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Extraocular ectoderm triggers dorsal retinal fate during optic vesicle evagination in zebrafish

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Abstract

Dorsal retinal fate is established early in eye development, via expression of spatially restricted dorsal-specific transcription factors in the optic vesicle; yet the events leading to initiation of dorsal fate are not clear. We hypothesized that induction of dorsal fate would require an extraocular signal arising from a neighboring tissue to pattern the prospective dorsal retina, however no such signal has been identified. We used the zebrafish embryo to determine the source, timing, and identity of the dorsal retina-inducing signal.

Extensive cell movements occur during zebrafish optic vesicle morphogenesis, however the location of prospective dorsal cells within the early optic vesicle and their spatial relationship to early dorsal markers is currently unknown. Our mRNA expression and fate mapping analyses demonstrate that the dorsolateral optic vesicle is the earliest region to express dorsal specific markers, and cells from this domain contribute to the dorsal retinal pole at 24 hpf.

We show that three *bmp* genes marking dorsal retina at 25 hpf are also expressed extraocularly before retinal patterning begins. We identified *gdf6a* as a dorsal initiation signal acting from the extraocular non-neural ectoderm during optic vesicle evagination. We find that *bmp2b* is involved in

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dorsal retina initiation, acting upstream of *gdf6a*. Together, this work has identified the nature and source of extraocular signals required to pattern the dorsal retina.

Keywords: *bmp*; dorsal; retina; initiation; *gdf6a*; morphogenesis

Introduction

Achieving precise retinotopic axon targeting during development requires the patterning of projecting (retina) and target (brain) tissues along anterior-posterior and dorsal-ventral axes, such that individual cells of both tissues acquire a molecularly specified positional identity (McLaughlin et al., 2003; McLaughlin and O'Leary, 2005). Regarding the first step in fate specification, we hypothesized that the dorsal retinal initiating signal would be a diffusible molecule, providing asymmetric positional information to prospective dorsal retina, from a location external to the eye field. However an extraocular initiator of dorsal retinal fate has not yet been identified.

Past research has identified several genes necessary for establishing dorsal retinal fate (Asai-Coakwell et al., 2007; Behesti et al., 2006; French et al., 2007; French et al., 2009; Gosse and Baier, 2009; McLaughlin et al., 2003; Murali et al., 2005; Plas et al., 2008; Sakuta et al., 2006) but has focused on expression and regulation within the retinal field. Historically, a simple model was set forth in which dorsal expression of *bmp4* initiates dorsal *tbx5* and downregulates

ventral *vax2* (McLaughlin et al., 2003). In support of this model, *bmp4* knockout mice never initiate expression of *tbx5* (Murali et al., 2005). Bmp4 gain of function experiments, and loss of function of receptors, also suggest Bmp-mediated regulation of *tbx5* in the dorsal retina (Behesti et al., 2006; Murali et al., 2005). However extraocular factors for initiating *bmp4* expression are unknown, and *bmp4* null mice die shortly after E9.5, precluding further analysis of gene expression and retinotectal topography in this model (Murali et al., 2005). Furthermore, recent work in zebrafish suggested that *bmp4* does not initiate dorsal-specific gene expression, whereas the *Bmp* family gene *gdf6a* plays a critical role (French et al., 2009). Gdf6a is a Bmp family member known to affect eye development in humans and mouse (Asai-Coakwell et al., 2007; Asai-Coakwell et al., 2009), yet its role in dorsal retinal patterning in these species has not yet been analyzed. In zebrafish, *gdf6a* is expressed extraocularly during optic vesicle evagination, and in the prospective dorsal retina from 15-48 hpf. *gdf6a* is critical for dorsal initiation, yet it has not been determined what time, and from what tissue, *gdf6a* acts. It is unknown whether *gdf6a* is an extraocular initiator of dorsal retinal fate, or acts only within the retinal field, suggesting another extraocular signal necessary for initiating *gdf6a* within the retina. We set out to determine the identity, time of action, and tissue source of an extraocular initiator, as well as the domain of the optic vesicle first initiated with dorsal-specific markers.

The first known manifestation of retinal dorsal identity in zebrafish is the expression of the T-box transcription factor genes, *tbx2a* and *tbx5a*, in restricted optic vesicle domains (Veien et al., 2008). The primary candidates for regulation of *tbx* genes, and thus initiation of dorsal identity, are the morphogens of the bone morphogenetic protein family (Bmps). Five *bmp* genes mark the dorsal domain of the zebrafish eye at 24 hpf—*bmp2a*, *bmp2b*, *bmp4*, *bmp7b* and *gdf6a* (Shawi and Serluca, 2008; Thisse, 2001; Thisse, 2005). The continuing expression of all known

retinal patterning genes from early to late stages of eye development led us hypothesize that these *bmps* were likely candidates for dorsal initiation signals. However it is unknown which of these candidate morphogens are expressed early, during optic vesicle evagination, in extraocular spatiotemporal domains where they could initiate the prospective dorsal retina transcription factors. Furthermore, it is not known which region of the early optic vesicle is poised to receive the dorsal initiation signal. Recent research analyzed the 24 hpf fates of early optic vesicle cells (Kwan et al., 2012), and found extensive cell movements during this time period. We theorized that extraocular dorsal initiation signals should be located adjacent to the region of the earliest polarized expression of dorsal fate within the retina. Tissues adjacent to the early optic vesicle that could give rise to the initiation signal include the neural tube (medial), non-neural ectoderm (dorsal and lateral), and prechordal plate mesoderm (anterior). In this study, we show that the lateral region of the early optic vesicle, which first expresses dorsal specific transcription factors, and is adjacent to non-neural ectoderm, is fated to give rise to the dorsal retina.

We analyzed the precise timing of dorsal initiation using a pharmacological inhibitor of Bmp signaling, and found that Bmps are required for initiating dorsal retinal markers prior to their expression within the optic vesicle, indicating that bmps are initiating dorsal retina by signaling from an extraocular tissue. We analyzed the spatiotemporal domains of genes encoding candidate initiators–*gdf6a*, *bmp2b*, and *bmp4*, and using mutants we determined the necessity of these genes for initiating dorsal fate in zebrafish.

Our work shows that dorsal retinal fate is initiated by extraocular *gdf6a*, arising from the non-neural head ectoderm at 11.5 hpf. Adjacent tissue of the dorsolateral optic vesicle leaflet receives this signal and upregulates T-box transcription factors in this domain. Additionally, we find that *bmp2b* is necessary for dorsal retinal initiation, acting upstream of *gdf6a*, possibly to

establish its expression in the extraocular ectoderm.

Materials and Methods

Animals

We maintained adult zebrafish (*Danio rerio*) on a 14-hour light, 10-hour dark cycle. We raised embryos in E3 embryo medium with methylene blue at 28.5 °C, anesthetized with 0.2mg/ml tricaine and fixed in 4 % paraformaldehyde unless otherwise noted. We staged embryos according to Kimmel et al., (1995), by counting somites (morphologically) and/or by *in situ* using the somite marker *myoD*. We used Tübingen strain embryos for Kaede fate map and Bmp inhibitor (LDN 193189) experiments.

Mutants

We used four mutant lines for this work: *bmp2b* ^{tc300} (Mullins et al., 1996), *bmp4* ^{st72} (Stickney et al., 2007), *gdf6a* ^{s327} (Muto et al., 2005) and *oep* ^{m134} (Schier et al., 1997; Zhang et al., 1998). The *bmp2b* ^{tc300} line was generated in the Tübingen strain background; *bmp4* ^{st72}, and *gdf6a* ^{s327} were generated in Tupfel long fin (TL) wild type strain and the *oep* ^{m134} line was generated in the AB wild type strain. We maintained all mutant lines on their original backgrounds.

