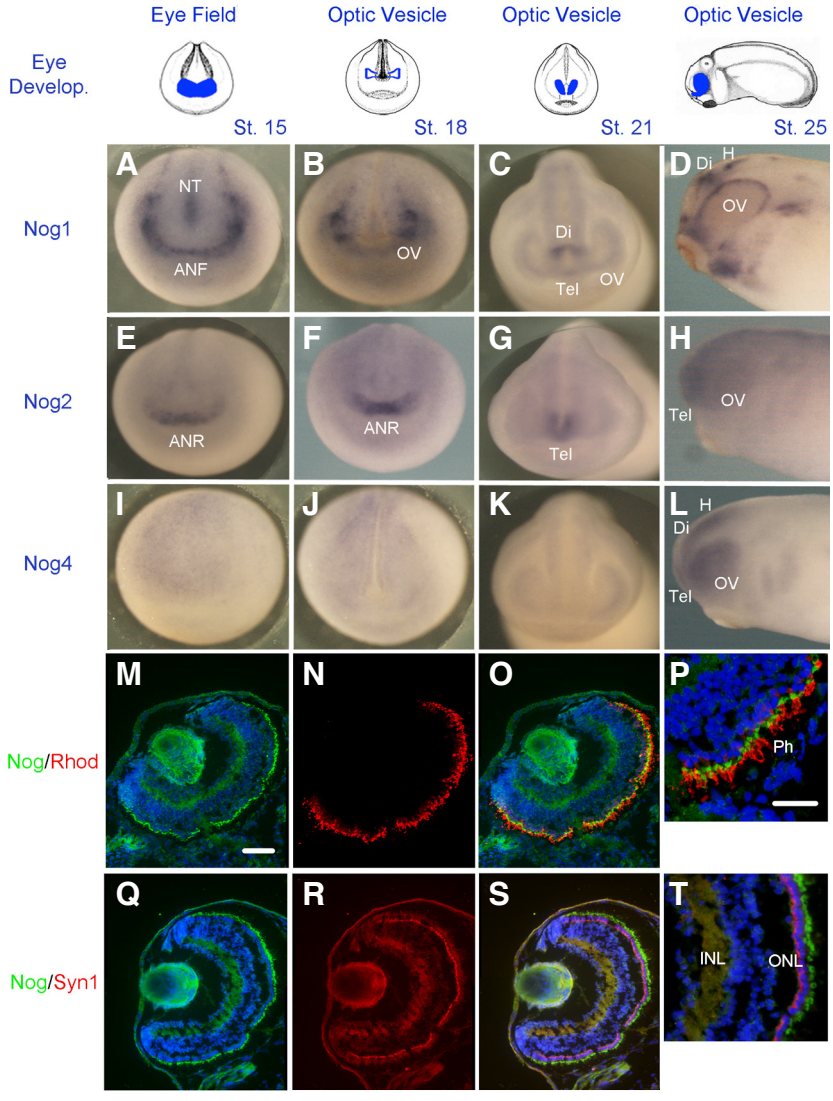

Fig. 2. *Noggin 1, noggin 2* and *noggin 4* expression during different *Xenopus* developmental stages by *in situ* hybridization and immunohistochemistry. (A-D) noggin 1 *expression at stage 15 (A), stage 18 (B), stage 22 (C) and stage 25 (D).* **(E-H)** noggin 2 *expression at stage 15 (E), stage 18 (F), stage 22 (G) and stage 25 (H).* **(I-L)** noggin 4 *expression at stage 15 (I), stage 18 (J), stage 22 (K) and stage 25 (L).* **(M-Q)** *Co-localization of noggin (M) with rhodopsin (N), merge (O) and magnification (P).* **(Q-T)** *Co-localization of noggin (Q) with synaptophysin (R), merge (S) and magnification (T). Scale bar 50 μm.* **(M-T)** *Nuclei are counterstained with DAPI. ANF, anterior neural fold; ANR, anterior neural ridge; Di, diencephalon; IPL, inner plexiform layer; NT, notocord; OPL, outer plexiform layer; OV, optic vesicle; Ph, photoreceptor; Tel, telencephalon.*

electroporation of neither GFP alone nor GFP + Noggin1 affected D/V or A/P axes with respect to wild-type controls at both stages analysed (Fig. 3G), suggesting that *noggin 1* overexpression did not alter the total size of the eye.

