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RESEARCH ARTICLE

Tbx3 represses *bmp4* expression and, with Pax6, is required and sufficient for retina formation

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ABSTRACT

Vertebrate eye formation begins in the anterior neural plate in the eye field. Seven eye field transcription factors (EFTFs) are expressed in eye field cells and when expressed together are sufficient to generate retina from pluripotent cells. The EFTF Tbx3 can regulate the expression of some EFTFs; however, its role in retina formation is unknown. Here, we show that Tbx3 represses *bmp4* transcription and is required in the eye field for both neural induction and normal eye formation in *Xenopus laevis*. Although sufficient for neural induction, Tbx3-expressing pluripotent cells only form retina in the context of the eye field. Unlike Tbx3, the neural inducer Noggin can generate retina both within and outside the eye field. We found that the neural and retina-inducing activity of Noggin requires Tbx3. Noggin, but not Tbx3, induces Pax6 and coexpression of Tbx3 and Pax6 is sufficient to determine pluripotent cells to a retinal lineage. Our results suggest that Tbx3 represses *bmp4* expression and maintains eye field neural progenitors in a multipotent state; then, in combination with Pax6, Tbx3 causes eye field cells to form retina.

KEY WORDS: Noggin, Eye field transcription factor, Retinal specification, Retinal determination, *Xenopus laevis*

INTRODUCTION

Normal brain development requires the coordinated activity of both extrinsic and intrinsic regulators. These factors first repress bone morphogenetic protein (BMP) signaling in the early ectoderm to induce the formation of multipotent neural progenitor cells, then specify and determine the neural plate to form distinct regions of the adult nervous system. High levels of BMP signaling specify epidermis, while low BMP signaling results in a neural fate. Excessive *bmp4* expression in the anterior neural plate results in a reduction or total absence of anterior neural structures, including eyes (Hartley et al., 2001, 2002). Noggin, and other BMP antagonists, bind BMP and prevent it from activating BMP receptors (Lamb et al., 1993; Re'em-Kalma et al., 1995). Noggin may also indirectly regulate *bmp4* transcription, since BMP4 protein can regulate its own transcription in a positive-autoregulatory feedback loop (Jones et al., 1992; Schmidt et al., 1995; Hammerschmidt et al., 1996; Piccolo et al., 1997; Gammill and Sive, 2000; Gestri et al., 2005). Together, these activities result in pluripotent ectoderm cells being determined to form multipotent neural, then retinal progenitors. Noggin not only drives pluripotent cells to form retina in the context of the eye field, but also determines cells to form retina on the embryonic flank and even in culture (Lan et al., 2009; Viczian et al., 2009; Wong et al., 2015).

In *Xenopus laevis*, the eye field transcription factor Tbx3 was originally identified as ET (eye T-box) (Li et al., 1997). Of the eye field transcription factors, Tbx3 has the most restricted eye field expression domain and is expressed prior to all EFTFs except Six3 (Zuber et al., 2003). Tbx3 functions downstream of Noggin and upstream of other EFTFs, and is a necessary component of the eye field transcription factor network sufficient to induce ectopic and functional eyes (Zuber et al., 2003; Viczian et al., 2009). In direct contrast to other EFTFs, Tbx3 misexpression does not induce ectopic retina or even enlarge the retina in *Xenopus* embryos (Mathers et al., 1997; Andreazzoli et al., 1999; Chow et al., 1999; Zuber et al., 1999; Bernier et al., 2000; Takabatake et al., 2002; Wong et al., 2002). Tbx3 is important for both the establishment and maintenance of stem cell pluripotency and can inhibit differentiation of progenitor cells, yet its role in early eye formation has not been determined (Davenport et al., 2003; Ivanova et al., 2006; Lu et al., 2011).

Here, we report that during early neural development, Tbx3 represses *bmp4* expression and maintains eye field cells in a multipotent neural progenitor state. Our results suggest that the local environment, not Tbx3, determines the differentiated fate of Tbx3-induced neural progenitor cells. We show that Tbx3, like Noggin, is a neural inducer, and the ability of Noggin to drive neural and retinal formation is Tbx3 dependent. The repressor activity of Tbx3 is required for proper patterning of the anterior neural plate at eye field stages and retinal progenitor cells lacking Tbx3 die during retinal development, resulting in abnormal eye formation. Lastly, we show that, together, Tbx3 and Pax6 are sufficient for retinal determination. Our results indicate that Tbx3 is important for maintaining neural progenitors of the early eye field in a multipotent state, allowing them to respond to local cues that determine a retinal fate.

RESULTS

Tbx3 and Pax6 are the only EFTFs sufficient to drive pluripotent cells to a retinal lineage in the context of the eye field

We asked which EFTFs can specify retina by injecting both blastomeres of 2-cell staged embryos with EFTFs and YFP, then transplanted donor animal cap cells to the stage 15 eye field of host embryos. We then sectioned the resulting retinas and analyzed the expression of the rod photoreceptor marker XAP-2 (animal cap transplant to eye field, ACT→EF, Fig. 1) (Harris and Messersmith, 1992; Viczian et al., 2009; Viczian and Zuber, 2010). Transplanted cells isolated from embryos expressing YFP only, or YFP with Otx2, Rax, Six3, Six6 or Nr2e1, formed only

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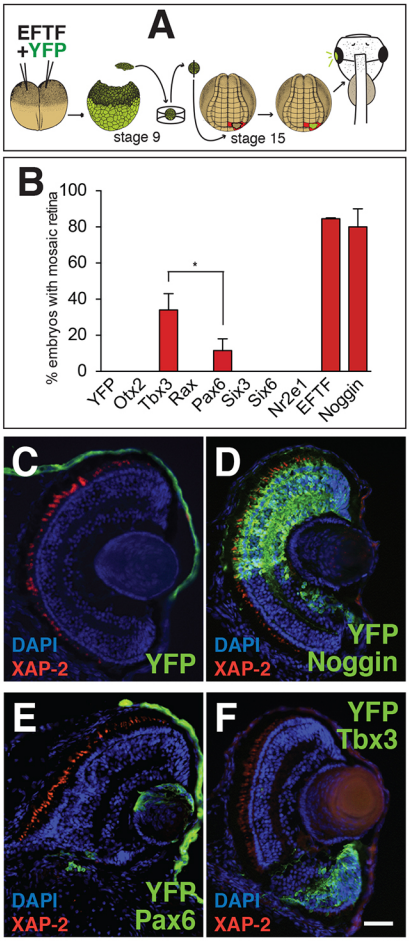

Fig. 1. Tbx3 is sufficient to specify pluripotent cells to a retinal lineage. (A) Schematic illustrating the animal cap transplant (ACT) to eye field assay (ACT→EF; Viczian and Zuber, 2010). (B) Histogram showing the percentage of tadpoles in which transplanted cells formed retina in response to the expression of YFP only, YFP with the indicated EFTF, an EFTF cocktail or Noggin (YFP only, 500 pg; Otx2, 25 pg; Tbx3, 50 pg; Rax, 50 pg; Pax6, 100 pg; Six3, 25 pg; Six6, 25 pg; Nr2e1, 25 pg; EFTF cocktail; Noggin, 2.5 pg). (C-F) Sections of tadpoles receiving transplants expressing (C) YFP and (D) Noggin, (E) Pax6 or (F) Tbx3. XAP-2 (red), DAPI (blue) and YFP (green) detect rod photoreceptors, nuclei and transplanted cells, respectively. Dorsal is up. N=2; n≥40; *P≤0.05. Scale bar: 50 µm.

epidermis (Fig. 1B,C and not shown). Only *tbx3* and *pax6* were sufficient to specify retinal cells (Fig. 1B,E,F). The number of embryos with donor cells forming retina was greater with *tbx3* than *pax6* (Fig. 1B, *tbx3* 35%; *pax6* 12%). In contrast, both *noggin* (*nog*) and the complete EFTF cocktail (*otx2* and the EFTFs *tbx3*, *pax6*, *rax*, *six3*, *six6* and *nr2e1*), efficiently specified retina (Fig. 1B,D; *noggin*, 80%; EFTF cocktail, 83%). Taken together, these results indicate that only Tbx3 and Pax6 are competent to specify pluripotent cells to a retinal lineage in the context of the eye field (Zuber et al., 2003; Viczian et al., 2009).

Tbx3 is required for normal eye formation

Tbx3 is expressed in the anterior neural plate at eye field stages (Li et al., 1997; Wong et al., 2002; Zuber et al., 2003; Weidgang et al., 2013). We used *in situ* hybridization to more precisely define the *tbx3* expression pattern (Fig. S1). Although detected in previously unreported tissues, the expression pattern of *tbx3* was consistent with a role in eye field specification (Fig. S1A-H). In addition, we discovered both *tbx3* homeologs are expressed in the developing eye field (Fig. S1I). We used *tbx3*-specific morpholinos (MOs) to determine whether Tbx3 is required for normal eye formation. Tbx3MO-LS targets a sequence predicted to inhibit translation of both *tbx3* homeologs (light blue, Fig. 2A; Fig. S2), whereas Tbx3MO-S only targets *tbx3.S* (dark blue, Fig. 2A; Fig. S2). Because antibodies recognizing *Xenopus laevis* Tbx3 are not available, we generated fusion constructs to test the translation blocking ability of the morpholinos (Fig. 2B; Fig. S2C-H″). Tbx3MO-LS inhibited translation of both Tbx3.L and Tbx3.S, while Tbx3MO-S only inhibited Tbx3.S expression (Fig. 2B; Fig. S2C-H″).