Genotyping

We genotyped adult fin clips and whole mount individual *in situ* embryos using Derived Cleaved Amplified Polymorphic Sequences (dCAPS) PCR. PCR primers for *gdf6a* ^{s327} and *bmp4* ^{st72} were previously described by (Gosse and Baier, 2009; Stickney et al., 2007) respectively. We generated additional primers for genotyping *bmp4* ^{st72} and *bmp2b* ^{tc300} using the web-based program dCAPS Finder 2.0 (Neff et al., 2002).

bmp4 ^{st72} Fwd: TGGTGAGGCACAACACCTCCAACTAG, Rev: CCGAGTCAGCGGGTGACTTTTGCCGTC. SpeI cuts mutant band.

bmp2b ^{tc300} Fwd: GAAGTATCCGAGGAGGCTGA Rev: CCTCCACCACCATGTCCT. HaeIII cuts mutant band.

In situ hybridization

We performed whole mount *in situ* hybridization to analyze mRNA expression as described by Thisse and Thisse, (2008) with the following modifications: we incubated and washed some of our samples using a Biolane HTI *in situ* machine (Huller and Huttner AG, Tübingen, Germany). We synthesized labeled riboprobes (fluorescein-UTP-labeled for *rx3*, all others labeled with Digoxigenin-UTP) using in vitro transcription RNA labeling kits from Roche. Probes were as follows: *tbx2a* (Dheen et al., 1999), *tbx5a* (Ruvinsky et al., 2000), *bmp4* (gift, M. Mullins, University of Pennsylvania), *tbx4*, *bmp2b* (Nikaido et al., 1997), *vax2* (Take-uchi et al., 2003), *pax2a*, *rx3*, *gdf6a* (Veien et al., 2008), *pitx3*, *ephrinB2a* (Durbin et al., 1998), *myoD* (Weinberg et al., 1996) and *isl1* (Inoue et al., 1994). Digoxigenin probes were developed with BM-Purple, and the fluorescein *rx3* probe was developed using INT-BCIP (Roche Applied Science). We cleared whole mount embryos in 50-80% glycerol and imaged them using an Olympus SZX 12 stereomicroscope, an Olympus SN1H045411-H camera, and Picture Frame™ imaging software version 2.3. Sectioned embryos were embedded in plastic according to (Sullivan-Brown et al.,

2011), and sectioned transversely at 12 µm thickness using a Reichert-Jung 2050 microtome. All sections were imaged on an Olympus BX51WI inverted microscope using the same camera and imaging software as for whole mount images. In cases where a particular mutant phenotype could not be identified, we imaged and genotyped individual embryos to confirm that the pictures presented contained the correct genotypes.

Kaede Fate Map

We prepared capped *NLS-Kaede* mRNA using the mMessage mMachine kit from Ambion. We injected 2 ng (500 ng/µL) *NLS-Kaede* mRNA into 1-cell stage zebrafish embryos according to (Hatta et al., 2006), and raised them in E2 with gentamycin (E2/GN). At 11 hpf we dechorionated and mounted embryos live in 1.5 % low melting temperature agarose to image them from the dorsal side on an FV1000-XY Olympus IX81 confocal microscope. Images shown are maximum intensity projections rendered using ImageJ.

Bmp inhibitor LDN 193189

LDN 193189 (Stemgent) was dissolved in 100% DMSO. We incubated dechorionated embryos in 1 µM LDN 193189, 1% DMSO in E2/GN or equal volume 1% DMSO/E2/GN as a control. We began our treatments at 4 or 9.5 hpf, and fixed at 13 hpf. We assayed treated embryos using *in situ* hybridization for expression of the early eye field marker *rx3* or the dorsal-specific transcription factor *tbx5a*.

Results

***tbx5a* expression is broad and dynamic during eye morphogenesis**

tbx5a expression is thought to mark prospective dorsal fate in the early optic vesicle. Because expression of this marker begins at 12 hpf (Veien et al., 2008), and is one of the earliest markers of dorsal fate, we hypothesized that the early expression domain of *tbx5a* may indicate the likely extraocular location of dorsal initiation signals. We therefore analyzed changes in the expression domain of *tbx5a* mRNA in the optic vesicle following its onset at 12 hpf, over time until 24 hpf. *tbx5a* expression is initiated in the lateral half of the dorsal optic vesicle leaflet at 12 hpf (Figure 1, A0-A3). The early dorsolateral expression domain begins to shift posteriorly and medially, and at 14 hpf occupies both the dorsal and ventral optic vesicle leaflets in the posterior optic vesicle, while remaining dorsolateral in the anterior domain (Figure 1 B0-B3; compare arrowheads in B3 and A3). *tbx5a* expression continues to move posteriorly over time (C0, D0), primarily occupying the prospective retinal (dorsal) leaflet (arrowhead in C3), with the posterior portion of expression in a more medial domain, and the anterior portion more lateral to create a teardrop shape in the whole mount dorsal view between 15-18 hpf (Figure 1 C0-C3 and D0-D3). At 24 hpf *tbx5a* expression is opposite to the choroid fissure in the dorsal retina, and also extends into the central retinal domain, medial to the lens (Figure 1 E0-E3). The dynamic expression of *tbx5a* suggests that early expression of this marker may mark the process of dorsal induction. Additionally, the early expression domain in the dorsolateral optic vesicle suggests an initiating signal could arise from the overlying extraocular ectoderm (arrow in Figure 1 A1).

Movements of prospective dorsal cells resemble *tbx5a* mRNA expression domain changes over time

Tissue patterning, via combinatorial expression and interactions of transcription factors, can commit specific cells to a particular cell fate early during development. According to this model, we hypothesized that a portion of the cells of the dorsolateral optic vesicle expressing the dorsal marker *tbx5a* at 12 hpf would undergo morphogenesis to reside in the dorsal retina at 24 hpf; To test this, we used the photoconvertible fluorescent protein Kaede to fate map a portion of the *tbx5a* 12 hpf expression domain from 14-22 hpf (Figure 2). We photoactivated the lateral optic vesicle of live 14 hpf embryos ubiquitously expressing Kaede (Figure 2 A, B), and took sequential confocal images to determine the fates of these lateral cells over time (Figure 2 C-D). In accordance with our hypothesis, cells within the Kaede activated lateral optic vesicle domain show a pattern of movement over time similar to the dynamic *tbx5a* mRNA domain (Figure 2 B'-D'). First, photoactivated lateral cells (magenta, Figure 2B) move to the posterior optic vesicle by 18 hpf (Figure 2C-C'), then populate the dorsal pole and central retinal domains extending from dorsal to behind the lens (medially) at 24 hpf (Figure 2D-D'). This final domain is triangular in shape, extending from the widest point dorsally, to a triangular tip more centrally and ventrally, very similar to the 24 hpf domain of *tbx5a* (Figure 1 E0-E3). These results indicate that dorsal retinal fate may be fixed over time—a portion of cells specified as dorsal and expressing *tbx5a* at 12 hpf populate the dorsal pole of the retina and maintain their expression of *tbx5a* at 24 hpf.

Dorsal-initiating Bmp signals are acting prior to expression of *bmps* within the retina

Previous work shows that *bmps* are necessary for initiating the expression of several dorsal markers, (French et al., 2009; Murali et al., 2005) however it is not known at what time these signals are acting. We performed pharmacological experiments using a selective Bmp inhibitor to analyze the time window in which Bmps are required for initiating dorsal marker expression. LDN193189 blocks phosporylation of Smad 1/5/8 proteins by type 1 Bmp receptors, and is therefore expected to block all

bmp signaling. We specifically tested whether Bmps are required for dorsal retinal initiation prior to their expression in the retina. Treatment with 1 µM LDN from 4-13 hours leads to strongly dorsalized phenotypes (Cannon et al., 2010). We found that these dorsalized embryos do form an eye field when assayed with the *rx3* eye field marker *in situ* probe (Figure 3 A-B). However these embryos fail to initiate the dorsal marker *tbx5a* (Figure 3 C-D). When we treated with 1 µM LDN beginning at 9.5 hpf, again fixing at 13 hpf, normal eye fields are formed as in the DMSO control (Figure 3 E-F), yet dorsal *tbx5a* is not detectable (Figure 3 G-H). These results show that Bmps are acting between 9.5 and 13 hpf to initiate dorsal retinal fate. Importantly, this is before expression of these genes is detected in the retina (Veien et al., 2008), indicating that Bmps are acting as extraocular initiators of dorsal fate.