In other model organisms, BMP signaling can induce retinal regeneration by proliferation of stem/progenitor cells (Haynes *et al.*, 2007). Moreover, our previous *ex vivo* experiments showed an increase in EdU incorporation in *noggin* overexpressing cells, indicating increased proliferation (Lan *et al.*, 2009). Therefore, we decided to analyse retinal precursors proliferation following *noggin 1* electroporation. In order to identify alterations in retinal precursors proliferation, we counted proliferating cells by labeling them with the phospho-histone H3 (PH3) antibody (Fig. 3 H-K). We then evaluated the percentages of GFP+/PH3+ cells with respect to the number of GFP+ cells at both stage 35 (Fig. 3 H,J,L) and stage 42 (Fig. 3 I,K,M) in eyes electroporated with both GFP (Fig. 3 H,I,L,M) and GFP + Noggin 1 (Fig. 3 J,K,L,M). *Noggin 1* electroporation did not affect proliferative state of retinal precursors at neither stage analysed with respect to GFP only controls (Fig. 3 L,M). These data support our previous work in which we showed a role for *noggin 1* in the acquisition of retinal identity in ACES cells, but not in the proliferation of retinal progenitors (Messina *et al.*, 2015).

Influence of noggin 1 *overexpression on retinal precursor differentiation*

We then tested the ability of *noggin 1* to influence the differentiation of retinal precursors by analyzing, at stage 42, electroporated retinal neurons. At this stage retinal neurons have acquired a mature phenotype, and it is thus possible to distinguish and count the different types of neuronal cells on the basis of their morphology and laminar localization (Fig. 4 A,B). GFP + *noggin 1* overexpressing retinae showed a slight but significant reduction in the number of bipolar cells (from 35% to 31%, p-value = 0.012; Fig. 4 D,E) with respect to GFP only (Fig. 4 C,E) overexpression. On the contrary, no significant effects were detected in other retinal populations. Bipolar cells and rod photoreceptors are the last retinal neurons to be born in the retina, while cones are generated among the first cell types (Andreazzoli, 2009). In order to visualize possible changes in the number of rods or cones in our electroporated retinae, we performed immunohistochemistry analyses using the rhodopsin 1D4 antibody, and specifically counted the number of GFP + rods in the electroporated retinae (Fig. 4 F-I). We show that in GFP only conditions the number of rods is 55% of the total photoreceptors number, as reported in literature (Hamm *et al.*, 2009). The overexpression of *noggin 1* does not alter the ratio of rods to cones, leaving the rod percentage unaltered (Fig. 4I), thus indicating no involvement of Noggin 1 in photoreceptors differentiation.

Discussion

The aim of this study was to investigate the role of *noggin 1* in the generation of retinal neurons during *Xenopus* development. *Noggin* genes are among the main BMP inhibitors, being involved in different developmental processes such as neural induction and mesoderm dorsalization, also contributing to the differentiation of specific brain areas (Smith and Harland, 1992; Smith *et al.*, 1993; Zimmerman *et al.,* 1996; Thomsen, 1997; Wilson and Houart, 2004, Delaune *et al.*, 2005; Bayramov *et al.*, 2011; Molina *et al.*,