Embryos unilaterally injected into one dorsal blastomere (D1) at the 8-cell stage with MOs were grown to stage 43 tadpoles for analysis (Fig. 2C-F). The eye on the injected side of tadpoles treated with 10 ng of CoMO or Tbx3MO-S morpholino were indistinguishable from control, wild-type embryos (Fig. 2C,D). In contrast, injection with 10 ng of Tbx3MO-LS morpholino reduced eye size in 94% of tadpoles (Fig. 2E).

Eye size varied little in wild-type, uninjected tadpoles or embryos injected with YFP, CoMO or Tbx3MO-S (Fig. 2G). In contrast, knockdown with Tbx3MO-LS reduced dorsoventral eye diameter by 29±1.6% (Fig. 2G). Similar effects were observed in the anteroposterior eye width (Fig. S2I). In addition to the reduction in overall eye diameter, the lens diameter and pigmentation of the RPE were also noticeably reduced. Injection into non-retinogenic blastomeres did not alter eye formation (V1, 0%, n=65; D2, 0%, n=58; V2, 0%, n=63, not shown) (Moody and Kline, 1990).

To determine if both homeologs were required, we co-injected MOs at suboptimal levels. When injected individually, 5 ng of either MO did not alter eye size significantly (Fig. 2H). Co-injection of CoMO with Tbx3MO-S or Tbx3MO-LS (10 ng total) also did not alter eye size (Fig. 2H). However, injection of Tbx3MO-S and Tbx3MO-LS together synergistically reduced both the dorsoventral and anteroposterior eye diameter relative to controls (Fig. 2H, DV 21±1.6%; Fig. S2J, AP 11±1.6%).

To confirm the reduction in eye size produced by Tbx3 knockdown was due to an eye field-specific reduction of Tbx3, we injected Tbx3MO-LS into the most retinogenic dorsal blastomeres of 16- and 32-cell staged embryos (Moody, 1987a, b; Huang and Moody, 1993). Tbx3MO-LS reduced eye size in 57% of embryos injected into blastomere D1.1 at the 16-cell stage (n=35) and 75% of embryos injected in D1.1.1 at the 32-cell stage (n=24; data not shown). Finally, as an independent test to confirm eye defects were caused by Tbx3 knockdown, we generated a MO targeting the exon 1 splice donor sites of both *tbx3.L* and *tbx3.S* (Tbx3MO-SP), resulting in an in-frame stop codon in the unspliced transcripts (Fig. S3A,B). Injection of Tbx3MO-SP increased the amount of unspliced *tbx3.L* and *tbx3.S* transcripts and resulted in eye defects similar to those observed with Tbx3MO-LS (Fig. S3C-H). Together, these results indicate that eye field expression of Tbx3 is required for normal eye formation, and either Tbx3.L or Tbx3.S may be sufficient for eye formation.

Tbx3 is required for the neural and retinal-specifying activity of Noggin

To determine whether Tbx3 knockdown altered the retina-specifying activity of Noggin, we repeated the experiments of Fig. 1, but co-injected Tbx3MO-LS (for simplicity, referred to as Tbx3MO from here on) with Noggin and asked if cells formed

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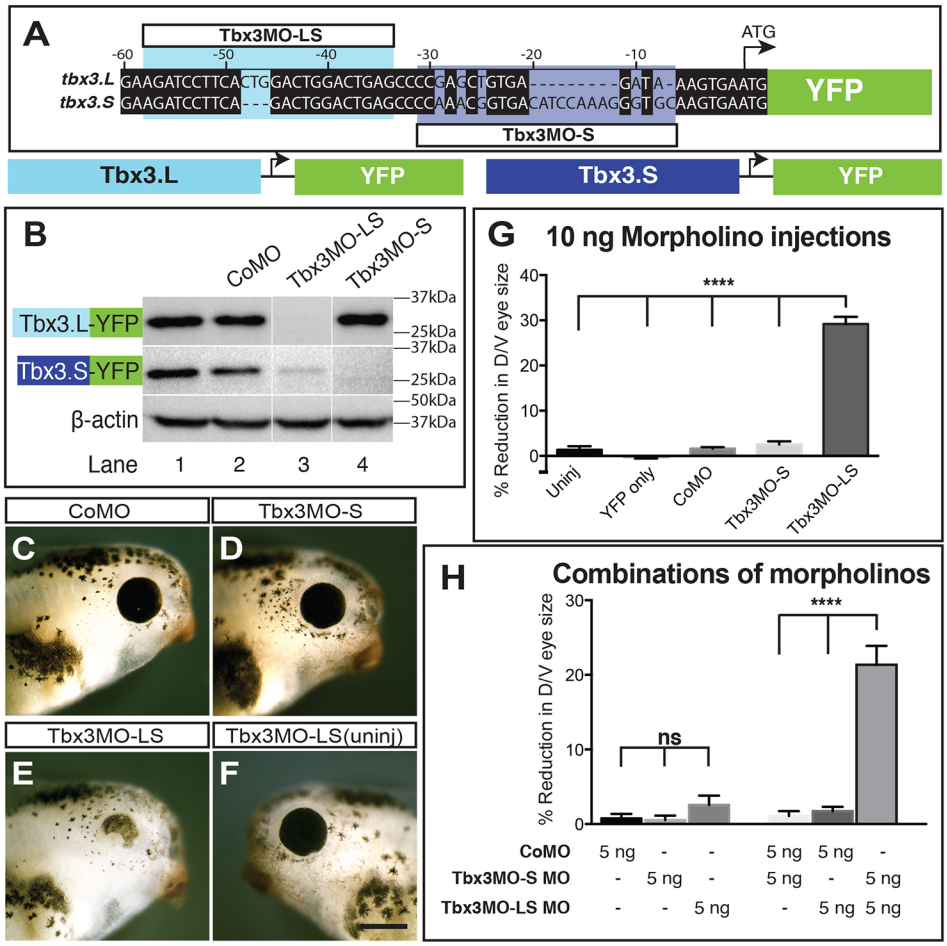

Fig. 2. *Tbx3* is required for normal eye formation. Design and test of Tbx3 morpholino activity. (A) Comparison of *X. laevis tbx3.L* and *tbx3.S* homeologs showing the position of morpholino target sequences. (B) Western blots were used to detect the expression of YFP and β-actin (loading control) in extracts prepared from embryos injected at the 2-cell stage (both blastomeres) with 10 ng of the indicated morpholino and Tbx3.L/.S-YFP RNA. (C-E) Injected side of tadpoles treated with 10 ng CoMO (C), Tbx3MO-S (D) or Tbx3MO-LS (E). (F) Uninjected side of tadpole in E. (G,H) The percentage reduction in eye size was determined by comparing the dorsoventral (D/V) eye diameter on the uninjected and injected sides. Histograms show eye diameter reduction in tadpoles injected with YFP RNA and the indicated morpholino (G) or combination of morpholinos (H). N=2; $n \geq 29$; ****P<0.0001; ns, not significant. Scale bar: 200 µm.

retina). Cells injected with YFP alone, with CoMO or with Tbx3MO never formed retina (Fig. 3A-C). Transplantation of donor cells expressing Noggin, however, generated mosaic retinas in 89% of animals (Fig. 3D,G). Although co-injection of CoMO did not significantly alter retina-inducing activity of Noggin (76%, P=0.32; Fig. 3E,G), Tbx3MO significantly reduced the number of embryos with YFP^{+} mosaic retinas (22%, $P \leq 0.001$; Fig. 3F,G). To determine if Tbx3MO blocked both the neural, as well as retinal-inducing activity of Noggin, we evaluated expression of Class II β-tubulin (Tubb2b) (Moody et al., 1996). Tubb2b protein was detected in the inner and outer plexiform layers (Fig. 3H,H′). The processes of retinal neurons generated from Noggin-expressing pluripotent cells expressed Tubb2b (Fig. 3I,I′). Surprisingly, Tbx3MO dramatically reduced the expression of Tubb2b in transplants derived from Noggin-expressing cells (Fig. 3J,J′). Together, these results suggest Tbx3 is required for the ability of Noggin to specify pluripotent cells to both a retinal and neural fate in the context of the eye field.

Tbx3 is a neural inducer, but unlike Noggin, is not sufficient to determine retina, yet is required for retinal determination of ectopically grafted eye fields

To further test the hypothesis that Tbx3 is required for both neural- and retinal-inducing activities of Noggin, we transplanted animal cap donor cells to the flank of stage 15 host embryos (ACT→Flank), which were then grown to tadpoles. YFP-expressing donor cells only generated epidermis (Fig. 4A,A′,F,K; Fig. S4A). *Tbx3*-expressing donor cells formed non-pigmented spheres that expressed the neural marker Tubb2b in 83% of transplants (Fig. 4B,B′,G,P; Fig. S4B), but never the rod photoreceptor marker XAP-2 (Fig. 4L,Q). Noggin-expressing controls generated pigmented, ectopic eye-like structures in 35% (YFP) and 33% (YFP+CoMO) of donor transplants (Fig. 4C-D′; Fig. S4C,D), which expressed both Tubb2b (Fig. 4H,I,P) and XAP-2, markers (Fig. 4M,N,Q), and had a morphology consistent with retina formation (Fig. 4M,N). In contrast, donor cells with Noggin and Tbx3MO formed a more lightly pigmented tissue mass, suggesting Tbx3 knockdown resulted in a change from a neural and retinal, to cement gland fate (Fig. 4E,E′,J,O; Fig. S4E). Consistent with this interpretation, transplants were labeled with the cement gland marker *Erythrina cristagalli* lectin (ECL; Fig. S5) (Turton et al., 2004). Tbx3 knockdown reduced the ability of Noggin to induce both neural (Fig. 4J,P) and retinal markers (Fig. 4O,Q) by 78-80%, resulting in the cells taking on a more anterior, non-neural, cement gland morphology.