Both *bmp2b* and *gdf6a* are necessary for initiation of dorsal retinal fate

Bmps are diffusible signaling molecules that can act in a gradient far from their source, and previous reports in several species implicate Bmps in dorsal fate initiation (Behesti et al., 2006; French et al., 2009; Murali et al., 2005; Plas et al., 2008). In zebrafish, several members of the *bmp* family are expressed surrounding the prospective dorsal retina, and therefore are likely candidates as extraocular initiators of dorsal fate. We first examined the 11-13 hpf expression patterns of *bmp* genes with known expression dorsally in the retina at 24 hpf. To determine whether these *bmps* might encode extraocular initiators of dorsal fate, we next examined expression of dorsal fate markers shortly after initiation in null mutants for each of these genes, allowing us to determine the complete loss of function phenotype.

The *bmp4* gene is expressed anteriorly to the evaginating optic vesicle at 12-14 hpf, in the prechordal plate (Supplementary Figure 1 A). However, we found that the prechordal plate is not necessary for dorsal retina initiation. The zebrafish mutant *one-eyed pinhead* (*oep* ^{m134}) does not form prechordal plate tissues, as evidenced by loss of *bmp4* and *isl1* expression in this region (Supplementary

Figure 1 A-D, arrows indicating prechordal plate in C and absent tissue in D). Yet the dorsal marker *tbx5a* is initiated normally in *oep* mutants (Supplementary Figure 1 E-F), suggesting that *bmp4* from this tissue does not participate in dorsal retinal fate specification. Furthermore, in agreement with previous morpholino studies (French et al., 2009) and contrary to results in mouse (Murali et al., 2005), the polarized dorsal-ventral markers *tbx5a*, *gdf6a*, *ephrinB2a*, and *vax2* showed normal expression in *bmp4* ^{st72} null mutants (Supplementary Figure 2 A-H).

The *bmp* family member *gdf6a* is expressed in the extraocular ectoderm between 11-15 hpf (see Figure 6) and in the prospective dorsal retina at 15-24 hpf (Thisse, 2001). Dorsal axons project to the ventral optic tectum in WT, but they mistarget dorsally in *gdf6a* ^{s327} null mutants, leaving the ventral optic tectum devoid of axons in this mutant (Gosse and Baier, 2009). Morpholino knockdown experiments have shown that *gdf6a* is important for establishing dorsal-ventral patterning within the retina, as assayed by expression domains of *tbx5a*, *bmp4*, *bambi* and *vax2* (French et al., 2007); yet the required time and location of this signal have not been determined. We found that the dorsal markers *tbx5a*, *bmp4* and *bmp2b* are never initiated in *gdf6a* mutants, and the dorsal markers *tbx2a* and *tbx4* are initiated in smaller domains and at lower intensity than in siblings (Figure 4 A-N). At later time points (18-26 hpf), the dorsal markers *tbx2a*, *tbx5a*, *tbx4*, *bmp2b*, *bmp4*, *ephrinB2a*, *ephrinB1* and *raldh2* are completely absent in *gdf6a* mutants [Supplementary Figure 3 A-P; see also (Gosse and Baier, 2009)]. Furthermore, markers of ventral retinal fate including *vax2* and *ephB2* are initiated normally in *gdf6a* mutants, then expand following their initiation (*vax2*, Figure 4 O-P), and are expressed throughout most of the optic vesicle at 18-26 hpf (*vax2*; *ephB2,* Supplementary Figure 3 Q-T). These data show that dorsal retinal fate is never properly initiated in *gdf6a* mutants, and confirm previous work showing that *gdf6a* is a critical initiator of dorsal retinal fate.

bmp2b is expressed surrounding the evaginating optic vesicle at 11-15 hpf in WT (Figure 5 A, arrow in Figure 5 C transverse section), and this expression domain is unaffected in *gdf6a* mutants (Figure 5 B). A null mutation in the *bmp2b* gene (*bmp2b* ^{tc300}) results in severe developmental defects, and an early death at 14-16 hpf (Mullins et al., 1996). We were able, however, to examine the earliest stages of dorsal eye specification in this mutant. First, expression of *rx3*, an early eye field marker, in *bmp2b* mutants and siblings showed that *bmp2b* mutants establish an eye field between 12-14 hpf (Figure 5 D-G). We next analyzed the expression of *tbx2a* at 12 hpf and *tbx5a* at 13 hpf, and found that *tbx2a* was initiated in a much smaller domain compared to wild type (Figure 5 H-I), and *tbx5a* was never initiated (Figure 5 J-K). Furthermore, the earliest marker of the prospective ventral optic stalk domain, *pax2a*, is normally initiated in *bmp2b* mutants (Figure 5 L-M, arrows mark retinal staining, arrowheads point to midbrain-hindbrain boundary). These results show that dorsal retinal markers are not properly initiated in *bmp2b* mutants, identifying a novel role for *bmp2b* in dorsal fate specification.

It is important to note that retinal expression of *bmp2b* is never present in *gdf6a* mutants (Figure 4 M, N; Supplementary Figure 3 G, H), suggesting that *gdf6a* acts upstream of *bmp2b* within the retinal field. However, our results showing that early extraocular *bmp2b* expression is normal in *gdf6a* mutants (Figure 5, A-C), suggest that the extraocular *bmp2b* necessary for dorsal initiation is acting upstream of *gdf6a*.

The dorsal initiation signal arises from the extraocular ectoderm adjacent to the dorsolateral optic vesicle at 11-12 hpf

Loss of the *gdf6a* initiation signal leads to near-complete loss of dorsally expressed genes and expansion of ventral fate toward the dorsal pole, but it was not clear in which tissue *gdf6a* is acting. To examine the precise location of *gdf6a* expression, we performed double *in situ* hybridization with *gdf6a*

and *rx3* probes developed in two colors, and imaged optic vesicles both in whole mount views and in tissue sections (Figure 6 A-H and K-N, *gdf6a* – blue, *rx3* - orange). *gdf6a* mRNA is expressed in the extraocular head ectoderm beginning at 11 hpf, immediately prior to and directly adjacent to the region of the lateral optic vesicle that expresses *tbx5a* (Figure 6 A, C, K, ; compare to Figure 1 A0-A3). The extraocular *gdf6a* expression pattern is highly dynamic between 11 and 13 hpf. At the 11 hpf time point, the strongest *gdf6a* expression is posterior and anterior to the optic vesicle, with only faint staining laterally (Figure 6 A, arrows for strong anterior and posterior staining, arrowhead for faint lateral staining). By 11.5 hpf, the lateral domain of *gdf6a* expression has intensified (arrowhead in Figure 6 C), and begins to expand over the dorsolateral half of the optic vesicle (Figure 6 C, arrow in K). This expansion and intensification continues, and at 12-13 hpf, *gdf6a* expression in the extraocular ectoderm covers the dorsal leaflet of the optic vesicle (Figure 6 E, G, L). The location and time of *gdf6a* expression extraocularly suggests that it is the final critical initiator of *tbx2a* and *tbx5a* expression in the cells of the dorsolateral optic vesicle (Figure 1 A0-A3), specifying dorsal fate within the retina.