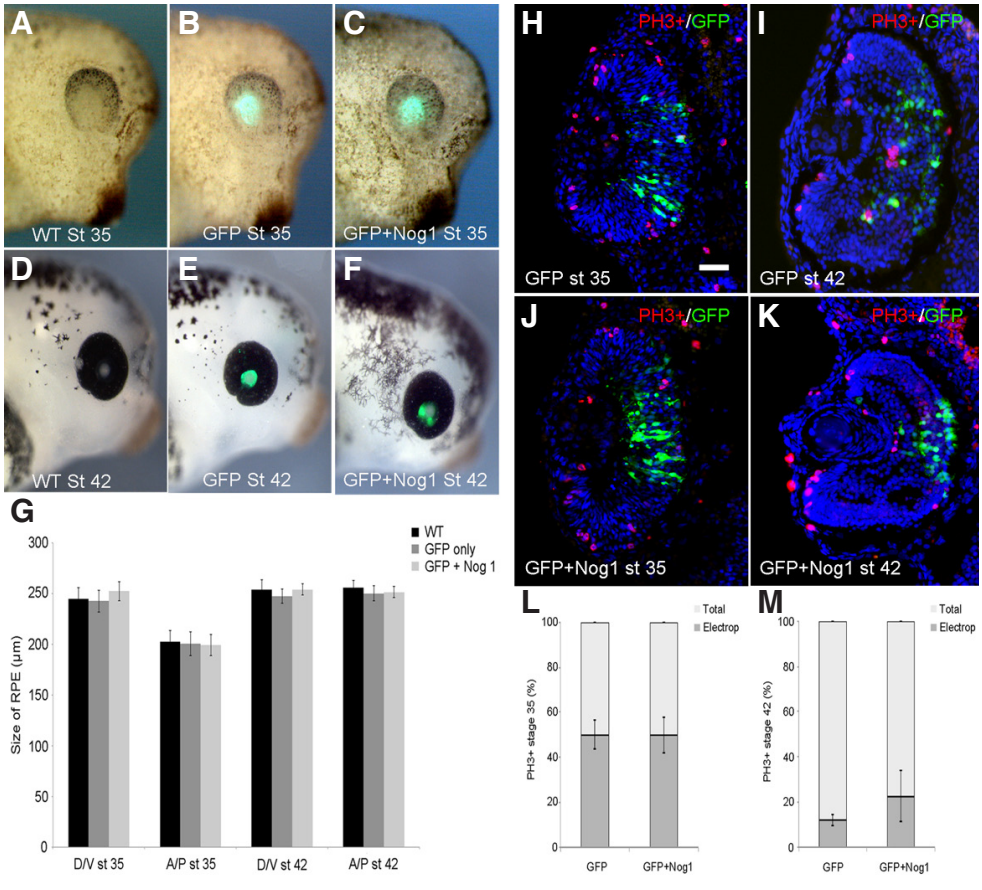

Fig. 3. Effects of *noggin 1* overexpression on eye size and on proliferation of retinal precursors (stage 35 and 42). (A,D) *Wild-type embryos at stage 35 (A) and stage 42 (D).* **(B,E)** *GFP electroporated embryos at stage 35 (B) and stage 42 (E).* **(C,D)** *GFP +* noggin 1 *electroporated embryos at stage 35 (C) and stage 42 (D). (G) Size of the RPE in wild-type (dark grey); GFP only (grey) and GFP +* noggin 1 *(light grey) samples. Values (mean ± s.d.) derive from the measure of the RPE size of 25 embryos for each condition. D/V st 35 (dorso-ventral axis stage 35); A/P st 35 (antero-posterior axis stage 35); D/V st 42 (dorso-ventral axis stage 42); A/P st 42 (antero-posterior axis stage 42).* **(H-K)** *Immunohistochemistry with anti-PH3 antibody (in red) and DAPI (in blue).* **(H, I)** *GFP electroporated embryos at stage 35 (H) and stage 42 (I).* **(J, K)** *GFP +* noggin 1 *electroporated embryos at stage 35 (J) and stage 42 (K).* **(H-K)** *Nuclei are counterstained with DAPI.* **(L,M)** *Percentage of PH3+ cells on GFP and GFP +* noggin 1 *electroporated eyes with respect to the total number of electroporated cells at stage 35 (L) and stage 42 (M). Scale bar 50 µm. Values (mean ± s.d.) derive from the count of GFP+/PH3+ cells in 19 eyes at stage 35 and 16 eyes at stage 42 electroporated with GFP only or GFP +* noggin 1.