We next asked whether Tbx3 knockdown has the same effect on intrinsic (rather than Noggin-induced) eye field cells (EF→Flank). Stage 15 eye field cells from embryos injected in one blastomere at the 8-cell stage with YFP alone, or with CoMO or Tbx3MO, were transplanted to the flank of host embryos. Eye field fragments isolated from YFP-only control or CoMO-injected embryos formed ectopic eyes, including retinal pigmented epithelium (RPE), in 90% and 85% of flank transplants, respectively (Fig. 4R,S). In contrast, YFP-positive donor eye field cells from Tbx3MO-injected embryos were never pigmented or laminated (Fig. 4T). Only 27% and 25% of the structures expressed Tubb2b or XAP-2, respectively (Fig. 4W,Z). Transplanted cells were disorganized and

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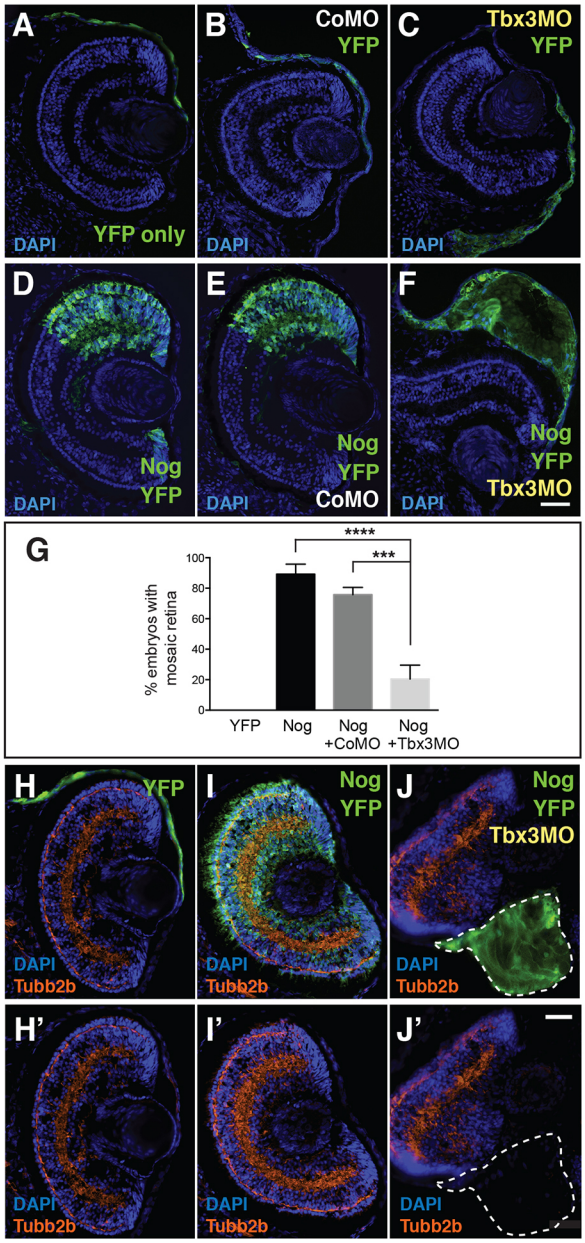

Fig. 3. Tbx3 knockdown inhibits the retinal- and neural-inducing activity of Noggin. (A-F) Retinal sections of tadpoles that received grafts at stage 15 (ACT→EF). (A-C) Donor cells expressed (A) YFP only, or were co-injected with (B) CoMO or (C) Tbx3MO-LS (Tbx3MO). (D-F) Donor cells expressed (D) YFP plus Noggin (Nog) alone, or in combination with (E) CoMO or (F) Tbx3MO. (G) Histogram showing the average percentage of tadpoles in which donor cells formed retina. (H-J′) Retinas of tadpoles receiving donor cells expressing (H,H′) YFP alone, or in combination with (I,I′) Noggin or (J,J′) Noggin with Tbx3MO. Staining marks nuclei (DAPI; blue), donor-derived cells (YFP; green) and neural tissue (Tubb2b; red). Dashed line in J and J′ indicates location of donor cells. Dorsal side up. N=3; n≥40; ***P<0.001, ****P<0.0001. Scale bar: 50 µm.

regions expressing either Tubb2b or XAP-2 were YFP negative. Eight-cell stage injection labels most, but not all donor eye field cells, therefore YFP-negative regions most likely originated from donor eye field cells that did not receive Tbx3MO. These results suggest that Tbx3 is a neural inducer, sufficient to determine pluripotent cells to a neural, but not retinal lineage. Furthermore, Noggin requires Tbx3 to generate both neural and retinal tissues from pluripotent cells.

Tbx3 specifies spinal cord but not retina, while Noggin-expressing cells remain determined to form retina in posterior neural plate transplants

Tbx3-expressing cells formed retina when transplanted to the stage 15 eye field, but not in the stage 15 flank. To test whether the neural plate provides a factor(s) necessary for retina formation that is not present in the flank, we generated ectodermal explants as before, but transplanted the Tbx3-expressing animal cap cells to the stage 15 posterior neural plate instead (ACT→PNP). Transplanted cells only generated epidermis when grafted into the posterior neural plate (Fig. 5A). Cells expressing Noggin generated ectopic eye-like structures in 61% of the transplants (Fig. 5B). Tbx3-expressing donor cells never generated ectopic eye-like structures (Fig. 5C).

To determine the differentiated fate of donor cells, we analyzed the presence of neural, retinal and spinal cord markers in stage 43 tadpoles. In controls, Tubb2b stains the bilaterally symmetrical spinal cord (Fig. 5D). In the enlarged Tubb2b-expressing spinal cord, in addition to ectopic eye-like structures, Noggin-expressing donor cells were present and often distorted the normal symmetry of the tissue (Fig. 5E,M). Although no ectopic eyes were detected in tadpoles that received transplants expressing Tbx3, 88% of the spinal cords were mosaic and 86% expressed Tubb2b (Fig. 5F,M). Noggin-expressing donor cells expressed XAP-2 and rod photoreceptor outer segments in 76% of transplants (Fig. 5H,M). Despite being transplanted to the neural plate, neither control nor Tbx3-expressing cells ever expressed XAP-2 and no evidence of RPE, rod outer segments or lamination was detected (Fig. 5G,I,M).

To determine if transplanted tissues were being specified to spinal cord, we looked for expression of Sox2 and islet proteins (Fig. 5J-L). In the spinal cord, Sox2 is expressed in the ventricular zone (Gaete et al., 2012) (Fig. 5J). Islet-1/2 is expressed in the dorsal Rohon-Beard cells and ventral motor neurons (MNs) (Diez del Corral and Storey, 2001; Olesnicky et al., 2010; Yajima et al., 2014) (Fig. 5J; Fig. S6). Noggin-expressing, YFP-positive donor cells were co-labeled with antibodies against both Sox2 and Islet-1/2 in 91% and 57% of transplants, respectively (Fig. 5K,M; Fig. S6). Islet-1/2-expressing cells are present at positions consistent with the location of motor neurons, as well as throughout the majority of the donor tissue (Fig. 5K; Fig. S6). Expression of the rod photoreceptor marker in these same regions (Fig. 5H) suggests the majority of the stained cells distant from the midline may be retinal ganglion, amacrine, bipolar and/or horizontal cells. Donor cells expressing Tbx3 also expressed Sox2 and Islet-1/2, but in a more restricted expression pattern that is consistent with the expected location of spinal neurons. $YFP^+/Sox2^+$ cells were detected in the ventricular zone in 85% of transplants, while $YFP^+/Islet\text{-}1/2^+$ cells (78% of transplants) were observed in regions consistent with the location of the ventral motor neurons (Fig. 5L,M; Fig. S6).

To determine whether the induction of neural markers occurs in culture, in contrast to grafting Tbx3-expressing cells into an embryo, explants were grown in culture and analyzed for the expression of the neural markers neural cell adhesion molecule 1 (*ncam1*) and *tubb2b* at the equivalent of stage 21. Tbx3 was sufficient to induce expression of both *ncam1* and *tubb2b*, while Noggin strongly induced only *ncam1* (Fig. 5N). To determine if Tbx3 induced neural markers directly, or indirectly through mesoderm induction, we analyzed the expression of the pan-mesodermal marker *xbra*, and the dorsal mesoderm marker cardiac muscle α-actin 1 (*actc1*). Neither Noggin, nor Tbx3 induced mesodermal markers, indicating that both are direct neural inducers (Fig. 5N).