Interestingly, *bmp2b* mutants have been shown to lack non-neural ectoderm (Nguyen et al., 1998), and therefore would not be expected to initiate *gdf6a* expression extraocularly, as this signal arises from the non-neural lateral head ectoderm. We therefore analyzed extraocular *gdf6a* expression in *bmp2b* mutants from 11-13 hpf. Indeed, we found that *bmp2b* mutants have increased anterior *gdf6a* expression (Figure 6 D, F arrows), but *gdf6a* expression surrounding the lateral and dorsal optic vesicle is undetectable or greatly downregulated (Figure 6 B, D, F, H arrowheads, arrows in M, N). Similarly, the lens placode marker *pitx3* is also absent in *bmp2b* mutants (Figure 6 I-J and O, arrows in I-J). These results are consistent with the lack of initiation of dorsal retinal fate in *bmp2b* mutants, due to a lack of a dorsal initiation signal from lateral non-neural ectoderm, again showing that extraocular *bmp2b* acts upstream of extraocular *gdf6a* in dorsal retina specification.

Discussion

Topographic connections of retinal axons with their brain targets allow us to perceive a spatially organized image of the visual world. Choreographing accurate axon targeting during development first requires the molecular patterning of retinal and tectal (brain) tissues along anterior-posterior and dorsal-ventral axes. Axial patterning often begins with a morphogen gradient that arises from a neighboring tissue, leading to initiation of transcription factors within the tissue of interest, and hence a graded fate along the axis. Our data demonstrate that dorsal retinal patterning employs such a mechanism (Figure 7). Initiation of dorsal retinal fate first requires expression of *bmp2b*. Previous research (Nguyen et al., 1998) and our experiments both indicate that *bmp2b* mutants never form non-neural ectoderm, the critical tissue for dorsal fate initiation by *gdf6a*. Non-neural ectoderm, itself expressing *bmp2b* as well as *gdf6a*, surrounds the evaginating optic vesicle between 10 and 15 hpf. *gdf6a* is likely the critical initiator of dorsal fate within the retina (French et al., 2009), and without this signal *tbx5a* and *tbx2a* fail to be initiated within the dorsolateral leaflet of the optic vesicle at 12 hpf. Our data show that the *gdf6a* initiation signal acts between 11-12 hpf, a time when this gene is not expressed within the retinal field but is expressed in the surrounding ectoderm. Pharmacological manipulations using the selective Bmp inhibitor LDN corroborate these results, showing that Bmps are necessary for dorsal initiation after 9.5 hpf.

Polarized expression of the prospective dorsal marker *tbx5a* is broad and dynamic during retinal morphogenesis. Initially, *tbx5a* marks the lateral half of the dorsal optic vesicle leaflet after dorsal initiation. Subsequently these cells undergo dramatic movements during morphogenesis of the optic cup. Kaede fate mapping shows that the dynamic pattern of *tbx5a* expression generally recapitulates the

movements of these cells. However, not all cells expressing this gene reside in the dorsal pole at 24 hpf. Thus, our data indicate that cells of the lateral optic vesicle at 12 hpf contribute to both the dorsal and central retinal domains at 24 hpf. Consistent with these findings, four dimensional cell tracking (Kwan et al., 2012) indicates that cells of the 12 hpf posterior-lateral dorsal optic vesicle leaflet give rise to dorsal pole cells at 24 hpf, and the central-lateral dorsal optic vesicle leaflet at 12 hpf gives rise to cells medial to the lens at 24 hpf. The expression domain of *tbx5a* is reduced to only a small triangle at the dorsal pole by 32 hpf, lending support to the idea that some cells eventually turn off this marker. Therefore, while early *tbx5a* expression is a marker of the potential for dorsal fate, many cells initially expressing this gene may ultimately reside in other regions.

While we believe that the primary role of *bmp2b* in dorsal fate initiation is in formation of non-neural ectoderm, we cannot rule out a later secondary role for this gene in initiation during optic vesicle evagination. *bmp2b* mRNA is expressed extraocularly in the lens placode at 11-13 hpf (Figure 5 A, C), overlapping with *gdf6a* expression (Figure 6 G), and could cooperate with *gdf6a* to initiate dorsal fate during evagination. Similarly, *bmp4* is expressed within the retinal field beginning at 14 hpf (Veien et al., 2008), and could be interacting with *gdf6a* at this later time point. Dorsal character is not entirely absent in *gdf6a* mutants (Figure 4 D, L arrowheads, Supplementary Figure 3 L, R arrowheads), suggesting the possibility that multiple Bmps could cooperate to initiate dorsal fate during optic vesicle evagination. However our analysis of different allelic combinations of *gdf6a*, *bmp2b*, and *bmp4* null mutations (not shown), was unable to conclusively demonstrate genetic interactions that would support combinatorial signaling mechanisms.

Despite the critical role of *gdf6a* for dorsal retina initiation in zebrafish, a similar role for this factor in other species has not been described. In other vertebrates, *Gdf6* is expressed in the dorsal retina and its loss results in a variety of ocular malformations including colobomata, microphthalmia and

anopthalmia (Asai-Coakwell et al., 2007; Asai-Coakwell et al., 2009; Hanel and Hensey, 2006). These gross anatomical phenotypes may be a result of defective dorsal-ventral patterning in these species as well. Additionally, while expression of *Bmp2* in dorsal retina is conserved across species, the function of this gene in dorsal fate specification is also incompletely understood. Intriguingly, *Bmp2* plays a critical role in maintenance of dorsal fate in chick, but has no apparent role in dorsal initiation (Sakuta et al., 2006). Characterization of later roles for *bmp2b* in dorsal retinal maintenance in zebrafish will require conditional inactivation, as global mutants die at 14-16 hpf. Interestingly, contrary to the observations in mouse (Murali et al., 2005), where *bmp4* plays a critical role in initiation of dorsal fate, we have not found any role for this gene in zebrafish retinal patterning. It is possible that different *Bmp* genes have exchanged functions in eye development through evolution, as has been shown previously during gastrulation (Sasai, 2001).

In summary, our study has identified the extraocular location and time of action of the dorsal retinal initiation signal. We have also demonstrated a novel role for *bmp2b* in dorsal initiation, acting in the formation of non-neural ectoderm, the critical source of the initiation signal. Our work defines a basic mechanism for retinal patterning using extraocular tissues, and while the precise location and identity of this signal may differ between vertebrate species, it is likely that the overall strategy is conserved.

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Figure Legends

Figure 1. The expression domain of prospective dorsal marker *tbx5a* is broad and dynamic. Expression of *tbx5a* in the developing retina at (A) 12, (B) 14, (C) 15, (D) 18 and (E) 24 hpf (blue). (0 wholemount; 1-3, transverse sections). (A0-D0 dorsal views; E0, lateral view). (A0-D0') Schematic diagram of orientation for A0-D0 whole-mount dorsal view images. Each image shows a single optic vesicle (red box in schematic), with the midline oriented toward the top. Dashed gray lines show approximate location of anterior, middle, and posterior sections in 1-3. (A) *tbx5a* mRNA is expressed in dorsolateral optic vesicle (arrow in A0, arrowhead in A2) at 12 hpf, and is absent from the ventral leaflet (arrowhead in A3). Extraocular ectoderm is directly overlying this portion of the optic vesicle (arrow in A1). (B) At 14 hpf expression extends to the ventral leaflet in posterior sections (arrowhead in B3). (C-D) *tbx5a* domain moves posteriorly from 15-18 hpf and resides primarily in the dorsal (prospective retinal) leaflet (arrowhead in C3). (E) *tbx5a* expression still covers much of the eye at 24 hpf, including prospective dorsal pole, central retinal domain medial to the lens (arrowhead in E2), and extending to the ventral domain (arrowhead in E3). Scale Bars = 50 µm. Dashed yellow lines outline optic vesicles in all images, also lens and choroid fissure in E0.