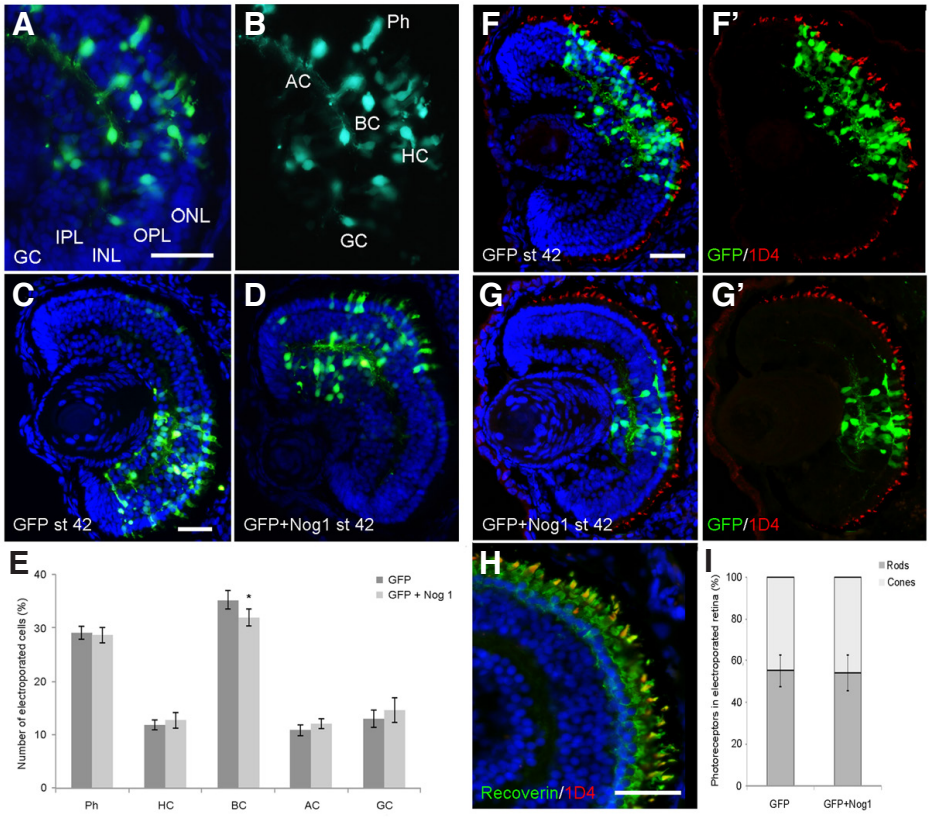

Fig. 4. Effect of *noggin 1* overexpression on retinal neuron specification (stage 42). (A) *Retinal layers;* **(B)** *retinal cells disposition in retinal layers.* **(C)** *GFP electroporated retina and* **(D)** *GFP +* noggin 1 *electroporated retina.* **(E)** *Percentages of different cell types in retinae electroporated with GFP (grey) and GFP +* noggin 1 *(light grey). Scale bar 50 μm. p≤0.05 (*).* **(F-H)** *Immunohistochemistry with rhodopsin 1D4 antibody (in red) on electroporated eyes. (F, F') GFP electroporated eye. (G, G') GFP +* noggin 1 *electroporated eye. (H) Co-localization between recoverin, a pan-photoreceptor marker (in green), and rhodopsin 1D4, a rod-specific marker (in red). (A, C, D, F, G, H) Nuclei are counterstained with DAPI.* **(I)** *Percentage of rods and cones in GFP and GFP +* noggin 1 *electroporated eyes. Scale bar 50 μm. AC, amacrine cell; BC, bipolar cell; GC, ganglion cell; HC, horizontal cell; INL, inner nuclear layer; IPL, inner plexiform layer; ONL, outer nuclear layer; OPL, outer plexiform layer; Ph, photoreceptor. Values (mean ± s.d.) derive from the count of cells in 16 eyes at stage 42 electroporated with GFP only or GFP +* noggin 1.

2011; Messina *et al*., 2015). On this aspect, in the last years new roles for *noggin* genes were discovered. In fact, recent data suggests that *noggin 2* is important for telencephalic development by inhibiting BMPs, but also TGF-βs and WNTs (Bayramov *et al*., 2011). *Noggin 4* was shown to be unable to antagonize BMPs and recover ventralized embryos (Molina *et al*., 2011). Instead, *noggin 1* contributes to forebrain organization and is able to drive ACES cells toward a diencephalic/retinal fate *in vitro* (Lan *et al*., 2009; Viczian *et al*., 2009; Messina *et al*., 2015). We also hypothesized that *noggin* may contribute to the maintenance of retinal structure and function in the adult vertebrate eye (Messina *et al*., 2014).

Previous studies showed that *noggin 1* was expressed both in the Spemann organizer and in the neural tube, but reported its expression in the eye as background (Smith and Harland, 1992). In the light of more recent evidence, we reconsidered *noggin 1* gene expression during *Xenopus* eye development in order to establish its possible contribution to the formation of retinal structures in *in vivo* experiments. Our expression studies support previously published data showing that *noggin 1* is expressed during *Xenopus* brain development and so is *noggin 2,* albeit at lower levels, and that *noggin 4* is expressed at low levels during the development of somites, branchial arches and central nervous system in *Xenopus* embryos (Smith and Harland, 1992; Fletcher *et al*., 2004; Eroshkin *et al*., 2006).

