Development 135, 4101-4111 (2008) doi:10.1242/dev.027367

Canonical Wnt signaling is required for the maintenance of dorsal retinal identity

Eric S. Veien[1,2], Judith S. Rosenthal[1], Renee C. Kruse-Bend[1,2], Chi-Bin Chien[1,2,3] and Richard I. Dorsky[1,2,3,*]

Accurate retinotectal axon pathfinding depends upon the correct establishment of dorsal-ventral retinal polarity. We show that dorsal retinal gene expression is regulated by Wnt signaling in the dorsal retinal pigment epithelium (RPE). We find that a Wnt reporter transgene and Wnt pathway components are expressed in the dorsal RPE beginning at 14-16 hours post-fertilization. In the absence of Wnt signaling, *tbx5* and Bmp genes initiate normal dorsal retinal expression but are not maintained. The expression of these genes is rescued by the downstream activation of Wnt signaling, and *tbx5* is rescued by Bmp signaling. Furthermore, activation of Wnt signaling cannot rescue *tbx5* in the absence of Bmp signaling, suggesting that Wnt signaling maintains dorsal retinal gene expression by regulating Bmp signaling. We present a model in which dorsal RPE-derived Wnt activity maintains the expression of Bmp ligands in the dorsal retina, thus coordinating the patterning of these two ocular tissues.

KEY WORDS: Wnt, Bmp, Dorsal retina, RPE, Zebrafish

INTRODUCTION

Vertebrate retinal development is a complex process that involves the coordination of morphogenetic tissue movements with gene expression. During this process, domains of gene expression are maintained despite large-scale changes in the size and shape of the retina and associated tissues. A key early step in eye development is the establishment of dorsal-ventral (DV) and nasal-temporal (NT) retinal polarity, manifested by the expression of specific genes in discrete retinal domains and leading to the accurate retinotopic targeting of retinal ganglion cell (RGC) axons to their targets in the brain. For example, in zebrafish, *tbx5* is expressed continuously in the presumptive dorsal retina starting from the early optic vesicle stage at 12 hours post-fertilization (hpf; Fig. 2M-P), whereas *vax2* is expressed in the ventral retina and the optic stalk starting from 12 hpf (Take-uchi et al., 2003). The activity of the transcription factors encoded by these and other genes ultimately leads to the correct DV topographical mapping of RGC axons to the optic tectum in anamniotes and avians, or to the superior colliculus in mammals, through the regulated expression of guidance molecules (reviewed by McLaughlin and O'Leary, 2005).

The sequence of events leading to ventral retinal identity is initiated when Sonic hedgehog (Shh) from the ventral midline triggers the expression of ventral retinal transcription factors, including *Vax2* (Ekker et al., 1995; Macdonald et al., 1995; Take-uchi et al., 2003; Zhang and Yang, 2001). Vax2 can exclude the expression of dorsal retinal genes from the ventral retina and also induce the graded expression of *Ephb2* and *Ephb3* (Schulte et al., 1999). This process leads to retinal progenitor cells that have been 'coded' with ventral positional identity in the form of EphB receptor tyrosine kinase expression (Barbieri et al., 2002; Mui et al., 2002; Schulte et al., 1999).

[1]Department of Neurobiology and Anatomy, [2]Program in Neuroscience, and [3]Brain Institute, University of Utah, 401 MREB, 20 N. 1900 E., Salt Lake City, UT 84132, USA.

*Author for correspondence (e-mail: richard.dorsky@neuro.utah.edu)

Accepted 22 October 2008

The establishment of dorsal retinal identity appears to be controlled by another family of growth factors. A current model of dorsal retinal patterning posits that Bmp4, expressed in the dorsal retina, triggers the graded dorsal expression of *tbx5*, which in turn leads to the graded expression of Ephrin B molecules (McLaughlin et al., 2003). Genetic inactivation of Bmp receptors and Bmp4 demonstrates the requirement of Bmp signaling for dorsal retinal identity in mouse (Murali et al., 2005), and misexpression of Bmp4 can dorsalize the ventral retina in chick and *Xenopus* (Koshiba-Takeuchi et al., 2000; Sasagawa et al., 2002). In zebrafish, multiple Bmp genes, as well as *tbx5*, are expressed in the dorsal retina (Rissi et al., 1995; Thisse and Thisse, 2005). Furthermore, at least one Bmp family member, Gdf6a, has been implicated in controlling the expression of dorsal retinal markers, including *tbx5*, in multiple vertebrate organisms (Asai-Coakwell et al., 2007; Delot et al., 1999; French et al., 2007; Hanel and Hensey, 2006). However, current models do not address whether Bmps or Tbx genes might act in distinct steps of dorsal patterning, such as initiation, maintenance or refinement, and leave open the possibility that other factors may also play key roles.

We were interested in whether canonical Wnt signaling acts in DV retinal patterning, based on several previous observations. The canonical Wnt pathway plays key roles in many important aspects of vertebrate CNS development, including the patterning of CNS structures (Bonner et al., 2008; Dorsky et al., 2003; Ille et al., 2007; Kapsimali et al., 2004; Kudoh et al., 2002; Muroyama et al., 2002). Several components of the canonical Wnt signaling pathway have also been shown to be expressed in developing vertebrate eye structures (reviewed by Van Raay and Vetter, 2004), suggesting their involvement in eye formation. Both Wnt reporter transgenes and Wnts themselves are expressed in the dorsal retinal pigmented epithelium (RPE) and in surrounding tissues during early eye development (Burns et al., 2008; Cho and Cepko, 2006; Fokina and Frolova, 2006; Liu et al., 2006). In the developing brain and spinal cord, both Bmp and Wnt signaling are required for proper dorsal patterning, and Shh induces ventral identities (for reviews, see Briscoe and Novitch, 2008; Ulloa and Briscoe, 2007; Zhuang and Sockanathan, 2006). The similarity in functions of Bmp and Shh in patterning both the neural tube and the retina raises the possibility that Wnt signaling may also have a conserved function in patterning

the dorsal retina. To date, we know of only one report suggesting a role for canonical Wnt signaling in DV retinal patterning. In analyzing *Lrp6*$^{-/-}$ mice, which lack expression of the Wnt reporter BAT-gal (Maretto et al., 2003), the authors observed that *Tbx5* is not expressed in the dorsal retina at E10.5, but they did not assay additional time points or other DV markers.

Here, we test the hypothesis that canonical Wnt signaling plays a role in the establishment of dorsal retinal identity. Through a combination of precisely timed in situ hybridization analyses and conditional misexpression experiments, we show that dorsal retinal identity in zebrafish is initiated at 12 hpf, very early in eye development, and then enters a maintenance phase between 14-16 hpf. We find that Wnt signaling is required for the maintenance of dorsal-specific retinal genes during this second phase, probably through the activation of Bmp signaling. We show that inhibition of the Wnt pathway leads to the loss of dorsal-specific retinal genes, with the concomitant expansion of ventral retinal genes. The loss of dorsal genes reflects a requirement for Wnt signaling in their maintenance, as they initiate their expression normally before Wnt signaling is active in the eye field. Finally, we show that Bmp signaling can rescue dorsal markers in the absence of Wnt signaling, but that activation of Wnt signaling cannot rescue dorsal markers in the absence of Bmp signaling, demonstrating that Wnts signal through Bmps to maintain the dorsal retinal domain.

MATERIALS AND METHODS

Animals

Zebrafish (*Danio rerio*) were maintained in a laboratory-breeding colony on a 14-hour/10-hour light/dark cycle. Embryos were maintained at 28.5°C and staged as described previously (Kimmel et al., 1995). The *Tg(TOP:GFP)*^{w25} stable transgenic line was generated by Dorsky et al. (Dorsky et al., 2002); the *Tg(hsp70l:dkk1-GFP)*^{w32} line was generated by Stoick-Cooper et al. (Stoick-Cooper et al., 2007); the *Tg(hsp70l:Tcf3-GFP)*^{w26} line was generated by Lewis et al. (Lewis et al., 2004); and the *Tg(hsp70l:nog3)*^{fr14} line was generated by Chocron et al. (Chocron et al., 2007). Wild-type fish and background of all transgenic lines were of the AB strain.