Figure 2. Cells of the lateral optic vesicle give rise to prospective dorsal and central retina. A Kaede fate map of 14 hpf lateral retina shows that cell movements generally recapitulate movements of *tbx5a* expression, indicating that dorsal fate initiation could be a discrete early event. (A-D) Dorsal view, maximum intensity projections of single optic vesicles, oriented as shown in the schematic (Figure 1 A0-D0') with the midline toward the top. Dashed yellow lines outline optic vesicles (A-D'), lenses (C-D'), and choroid fissure (D-D'). (A) Pre-activation, ubiquitous NLS green Kaede, 13.5 hpf. (B) 14 hpf, immediately

after photoactivation, cells of lateral optic vesicle express photoactivated Kaede (magenta). (B') 14 hpf whole mount *tbx5a* expression, dorsal view (C) 18 hpf, photoactivated Kaede cells from lateral optic vesicle have moved posteriorly. (C') 18 hpf whole mount *tbx5a* expression, dorsal view (D) 22 hpf, photoactivated magenta cells reside primarily in the dorsal and central retinal domains, as well as in the lens. (D') 24 hpf whole mount *tbx5a* expression, lateral view. Movements of photocleaved Kaede 14 hpf lateral retinal cells are generally consistent with the pattern of movement of *tbx5a*—compare magenta domains in A-D to A'-D' insets and to Figure 1 sections. White arrowheads in C and D mark extraocular ectodermal cells overlying the retina and lens.

Figure 3. bmp signals necessary for dorsal fate initiation are acting between 9.5 hpf and 13 hpf

Pharmacological inhibition of *bmp* signaling using the small molecule inhibitor LDN 193189 prevents initiation of dorsal fate in the retina. (A, B) *rx3* expression following 4-13 hpf continuous treatment with 1% DMSO in E2/GN (control) or 1 µM LDN 193189 in 1% DMSO E2/GN shows that treatment with LDN 193189 leads to severely dorsalized embryos, yet these embryos still form an eye field. (C, D) Contrary to DMSO controls, eye fields in LDN treated embryos fail to initiate expression of the prospective dorsal marker *tbx5a*. (E, F) Embryos treated from 9.5-13 hpf with 1% DMSO in E2/GN (control) or 1 µM LDN 193189 in 1% DMSO E2/GN both show normal *rx3* expression, indicating normal eye field formation. (G, H) However, contrary to controls, optic vesicles in treated embryos fail to initiate expression of the prospective dorsal marker *tbx5a*, suggesting that dorsal fate is never initiated if *bmp* signaling is inhibited after 9.5 hpf. All images are dorsal views, anterior to the left. Scale bar = 50 µm. Dashed yellow lines outline optic vesicle domains.

Figure 4. *gdf6a* is necessary for establishing dorsal retinal fate.

In *gdf6a* mutants, dorsal retinal patterning genes either fail to express, or have greatly downregulated expression. Ventral genes are turned on normally, but expand shortly after to fill almost the entire retinal field. Expression of dorsally or ventrally expressed genes is shown at stages shortly after they are first detectable in wild type. (A-D) Robust 12 hpf *tbx2a* in *gdf6a* siblings is only faintly detectable in *gdf6a* mutants (arrowhead in D). (E-H) Expression of *tbx5a* begins at 12 hpf (14 hpf shown in figure) in sibling embryos, but is never seen in *gdf6a* mutants (F, H). (I-N) Similarly, *bmp4* and *bmp2b* are completely lost in *gdf6a* mutants, while *tbx4* expression is initiated at very low levels (arrowhead in L). (O-P) Expression of *vax2* is only slightly expanded at 14.5 hpf, and continues to expand to fill most of the retina by 24 hpf (See supplementary Figure 3 R) Anterior to the left; A, B, E, F are dorsal views; C, D, G-R are lateral views. Scale bars = 50 µm. Dashed yellow lines outline optic vesicles.

Figure 5. *bmp2b* is necessary for dorsal retina initiation.

Despite severe morphological defects, *bmp2b* mutants develop *rx3* expressing eye fields. They fail, however to initiate expression of several dorsal markers. (A-C) *bmp2b* is expressed in the extraocular ectoderm in 13 hpf *gdf6a* siblings (A) and this expression domain is unchanged in *gdf6a* mutants (B). (D-G) *rx3* expression in siblings and *bmp2b* mutants at 12 and 14 hpf. (H, I) The earliest dorsal fate marker, *tbx2a*, is expressed in siblings beginning at 11 hpf (12 hpf shown, arrowhead). *tbx2a* is greatly downregulated in *bmp2b* mutants (H, arrowhead). (J, K) *tbx5a* expression is initiated at 12 hpf in siblings (13 hpf shown, arrowhead in I), but never turns on in *bmp2b* mutants (arrowhead in K where staining is absent). (L, M) In 12 hpf siblings, *pax2a* marks the prospective ventral retinal domain (*arrow*, L), which is also present in *bmp2b* mutants at this time point (*arrow*, M), despite severe dorsalization, which leads to a circular midbrain-hindbrain boundary (arrowheads; compare M with L). A-B, D-I, L-M are dorsal

views, anterior to the left. J-K are lateral views, anterior to the left; C is a transverse section. Scale Bars = 50 µm. Dashed yellow lines outline optic vesicles where boundaries are visible.

Figure 6. The dorsal initiation signal arises from non-neural ectoderm at 11-12 hpf.

Extraocular *gdf6a* is expressed in the correct time and place to initiate dorsal fate within the retina, and expression is absent in *bmp2b* mutants, lacking dorsal fate. These results suggest that lateral head ectoderm is the tissue responsible for initiating dorsal fate within the retina. (A-H) *gdf6a* expression in blue, *rx3* expression in orange. (A, C, K) *gdf6a* in *+/?* siblings is expressed in the non-neural head ectoderm, and is initially strongest anterior and posterior to the optic vesicle (arrows in A, arrowhead in A marks weaker expression lateral to the optic vesicle). At 11.5 hpf *gdf6a* expression has intensified covering the lateral portion of the optic vesicle (C, arrowhead; K transverse section, arrow). (E, G, L) *gdf6a* later expands to cover the majority of the dorsal leaflet of the optic vesicle (G; L transverse section, arrow). (K, L) Extraocular *gdf6a* at 11.5-13 hpf is directly adjacent to optic vesicle *tbx5a*, initiated at 12 hpf (Compare K, L to Figure 1 A0-A3).

(B, D, F, H, M, N) In *bmp2b* mutants the lateral extraocular domain of *gdf6a* is not detectable or greatly downregulated (arrowheads in B, D, F, H, arrows in M, N). Expression of *gdf6a* anterior to the optic vesicle is upregulated in mutants (arrows in D and F). (I, J, O) The extraocular lateral domain of the non-neural ectoderm and lens placode marker *pitx3* is also absent in *bmp2b* mutants (arrows in I, J), indicating that *bmp2b* mutants do not form the non-neural ectodermal tissue necessary for *gdf6a* expression and subsequent initiation of dorsal fate. (A-J) whole mount dorsal view, anterior to the left. (K-O) transverse sections. Scale Bars = 50 µm. Dashed yellow lines outline optic vesicles.

Figure 7. Lateral head ectoderm initiates dorsal retinal fate starting at 11 hpf

We propose that *bmp2b* acts upstream of *gdf6a* to pattern the non-neural ectoderm, the critical tissue for initiating dorsal retinal fate. *gdf6a* expressed in the non-neural ectoderm (prospective lens placode) during optic vesicle evagination, acts to initiate the expression of prospective dorsal markers within the lateral presumptive retinal field. The first polarized prospective dorsal markers, *tbx2a* and *tbx5a*, turn on at 11 and 12 hpf respectively (12 hpf shown in model) in the dorsolateral leaflet of the optic vesicle. Many cells expressing these dorsal markers undergo morphogenesis to populate the dorsal and central retina at 24 hpf.