In this work, we also showed a specific localization of *noggin 1* mRNA during different phases of eye development by *in situ* hybridization and RT-qPCR experiments, supporting our hypothesis that *noggin 1* can also contribute to retinal development *in vivo*. Our gain-of-function experiments confirm this hypothesis. We showed that *noggin 1* overexpression in the optic vesicle did not alter eye size with respect to GFP-only controls, and did not affect proliferation of retinal progenitors in the electroporated regions, but it was able to influence the differentiation of retinal progenitors. We reported a slight but significant reduction in the number of bipolar cells in the INL of *noggin 1*-electroporated retinae with respect to GFP only retinae, but no significant differences were detected in the differentiation of other retinal subpopulations. Recently, two independent studies showed that a BMP wave is necessary to specify cells located in the INL of the mouse retina (Kuribayashi *et al*., 2014; Ueki *et al*., 2015), but no data are available for other vertebrates. Kuribayashi and collaborators (2014) showed that the overexpression of BMPs or BMP receptors increased the number of Müller glia and bipolar cells at the expense of rods, supporting the idea that BMPs play a pivotal role in the maturation of these two retinal cell types. Our data show that in *Xenopus* embryos, blocking BMP signaling by *noggin 1* overexpression reduces the number of bipolar cells, thus suggesting that the putative role for BMPs in bipolar cell maturation might be conserved among vertebrates. The reduction we observe is not very strong, one reason for this could be that we overexpress an amount of *noggin 1* sufficient to only partially inhibit BMP signaling. We speculate that during late retinal development, regulation of BMP signaling mediated by *noggin 1* might be necessary for the specification of the latest born retinal cells. In this case, loss-of-function experiments for *noggin 1* should result in an increase in bipolar cells, as already observed following BMP signaling up-regulation.

To summarize, the data presented in this work support the idea that a wave of BMP signaling, regulated by *noggin 1*, could be involved in the maturation of the vertebrate retina and in the differentiation of specific retinal subpopulations during eye development.

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 as previously reported (Messina *et al.*, 2014). All efforts were made to minimize animal suffering. Embryos were staged according to Nieuwkoop and Faber (1956).

Whole mount in situ *hybridization*

Whole mount *in situ* hybridization (WM-ISH) was performed as described (Casarosa *et al.*, 1997) using the following probes: *noggin 1*, *noggin 2*, *noggin 4*. *Noggin 1* probe was transcribed using pCS2-Noggin 1 plasmid linearized with Cla I and Transcribed with T7 promoter. *Noggin 2* and *noggin 4* probes were cloned by RT-PCR using the following primers: T7-Noggin2-For 5'- AATACGACTCACTATAGGGTTTGCTTGTGGCTGTTCTG-3' and SP6-Noggin2-Rev 5'-GATTTAGGTGACACTATGACTGACTTGATGGGCTTG-3'; T7-Noggin4-For 5'-TAATACGACTCACTATAGGGACTCTTGGGACTTGGGGACT-3' and SP6-Noggin4-Rev 5'-GATTTAGGTGACACTATAGCCAGGTACATTGCTGTGTGG-3'. Images were acquired using a Leica ZM16F Stereomicroscope.

RT-qPCR experiments

Whole embryos (stage 9 and stage 15), optic vesicle (stage 25) and eyes (stage 42) were collected and frozen in dry-ice for total RNA extraction. RNA was extracted using Nucleospin RNA/Protein Kit (FC140933N; Macherey-Nagel). RNAs were quantified with NanoDrop (Thermo Scientific) and checked for integrity by 1% agarose gel electrophoresis. cDNAs (1 μg) were prepared using SuperScript VILO cDNA synthesis Kit (11754250; Life Technologies). RT-qPCR experiments were performed in a CFX384 thermocycler (Bio-Rad) using SYBR Fast Universal Ready Mix Kit (KK4601; Kapa Biosystems) according to Messina *et al.,* (2015). Data were analysed using Bio-Rad CFX manager software (Bio-Rad). RT-qPCR primers used are listed in Messina *et al.* (2014).

Electroporation in Xenopus *embryos*

pCS2-GFP and pCS2-Noggin1 were electroporated in *Xenopus laevis* embryos according to Falk *et al.,* (2007). Briefly, vitelline membranes were removed and stage 25 embryos were placed in 0.1× MBS (88 mM NaCl/1 mM KCl/2.4 mM NaHCO3/10 mM Hepes/0.8 mM MgSO4/0.33 mM Ca(NO3)2/0.4 mM CaCl2) containing 0.4 mg/ml MS222 (E10521, Sigma-Aldrich). Anaesthetized embryos were transferred into the transfection T chamber. Platinum electrodes were placed into the transverse channel of T chamber. Needle was charged with pCS2-GFP (1 μg/μl) only or pCS2-GFP plus pCS2-Noggin 1 (1 μg/μl), 30 nl of DNA(s) solution was injected in the optic vesicle lumen using an air-pressured injector (Picospritzer II, Intracel) and an electric pulse was applied using a TSS20 OVODYNE electroporator (Intracel). The electroporated embryos were collected and transferred in a new dish containing fresh 0.1xMBS and grown at 18°C until the desired stage. Both the capillary and electrodes were manipulated using manual micromanipulators (Fine Science Tools).

Eye size measurements

Size of the wild type and electroporated eyes was evaluated measuring the length of the D/V (dorso-ventral) and A/P (antero-posterior) axes of the RPE using the ruler function of PhotoshopCS3 as previously reported (Messina *et al.*, 2015). Statistically significant differences were evaluated with respect to D/V and A/P eye of wild-type embryos.

Immunohistochemistry

Fifteen μm-thick cryostat sections were prepared from embryos at stage 35 and stage 42 and used for immunohistochemistry as described by Messina *et al.,* (2014). Sections were washed in 1xPBS and blocked for 1 hr in 1xPBS containing 0.3% bovine serum albumin and 0.1% Triton X-100. Sections were incubated for 2 h with primary antibodies (Table 1) diluted in blocking solution and then washed three times in 1xPBS plus 0.1% Tween-20. Sections were then incubated with secondary antibodies (Table 1) for 1 hr and again washed three times in 1xPBS containing 0.1% Tween-20. Nuclear staining was performed with DAPI (1:10,000 dilution in 1x PBS). Images were acquired at 20x or 40x magnification using a Zeiss Axio Observer z1 fluorescence microscope (Zeiss; Oberkochen, Germany) and processed using the Zeiss AxioVision software (v4.3.1).

TABLE 1

PRIMARY AND SECONDARY ANTIBODIES USED FOR IMMUNOHISTOCHEMISTRY EXPERIMENTS

Antigen	Antiserum	Source (cat. No.)	Dilution
Primary antibodies			
noggin	rabbit polyclonal	ABCAM (ab 16054)	1:50
phospho-H3	rabbit polyclonal	Millipore (06-570)	1:500
recoverin	rabbit polyclonal	Millipore (AB 5585)	1:750
rhodopsin	mouse monoclonal	Sigma-Aldrich (O 4886)	1:500
rhodopsin 1D4	mouse monoclonal	Sigma-Aldrich (R 5403)	1:500
synaptophysin 1	rabbit polyclonal	Synaptic System (101 002)	1:500
Secondary antibodies			
Biotin-conj. anti-rabbit	goat polyclonal anti-rabbit	DBA (BA-1000)	1:500
Alexa 594	goat polyclonal anti-rabbit	Invitrogen srl (A11037)	1:1000
Alexa 594	goat polyclonal anti-mouse	Invitrogen srl (A11032)	1:1000
Strept-conj. Alexa 488		Invitrogen srl (S11223)	1:500

GFP positive cell count

Electroporated cells were counted, scoring the different cell types, on the basis of their morphology and laminar position as reported by Ohnuma *et al.,* (2002) and Casarosa *et al.,* (2003).

PH3 and Rhod1D4 positive cell count

GFP+/PH3+ cells were counted in the central sections derived from 19 eyes at stage 35 and 16 eyes at stage 42 electroporated with GFP only or GFP + *noggin 1*. Rhod1D4 positive cells were counted on all sections derived from 28 eyes (stage 42) electroporated with GFP only or GFP + *noggin 1* in order to establish the percentage of cones and rods influenced by the electroporation. Antibodies used are reported in Table 1.

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 $p < 0.05$.

Acknowledgements

We are grateful to Tania Incitti for helpful discussions. We thank the Model Organism Facility and Advanced Imaging Core Facility of the University of Trento for help in animal management and technical procedures, and Patrizia Paoli and Giorgia Moser for administrative support. This work was funded by a University of Trento Startup Grant to YB and SC and Cassa di Risparmio di Trento e Rovereto Grant n. 2011.0251 (SC). This work was funded by the University of Trento Statup Grant to YB and SC, Cassa di Risparmio di trento e Rovereto Grant n.2011.0251 (SC), G. Armenise-Harvard Foundation (MLB) and Marie Curie CIG grants (MLB).

References

ANDREAZZOLI M (2009). Molecular regulation of vertebrate retina cell fate. *Birth Defects Res C Embryo Today* 87: 284-295.

BAUDET ML, ZIVRAJ KH, ABREU-GOODGER C, MULDAL A, ARMISEN J, BLENKIRON C, GOLDSTEIN LD, MISKA EA, HOLT CE (2011). miR-124 acts through

CoREST to control onset of Sema3A sensitivity in navigating retinal growth cones. *Nat Neurosci* 15: 29-38.

BAYRAMOV AV, EROSHKIN FM, MARTYNOVA NY, ERMAKOVA GV, SOLOVIEVA EA, ZARAISKY AG (2011). Novel functions of Noggin proteins: inhibition of Activin/Nodal and Wnt signaling. *Development* 138: 5345-5356.

CASAROSA S, ANDREAZZOLI M, SIMEONE A, BARSACCHI G (1997). *Xrx1*, a novel *Xenopus* homeobox gene expressed during eye and pineal gland development. *Mech Dev* 61: 187-198.

CASAROSA S, AMATO MA, ANDREAZZOLI M, GESTRI G, BARSACCHI G, CREMISI F (2003). *Xrx1* controls proliferation and multipotency of retinal progenitors. *Mol Cell Neurosci* 22: 25-36.

DELAUNE E, LEMAIRE P, KODJABACHIAN L (2005). Neural induction in *Xenopus* requires early FGF signalling in addition to BMP inhibition. *Development* 132: 299-310.

DORSKY RI, CHANG WS, RAPAPORT DH, HARRIS WA (1997). Regulation of neuronal diversity in the *Xenopus* retina by Delta signalling. *Nature* 385: 67-70.

EROSHKIN FM, ERMAKOVA GV, BAYRAMOV AV, ZARAISKY AG (2006). Multiple noggins in vertebrate genome: cloning and expression of noggin2 and noggin4 in *Xenopus laevis*. *Gene Expr Patterns* 2: 180-186.

FALK J, DRINJAKOVIN J, LEUNG KM, DWINVEDY A, REGAN AG, PIPER M, HOLT CE (2007). Electroporation of cDNA/Morpholinos to targeted areas of embryonic CNS in *Xenopus*. *BMC Dev Biol* 7: 107.

FLETCHER RB, WATSON AL, HARLAND RM (2004). Expression of *Xenopus tropicalis* noggin1 and noggin2 in early development: two noggin genes in a tetrapod. *Gene Expr Patterns* 5: 225-230.

HAMM LM, TAM BM, MORITZ OL (2009). Controlled rod cell ablation in transgenic *Xenopus laevis*. *Invest Opthalmol Vis Sci* 50: 885-892.

HAYNES T1, GUTIERREZ C, AYCINENA JC, TSONIS PA, DEL RIO-TSONIS K. (2007). BMP signaling mediates stem/progenitor cell-induced retina regeneration. *Proc Natl Acad Sci U S A* 104: 20380-20385.

KURIBAYASHI H, BABA Y, WATANABE S (2014). BMP signaling partipates in late phase differentiation of the retina, partly via upregulation of Hey 2. *Dev Neurobiol* 74: 1172-1183.

LAN L, VITOBELLO A, BERTACCHI M, CREMISI F, VIGNALI R, ANDREAZZOLI M, DEMONTIS GC, BARSACCHI G, CASAROSA S (2009). Noggin elicits retinal fate in *Xenopus* animal cap embryonic stem cell. *Stem Cells* 27: 2146-2152.

MESSINA A, INCITTI T, BOZZA A, BOZZI Y, CASAROSA S (2014). Noggin expression in vertebrate retina suggests a conserved role during vertebrate evolution. *J Histochem Cytochem* 62: 532-540.

MESSINA A, LAN L, INCITTI T, BOZZA A, ANDREAZZOLI M, VIGNALI R, CREMISI F, BOZZI Y, CASAROSA S (2015). Noggin-mediated retinal induction reveals a novel interplay between BMP inhibition, TGFβ and SHH Signaling. *Stem Cells* 33:2496-2508 (doi: 10.1002/stem.2043).

MOLINA MD, NETO A, MAESO I, GÓMEZ-SKARMETA JL, SALÓ E, CEBRIÀ F (2011). Noggin and noggin-like genes control dorsoventral axis regeneration in planarians. *Curr Biol* 21: 300-305.

MOORE KB, MOODY SA. (1999). Animal-vegetal asymmetries influence the earliest steps in retina fate commitment in *Xenopus*. *Dev Biol* 212: 25-41.

NIEUWKOOP PD, FABER J (1956). Normal Table of *Xenopus laevis* (Daudin). Elsevier-North Holland Publishing Co.

OHNUMA S, MANN F, BOY S, PERRON M, HARRIS WA (2002). Lipofection strategy for the study of *Xenopus* retinal development. *Methods* 28: 411-419.

SMITH WC, HARLAND RM (1992). Expression cloning of noggin, a new dorsalizing factor localized to the Spemann organizer in *Xenopus* embryos. *Cell* 70: 829-840.

SMITH WC, KNECHT AK, WU M, HARLAND RM (1993). Secreted noggin protein mimics the Spemann organizer in dorsalizing *Xenopus* mesoderm. *Nature* 361: 547-549.

THOMSEN GH (1997). Antagonism within and around the organizer: BMP inhibitors in vertebrate body patterning. *Trends Genet* 13: 209-211.

UEKI Y, WILKEN MS, COX KE, CHIPMAN LB, BERMINGHAM-MCDONOGH O, REH TA (2015). A transient wave of BMP signaling in the retina is necessary for Muller glia differentiation. *Development* 142: 533-543.

VICZIAN AS, SOLESSIO EC, LYOU Y, ZUBER ME (2009). Generation of functional eyes from pluripotent cells. *PLoS Biol* 7:e1000174.

WILSON SW, HOUART C (2004). Early step in development of forebrain. *Dev Cell* 6: 167-181.

WONG KA, TREMBLEY M, ABD WAHAB S, VICZIAN AS (2015). Efficient retina formation requires suppression of both Activin and BMP signaling pathways in pluripotent cells. *Biol Open* 4: 573-583. (doi: 10.1242/bio.20149977)

ZAGOZEWSKI JL, ZHANG Q, EISENSTAT DD (2014). Genetic regulation of vertebrate eye development. *Clin Genet* 86: 453-60.

ZIMMERMAN LB, DE JESÚS-ESCOBAR JM, HARLAND RM (1996). The Spemann organizer signal noggin binds and inactivates bone morphogenetic protein 4. *Cell* 86: 599-606.

ZUBER ME, GESTRI G, VICZIAN AS, BARSACCHI G, HARRIS WA (2003). Specification of the vertebrate eye by a network of eye field transcription factors. *Development* 130: 5155-5167.

Further Related Reading, published previously in the *Int. J. Dev. Biol.*

A change in response to Bmp signalling precedes ectodermal fate choice
Chris T. Dee, Abigail Gibson, Andrea Rengifo, Shun-Kuo Sun, Roger K. Patient and Paul J. Scotting
Int. J. Dev. Biol. (2007) 51: 79-84
http://dx.doi.org/10.1387/ijdb.062204cd

Retinal stem cells in vertebrates: parallels and divergences
Marcos A. Amato, Emilie Arnault and Muriel Perron
Int. J. Dev. Biol. (2004) 48: 993-1001
http://dx.doi.org/10.1387/ijdb.041879ma

Regulation of vertebrate eye development by *Rx* genes
Travis J. Bailey, Heithem El-Hodiri, Li Zhang, Rina Shah, Peter H. Mathers and Milan Jamrich
Int. J. Dev. Biol. (2004) 48: 761-770
http://dx.doi.org/10.1387/ijdb.041878tb

Retinal stem cells and regeneration
Ala Moshiri, Jennie Close and Thomas A. Reh
Int. J. Dev. Biol. (2004) 48: 1003-1014
http://dx.doi.org/10.1387/ijdb.041870am

The BMP antagonists cerberus-like and noggin do not interact during mouse forebrain development
A C Borges, S Marques and J A Belo
Int. J. Dev. Biol. (2001) 45: 441-444
http://dx.doi.org/10.1387/ijdb.11330864

5 yr ISI Impact Factor (2013) = 2.879