Together, these results indicate that Tbx3, like Noggin, induces neural tissue directly. However, unlike Noggin, Tbx3 is unable to

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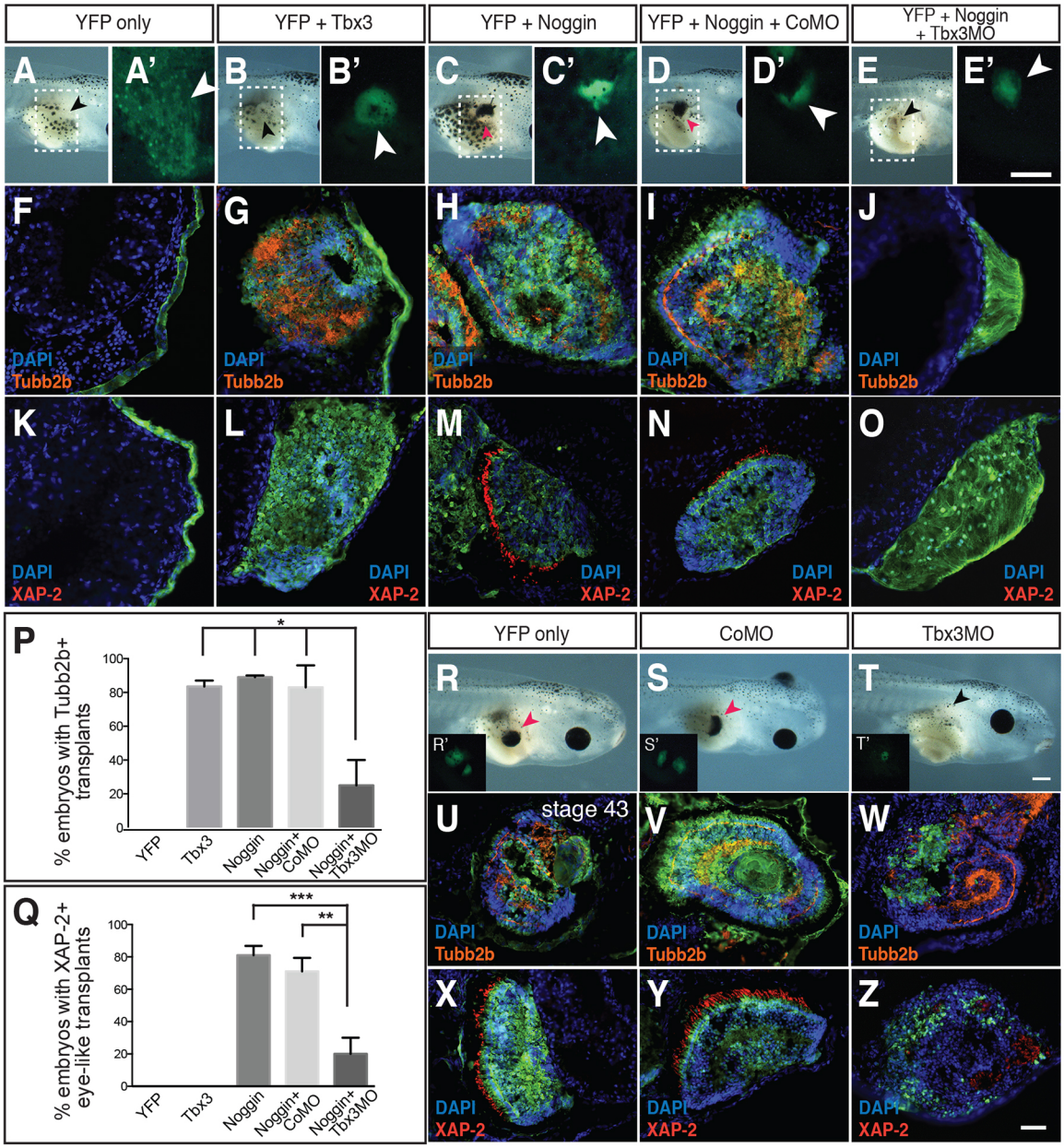

Fig. 4. Tbx3 induces neural but not retinal tissue and is required for Noggin to determine pluripotent cells to a neural and retinal fate. (A-O) Donor cells expressing the indicated RNAs and morpholinos were grafted to the flank of stage 15 host embryos (ACT→Flank). Arrowheads (A-E′) indicate location of grafted cells in brightfield (A-E) and YFP fluorescence (A′-E′) images. Dashed boxes in A-E outline magnified regions in A′-E′. Sections are stained for YFP (F-O, green), the neural marker Tubb2b (F-J, orange) and rod photoreceptor marker, XAP-2 (K-O, red). (P,Q) Histograms showing percentage of donor transplants that were $YFP^+/Tubb2b^+$ or $YFP^+/XAP\text{-}2^+$. (R-Z) Donor eye fields expressing YFP only, with CoMO or Tbx3MO were transplanted to the flank of stage 15 host embryos (EF→Flank). R′-T′ show the location of YFP fluorescence in the flank of embryos in R-T. DAPI-labeled tadpole sections were stained for YFP (green) and Tubb2b (U-W, orange) or XAP-2 (X-Z, red). N=2; $n \geq 39$; $^*P \leq 0.05$, $^{**}P<0.01$, $^{***}P<0.001$. Scale bars: 400 µm (E′,T), 50 µm (Z).

determine pluripotent cells to a retinal lineage outside the eye field, even when cells are transplanted to other regions of the neural plate (ACT→PNP).

Tbx3 represses *bmp4* expression in pluripotent cells and the anterior neural plate during eye field specification

Noggin can repress *bmp4* expression. Since Tbx3 is required for the neural- and retinal-inducing activity of Noggin and both Noggin and Tbx3 are neural inducers, we asked if Tbx3 could also repress *bmp4*. All YFP-expressing explants express *bmp4* (Fig. 6A). In contrast, *bmp4* expression was reduced in explants expressing either Noggin or Tbx3 (Fig. 6B,C). Prior to gastrulation, *bmp4* expression is detected in the dorsal ectoderm (future neural plate) but by stage 12.5, expression is excluded from the neural plate and detected in more anterior and ventrolateral regions of the embryo (Fig. 6D). Unilateral expression of either Noggin or Tbx3 reduced *bmp4* expression on the injected side of embryos (Fig. 6E,F).

To determine if the ability of Noggin to repress *bmp4* expression is also dependent on Tbx3, we isolated ectodermal explants from embryos expressing Noggin in the presence or absence of Tbx3 MOs. Neither control MO nor Tbx3MO alone altered the expression of *bmp4* relative to YFP-expressing explants (Fig. S7A-C). Noggin repressed *bmp4* expression in 91% and 81% of explants when expressed alone or with control MO, respectively (Fig. S7D,E).

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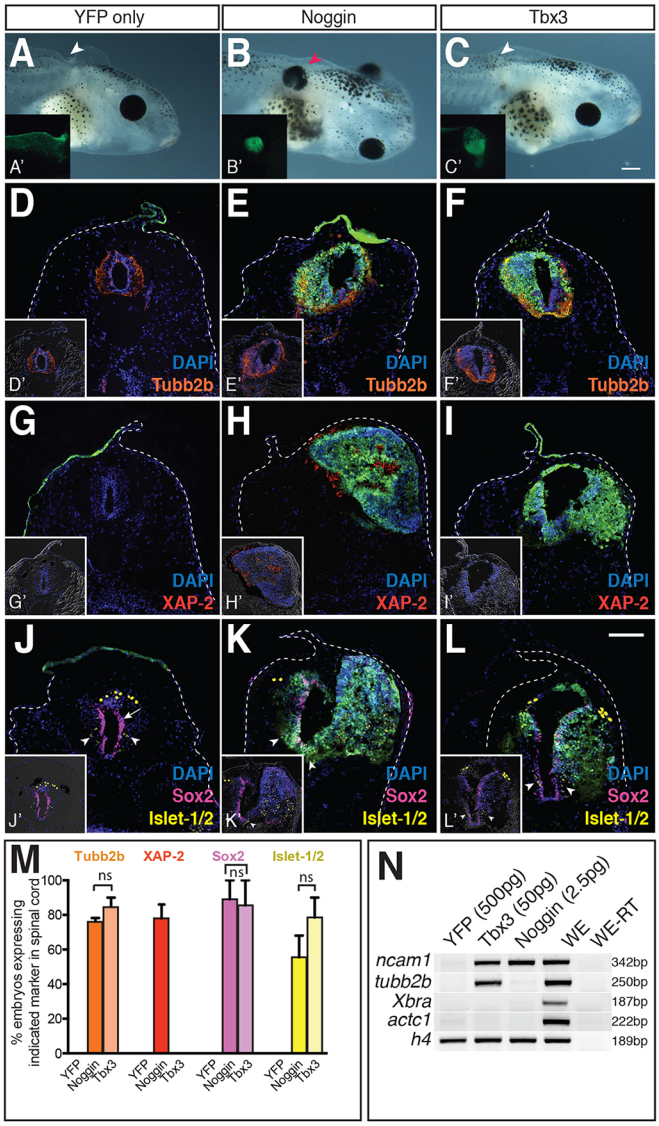

Fig. 5. Tbx3-expressing cells are specified to a spinal cord, not retinal fate when transplanted to the posterior neural plate. (A-L) Ectodermal explants expressing the indicated RNAs were transplanted to the posterior neural plate of stage 15 embryos and grown to tadpoles (ACT→PNP). Arrowheads (A-C) indicate location of YFP donor tissue. (D-L) Donor cells expressing YFP alone (D,G,J) or with Noggin (E,H,K) or Tbx3 (F,I,L). Sections were stained to detect neural (Tubb2b, orange, D-F), retinal (XAP-2, red, G-I) and spinal cord (Sox2, magenta, ventricular zone; Islet-1/2, yellow, Rohon-Beard and motor neuron, J-L) cells. DAPI (blue) and YFP (green) label nuclei and donor cells, respectively. To better visualize cell fate markers, D′-L′ insets show D-L without YFP channel. (M) Histogram showing the percentage of host embryos expressing the indicated markers in mosaic spinal cords. (N) Ectodermal explants isolated from stage 9 embryos injected bilaterally at the 2-cell stage with the indicated RNA were cultured *in vitro* to stage 21. RT-PCR was used to detect *ncam1*, *tubb2b*, *t* (*xbra*), *actc1* and histone H4 (*h4*: loading control). Whole embryos processed with (WE) and without (WE-RT) reverse transcriptase served as positive and negative controls, respectively, $N=2$; $n\geq32$; ns, not significant. Scale bars: 400 µm (C), 100 µm (L).

When Noggin was injected with Tbx3MO, however, *bmp4* expression recovered and was repressed in only 37% of explants (Fig. S7F), indicating that Tbx3 is also necessary for the ability of Noggin to repress *bmp4* expression.