Supplementary Figure 1. The prechordal plate, which expresses *bmp4*, is not necessary for dorsal retina initiation. (A-B) *One eyed pinhead* (*oep* ^{m134}) mutants do not initiate expression of *bmp4* in the prechordal plate region (C-D) *oep* mutants do not form prechordal plate tissues as assayed by expression of the prechordal plate marker *isl1* at 14 hpf (arrows in C and D). (E-F) However, dorsal retina develops normally in these mutants as assayed by expression of *tbx5a* at 22 hpf.

Supplementary Figure 2. *bmp4* is not necessary for initiation or maintenance of dorsal retinal fate.

(A-F) The dorsal-specifying genes *tbx5a*, *gdf6a*, and *ephrinB2a* are expressed normally in *bmp4*^{st72} mutants at 25 hpf, as is the ventral specifying gene *vax2*. (G-H) The ventral patterning gene *vax2* is also normally expressed in *bmp4* mutants at 25 hpf. mRNA expression of polarized dorsal and ventral genes (*tbx2a, tbx5a*, *gdf6a*, and *vax2)* is also initiated normally in *bmp4*^{st72} mutants at 12-15 hpf (data not shown). Scale Bar = 50 µm.

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Supplementary Figure 3. Gdf6a is necessary for maintenance of dorsal retinal fate.

In *gdf6a* mutants, dorsal retinal patterning genes are absent or greatly downregulated at 25 hpf. Ventral genes are expanded to fill the majority of the retinal field. (A-P) Polarized dorsal 25 hpf expression of *tbx2a, tbx5a, tbx4, bmp2b, bmp4,ephrinB2a, ephrinB1 and raldh2* in *gdf6a* siblings is absent in *gdf6a* mutants or faintly detectable in the case of *ephrinB2a* (arrowhead in L). (Q-T) Expression of *vax2* and *ephB2* ventral markers is robustly expanded at 25 hpf in gdf6a mutants to fill most of the retinal field (arrowhead in R marks small portion without *vax2* expression). (A-T) Anterior to the left; lateral views. Scale Bar = 50 µm.

Highlights

- Early optic vesicle cells expressing dorsal markers contribute to dorsal retina
- Bmps are required for initiating dorsal fate prior to *bmp* expression in retina
- Two *bmps* are required for dorsal retinal fate initiation, acting extraocularly
- Extraocular *bmp2b* is required, acting upstream of *gdf6a* in dorsal retina initiation
- Our work defines a basic mechanism for retinal patterning using extraocular signals

Figure 1:

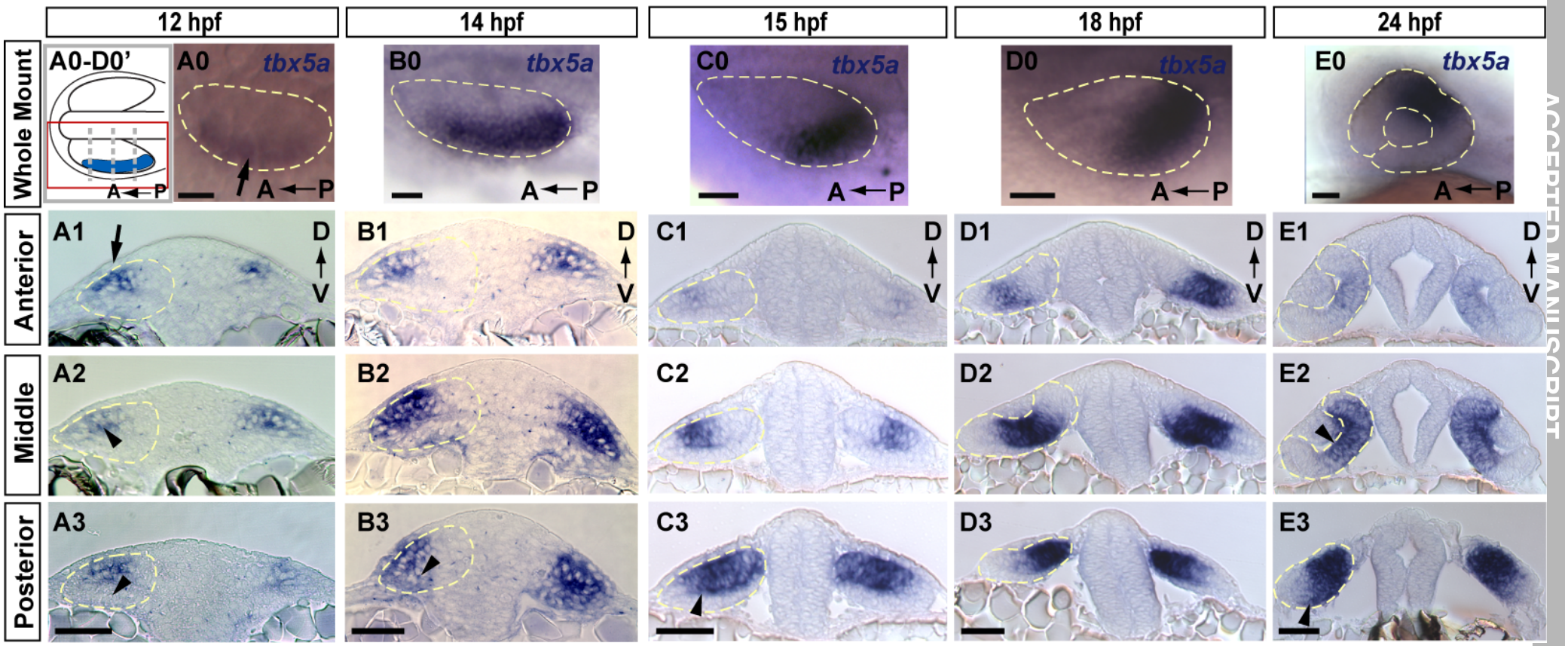
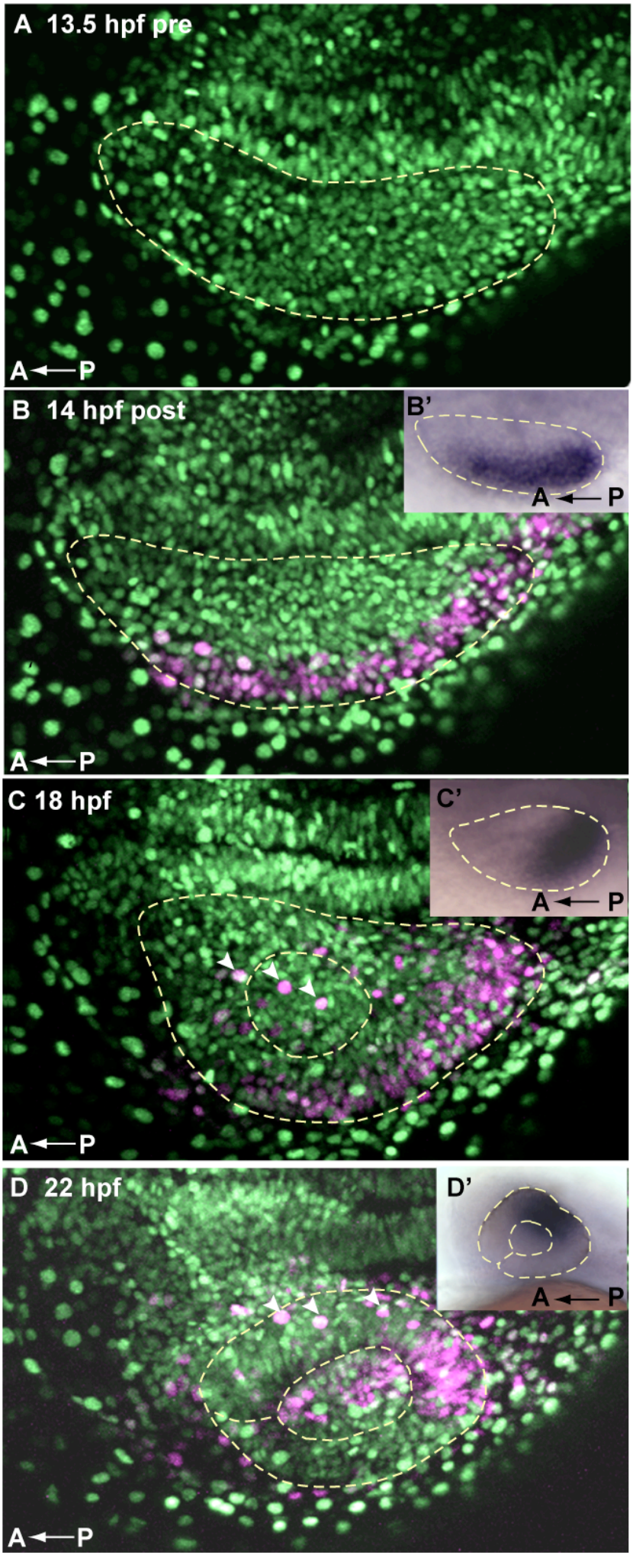