In situ hybridization

Digoxigenin–UTP-labeled riboprobes for *tbx5*, *bmp4*, *bmp2b*, *gdf6a*, *vax2*, *pax6a*, *pax6b*, *vsx2*, *egfp*, *efnb2a* and *ephb2*, and fluorescein–UTP-labeled riboprobe for *rx3* were synthesized by in vitro transcription. Probes for *vsx2* and *egfp* were synthesized in our laboratory. References for other probes are as follows: *tbx5* (Ruvinsky et al., 2000), *bmp4* (gift from M. Mullins, University of Pennsylvania), *bmp2b* (Nikaido et al., 1997), *gdf6a* (Open Biosystems EDR1052-524137; GenBank BI475848), *vax2* (Take-uchi et al., 2003), *pax6a* (Puschel et al., 1992), *pax6b* (Krauss et al., 1991), *efnb2a* (Durbin et al., 1998), *ephb2* (IMAGE Consortium clone 3714371). Whole-mount in situ hybridization and double in situ hybridization were performed as previously described (Jowett and Lettice, 1994). For histological analysis, embryos were stained for 40 hours in BM Purple (Roche Applied Sciences), re-fixed for 4 hours in 4% paraformaldehyde (PFA) in phosphate buffer, dehydrated, embedded in plastic and sectioned.

Transgenic heat-shock experiments

Adults heterozygous for the ΔTcf and Dkk1 transgenes were outcrossed to AB strain fish, and Noggin transgenic fish were outcrossed to TL strain fish. The resulting clutches were heat shocked at various times for 1 hour at 39°C (2 hours at 39°C for hs:Dkk1 and hs:Noggin), sorted for GFP expression under a fluorescent dissecting microscope, and fixed in 4% PFA at the required stages. Because the hs:Noggin transgene is untagged, these embryos were not sorted for GFP.

Lithium chloride treatment

For Dkk1 rescue, embryos were transferred to embryo water containing 150 mM LiCl at 11 hpf and removed to fresh water at 14 hpf. Heat-shock was performed at 12 hpf and embryos fixed at 24 hpf. For hs:Noggin rescue, embryos were transferred to embryo water containing 200 mM LiCl at 18 hpf until 24 hpf. Heat shock was performed at 18-20 hpf and embryos fixed at 24 hpf.

Bmp rescue experiments

The DNA construct *pDestTol2pA2;hsp70l:bmp4-IRES-GFP* was generated using the Tol2kit (Kwan et al., 2007). The construct (25 pg), along with 15 pg *tol2* transposase mRNA, was injected into one-cell stage embryos. Embryos were heat shocked at 12 hpf and fixed at 24 hpf.

RESULTS

Wnt signaling becomes active in the developing eye field between 14-16 hpf

Reasoning that the spatial and temporal domains of expression of Wnt pathway components in and around the developing eye field could provide clues to the function of Wnt signaling in establishing DV polarity, we sought to determine where and when the Wnt reporter TOP:dGFP is expressed. The *Tg(TOP:GFP)*^{w25} transgenic line expresses this reporter, which carries four LEF/TCF-binding sites driving destabilized EGFP, and has been shown to be a reliable readout of active Wnt signaling (Dorsky et al., 2002). To increase sensitivity, we detected the reporter by using in situ hybridization for *gfp* mRNA (Fig. 1A-F).

The eyes in zebrafish develop as a bilateral evagination of the anterior neural keel beginning at approximately 11 hpf to form the optic vesicles. At this stage, the optic stalk is located at the anterior of the optic vesicle, and the future dorsal retina is located posteriorly. At 16 hpf, the optic vesicle begins to invaginate to form the optic cup, and the lens placode forms from the surface ectoderm in contact with the presumptive neural retina. Finally, at about 22 hpf, the entire optic cup rotates approximately 90°, so that the posterior part of the optic cup becomes dorsal. At 12 hpf [6 somite stage (ss)], during early optic vesicle evagination, the rostral limit of active Wnt signaling is at the midbrain-hindbrain boundary (Fig. 1A), several cell diameters caudal to the eye field. At 14 hpf (10 ss), the *gfp* signal has extended rostrally along the neural tube to the presumptive telencephalon, but still appears to be excluded from the optic vesicles (Fig. 1B,C). By 16 hpf (14 ss), Wnt signaling activity is clearly evident in the optic vesicles and is restricted to the dorso-posterior presumptive RPE (Fig. 1D,F). In embryos sectioned coronally through the midbrain, TOP:dGFP expression is absent from the optic vesicles at 14 hpf, and is present in the presumptive RPE but not in the neural retina at 16 hpf (Fig. 1C,F). As development proceeds, TOP:dGFP expression becomes stronger in the developing eye, remaining in the dorsal RPE (Fig. 1E). By 24 hpf, TOP:dGFP is expressed throughout the entire RPE and ciliary marginal zone (Dorsky et al., 2002). This expression analysis shows that Wnt signaling becomes active in the dorsoposterior RPE between 14-16 hpf. Thus, any role played by Wnt signaling in the establishment of DV retinal polarity probably begins at this time. Furthermore, it suggests that the reception of Wnt signaling is localized to the presumptive RPE and excluded from the neural retina at optic vesicle stages.

We next analyzed the expression of Tcf transcription factors and Wnt ligands by in situ hybridization at 12 hpf (6 ss) and 18 hpf (18 ss). There are five Tcf transcription factor family members in zebrafish: Tcf7, Lef1, Tcf3a (Headless; Tcf7l1a), Tcf3b (Tcf7l1b) and Tcf4 (Tcf7l2) (Dorsky et al., 1999; Kim et al., 2000; Veien et al., 2005). At 12 hpf, during optic vesicle evagination, *tcf3a*, *tcf3b* and *tcf4* are expressed throughout the anterior neural tube and optic vesicle primordia, while the other family members are not expressed in this region (Fig. 1G-J; data not shown). By 18 hpf, *tcf3a* and *tcf3b*

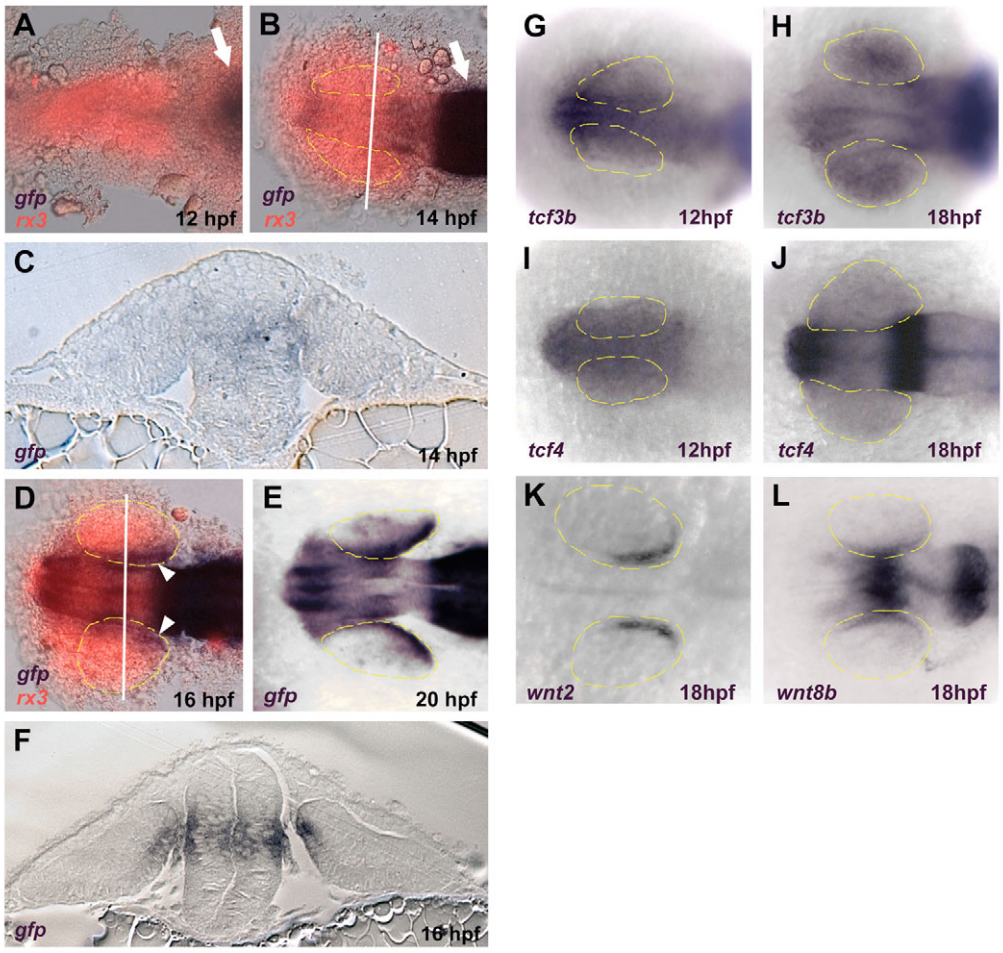

Fig. 1. Wnt signaling becomes active in the dorso-posterior retinal pigmented epithelium (RPE) between 14 and 16 hpf. (**A-F**) Expression of the TOP:dGFP Wnt reporter detected using in situ hybridization for *gfp* (blue). In A, B and D, the embryos were also probed for *rx3* expression (red) which marks the eye field. (A,B) Dorsal views, anterior left. Active Wnt signaling does not extend rostrally past the midbrain-hindbrain boundary (arrows) at 12 hpf, and approaches but does not enter the eye field at 14 hpf. (C,F) Coronal sections through caudal midbrain/posterior optic vesicles, dorsal up. The lines in B and D indicate the planes of section in C and F, respectively. Active Wnt signaling is seen in the dorso-posterior RPE at 16 hpf, but not at 14 hpf. (D,E) Dorsal views, anterior left. Active Wnt signaling is clearly present in the dorso-posterior eye field at 16 and 20 hpf. (**G-L**) Dorsal views, anterior left. (G-J) Expression of *tcf3b* and *tcf4* is present in the early eye-field during optic vesicle evagination (12 hpf) and throughout the eye at 18 hpf. (K) The Wnt ligand *wnt2* is expressed in the dorsal RPE at 18 hpf. (L) Expression of *wnt8b* in the midbrain and RPE at 18 hpf.

are expressed at high levels throughout the optic vesicles, and expression of *tcf4* is present at somewhat lower levels in the same region. These expression patterns persist through 24 hpf (not shown). The expression of *tcf7* initiates at 16 hpf, specifically in the dorsal retina, and is maintained in this region through 36 hpf (Veien et al., 2005). We found no *lef1* expression in the optic vesicles at any stage examined (not shown). Of the approximately 20 Wnt ligands present in zebrafish, at least two, Wnt2 and Wnt8b, are expressed in or around developing eye structures. Expression of *wnt8b* has been previously observed in the dorsal RPE as early as 16 hpf (Kelly et al., 1995). We observed expression of both *wnt2* and *wnt8b* in the dorsal RPE at 18 hpf (Fig. 1K,L). Therefore, multiple Wnt ligands and Lef/Tcf factors are expressed in the right place and at the right time to mediate Wnt activation in the dorsal RPE during mid-somitogenesis stages.

Bmp ligands are expressed in developing eye structures (Behesti et al., 2006; Delot et al., 1999; French et al., 2007; Hocking and McFarlane, 2007; Liu et al., 2003; Lupo et al., 2005; Murali et al., 2005; Sakuta et al., 2006; Sasagawa et al., 2002). Because Bmp signaling can control DV retinal polarity in other vertebrates (Behesti et al., 2006; Liu et al., 2003; Murali et al., 2005; Plas et al., 2008), we wanted to determine which Bmp ligands might play a role in zebrafish dorsal retinal patterning. We therefore examined the expression patterns of the Bmp ligands *bmp4*, *gdf6a* and *bmp2b* during the initial stages of zebrafish eye development. *bmp4* does not appear in the optic vesicle until 14 hpf, when it begins to be expressed most strongly in the presumptive dorsal retina (Fig. 2A-C). At 24 hpf, *bmp4* expression is restricted to the dorsal retina (Fig. 2D). Although *gdf6a* and *bmp2b* are expressed in the ectoderm overlying the anterior neural plate at 12 hpf, they are not expressed in the retina until 16 and 14 hpf, respectively (Fig. 2E-G,I-K). These ligands also become restricted to the dorsal retina by 24 hpf (Fig. 2H,L). Interestingly, the putative Bmp target *tbx5* begins its expression in the retina earlier than do these Bmp ligands, initially in a lateral ocular domain at 12 hpf, immediately after optic vesicle

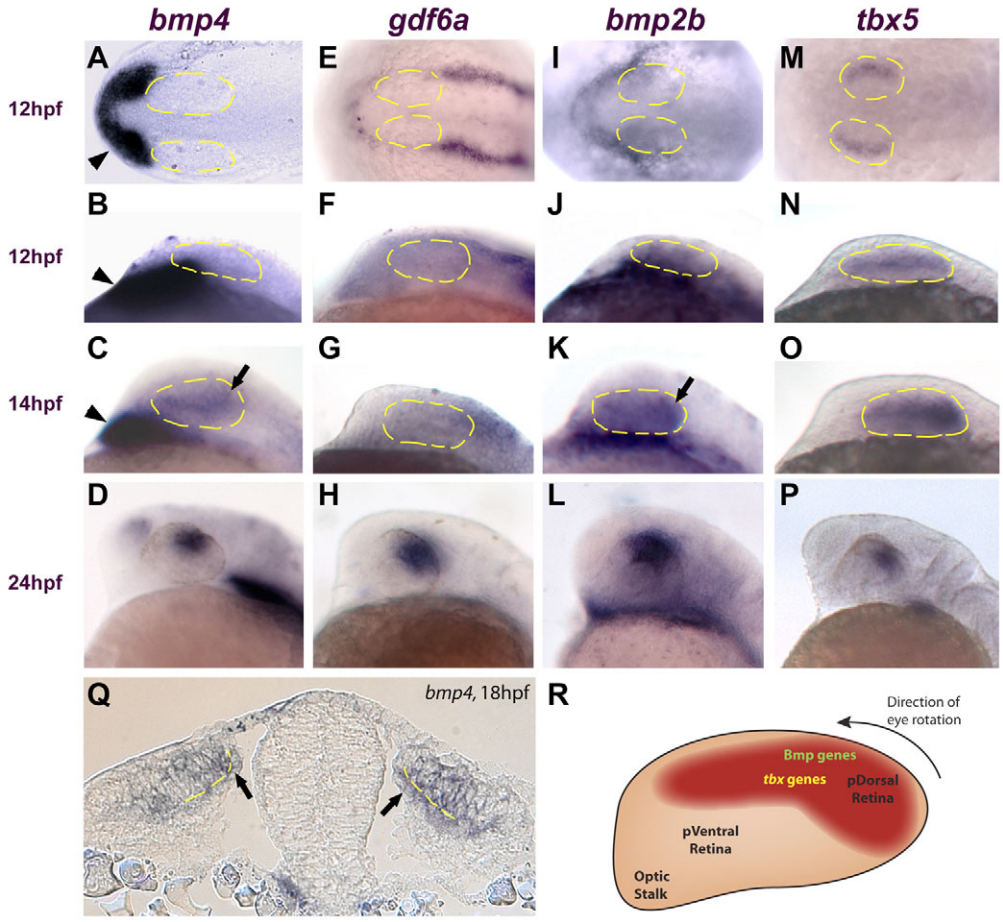

Fig. 2. Multiple Bmp genes and *tbx5* are expressed in the retina before canonical Wnt activity. (A,E,I,M) Dorsal views, anterior left. (B-D,F-H,J-L,N-P) Lateral views, dorsal up, anterior left. (**A-D**) *bmp4* is expressed in the prechordal mesoderm at 12 and 14 hpf (arrowheads in A-C) but is not expressed in the optic vesicle until 14 hpf (arrow in C). At 24 hpf, *bmp4* expression is restricted to the dorsal retina (D). (**E-L**) *gdf6a* and *bmp2b* are not expressed in the optic vesicle at 12 hpf (expression of these genes is restricted to the surface ectoderm). Expression of *bmp2b* is present in the retina at 14 hpf (arrow in K), but *gdf6a* does not appear in the optic vesicle until 16 hpf (not shown). Both genes are expressed in the dorsal retina at 24 hpf (H,L). (**M-P**) *tbx5* expression begins in the optic vesicle at 12 hpf and becomes progressively restricted to the dorsal retina by 24 hpf. (**Q**) Transverse section through the midbrain at 18 hpf. *bmp4* is expressed in the presumptive dorsal neural retina and RPE (arrows). Broken yellow lines indicate the interface between the neural retina and the RPE. (**R**) Diagram of the zebrafish retina at approximately 14 hpf, showing the expression domains of Bmp genes and *tbx5* at this timepoint. At approximately 22 hpf, the entire eye rotates 90° in the direction indicated. Anterior left, dorsal up.

evagination (Fig. 2M,N). As the eye undergoes morphogenetic changes, the *tbx5* domain becomes reoriented, coming to occupy the presumptive dorsal retina at 14 hpf (Fig. 2O) and eventually the dorsal retina at 24 hpf (Fig. 2P). A cross-section through the midbrain and optic vesicles reveals that *bmp4* is expressed in both the presumptive RPE and the retina at 18 hpf (Fig. 2Q, arrows). Thus, multiple Bmp ligands and *tbx5* are expressed in and around in the optic vesicle during mid-somitogenesis, including in the presumptive dorsal retina (Fig. 2R). These data suggest that Bmp factors in either the RPE or the retina could act to maintain *tbx5* expression in the dorsal retina, and that both *bmp* and *tbx5* expression in the retina precede Wnt activity.

Repression of Wnt target genes leads to the loss of *tbx5*

To examine the role of canonical Wnt signaling in the establishment of DV retinal polarity, we used a zebrafish line that expresses a dominant-repressor form of Tcf3 (ΔTcf) fused to GFP under the control of the *hsp70* promoter [*Tg(hsp70l:Tcf3-GFP)*^{w26}]. This transgene has been shown to reliably repress Wnt target genes in an inducible manner (Lewis et al., 2004). A heterozygous outcross of these fish was heat shocked and embryos were sorted for GFP fluorescence to examine the effect of ΔTcf expression on retinal patterning. Activation of the ΔTcf transgene at any of multiple timepoints resulted in the abolition of *tbx5* expression, with no effect on *tbx5* expression in non-transgenic embryos (Fig. 3A-H). When the transgene was activated (HS) at 10 hpf and embryos were fixed (F) at 18 hpf (HS10; F18), *tbx5* expression was strongly downregulated in 100% (n=37) of embryos. In HS10; F24 and HS18; F24 experiments, 100% (n=40, 49) of embryos showed a similarly strong reduction of *tbx5* expression. At these later timepoints, *tbx5* expression was maintained in non-ocular areas such as the heart and pectoral fin buds (data not shown). When the transgene was activated as late as 24 hpf (HS24; F30), *tbx5* expression was still strongly reduced in 93% (n=109) of embryos. Thus,

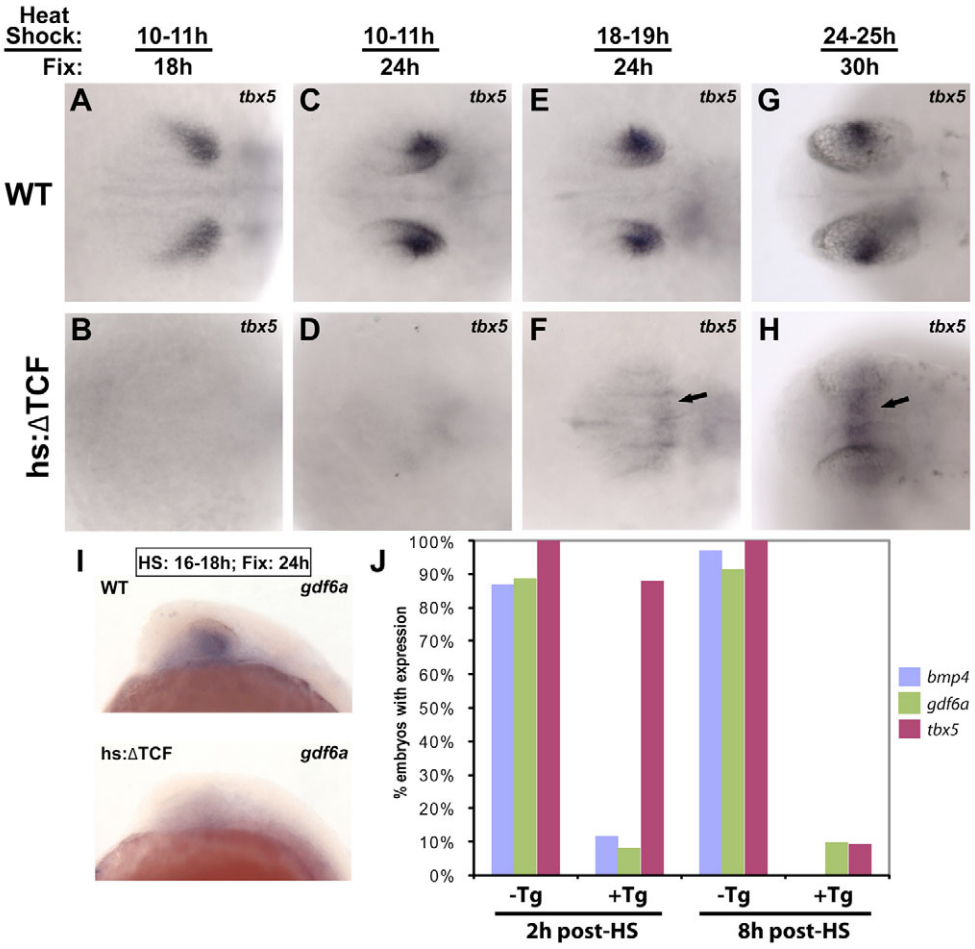

Fig. 3. Expression of dorsal retinal genes is lost following the repression of Wnt signaling. The *Tg(hsp70l:Tcf3-GFP)*^{w26} transgenic zebrafish line, which expresses a dominant-repressor form of Tcf3 (ΔTcf-GFP) upon heat shock, was used for these experiments. Dorsal views, anterior left. (**A-H**) Embryos were heat shocked and fixed at the indicated times, and sorted for GFP expression. The repression of Wnt targets led to the downregulation of *tbx5* in the dorsal retina at every timepoint. *tbx5* expression was upregulated in the dorsal diencephalon at later timepoints (arrows in F,H). (**I**) Expression of *gdf6a* was also eliminated in embryos expressing ΔTcf at 18 hpf and fixed at 24 hpf. (**J**) To determine the times at which *bmp4*, *gdf6a* and *tbx5* are lost in the dorsal retina following the repression of Wnt targets, embryos were heat shocked at 16 hpf and fixed 2 and 8 hours later. *bmp4* and *gdf6a* were strongly reduced at the 2 hour timepoint, whereas *tbx5* was still expressed. By 8 hours, the expression of all three genes was lost.

regardless of when the heat shock was performed or when the embryos were fixed, activation of the ΔTcf transgene eliminated *tbx5* expression in the dorsal retina, suggesting that *tbx5* is downstream of Wnt signaling in this region.

At later heat-shock timepoints, we noticed that *tbx5* was ectopically expressed in the dorsal diencephalon (Fig. 3F,H), perhaps because ΔTcf represses a gene that normally represses *tbx5* in this region. Together with maintained *tbx5* expression in non-ocular areas, this result suggests that *tbx5* may be an indirect transcriptional target of Wnt signaling. In the course of examining other dorsal markers, we observed that *gdf6a* and *bmp4* expression were also reduced following ΔTcf expression (Fig. 3I; not shown). This suggested that the downregulation of *tbx5* could be a result of decreased Bmp signaling. To further investigate this possibility, we examined the expression of Bmp genes and *tbx5* in more detail, focusing on the timecourse of downregulation of these genes following ΔTcf expression. Following heat shock at 16 hpf, we found that *bmp4* expression was present in 12% (n=17) of optic vesicles at 2 hours post-heat shock (18 hpf) and in 0% (n=44) at 8 hours post-heat shock (24 hpf). *gdf6a* was present in 9% (n=11) at 2 hours post-heat shock and in 11% (n=9) at 8 hours post-heat shock. By contrast, *tbx5* expression was present in 88% (n=25) of embryos at 2 hours post-heat shock, and in 9% (n=67) at 8 hours post-heat shock (Fig. 3J), indicating that Bmp genes are downregulated before *tbx5*. These data are consistent with a model in which *tbx5* is indirectly regulated by Wnt signaling through Bmp activity.

Wnt signaling is required for the maintenance of dorsal retinal genes

A potential concern with the ΔTcf transgene is that it might repress targets that contain Tcf-binding sites but are not controlled by endogenous Wnt activity. Thus, we used a second transgenic fish line that expresses a secreted inhibitor of Wnt signaling, Dickkopf 1 (Dkk1), upon heat-shock stimulation [*Tg(hsp70l:dkk1-GFP)*^{w32}] (Stoick-Cooper et al., 2007). This transgene inhibits Wnt signaling at the receptor level instead of at the transcriptional level, and thus is expected to block only active Wnt signaling. Embryos in which Dkk1 is activated early (9 hpf) display an enlarged head and a truncated tail (not shown), phenotypes associated with the loss of Wnt signaling, and downregulation of the Wnt reporter TOP:dGFP (Stoick-Cooper et al., 2007). When Dkk1 expression was activated at 9 hpf and embryos were fixed at 15 hpf, the dorsal marker *tbx5* was expressed normally (Fig. 4A,B). This result was consistent with our findings that active Wnt signaling begins in the eye field between 14 and 16 hpf, after *tbx5* expression has been initiated at 12 hpf. However, Dkk1 misexpression resulted in the strong downregulation of *tbx5* in the dorsal retina at 18 hpf and 24 hpf, which is similar to the results obtained using ΔTcf; at later timepoints, *tbx5* was reduced, although not completely absent (Fig. 4C-J). At the HS10; F18 timepoint, 96% (n=71) of embryos had strongly reduced *tbx5* expression. At HS10; F24 and HS18; F24, *tbx5* expression was strongly reduced in 88% (n=65) and 83% (n=71) of embryos, respectively. By contrast, at the last timepoint (HS24; F30), only 26% (n=19) of embryos showed reduced *tbx5*

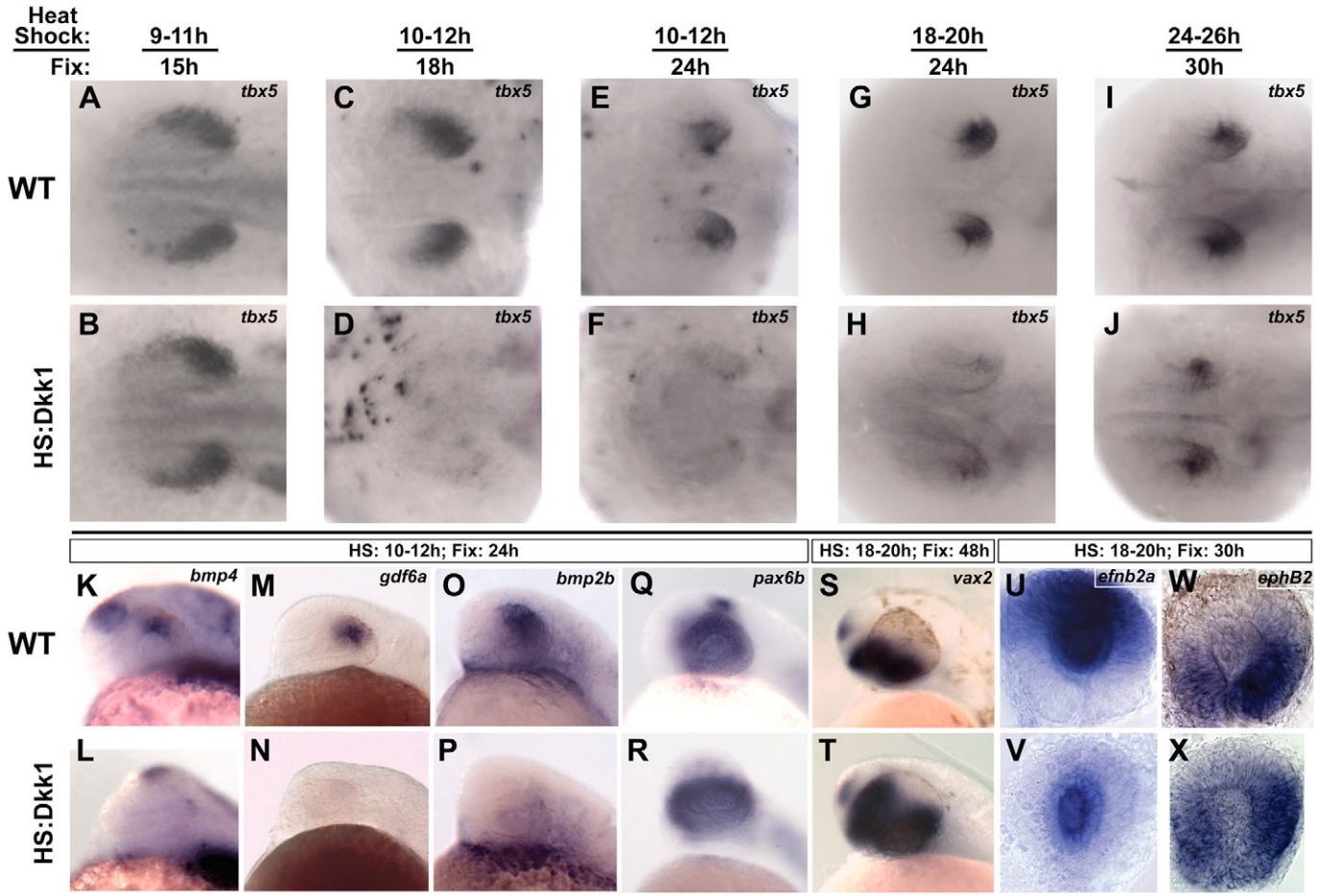

Fig. 4. Wnt signaling is required for the maintenance of dorsal retinal identity. The *Tg(hsp70l:dkk1-GFP)*^{w32} transgenic zebrafish line, which expresses the secreted Wnt pathway inhibitor Dkk1 upon heat shock, was used for these experiments. (A-J) Dorsal views, anterior left. Embryos were heat shocked and fixed at the indicated times, and sorted for GFP expression. (**A**,**B**) Embryos fixed just before Wnt signaling becomes active in the dorsal RPE express *tbx5* normally, showing that *tbx5* expression initiates properly in the absence of Wnt signaling. (**C-J**) Inhibition of Wnt signaling caused a strong downregulation of *tbx5* at the early timepoints, with a weaker effect at the last timepoint. This demonstrates a requirement for Wnt signaling in the maintenance of *tbx5*. (K-T) Lateral views, dorsal up, anterior left. (**K-P**) Expression of the Bmp ligands *bmp4*, *gdf6a* and *bmp2b* are lost from the dorsal retina following Wnt inhibition, suggesting a loss of dorsal character. (**Q-T**) *pax6b* is expressed normally and *vax2* expands dorsally, suggesting a ventralized retina. (**U-X**) Whole eyes, dorsal up. Following Wnt inhibition, the expression of *ephrin B2a* (*efnb2a*) is downregulated in the dorsal retina, but maintained in the lens, and *ephb2* expands dorsally.

expression in the dorsal retina. This may indicate that Wnt signaling is required for the expression of dorsal retinal genes during a time window of approximately 14-24 hpf, a developmental period in which the eye goes through dramatic morphological changes (C.-B.C. and K. Kwan, unpublished), and when genes that are initially expressed in a broad retinal domain refine their expression to the dorsal retina. The finding that *tbx5* expression in Dkk1-expressing embryos initiates normally and then later disappears suggests that Wnt signaling is necessary for the maintenance of *tbx5*, but not for its initiation.

We next examined whether the expression of Bmp ligands was affected by Dkk1 misexpression. Similar to *tbx5*, *bmp4* (91%, *n*=46), *gdf6a* (95%, *n*=58) and *bmp2b* (96%, *n*=25) were all reduced when the transgene was activated at 10 hpf and embryos were fixed at 24 hpf (Fig. 4K-P). To rule out the possibility that blocking Wnt signaling grossly perturbs eye development, we looked at the pan-retinal markers *pax6a*, *pax6b* and *vsx2* in embryos heat shocked at 10 hpf and fixed at 24 hpf. These markers were unaffected in transgenic embryos (Fig. 4R; data not shown), indicating that the retina is specified correctly and that Wnt signaling specifically acts on dorsally restricted retinal markers. Recent studies have shown that the loss or inhibition of dorsal-specific retinal genes such as *bmp4* and *tbx5* correlates with a concomitant expansion of ventral genes into the dorsal retinal domain (Behesti et al., 2006; Koshiba-Takeuchi et al., 2000; Murali et al., 2005; Sasagawa et al., 2002); thus, we examined the expression of the ventral retinal gene *vax2* in embryos induced to express Dkk1 at 18 hpf and fixed at 48 hpf. The expression of *vax2* expanded significantly into the dorsal retina in 76% (*n*=13) of these embryos (Fig. 4J). These results demonstrate that, in the absence of Wnt signaling, the retina forms correctly but is ventralized.

Because experimental manipulations of Bmp and Tbx5 levels have been shown to perturb the expression of the Ephrin B and EphB axon guidance molecules (Koshiba-Takeuchi et al., 2000; Murali et al., 2005), we examined the dorsal gene *ephrin B2a* (*efnb2a*) and the ventral gene *ephb2* in Dkk1-expressing embryos heat shocked at 18 hpf and fixed at 30 hpf. In accord with the observed reduction in *tbx5* expression and expansion of *vax2*

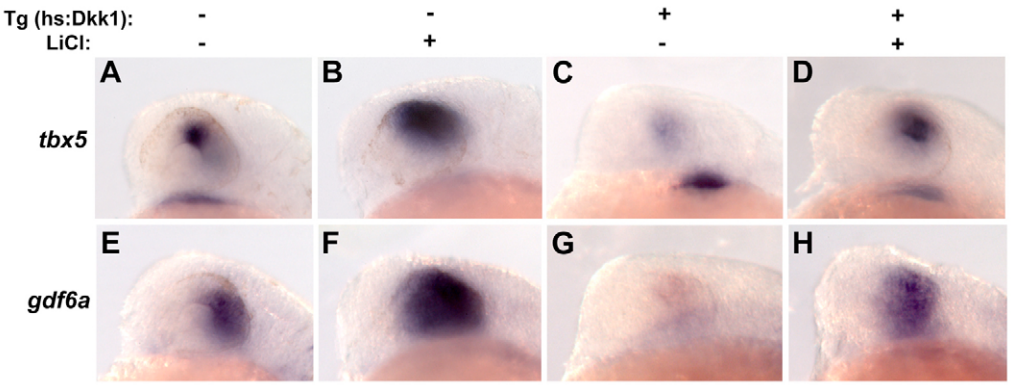

Fig. 5. Activation of Wnt signaling rescues loss of dorsal eye markers in Dkk1-expressing embryos. (**A-H**) Dkk1-expressing embryos were treated with the Wnt pathway activator LiCl (150 mM) from 11-14 hpf, heat shocked at 12 hpf, and fixed at 24 hpf. The expression of *tbx5* (A-D) and *gdf6a* (E-H) were analyzed by in situ hybridization. LiCl led to an expansion of *tbx5* and *gdf6a* expression in embryos not expressing Dkk1 (B,F), and a rescue of *tbx5* and *gdf6a* in embryos expressing Dkk1 (D,H). Lateral views, dorsal up, anterior left.

expression, *efnb2a* was strongly reduced in 93% (*n*=29) of embryos, and *ephb2* was modestly expanded dorsally in 84% (*n*=19) of embryos (Fig. 4U-X). *efnb2a* is also expressed in the lens, and this domain of expression was still present after Dkk1 misexpression, again demonstrating the specific requirement of Wnt signaling for dorsal retinal gene expression. An obvious prediction from these results is that the retinotectal map will be perturbed in a predictable way. However, Dkk1-expressing embryos did not survive until 5 dpf, when retinotectal pathfinding could be assayed, thereby precluding such an analysis. In addition, multiple Wnt pathway components, including Wnt3 and Sfrp5, are expressed in the tectum and Wnt signaling is also required for axon guidance in this target tissue (Schmitt et al., 2006; Tendeng and Houart, 2006). Tissue-specific modulation of Wnt signaling in the eye is thus required to determine the ultimate role of this pathway in pathfinding. At this point, our data suggest that Wnt signaling is specifically required for the maintenance of dorsal retinal genes, the loss of which results in a dorsal expansion of ventral retinal genes.

Activation of Wnt signaling downstream of Dkk1 rescues the hs:Dkk1-GFP phenotype

If Wnt signaling is required for dorsal retinal identity, Wnt pathway activation downstream of the Dkk1-Lrp6 interaction should rescue the expression of dorsal retinal genes that is lost in embryos misexpressing Dkk1. To test this hypothesis in a temporally controlled manner, we used LiCl, which is known to inhibit glycogen synthase kinase 3β (Gsk3β), resulting in the accumulation of unphosphorylated β-catenin and the amplified transcription of Wnt target genes (Hedgepeth et al., 1997). LiCl (150 mM) was applied to embryos at 11 hpf, which were then heat shocked to induce Dkk1 transgene activation at 12 hpf. LiCl was then removed at 14 hpf and embryos were fixed at 24 hpf. We found that the expression of both *gdf6a* (30%, *n*=47) and *tbx5* (33%, *n*=108) were expanded in wild-type embryos treated with LiCl (Fig. 5B,F). Importantly, application of LiCl rescued *tbx5* and *gdf6a* expression to wild-type levels in 26% (*n*=69) and 51% (*n*=63) of Dkk1-expressing embryos, respectively (Fig. 5D,H). Although LiCl application at 11 hpf resulted in a highly variable phenotype, this rescue was significant because wild-type expression levels of *tbx5* and *gdf6a* were never seen in untreated Dkk1-expressing embryos. These results confirm a specific role for canonical Wnt signaling in the maintenance of dorsal retinal gene expression.

Loss of dorsal identity downstream of Wnt inactivation can be rescued by Bmp signaling

In order to examine the relationship between Wnt and Bmp signaling during the establishment of DV retinal polarity, we investigated whether the activation of Bmp signaling could substitute for the loss of Wnt signaling. We injected one-cell stage embryos with DNA for a Bmp4 construct driven by the *hsp70* promoter (*pDestTol2pA2;hsp70l:bmp4-IRES-GFP*). When injected into wild-type embryos heat shocked at 12 hpf, this construct led to the widespread clonal expression of *bmp4* and *gfp* throughout the embryos, and *gfp*-expressing clones were found within the retina in 85% (*n*=54) of these embryos, as assayed by in situ hybridization (Fig. 6A,B). We next injected this construct into *Tg(hsp70l:dkk1-GFP)*^{w32} embryos at the one-cell stage and heat shocked them at 12 hpf, which simultaneously blocked Wnt signaling and stimulated the clonal expression of Bmp4. In wild-type embryos, Bmp4 expression led to an expansion of *tbx5* into the ventral retinal domain in 38% (*n*=46) of embryos (Fig. 6D), showing that Bmps can upregulate *tbx5* in the eye. This result is consistent with a recent study which showed that implantation of Bmp4-soaked beads into the mouse eye causes a ventral expansion of *tbx2*, *tbx3* and *tbx5* (Behesti et al., 2006). In embryos expressing Dkk1, activation of Bmp4 rescued *tbx5* expression in 44% (*n*=62) of embryos (Fig. 6F). This rescue was specific to the eye, as no other part of the embryo displayed ectopic *tbx5* staining. We next examined whether the loss of *gdf6a* expression in embryos expressing Dkk1 could be rescued by the activation of Bmp4. No significant rescue was seen in this case (*n*=49; data not shown). Together with the rescue of dorsal genes by Wnt pathway activation described previously, these results show that: (1) *tbx5* is downstream of both Wnt and Bmp signaling; and (2) the activation of Bmp signaling rescues *tbx5* but not *gdf6a* expression. This supports a model in which Wnt signaling maintains dorsal retinal identity through the regulation of Bmp signaling.

Activation of Wnt signaling does not rescue dorsal identity lost from Bmp inhibition

Our results suggest that Wnt signaling maintains dorsal retinal markers by activating Bmp signaling, but another formal possibility is that Wnts and Bmps act in parallel. To address this point, we first confirmed that *tbx5* expression is lost following Bmp inhibition, then tried to rescue *tbx5* expression by activating Wnt signaling. The implantation of beads soaked with the Bmp inhibitor Noggin just

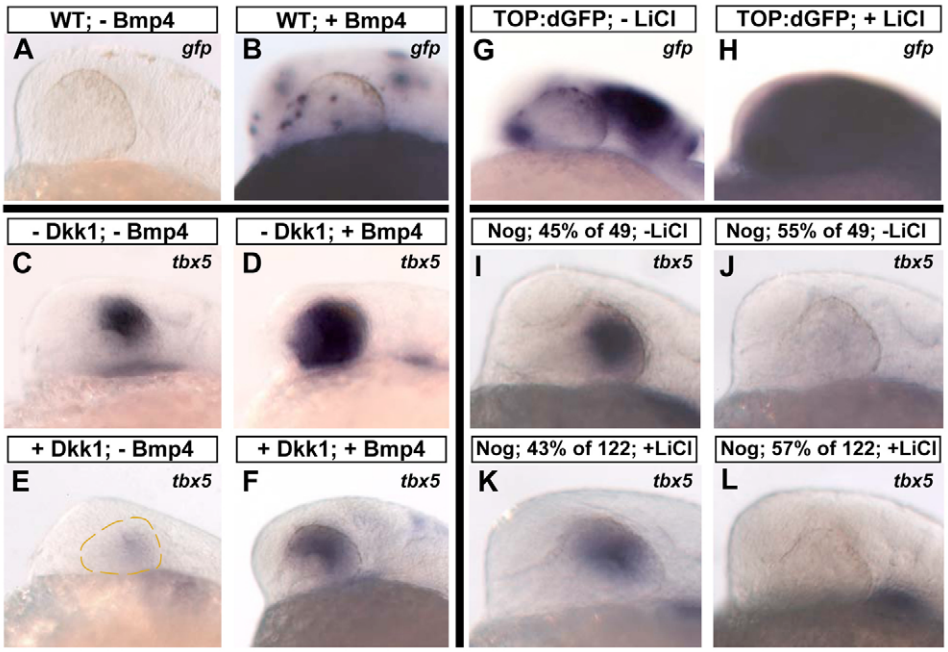

Fig. 6. Bmp signaling is downstream of Wnt signaling in the maintenance of dorsal retinal markers. (**A-F**) Bmp4 can rescue dorsal retinal markers in the absence of Wnt signaling. hs:Dkk1 and control wild-type (WT) embryos at the one-cell stage were injected with a construct that expresses Bmp4 upon heat shock (*pDestTol2pA2;hsp70l:bmp4-IRES-GFP*), heat shocked at 12 hpf, and fixed at 24 hpf. (A,B) To illustrate transgene expression following heat shock, in situ hybridization was performed for *gfp*. Widespread clonal expression was observed in the retinas in 85% of embryos. (C-F) Expression of Bmp4 caused a clear expansion of *tbx5* in embryos not expressing Dkk1 (D) and the rescue of *tbx5* in embryos expressing Dkk1 (F). (**G-L**) The activation of Wnt signaling does not rescue dorsal markers in the absence of Bmp signaling. Embryos heterozygous for the *Tg(hsp70l:nog3)*^{fr14} transgene, which express the Bmp pathway inhibitor Noggin upon heat shock, were outcrossed to TL strain fish and placed in 200 mM LiCl at 18 hpf. A 2-hour heat shock was performed at 18 hpf, and embryos were fixed at 24 hpf. To illustrate Wnt pathway activation, TOP:dGFP embryos were similarly treated with LiCl from 18-24 hpf and *gfp* detected by in situ hybridization (G,H). For hs:Noggin embryos untreated with LiCl, 55% of 49 embryos lost expression of *tbx5* (J) and, for embryos treated with LiCl, 57% of 122 embryos lost expression of *tbx5* (L), showing that the activation of Wnt signaling cannot rescue *tbx5* in the absence of Bmp signaling. Lateral views, dorsal up, anterior left.

dorsal to the optic vesicle was recently shown to abolish *tbx5* expression (Behesti et al., 2006). We used the transgenic fish line *Tg(hsp70l:nog3)*^{fr14}, which expresses Noggin upon heat-shock stimulation (Chocron et al., 2007). To achieve robust activation of Wnt signaling, embryos were placed in 200 mM LiCl from 18-24 hpf and heat-shocked at 18 hpf for 2 hours to activate Noggin expression. Embryos were fixed at 24 hpf and processed for *tbx5* expression by in situ hybridization. To confirm that these embryos had increased Wnt signaling, we treated the TOP:dGFP reporter line with LiCl from 18-24 hpf, which revealed strongly upregulated reporter expression (Fig. 6G,H). Because these embryos were obtained from a heterozygous outcross, we expected 50% of them to express Noggin. For embryos untreated with LiCl, 55% (n=49) embryos lost *tbx5* expression whereas, for embryos treated with LiCl, 57% (n=122) embryos lost *tbx5* expression (Fig. 6I-L). No significant rescue was seen; thus, inhibiting Bmp signaling indeed abolishes dorsal identity, and this effect is downstream of the dorsal-promoting effect of Wnt activation. Taken together, these experiments reveal a linear pathway in which Wnt signaling in the RPE maintains the identity of the dorsal retinal domain through the activation of Bmp signaling in the RPE and retina.

DISCUSSION

The activation of region-specific genetic cascades during early retinal development is thought to lead to the accurate topographic targeting of RGC axons to the optic tectum. In this study, we have shown for the first time that Wnt signaling is required for the proper development of DV retinal polarity. Our expression analysis suggests that Wnt signaling functions in the RPE, while Bmp ligands are expressed in both the RPE and retina (Figs 1, 2). Our results demonstrate that dorsal retinal genes initiate their expression normally at around 12 hpf in the absence of Wnt signaling, but soon thereafter require Wnt signaling for their maintained expression in the dorsal retinal domain (Figs 3, 4). The expression of Bmp ligands in the dorsal retina is dependent on Wnt signaling, and following Wnt inhibition the loss of at least one Bmp ligand, *gdf6a*, can be rescued by activation of Wnt signaling (Fig. 5). In addition, *tbx5*, an early marker of dorsal identity, is rescued by the activation of either Wnt or Bmp signaling following Wnt inhibition. By contrast, *tbx5* cannot be rescued by the activation of Wnt signaling in the absence of Bmp signaling (Figs 5, 6). These data together suggest a model for the maintenance of DV retinal identity in which Wnt signaling in the dorsal RPE transcriptionally maintains Bmp expression in the dorsal RPE and retina, which regulates the expression of downstream DV axis genes, including *tbx5* and Ephrin B axon guidance molecules (Fig. 7). This mechanism provides a conduit through which a Wnt signal in the RPE can exert its effects in the neural retina. It is likely that this mechanism functions to maintain the integrity of the dorsal retinal domain by coordinating its patterning with the dorsal RPE, but detailed fate-mapping in the developing retina and RPE is needed to show this conclusively.

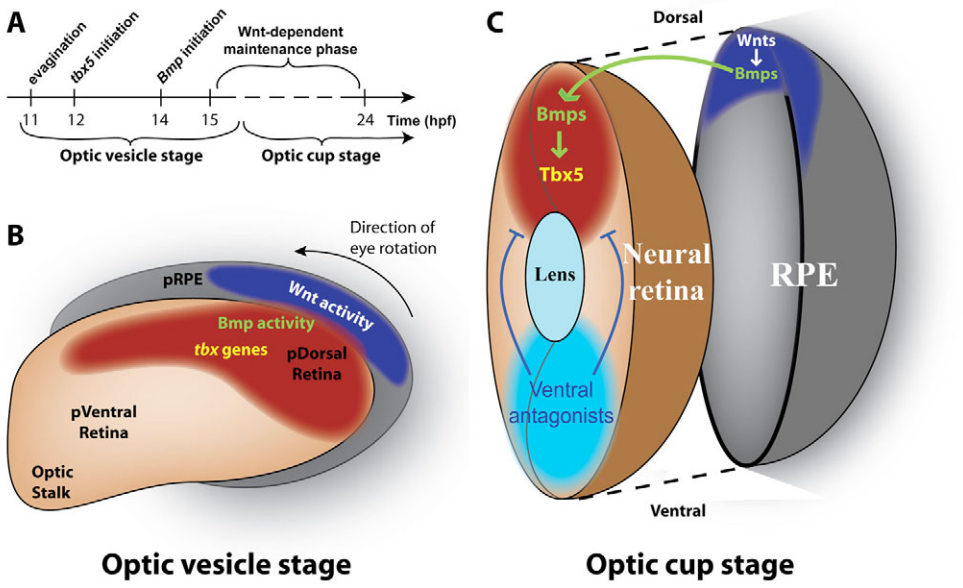

Fig. 7. Model for Wnt-dependent maintenance of dorsal identity. (**A**) Timeline of dorsal identity establishment. Optic vesicles evaginate from the anterior neural tube at 11 hpf. Soon thereafter, at 12 hpf, the first dorsally restricted marker, *tbx5*, begins to be expressed. Expression of Bmp ligands within the dorsal retina and RPE begins at 14 hpf, and a phase of Wnt-dependent dorsal identity maintenance begins between 14 and16 hpf. (**B**) Diagram of the optic vesicle at approximately 14 hpf. Wnt signaling becomes active in the dorso-posterior presumptive RPE at this point, Bmp signaling is active in the presumptive dorsal retina and the presumptive RPE, and *tbx5* is expressed in the presumptive dorsal retina. At about 22 hpf, the entire eye undergoes an approximate 90° rotation so that the posterior eye assumes its final dorsal position. (**C**) Model of Wnt-mediated maintenance of dorsal retinal identity. A Wnt signaling center in the dorsal RPE maintains Bmp expression in the dorsal RPE and retina. Bmp signaling then maintains *tbx5* expression in the dorsal retina. Simultaneously, inhibitors such as Bmp antagonists and transcription factors like Vax2 act to limit the extent of dorsal identity. pRPE, presumptive retinal pigmented epithelium; pDorsal Retina, presumptive dorsal retina; pVentral Retina, presumptive ventral retina.

Our expression analysis revealed that several Wnt pathway components are expressed in and around the developing eyes from the optic vesicle stage through 24 hpf. Of the five Tcfs present in zebrafish, only *tcf3a*, *tcf3b* and *tcf4* are present in or around the optic vesicles at the stage when Wnt signaling becomes active in the dorsal RPE (14-16 hpf). *tcf3a* and *tcf3b* are both expressed at high levels in the evaginating optic vesicles, and *tcf4* is expressed in the same domain but at a slightly lower level (Fig. 1G-J). Although Tcf3 is usually referred to as a 'repressor' in the literature (for a review, see Arce et al., 2006), it may also function as an activator under conditions in which β-catenin is stabilized, and therefore Tcf3 and/or Tcf4 are likely to be the transcription factors through which Wnt signaling maintains the expression of dorsal retinal genes. A practical difficulty in testing this idea stems from the fact that Tcf3 loss of function results in embryos lacking anterior forebrain structures, including eyes (Kim et al., 2000), whereas fish mutant for *tcf4* do not have a retinal phenotype on their own (Muncan et al., 2007) (our unpublished observations). Thus, the unique contribution of Tcf3a, Tcf3b and/or Tcf4 in mediating Wnt signaling relevant to the expression of dorsal retinal genes is still unknown. *tcf7* is also expressed in the dorsal retina, but it appears after the initial onset of Wnt signaling in the dorsal neural retina, a domain slightly different from that of the Wnt reporter (Veien et al., 2005). Therefore we believe that *tcf7*, rather than mediating Wnt function in the dorsal RPE, may in fact be a downstream target of the pathway, and that it is possibly regulated through Bmp activity.

We identified Wnt2b and Wnt8b as being expressed in the dorsal RPE at 18 hpf, which suggested that Wnt activity from these two ligands might be responsible for the maintenance of the dorsal retinal domain. We thus used morpholino oligonucleotides targeted against these genes, both alone and in combination, to knock down their expression. Although the embryos were strongly affected by these manipulations, *tbx5* expression was still seen in the dorsal retina, but at lower levels (data not shown). These results point to the difficulty in studying Wnt ligands during development: they are often expressed in highly redundant, overlapping patterns. Other Wnts have been identified in the RPE of mouse and chick, including Wnt5a and Wnt5b (Fokina and Frolova, 2006; Rossi et al., 2007; Van Raay and Vetter, 2004). Further work is necessary to identify other members of the Wnt family that are expressed in the RPE and their individual contributions to DV retinal patterning.

Both the ΔTcf and Dkk1-expressing zebrafish lines are powerful tools with which to study the loss of Wnt signaling in a temporally-controlled manner, acting through distinct mechanisms. ΔTcf directly represses target genes containing Wnt response elements (WREs) within their promoters, and Dkk1 specifically inhibits Wnts from signaling through the canonical pathway by competing for Lrp receptor occupancy (reviewed by Arce et al., 2006). The early activation of either transgene resulted in the loss of *tbx5* and Bmp genes in the dorsal retina, providing evidence that the observed phenotype is not a result of ectopic repression of genes that are not normally responsive to Wnt signaling. The downregulation of multiple Bmp ligands suggests two possible nonexclusive mechanisms: Wnt signaling may transcriptionally regulate multiple Bmp genes, or there may be a positive-feedback mechanism through which one Wnt-dependent Bmp molecule positively regulates the expression of other Bmp genes. At later heat-shock timepoints, the activation of ΔTcf still led to the loss of dorsal markers, but *tbx5* expression was seen ectopically in the dorsal diencephalon. This suggests that a factor(s) present in the diencephalon normally

represses *tbx5* in this region, and is itself repressed by ΔTcf. In addition, this finding, together with maintained non-ocular *tbx5* expression and the downregulation of Bmp genes before *tbx5* in ΔTcf embryos, suggests that *tbx5* is an indirect target of Wnt signaling and supports our hypothesis in which Wnt signaling maintains the dorsal retinal domain through the regulation of Bmp signaling. However, we cannot completely rule out the possibility that *tbx5* is also a direct target of Wnt signaling in the retina, and direct analysis of *tbx5* regulatory elements is required to further address this issue. The activation of Dkk1 at 24 hpf led to a modest downregulation of *tbx5* in the dorsal retina at 30 hpf, suggesting that Wnt signaling is required during a specific time window, approximately 14-24 hpf, as the dorsal retinal domain is being established.

Wnt and Bmp signaling are known to co-regulate gene expression in several parts of the developing vertebrate embryo. For example, in zebrafish, Wnt8 and Bmp2b have recently been shown to be required for the establishment of ventrolateral mesoderm through their cooperative regulation of *vent*, *vox* and *ved* (Ramel et al., 2005), and Wnt and Bmp signals also function cooperatively in the formation of posterior structures through their regulation of genes such as *tbx6* (Szeto and Kimelman, 2004). Wnt and Bmp signals coordinately control the specification of dorsal spinal cord neurons by regulating *Olig3* transcription in mice (Zechner et al., 2007). Conditional ablation of the Bmp receptor 1a and β-catenin in the mouse heart revealed that Bmp signaling is required for the expression of *Tbx5* and specification of the first heart field, and that Wnt signaling is required for the expression of *Bmp4* and specification of the second heart field (Klaus et al., 2007). Thus, Wnt/Bmp co-regulation of gene expression and pattern formation is a general mechanism used in multiple places and at multiple times in developing embryos. Our results show that this mechanism is also used during maintenance of the dorsal retinal domain, and that Wnt signaling is itself required for Bmp pathway activity in this region. In addition to the possibility that Bmp genes may be direct targets of Wnt signaling, another possible mechanism by which Wnt signaling could regulate the Bmp pathway is through the Gsk3-dependent phosphorylation of Smad proteins (Fuentealba et al., 2007). The presence of differential Smad phosphorylation in the developing optic vesicle, particularly in the dorsal versus ventral presumptive RPE, would support such a model. The use of multiple signaling pathways for patterning a complex organ such as the eye has obvious advantages. If Bmp signaling alone regulated the expression of dorsal retinal genes, it would be difficult to maintain this expression in a static domain during morphogenetic tissue movements. Localized to the dorsal RPE, Wnt signaling could stabilize dorsal retinal identity by coordinating the development of these two tissues during eye patterning.

We thank Sabine Fuhrmann, David Grunwald and Corinne Houart for helpful discussions, and the Centralized Zebrafish Animal Resource (CZAR) for fish care. This work was supported by NIH T32 HD007491 (E.S.V.), NIH T32 DC008553-03 (R.C.K.-B.), and by NIH P01 HD048886 (R.I.D. and C.-B.C.).

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