To determine how Tbx3 regulates *bmp4* expression, we generated repressor and activator versions. To avoid disrupting possible roles of Tbx3 function prior to eye field stages, hormone-inducible versions were generated using the ligand binding domain of the glucocorticoid receptor (GR) and activated by dexamethasone treatment (Kolm and Sive, 1995). Dexamethasone did not alter *bmp4* expression in explants of pluripotent cells (compare Fig. 6G with K). Fusion of the entire coding region of Tbx3 to GR (Tbx3-GR) renders Tbx3 activity dexamethasone dependent. *Bmp4* expression was unaltered by Tbx3-GR expression (Fig. 6H), but hormone treatment reduced expression in 87% of explants (Fig. 6L). Similar results were obtained using only the DNA-binding domain (DBD) of Tbx3 fused to the engrailed repressor domain and GR (DBD-EnR-GR). In explants expressing DBD-EnR-GR, *bmp4* expression was reduced in only 8% of explants (Fig. 6I), but reduction of expression increased to 91% when treated with hormone (Fig. 6M). No change in *bmp4* expression was detected when Tbx3 was fused to the transactivation domain of VP16 (VP16-DBD-GR) (Fig. 6N). In explants expressing VP16-DBD-GR, *Bmp4* was detected throughout explants with or without dexamethasone treatment (compare Fig. 6J with N). Together, these results suggest that Tbx3 functions as a transcriptional repressor and is necessary for Noggin to repress *bmp4* expression in cultured explants.

We next asked if Tbx3-GR, DBD-EnR-GR and VP16-DBD-GR can regulate *bmp4* expression *in vivo*. Embryos were injected unilaterally, treated with hormone to activate the fusion constructs and analyzed at early eye field stage (12.5). Dexamethasone did not alter the expression pattern of *bmp4* in control, YFP-injected embryos (compare Fig. 6O with S). In contrast, the frequency of *bmp4* repression was nearly 5-fold greater with hormone treatment in embryos expressing either Tbx3-GR (compare Fig. 6P with T) or DBD-EnR-GR (compare Fig. 6Q with U). VP16-DBD-GR did not alter the expression pattern of *bmp4* in any of the untreated embryos (Fig. 6R). In contrast to explants, however, dexamethasone treatment dramatically altered the expression pattern of *bmp4* in VP16-DVD-GR-expressing embryos. We observed ectopic expression in the posterior neural plate and reduced expression anterior to the neural plate (Fig. 6V). These results suggest that both Noggin and Tbx3 repress *bmp4* expression, and can do so both in isolated ectodermal explants as well as in the anterior neural plate during eye field specification.

The repressor activity of Tbx3 is required for normal neural patterning during eye field stages and for eye formation

Continuous inhibition of BMP signaling is required for normal anterior neural development (Hartley et al., 2001; Gestri et al., 2005) and Tbx3 represses *bmp4* transcription (Fig. 6). To determine if normal anterior neural patterning is regulated by Tbx3 activity, we assessed the effects of DBD-EnR-GR and VP16-DBD-GR on eye field (*rax* and *pax6*), forebrain and midbrain (*otx2*), prospective telencephalon (*foxg1*) and cement gland (*ag1*) markers. In the absence of dexamethasone, marker expression patterns were unaltered (Fig. 7 and not shown). In contrast, activation of DBD-EnR-GR by dexamethasone treatment starting at stage 12.5 resulted in an expansion of the *rax*, *pax6*, *otx2* and to a lesser extent *foxg1* expression domains (Fig. 7H-K), while the expression domain of the cement gland marker *ag1* was reduced in most embryos (Fig. 7L). Activation of VP16-DBD-GR had the opposite effect. *rax*, *pax6*, *otx2* and *foxg1* expression domains were either reduced or completely lost (Fig. 7N-Q), while the *ag1* expression domain appeared diffuse in most embryos (Fig. 7R).

To determine if, and when, the repressor activity of Tbx3 was required for normal eye formation, we activated the VP16-DBD-GR protein in embryos at different time points, grew embryos to

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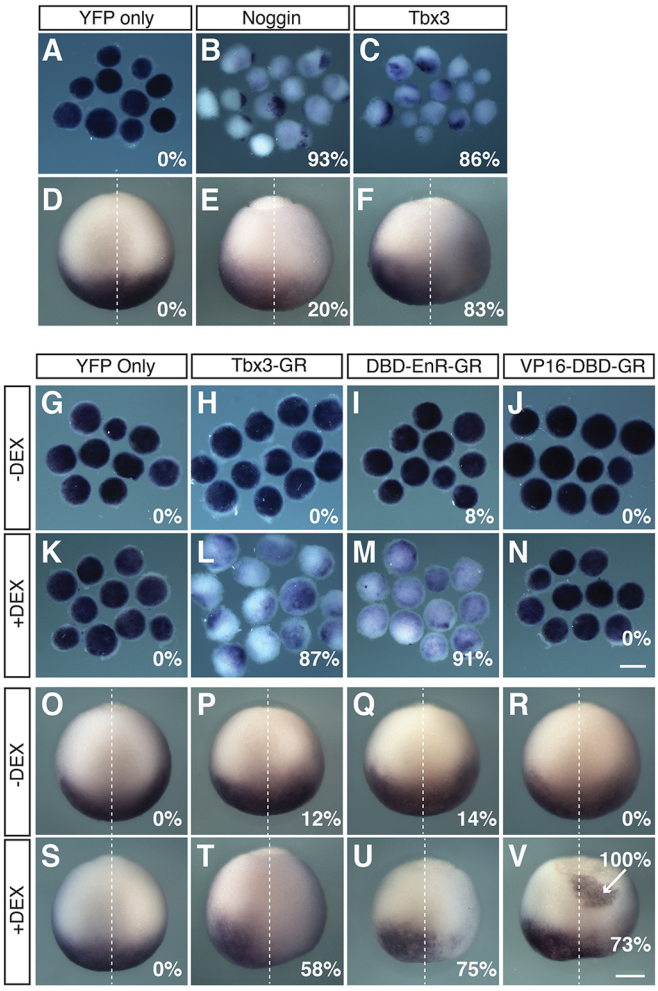

Fig. 6. Noggin and Tbx3 repress *bmp4* expression *in vitro* and *in vivo*. *bmp4* expression was detected by *in situ* hybridization in ectodermal explants and intact embryos. (A-C,G-N) Ectodermal explants were isolated at stage 9 from embryos injected bilaterally at the 2-cell stage with the indicated RNA. Explants were left untreated (A-C,G-J) until stage 22, or treated from stage 15 with dexamethasone (K-N). The percentage of explants with reduced *bmp4* expression is indicated, N=4; $n \geq 36$. (D-F,O-V) 4-cell stage embryos were injected in one dorsal blastomere with the indicated RNA. Embryos were left untreated (D-F,O-R) until stage 12.5, or treated from stage 9 with dexamethasone (S-V). The percentage with reduced *bmp4* expression is indicated, N=3; $n \geq 48$. (V) 100% of embryos showed posterior ectopic *bmp4* expression (arrow), while 73% showed reduced *bmp4* expression anteriorly. RNA injected: 500 pg YFP, 2.5 pg Noggin, 50 pg Tbx3, 100 pg Tbx3-GR, 250 pg DBD-EnR-GR, 5 pg VP16-DBD-GR (doubled for explants). Dorsal view, anterior toward the bottom. Scale bars: 200 µm (N), 400 µm (V).

tadpoles, and determined the effect on eye formation. YFP alone had no detectable effect on eye formation, both in the absence and presence of dexamethasone (Fig. 7S-V). The eyes of embryos injected with VP16-DBD-GR without dexamethasone were only slightly smaller on the injected side in some tadpoles (Fig. 7W,AA). By contrast, VP16-DBD-GR activation with dexamethasone starting at stage 12.5, resulted in eyeless embryos 77% and coloboma 23% of the time, respectively (Fig. 7X,AA). The frequency and severity of eye defects was reduced when dexamethasone treatment was started at later developmental stages (stg. 15, 20, 24), with relatively little effect on eye formation after eye field stages (Fig. 7Y,Z,AA). Therefore, the repressor activity of Tbx3 is required at eye field stages (stg. 12.5-15) not only for reducing *bmp4* expression, but also for normal anterior neural patterning and eye formation.

To address why Tbx3 knockdown in eye field cells results in eye defects, we grafted eye field cells from YFP$^+$ donor embryos injected at the 8-cell stage to wild-type host eye fields when they reached stage 15 (Fig. S8). When eye field cells containing YFP and YFP/CoMO were grafted, the eyes developed normally and YFP$^+$ donor cells could be detected in the eyes of living and sectioned retinas (Fig. S8A-L). In contrast, eyes were smaller and YFP fluorescence was significantly reduced with grafts of YFP/Tbx3MO-expressing eye field cells (Fig. S8M-R). By stage 39, YFP fluorescence was noticeably reduced in live embryos and by stage 43, the total volume of YFP$^+$ donor cells in sectioned retinas was reduced to less than 5% of that in controls (Fig. S8S,T). No increase in YFP fluorescence was detected elsewhere in the embryos, suggesting the reduction in retinal YFP expression observed in YFP/Tbx3MO grafts, was not due to cell migration.

To determine if cell death might explain the loss of donor eye field cells following Tbx3 knockdown, we tested for apoptosis using TUNEL (Fig. 8). At optic vesicle stage (stg. 22) YFP-positive donor cells were present, vesicle morphology appeared normal, and no TUNEL-positive cells were detected in transplants derived from untreated, CoMO- or Tbx3MO-LS-injected hosts (Fig. 8A,A′,E,E′, I,I′,M). In contrast, from stage 25 to 39, there was a significant increase in the number of TUNEL-positive donor eye field cells transplanted from host embryos injected with Tbx3MO-LS (Fig. 8M). In addition, lens and eye formation appeared delayed at these stages, and eyes were smaller in embryos receiving YFP/Tbx3MO-LS eye field transplants (Compare Fig. 8B-D′ and F-H′ to J-L′). A similar number of TUNEL-positive cells were present at stage 35 when the splice-blocking morpholino Tbx3MO-SP was used to knockdown Tbx3 expression in donor eye field cells (Fig. 8N). We conclude that knockdown of Tbx3 in eye field cells resulted in their death during the late optic vesicle and optic cup stages of eye development.

Neither is sufficient, but together Tbx3 and Pax6 can determine retina

We previously demonstrated that Noggin induces *pax6* transcription, while Tbx3 does not (Zuber et al., 2003). We asked if together, Tbx3 and Pax6 could generate retina from pluripotent cells (Fig. 9). Similar to YFP alone (Fig. 9A,F), Pax6-expressing cells generated skin epidermis in flank transplants (Fig. 9B,G). Neither the neural marker Tubb2b nor the rod photoreceptor marker rod transducin were present in YFP (Fig. 9K,P,U) or Pax6-expressing cells (Fig. 9L,Q,U). Despite the fact that Tbx3 induced Tubb2b, rod transducin (Gαt1) was never detected (Fig. 9C,H,M,R, U). In striking contrast, co-expression of Pax6 with Tbx3 not only induced the expression of rod transducin, but cells expressing Tbx3 and Pax6 organized into an eye-like structure (Fig. 9D,I,N). Rod transducin-expressing cells were detected adjacent to the pigmented RPE (Fig. 9I,N,S), similar to expression observed in the ectopic eyes generated from Noggin-expressing cells (Fig. 9E,J,O,T,U). These results suggest, that in addition to inhibiting BMP signaling, Noggin (but not Tbx3) also induces Pax6 expression, and this induction is sufficient, when combined with Tbx3, to drive retinal determination (Fig. 9V).

DISCUSSION

We previously proposed Noggin functions upstream of Tbx3 and other EFTFs, yet little was known about the role of Tbx3 in early eye field formation (Zuber et al., 2003). Our results suggest that Noggin

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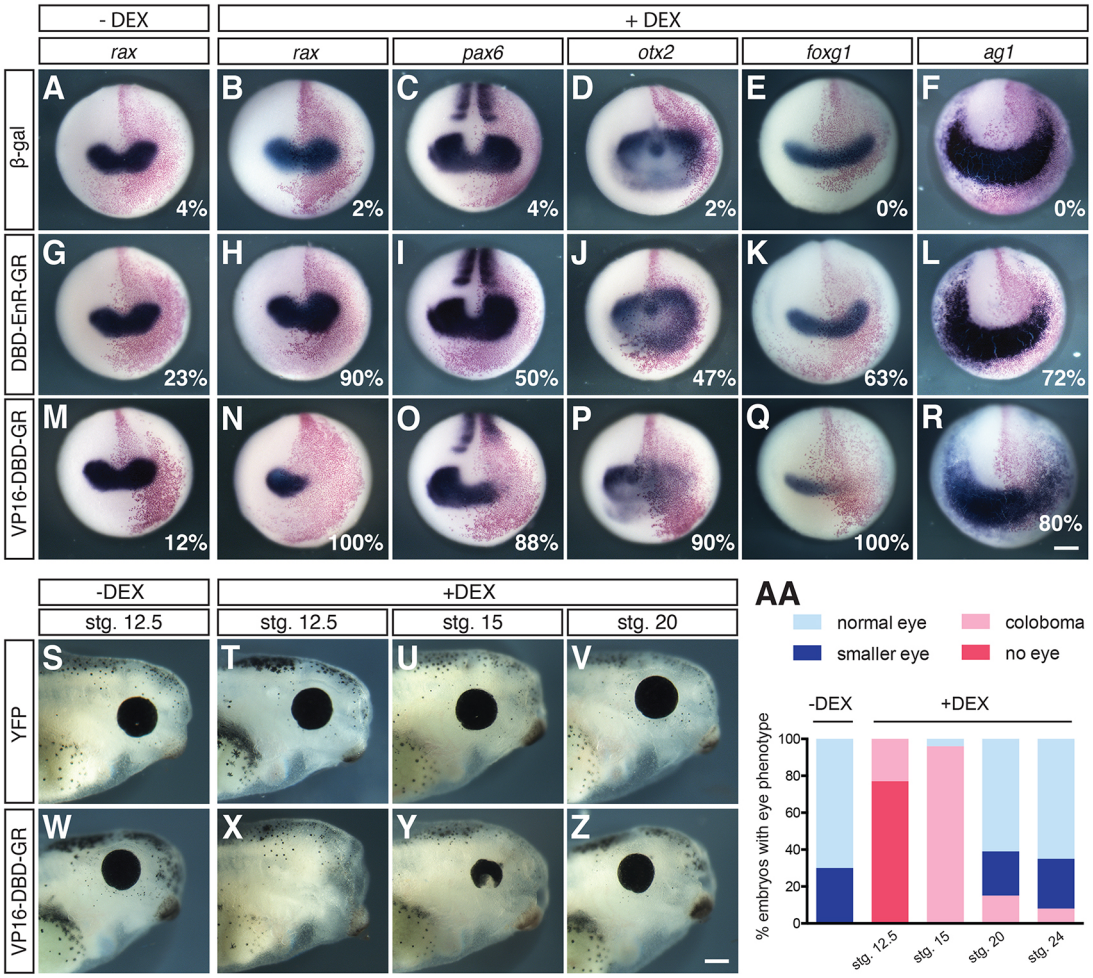

Fig. 7. Tbx3 repressor activity is required at eye field stages for normal neural patterning and eye formation. (A-R) *In situ* hybridization was used to detect changes in *rax*, *pax6*, *otx2*, *foxg1* and *ag1* transcript levels at stage 15. Eight-cell embryos were injected in one blastomere with β-gal alone (150 pg, A-F), and in combination with DBD-EnR-GR (12.5 pg, G-K; 50 pg, L) or VP16-DBD-GR (5 pg, M-R) RNA. At stage 12.5, embryos were untreated (A,G,M) or treated with dexamethasone (B-F, H-L and N-R). The percentage showing a change in expression is indicated, N=2; $n \geq 41$. (S-Z) The repressor activity of Tbx3 is required at eye field stages for normal eye formation. Control (S-V)- and VP16-DBD-GR (W-Z)-injected embryos were untreated (S,W) or treated with dexamethasone starting at the indicated stage. (AA) Histogram shows the percentage of tadpoles with the indicated eye defects, N=2; $n \geq 35$. Scale bars: 300 µm (R), 400 µm (Z).

may repress *bmp4* expression and drive retina formation by inducing *tbx3* expression. However, Noggin instead represses *tbx3* expression in ectodermal explants [Fig. S9A and Zuber et al. (2003)]. Otx2 blocks the repression of *tbx3* by Noggin in ectodermal explants, and is coexpressed with Noggin and Tbx3 in the dorsal blastopore lip and early anterior neural plate suggesting that *tbx3* might be regulated by Otx2 in these regions (Fig. S1; Pannese et al., 1995; Kablar et al., 1996; Zuber et al., 2003). However, *otx2* expression does not overlap that of *tbx3* during eye field specification or determination (Zuber et al., 2003). Noggin can expand the eye field expression domain of *tbx3 in vivo* (Fig. S9B,C). Together, these results indicate that unidentified regulator(s) are required *in vivo* (possibly with Noggin) to maintain Tbx3 expression in the eye field (Fig. 10). It will also be important to determine if Tbx3 is regulated by and/or required for the neural-inducing activity of other known direct neural inducers (Ozair et al., 2013).

Repression of BMP signaling during neural development

Acquisition and stabilization of a neural fate requires constant inhibition of BMP signaling in the neural plate. Since Tbx3 expression is restricted, could other T-box family members also repress BMP signaling in the neural plate? *Xenopus* Tbx1-Tbx5 are all expressed during neural development and have non-identical expression patterns (Li et al., 1997; Takabatake et al., 2000; Sasagawa et al., 2002; Showell et al., 2006). Tbx1 suppresses BMP signaling in the mouse hair follicle stem cell niche and cardiomyocytes, while Tbx4 and Tbx5 both repress BMP2 during chick limb development (Rodriguez-Esteban et al., 1999; Fulcoli et al., 2009; Chen et al., 2012). Tbx2, in contrast, activates BMP signaling in the mouse limb bud (Farin et al., 2013). T-box family members have complex and sometimes combinatorial roles in multiple tissues, developmental processes and species (Washkowitz et al., 2012; Papaioannou, 2014). Therefore, additional T-box family members may also regulate BMP signaling to specify and determine other regions of the developing nervous system (including the eye) in *Xenopus* and other species. In addition to the EFTFs Six3 and Tbx3, there is a growing number of transcription factors expressed in the early neural plate that can repress BMP signaling (Gestri et al., 2005; Moody et al., 2013; Lee et al., 2014). What remains to be determined is if other inhibitors of

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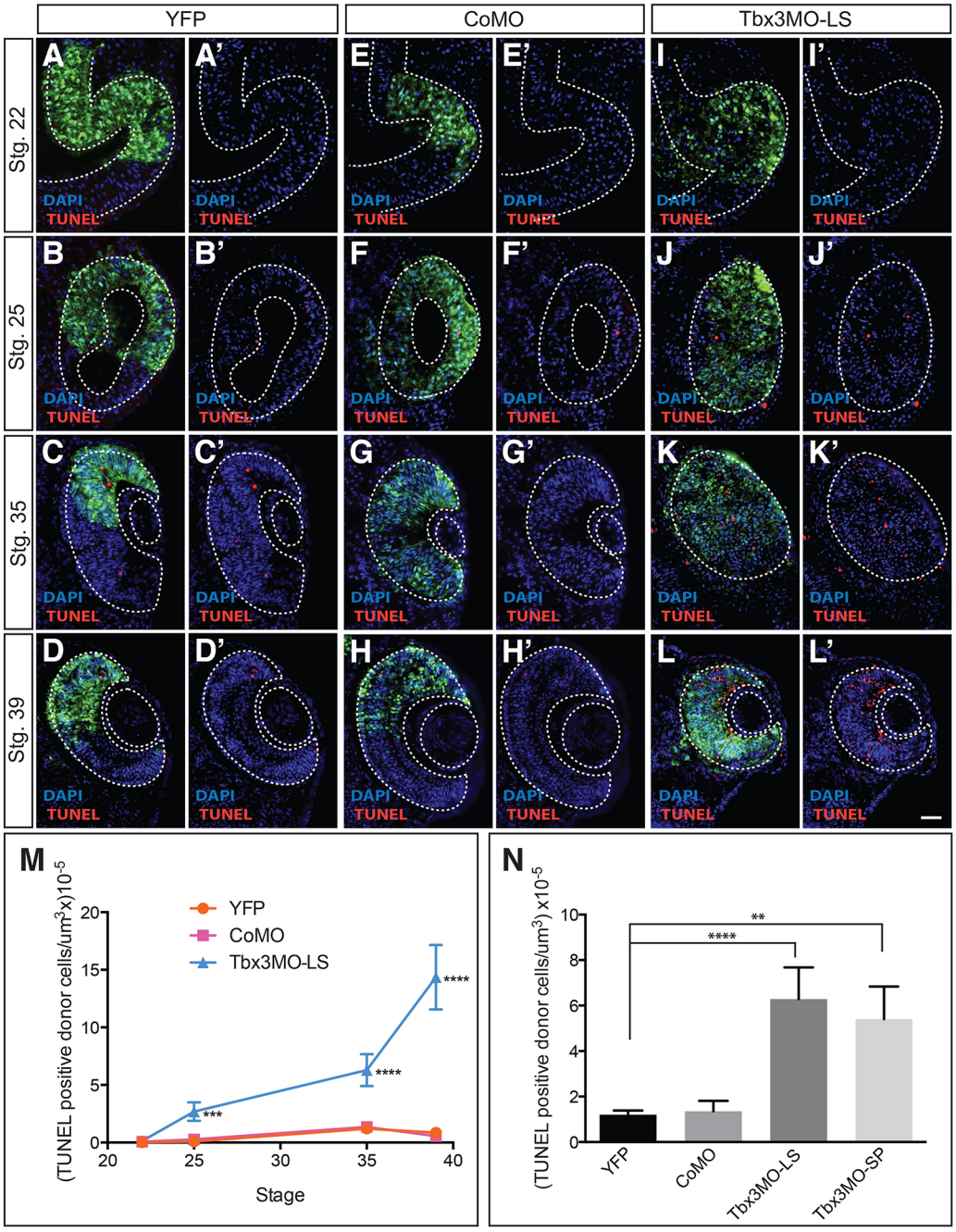

Fig. 8. Tbx3 knockdown results in retinal progenitor apoptosis and eye defects. Eye field cells isolated from embryos expressing YFP (A-D′), CoMO (E-H′) or Tbx3MO-LS (I-L′) were grafted into the eye field of untreated embryos (EF→EF). TUNEL staining was used to detect cell death of the transplanted (YFP-positive) cells at stage 22 (A,A′,E,E′,I,I′), 25 (B,B′,F,F′,J,J′), 35 (C,C′,G,G′,K,K′), and 39 (D,D′,H,H′,L,L′). Dotted lines indicate the outline of the optic vesicle (stgs 22 and 25), optic cup and lens (stgs 35 and 39). (M) Line graph indicates the number of TUNEL-positive donor (YFP-positive) cells per unit volume of transplanted cells as a function of developmental stage. (N) Number of TUNEL/YFP double-positive cells per unit volume that were detected in the stage 35 retina of tadpoles that received eye field transplants from embryos injected with YFP only, CoMO, Tbx3MO-LS and Tbx3MO-SP at stage 15. Dorsal retina is the top of each panel. Graphs show mean±s.e.m.; N=3, $n\geq9$; **$P\leq0.01$, ***$P\leq0.001$, ****$P\leq0.0001$. Scale bar: 50 µm.

BMP signaling serve a Tbx3-like function during the specification and determination of other neural plate regions.

Tbx3 expression in the presumptive cement gland is also consistent with a role in BMP repression. A dorsoventral BMP signaling gradient determines neural (low signaling) versus cement gland (intermediate signaling) versus epidermal tissues (high signaling) (reviewed in Eivers et al., 2008 and Gammill and Sive, 2000; Wardle and Sive, 2003; Dickinson and Sive, 2007). Noggin strongly represses *bmp4* levels in ectodermal explants, which form retina when grafted to embryos (Figs 4,6; Figs S4,S7). Co-injection of Tbx3MO with Noggin resulted in intermediate *bmp4* expression and generated cement gland (Fig. 4, Figs S4,S5,S7). We hypothesize that Tbx3 expression anterior to the neural plate reduces BMP signaling to intermediate levels, thereby specifying cement gland.

Tbx3 regulates a diverse list of developmental processes, and cofactors often function with Tbx3 in a tissue-specific manner

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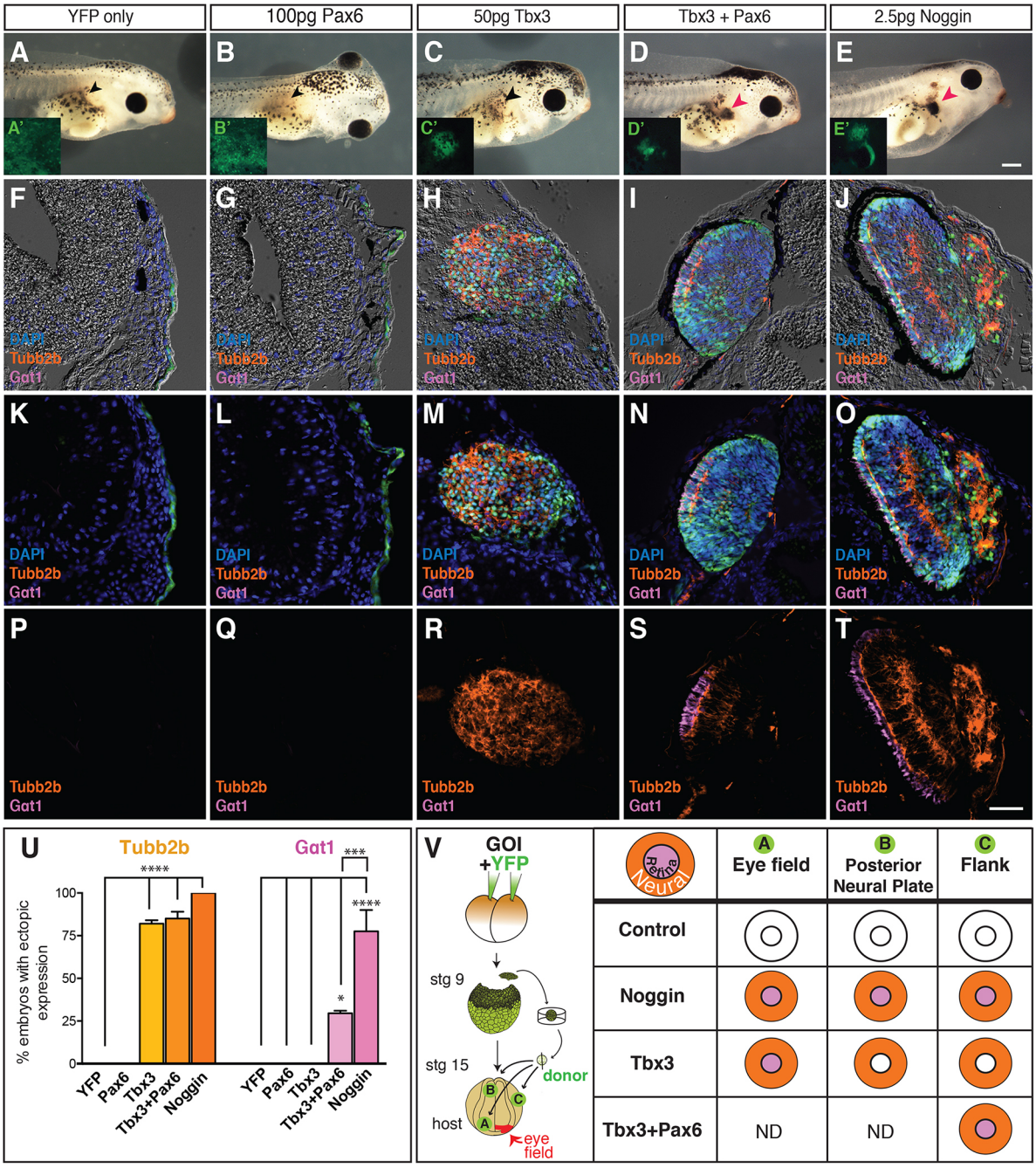

Fig. 9. Together, Tbx3 and Pax6 are sufficient to determine retina from pluripotent cells. (A-T) Pluripotent cells isolated from embryos injected with the indicated RNAs were transplanted to the flank of stage 15 embryos then grown to tadpoles (ACT→Flank). Arrowheads (A-E) show location of transplant (green fluorescence, A′-E′). (F-T) Sections were stained for a neural marker (Tubb2b, orange), rod photoreceptor marker transducin (Gαt1, magenta) and nuclei (DAPI, blue). (U) Percentage of transplants with YFP$^+$/Tubb2b$^+$ and YFP$^+$/Gαt1$^+$ cells, N=2; $n\geq29$; *$P\leq0.05$, ***$P<0.001$, ****$P\leq0.0001$. (V) Schematic graphically summarizing results obtained from transplants performed in Figs 1,3-5 and 9; GOI, gene of interest. Scale bars: 400 µm (E), 50 µm (T).

(reviewed in Washkowitz et al., 2012). Similar to our results, Tbx3 represses *bmp4* expression in the mesenchyme of the mouse anterior palatal shelf and mammary gland – although direct repression was not demonstrated (Cho et al., 2006; Lee et al., 2007b). In contrast, Tbx3 activates *bmp2* and *bmp7* transcription during chick digit formation (Suzuki et al., 2004). Clearly, additional work is necessary to determine if Tbx3 represses *bmp4* directly or via expression of a *bmp4* transcriptional activator and if Tbx3 requires cofactors to reduce *bmp4* levels during eye field specification and determination (Fig. 10).

Maintaining neural progenitors in a proliferative and multipotent state

For the eye to reach its normal size, neural progenitor cells of the anterior plate that eventually form the retina must remain pluripotent and continue to proliferate (reviewed in Zuber, 2010; Sinn and Wittbrodt, 2013). Tbx3 plays important roles in establishing and maintaining the pluri- and multipotency of stem and progenitor cells (Ivanova et al., 2006; Lee et al., 2007a; Lüdtke et al., 2009; Niwa et al., 2009; Han et al., 2010; Lu et al., 2011; Esmailpour and Huang, 2012). The ability of Tbx3-expressing *Xenopus* cells to

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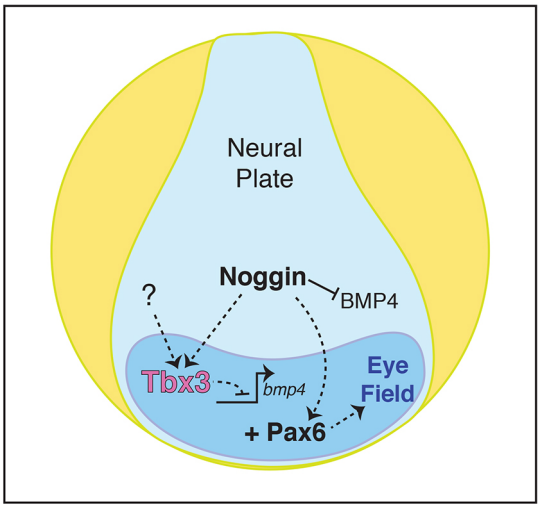

Fig. 10. Model illustrating the role of Tbx3 during eye field determination. Noggin protein binds BMP4 directly blocking BMP receptor activation in the early neural plate (light blue), which includes the presumptive eye field (dark blue). Tbx3 is required to repress *bmp4* transcript levels, resulting in continued BMP inhibition in the eye field. The combined activity of Tbx3 and Pax6 is sufficient to determine the eye field to form retina. Solid and dashed lines indicate direct and possible indirect interactions, respectively.

integrate into and differentiate as anterior (retinal) or posterior (spinal cord) neural plate cells, suggests that Tbx3 maintains neural cells in a multipotent neural progenitor state. We propose that, in addition to its function as a repressor of *bmp4* transcription, Tbx3 also prevents premature differentiation of eye field cells. Once a sufficient number of cells to form a normal-sized eye are generated, eye field cells can then still respond to local cues, which determine them to a retinal lineage.

Signaling required for retinal determination

Noggin is sufficient to specify and determine retina, while Tbx3 can only specify retina. We previously proposed that the EFTFs form a self-regulating feedback network that is required for retina formation (Zuber et al., 2003). The work presented here indicates that Tbx3 and Pax6 are together sufficient to initiate this network (Fig. 10). As a result of the more potent activity of Noggin, however, we propose that Tbx3 and Pax6 lack an activity provided by Noggin that potentiates and possibly secures retinal determination.

Noggin1, -2 and -4 are all expressed in the *Xenopus* anterior neural plate (Fletcher et al., 2004; Eroshkin et al., 2006). The expression domains and relative expression levels of the three Noggin proteins are distinct, suggesting they may have non-redundant roles during eye field determination. In addition, the ligand binding affinity of the Noggin proteins are distinct (Bayramov et al., 2011). Although Tbx3, through its role in *bmp4* inhibition, may be sufficient to inhibit BMP signaling, Noggin(s) repression of Activin, Nodal and possibly Wnt signaling may be required as well (Bayramov et al., 2011; Eroshkin et al., 2016; Wong et al., 2015). It will be important to discover which Noggin proteins can determine retina in the developing embryo, the mechanisms by which they induce expression of the EFTFs, and the signaling pathways regulated by Noggin(s) that stabilize the EFTF network. In addition, identifying the distinct and possibly common transcriptional targets of Tbx3 and Pax6 will be necessary to fully understand the molecular and genetic basis of retinal determination.

MATERIALS AND METHODS

Animals

Outcrossed *Xenopus laevis* were obtained from Nasco (Fort Atkinson, WI). Embryos and tadpoles were obtained by *in vitro* fertilization or natural mating and staged according to Nieuwkoop and Faber (1994). The State University of New York Upstate Medical University Committee for the Humane Use of Animals approved all procedures.

Plasmids

Tbx3MO target sequences from 5′UTR of *tbx3.L* and *tbx3.S* were PCR amplified (see Table S1 for primers) from *X. laevis* genomic DNA (gDNA) and cloned in-frame with YFP to generate pCS2R.Tbx3.L-YFP and pCS2R.Tbx3.S-YFP. To generate pCS2R.XlTbx3GR, GR was amplified from pCS2+Tbx5-EnR-GR (Horb and Thomsen, 1999) and inserted in-frame with XlTbx3. *tbx3.L* DNA-binding domain amplified from pCS2.Tbx3L and EnR-GR domain from pCS2+Tbx5-EnR-GR (Horb and Thomsen, 1999) were cloned into pCS2R to create pCS2R.Tbx3LDBD-EnR-GR. RN3P-VP16-DBD-GR was obtained from Dr Takeshima (Takabatake et al., 2002). Detailed maps, construct sequences and plasmids are all available upon request.

Microinjection and tissue transplants

Tbx3 (Table S1) and control (25-N random control oligo) morpholinos were obtained from Gene Tools LLC (Philomath, OR). Capped RNA was synthesized using SP6 mMessage Machine Kit (Thermo Fisher Scientific, Waltham, MA). Figure legends contain developmental stages and amounts of RNA/morpholino injected. For *in situ* hybridization, stage 15 caps [collected in 0.7× Marc's Modified Ringer's (MMR) at stage 9, from bilaterally injected 2-cell-stage embryos] were transferred to 0.1× MMR (with or without 10 µM dexamethasone) and fixed at stage 22 (Kolm and Sive, 1995); 10 µM dexamethasone was also used on whole embryos. Animal cap transplant (ACT) was performed at stage 15, as previously described (Viczian and Zuber, 2010). For eye field transplants, dorsal animal blastomeres (D1) of 8-cell staged embryos were unilaterally injected with YFP and gene(s) of interest. A central region of stage 15 donor eye field (~1/3 of eye field) was transplanted to host after removing tissue of a similar size from the graft location.

In situ hybridization

In situ hybridizations were performed as previously described (Zuber et al., 2003). RNA probes were generated from pCS2R.Tbx3L, pBSSKII.Bmp4, pGEMTEZ.Rax, pCS2R.Pax6, pCS2.Otx2, pBSSKII.Xag1, and pCS2+.XlFoxG1 using RNA Polymerase-Plus (Thermo Fisher Scientific).

Reverse transcription PCR

Total RNA was extracted from animal caps (10 per condition), dissected tissue (20 per condition) or whole embryos (5 per condition) using RNAzol RT (Molecular Research Center, Cincinnati, OH) and cDNA was synthesized with M-MLV Reverse Transcriptase (Promega, Madison, WI). See Table S1 for full list of primers used.

Western blotting

Samples were prepared as previously described using 30 µg total protein (Wong et al., 2015). Details of all primary antibodies can be found in Table S2.

Immunostaining and imaging

Sections (12 µm) of stage 43 tadpoles were stained as previously described (Viczian et al., 2003; Martinez-De Luna et al., 2013), or according to conditions in Table S2. Whole embryo and section images were captured and processed as previously described (Viczian et al., 2009). TUNEL was performed using an ApopTag(R) Red In Situ Apoptosis Detection Kit (EMD Millipore).

Statistical analysis

All statistical analyses were performed with Prism version 6.0f (GraphPad Software, La Jolla, CA) using an ordinary one-way ANOVA, with a

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Bonferroni's multiple comparison test. Statistical significance was determined by $P \leq 0.05$. All graphs show mean±s.e.m. normalized to controls. Significance denoted by ns (not significant, $P > 0.05$), *$P \leq 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$. All successful transplants (YFP^+ donor cells observed in host) were included in analyses. All analyses were performed by an experimenter blind to sample groups. *N*=biological replicates, *n*=animal number.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: M.E.Z. and A.S.V.; Methodology: M.E.Z. and A.S.V.; Investigation: Z.M.; Writing - Original draft: Z.M. and M.E.Z.; Writing - Review & Editing: R.I.M-DL., A.S.V. and M.E.Z.; Visualization: Z.M., A.S.V. and M.E.Z.; Funding Acquisition: A.S.V. and M.E.Z.; Supervision: A.S.V. and M.E.Z.

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Supplementary information

Supplementary information available online at
http://dev.biologists.org/lookup/doi/10.1242/dev.130955.supplemental

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