Figure 2:



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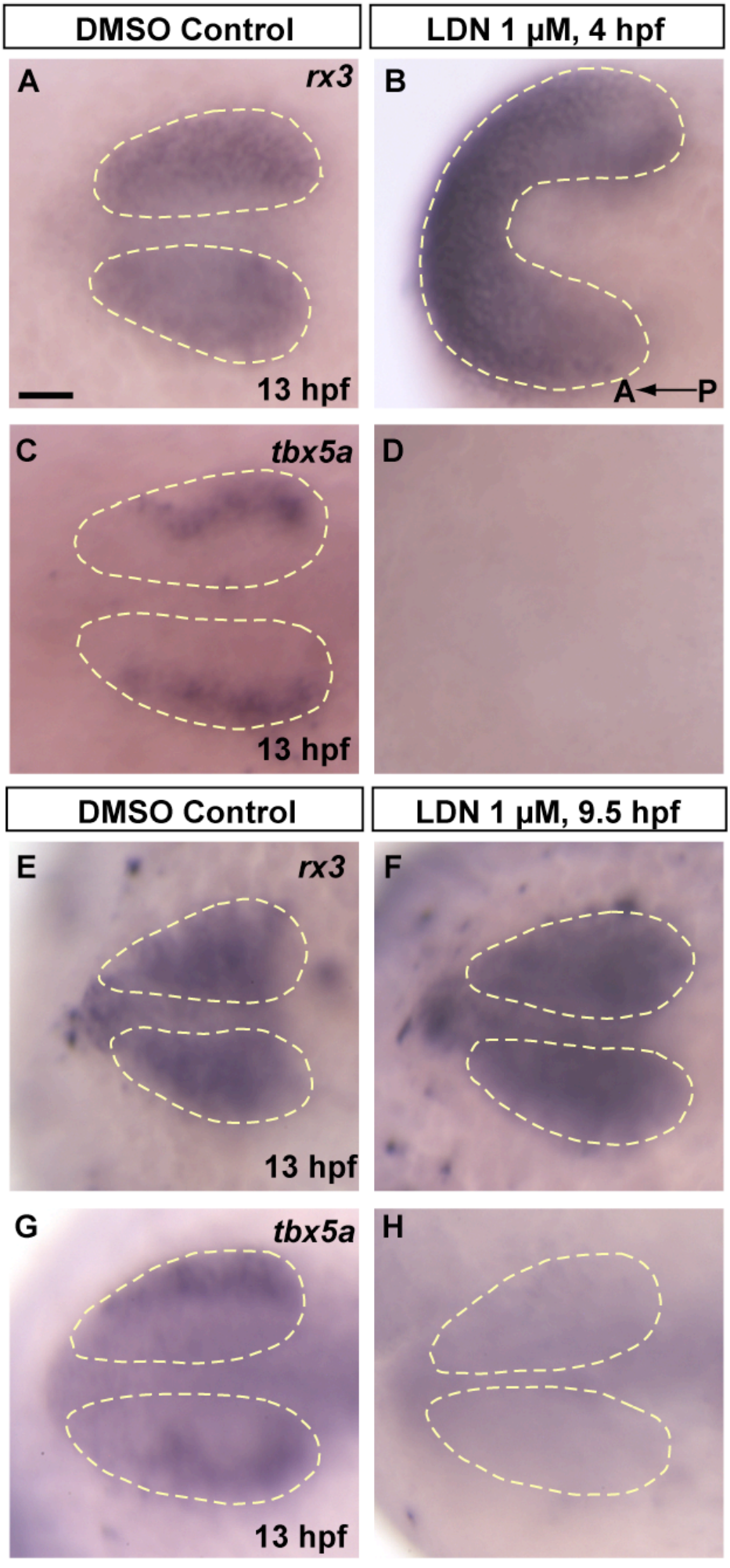

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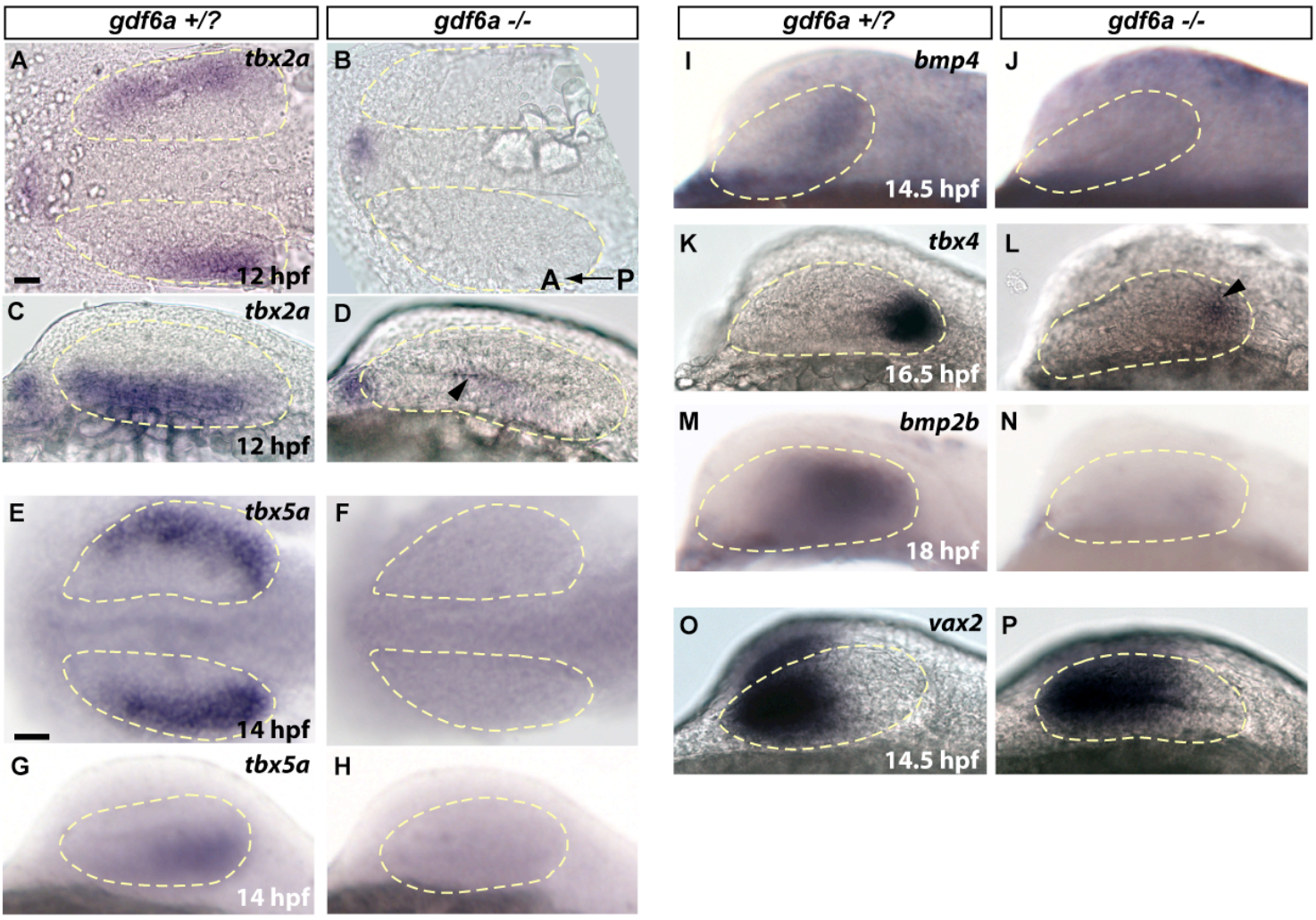

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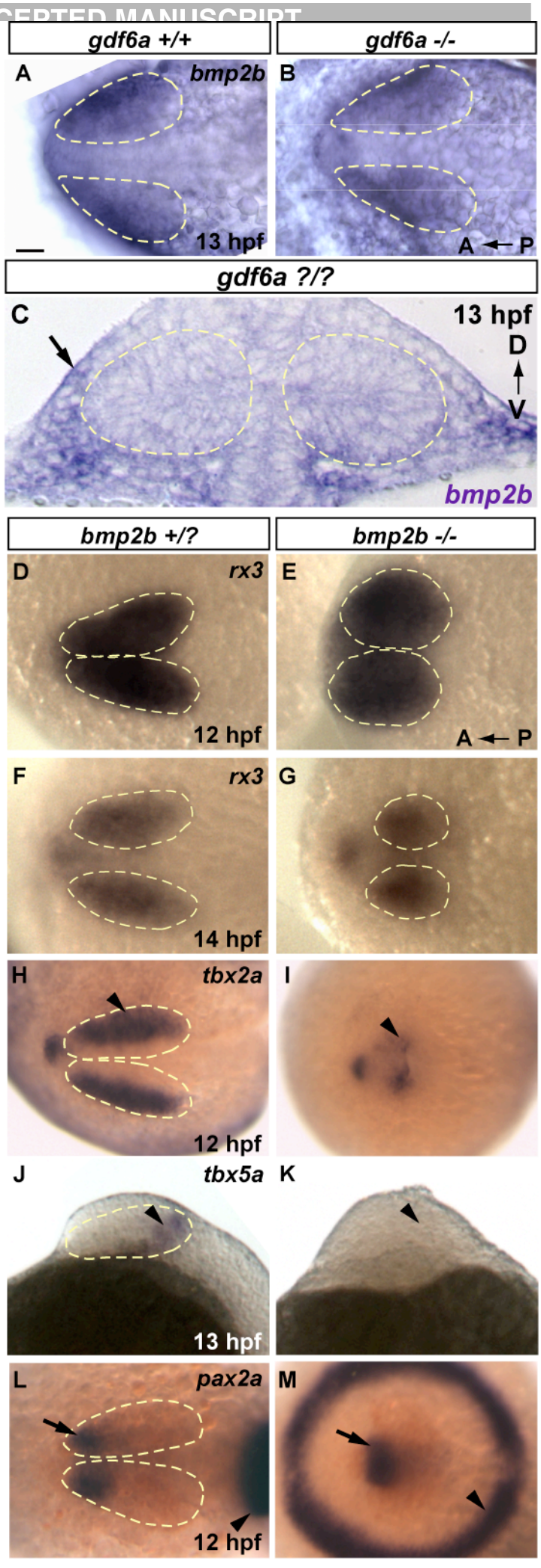

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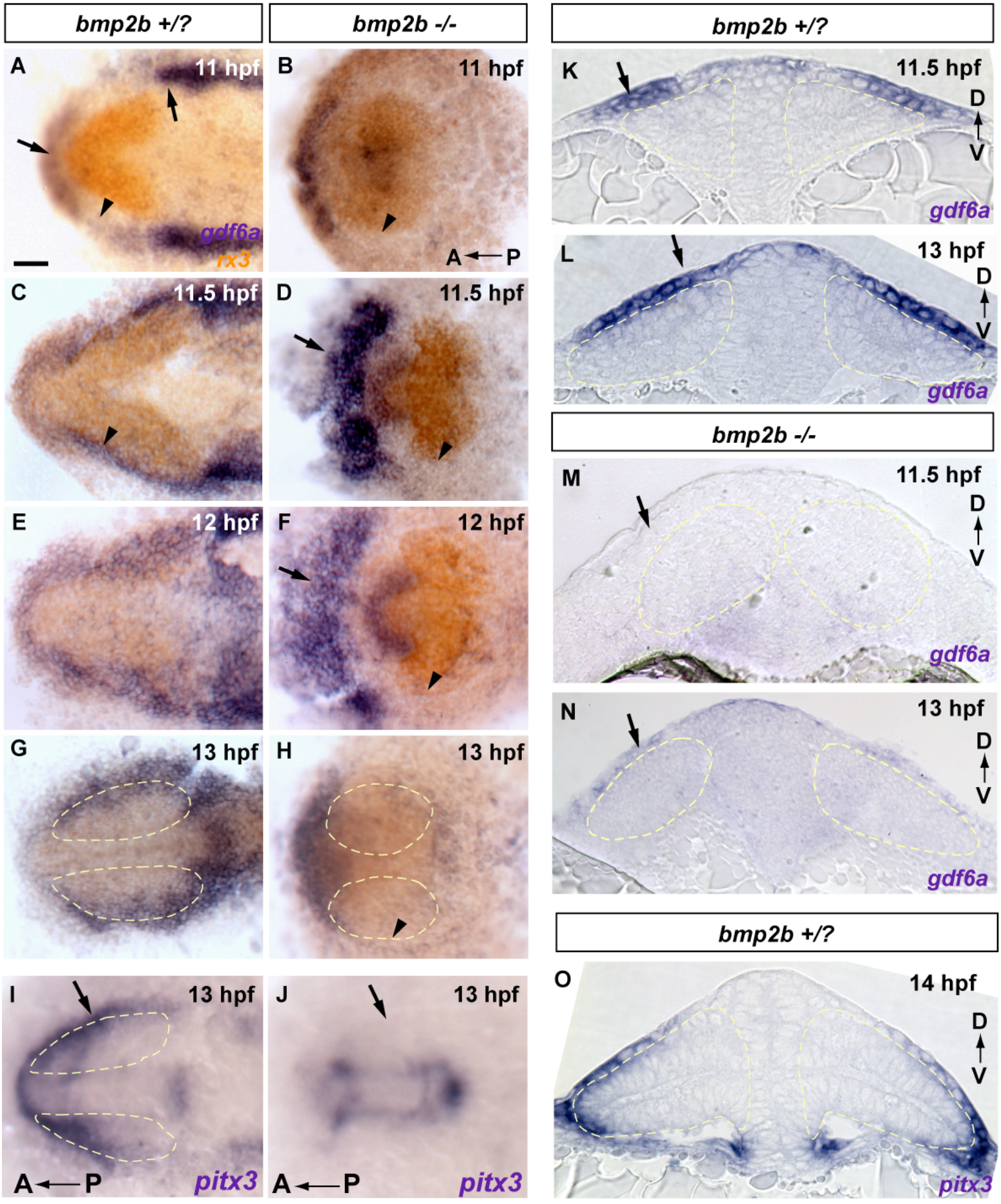

Figure 7: