

# Distinct developmental programs require different levels of Bmp signaling during mouse retinal development

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## Summary

The Bmp family of secreted signaling molecules is implicated in multiple aspects of embryonic development. However, the cell-type-specific requirements for this signaling pathway are often obscure in the context of complex embryonic tissue interactions. To define the cell-autonomous requirements for Bmp signaling, we have used a *Cre-loxP* strategy to delete Bmp receptor function specifically within the developing mouse retina. Disruption of a Bmp type I receptor gene, *Bmpr1a*, leads to no detectable eye abnormality. Further reduction of Bmp receptor activity by removing one functional copy of another Bmp type I receptor gene, *Bmpr1b*, in the retina-specific *Bmpr1a* mutant background, results in abnormal

retinal dorsoventral patterning. Double mutants completely lacking both of these genes exhibit severe eye defects characterized by reduced growth of embryonic retina and failure of retinal neurogenesis. These studies provide direct genetic evidence that *Bmpr1a* and *Bmpr1b* play redundant roles during retinal development, and that different threshold levels of Bmp signaling regulate distinct developmental programs such as patterning, growth and differentiation of the retina.

Key words: *Bmpr1a*, *Bmpr1b*, Bmp signaling, Mutant mouse, Retinal patterning, Retinal growth, Retinal neurogenesis

## Introduction

Vertebrate eye development proceeds through a series of inductive processes involving multiple tissue components and has been studied as a model system to explore the general mechanisms underlying embryonic tissue interactions (Jacobson and Sater, 1988). Several families of secreted signaling molecules including the bone morphogenetic protein (Bmp) family are implicated in the control of inductive processes during normal eye development (Chow and Lang, 2001). Previous studies have implicated at least two members of the Bmp gene family, *Bmp4* and *Bmp7*, in mouse eye development. Both *Bmp4*<sup>-/-</sup> and *Bmp7*<sup>-/-</sup> null mutants exhibit defects in lens induction (Furuta and Hogan, 1998; Wawersik et al., 1999). In addition, *Bmp4*<sup>+/-</sup> heterozygotes show a spectrum of eye abnormalities in the adult (Chang et al., 2001). However, the precise genetic mechanisms by which Bmp signaling regulates these developmental processes are obscured by the embryonic lethality and variably penetrant eye phenotypes in these conventional null mutants.

Within the optic cup, Bmp signaling mediated by the

dorsally localized *Bmp4* ligand appears to control the patterning of the dorsoventral axis of the developing retina. Ectopic expression of *Bmp4* in the embryonic chick retina leads to the upregulation of the dorsal transcription factor, *Tbx5*, throughout the optic cup, and downregulation of the ventral markers, *Vax* and *Pax2* (Koshiba-Takeuchi et al., 2000). *Bmp4* signaling may be subject to negative regulation by the ventrally localized *Bmp4* antagonist, ventroptin (*Chrd11* – Mouse Genome Informatics) (Sakuta et al., 2001). Forcing dorsal expression of ventroptin in the early chick retina represses dorsal *Bmp4* expression and expands *Vax* expression. These molecular changes result in severely abnormal projection patterns of retinal ganglion cell axons. Bmp signaling has also been implicated in other aspects of retinal development. *Bmp4* can influence proliferation and cell death in the chick retina (Trousse et al., 2001). Inhibition of Bmp signaling in the chick retina by overexpression of the Bmp antagonist, Noggin, leads to disruption of ventral retinal structures (Adler and Belecky-Adams, 2002). At present, however, the possible involvement of Bmp signaling in retinal cell differentiation in vertebrates has not been established,

whereas the Bmp homolog *decapentaplegic* (*Dpp*) is a principal regulator of retinal neurogenesis in *Drosophila*. Several Bmp ligands and cognate receptors are expressed in the developing mouse eye (Dudley and Robertson, 1997; Furuta and Hogan, 1998). One possible model to account for the diverse effects of Bmps in the eye is that the different ligand-receptor combinations can result in qualitative differences in signaling output. Alternatively, different developmental processes may require distinct levels of signaling activity as described in a morphogen model (Freeman and Gurdon, 2002).

The Bmp ligands signal via heteromeric complexes composed of type I and type II transmembrane serine/threonine kinase receptors (Mishina, 2003). In the ligand-activated complex, the type II receptors phosphorylate and activate type I receptors. Activated type I receptors in turn trigger downstream signaling by Smad proteins, which are responsible for transduction of the extracellular signal to the nucleus. In this system, the activity of type I receptors primarily dictates the level and specificity of the intracellular signaling. Among various type I receptors, *Acvr1* (Alk2), *Bmpr1a* (Alk3) and *Bmpr1b* (Alk6) are capable of mediating Bmp ligand signal, although the affinity for different ligands varies among these receptors (Mishina, 2003). Of these, *Bmpr1a* and *Bmpr1b* are expressed within the developing mouse retina (Furuta and Hogan, 1998). Sources of Bmp ligand that can potentially signal to the retina via *Bmpr1a* and/or *Bmpr1b* include *Bmp7* in the lens and peri-ocular mesenchyme, *Bmp4* in the dorsal retina, and *Bmp2* and *Bmp3* in the retinal pigment epithelium and surrounding mesenchyme, respectively (Dudley and Robertson, 1997; Furuta and Hogan, 1998; Wawersik et al., 1999). In order to resolve this complex signal transduction apparatus, it is crucial to evaluate the cell type specific roles of the signaling pathway in vivo. Moreover, although multiple receptor-ligand pairs have been suggested to exist by biochemical experiments, genetic evidence for redundancy among the various receptors is lacking.

Here, we report the generation of retina-specific Bmp type I receptor mutant mice to investigate the role of Bmp signaling within the developing retina. A graded diminution of Bmp signaling activity, achieved by combining mutations of *Bmpr1a* and *Bmpr1b* genes causes defects in multiple aspects of retinal development, including dorsoventral patterning, growth and differentiation. Our studies provide direct genetic evidence that *Bmpr1a* and *Bmpr1b* play largely redundant roles during early retinal development, and that different thresholds of Bmp signaling regulate distinct developmental programs in the retina.

## Materials and methods

### Mice

Mice carrying null and conditional mutant alleles of the *Bmpr1a* gene [*Bmpr<sup>-</sup>* (Mishina et al., 1995), referred to as *Bmpr1a<sup>-</sup>*; and *Bmpr(fx)* (Mishina et al., 2002), referred to as *Bmpr1a<sup>fx</sup>* in this paper], a null allele of *Bmpr1b* [*Bmpr1b<sup>tm1Kml</sup>* (Yi et al., 2000), referred to as *Bmpr1b<sup>-</sup>*], a null allele of *Bmp4* (Winnier et al., 1995) and a *Six3Cre* transgene to drive Cre recombinase expression in the developing retina (Furuta et al., 2000) have been previously described. Among multiple lines of *Six3Cre* transgenic mice generated, line 69 [designated TgN(*Six3Cre*)69Frty, according to Standardized Genetic Nomenclature for Mice ([www.informatics.jax.org/mgihome/nomen/gene.shtml](http://www.informatics.jax.org/mgihome/nomen/gene.shtml))] was

used to conditionally disrupt the *Bmpr1a<sup>fx</sup>* allele, owing to consistency of tissue-specific Cre expression and absence of abnormal phenotypes in homozygotes.

### Tissue preparations for marker analyses

Embryonic tissue explant culture using filters and BMP4-soaked beads was performed essentially as described (Furuta and Hogan, 1998). Recombinant human BMP4 protein was kindly provided by the Genetics Institute (Cambridge, MA). Embryos or cultured explants were fixed in 4% paraformaldehyde in PBS, dehydrated through a graded series of methanol, followed by whole-mount in situ hybridization (see below) or histological sectioning. Sections were processed for Hematoxylin and Eosin staining, in situ hybridization or immunohistochemistry (see below). For analyses of retinal cell type markers, fixed retinal tissues were embedded in polyester wax (Electron Microscopy Sciences, PA) for sectioning. For all the experiments described here, three or more animals/embryos of each genotype were examined.

### In situ hybridization

In situ hybridization using [<sup>35</sup>S] UTP-labeled riboprobes on sections or digoxigenin (DIG)-labeled riboprobes on sections or whole-mount embryos was performed essentially as described previously (Nagy, 2003). Templates for *Rldh1a1*, *Rldh1a3*, *Chx10*, *Brn3b* and *Math5* (*Atoh7* – Mouse Genome Informatics) RNA probes were obtained from a retinal EST library (Mu et al., 2001). Photos for radioactive in situ sections were taken by double exposures, digitally combining dark field images under a red channel and bright-field images under a blue channel. Pictures for DIG in situ sections were photographed under differential interference contrast illumination.

### Immunohistochemistry

Antibodies used for immunohistochemical detection were anti-Syntaxin monoclonal antibody (mAb) (HPC-1, Sigma), anti- $\beta$ -tubulin III mAb (TU-20, Chemicon International), anti-phospho-smad1(ser463/465)/smad5(ser463/465)/smad8(ser426/428) rabbit polyclonal (Cell Signaling Technologies), anti-cyclinD1 mAb (Santacruz Biotechnology) and rabbit antisera to protein kinase C- $\alpha$  (Sigma). Secondary antibodies were biotin conjugated anti-mouse IgG (goat), Cy2-conjugated anti-rabbit IgG and Cy3-conjugated anti-mouse IgG (Jackson Immuno Research Laboratories) in appropriate combination with primary antibodies. For detection of biotin-conjugated antibodies, specimens were incubated with peroxidase-conjugated streptavidin (Vectastain ABC kit; Vector Laboratories), followed by chromogenic reactions using diaminobenzidine (0.6 mg/ml in 50 mM Tris-Cl, pH=7.6) as a substrate in the presence of 0.03% H<sub>2</sub>O<sub>2</sub>.

### Analyses for cell proliferation and cell death

Pregnant mice were injected intraperitoneally with 1 ml/100 g body weight of Cell Proliferation Labeling Reagent (Amersham) 2 hours before embryo dissection. Sections of BrdU-labeled embryos were processed for immunohistochemical analysis using an anti-BrdU monoclonal antibody (Chemicon International). A biotin-conjugated anti-mouse secondary antibody, bound by extravidin-FITC was used for fluorescence detection. BrdU-positive cells were counted manually, and expressed as a percentage of total cell number. TUNEL apoptosis detection was performed using Apoptag Cell Death Detection Kit (Chemicon International) according to the manufacturer's instructions.

### Retinotopic mapping analyses

Anesthetized P7-P10 pups were injected with DiI (Molecular Probes) applied focally to dorsal or ventral region of the retina using a Nanoject (Drummond). For each labeling, ~9.2 nl of 10% DiI dissolved in dimethyl formamide was injected. The brain of labeled pups was dissected 2 days after injection. The superior colliculus was

exposed by removing cerebral hemispheres, observed and photographically documented under fluorescent stereoscopes.

## Results

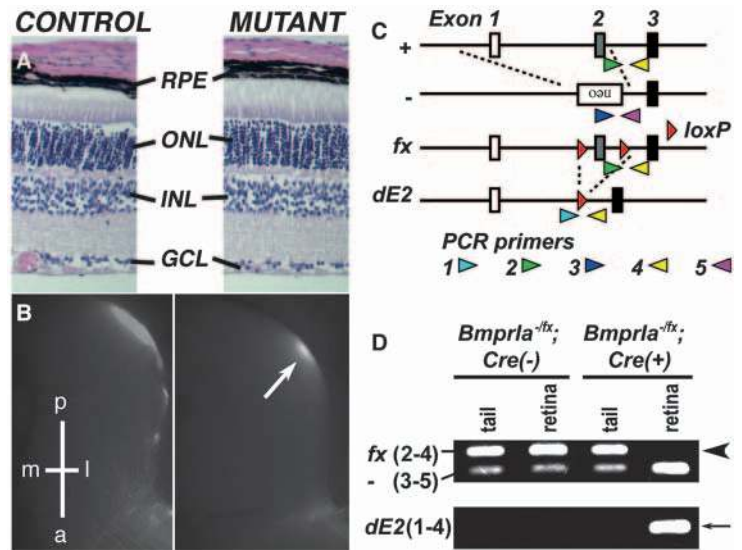
### Bmpr1a function is not essential for retinal development

*Bmpr1a* is expressed ubiquitously during early embryonic development (Mishina et al., 1995), including the developing eye (Furuta and Hogan, 1998). Mice homozygous for a *Bmpr1a*-null mutation die prior to gastrulation (Mishina et al., 1995). To directly test a role for this receptor in the developing retina, we specifically disrupted *Bmpr1a* gene function using the *Six3Cre* transgene, which drives Cre recombinase expression in the retina and ventral forebrain (Furuta et al., 2000). The conditional *Bmpr1a* allele results in a null allele upon Cre mediated recombination (Bartlett et al., 2002). In our mating scheme, we employ a combination of the null allele (Mishina et al., 1995) and conditional allele; mice transheterozygotes for these alleles (referred to as *Bmpr1a*<sup>-fx</sup> below) are thus hemizygous for the *Bmpr1a* locus prior to Cre-mediated recombination.

Animals lacking retinal *Bmpr1a* function (*Bmpr1a*<sup>-fx</sup> in the *Six3Cre* transgenic background; referred to as *Bmpr1a*<sup>-fx;Cre</sup> below) develop normally without overt ocular abnormalities. In the adult retina, the characteristic layered structure is retained in the mutant (Fig. 1A), without significant qualitative changes in the distribution of major retinal cell types examined (data not shown). Bmp signaling is implicated in dorsoventral patterning of the retina, which is reflected later in the retinotectal projection maps formed by retinal ganglion cell axons. We analyzed the trajectory of retinal ganglion cell axons from the retina to the superior colliculus (mid-brain) using focal injection of the lipophilic dye, DiI. In wild-type mice, dorsal retina-derived ganglion cell axons project to the lateroposterior quadrant of the contralateral mid-brain (e.g. Fig. 1B, left) and ventral ganglion cell axons, to the medioanterior domain (not shown). In the mutants, the dorsal axons project to their termination zones in a wild-type pattern, suggesting that retinotectal projections are unaffected (Fig. 1B, right). Genomic PCR analyses confirm extensive Cre-mediated recombination of the *Bmpr1a*<sup>fx</sup> allele throughout the differentiated retina in the adult (Fig. 1C,D), suggesting that the retina of *Bmpr1a* conditional mutants is entirely composed of *Bmpr1a*-deficient cells. As discussed below (Fig. 3), loss of *Bmpr1a* function appears to take place early during retinal development. Therefore, we conclude that *Bmpr1a* function is not essential for gross patterning, morphogenesis and differentiation of the developing retina.

### Both Bmpr1a and Bmpr1b function to maintain dorsoventral patterning of the retina

Transcripts of another Bmp type I receptor, *Bmpr1b*, are present in a ventral-high-to-dorsal-low gradient in the developing retina (Furuta and Hogan, 1998). *Bmpr1b* null mutant mice are viable, although they exhibit defects in chondrogenesis and female fertility (Baur et al., 2000; Yi et al., 2000). These animals also exhibit abnormal ventral retinal



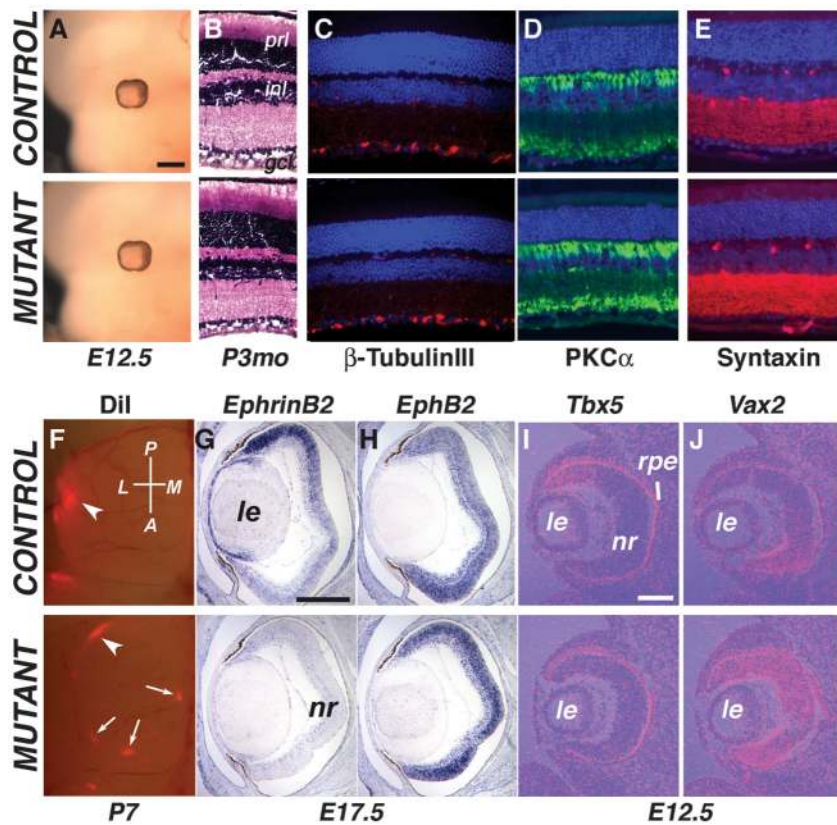
**Fig. 1.** *Bmpr1a* function is not required for embryonic retinal development. Control samples are from *Bmpr1a*<sup>+/fx</sup>; *Cre* mice and mutants are from *Bmpr1a*<sup>-fx</sup>; *Cre* mice. (A) Hematoxylin and Eosin stained sections of the adult retina from 3-month-old mice. Retinal lamination pattern and cell types are unaltered in the *Bmpr1a*<sup>-fx</sup>; *Cre* mutants (right), compared with control littermates. (B) Anterograde tracing of retinal ganglion cell axons into the superior colliculus of the midbrain of control (left) and mutant (right) 9-day-old pups that have received a focal injection of DiI into the right dorsal retina, arrow indicates termination zone of dorsal axons. The axes in the left superior colliculus are indicated; a, anterior; l, lateral; m, medial; p, posterior. (C) Schematic representation of the *Bmpr1a* genomic locus and various mutant alleles. (D) Genomic PCR to detect *Bmpr1a* alleles using primers indicated in C. The genotypes of the sample DNAs are indicated at the top. In the retina of adult mice carrying the Cre transgene, the amplicon for the conditional allele (*fx*) is undetectable (arrowhead), whereas in the tail the PCR product corresponding to the unrecombined conditional allele is readily detected. Consistent with recombination of the floxed allele occurring only in the retina, the *dE2* amplicon, which represents the recombined allele with exon 2 removed, is present only in the retina of mice carrying the Cre transgene. gcl, ganglion cell layer; inl, inner nuclear layer; onl, outer nuclear layer; rpe, retinal pigment epithelium.

ganglion cell axon behavior (Liu et al., 2003). To test whether *Bmpr1a* and *Bmpr1b* function redundantly in the retina, a null allele of *Bmpr1b* was introduced into the conditional *Bmpr1a* background. Animals lacking *Bmpr1a* and one copy of *Bmpr1b* (*Bmpr1a*<sup>-fx</sup>; *Bmpr1b*<sup>+/-</sup>; *Cre*) have normal eye size (Fig. 2A) and layered retinal morphology (Fig. 2B), without obvious changes in the distribution of major retinal cell types (Fig. 2C-E). However, analyses of retinotectal mapping revealed that in all mutant animals examined, many dorsal retinal ganglion cell axons form ectopic termination zones (Fig. 2F, bottom, arrows). The transmembrane receptor tyrosine kinases EphBs and the ephrin B (*Efnb* – Mouse Genome Informatics) ligands are expressed in countergradients along the retinal dorsoventral axis, and are implicated in ganglion cell axonal guidance during retinotectal map formation (McLaughlin et al., 2003). At embryonic day 17.5 (E17.5), dorsal *Efnb2* expression is completely abolished in the *Bmpr1a*<sup>-fx</sup>; *Bmpr1b*<sup>+/-</sup>; *Cre* embryos (Fig. 2G). Conversely, the ventral-high-to-dorsal-low gradient of *Ephb2* is no longer seen, with uniform transcript

levels throughout the retina (Fig. 2H). Earlier during development (E12.5), expression of a T-box transcription factor, *Tbx5*, implicated in dorsal specification of the retina (Koshiba-Takeuchi et al., 2000), is abolished (Fig. 2I). Furthermore, the expression domain of a ventral marker *Vax2* (Barbieri et al., 1999) is expanded throughout the entire retina (Fig. 2J). These observations are consistent with the notion that dorsal retinal character is lost in these mutants.

*Bmp4* expression in the optic vesicle initiates at around the 12-somite stage in the mouse and concomitant with lens formation, becomes progressively localized to the dorsal quadrant of the optic cup (Furuta and Hogan, 1998). Initiation of *Tbx5* expression at around the 16-somite stage (Bruneau et

al., 1999) is thus preceded by that of *Bmp4*. In *Bmp4*<sup>-/-</sup> mutants, *Tbx5* expression in the optic vesicle is absent from the 16-somite to >25-somite stages (Fig. 3A). This indicates that among multiple *Bmps* expressed in the developing mouse eye, *Bmp4* is the primary mediator of dorsal-specific gene expression. Although dorsoventral patterning is disrupted in *Bmpr1a*<sup>-f/x</sup>;*Bmpr1b*<sup>+/-</sup>;*Cre* embryos by E12.5, *Tbx5* expression appears normal until E10.5 (Fig. 3B). However, its expression is rapidly downregulated, and completely lost by E11.5 (Fig. 3C). These observations strongly indicate that a certain level of Bmp signaling is required not only for the induction, but also for the maintenance of *Tbx5* expression. Moreover, this loss of *Tbx5* expression between E10.5 and E11.5 probably



**Fig. 2.** Retinal dorsoventral patterning defects in *Bmpr1a*<sup>-f/x</sup>;*Bmpr1b*<sup>+/-</sup>;*Cre* mutant mice. Control littermates shown are representatives of the *Bmpr1a*<sup>+f/x</sup>;*Bmpr1b*<sup>+/-</sup>;*Cre* or *Bmpr1a*<sup>+f/x</sup>;*Bmpr1b*<sup>+/-</sup>;*Cre* genotypes. (A) The *Bmpr1a*<sup>-f/x</sup>;*Bmpr1b*<sup>+/-</sup>;*Cre* mutants show no overt morphological defects in the eye. (B–E) Hematoxylin and Eosin-stained sections (B),  $\beta$ -TubulinIII antibody staining for retinal ganglion cells (C), anti-PKC $\alpha$  for bipolar cells (D) and anti-Syntaxin for amacrine cells (E) reveal that the retinal lamination pattern and cell types are unaltered in the *Bmpr1a*<sup>-f/x</sup>;*Bmpr1b*<sup>+/-</sup>;*Cre* mutants (bottom) compared with control littermates (top). (F) Analyses of retinotectal axon projection in the superior colliculus of the midbrain in postnatal day 7 (P7) animals. Focal injection of DiI into the dorsal retina of the left eye reveals a single termination zone in a lateral-posterior region of the contralateral (right) superior colliculus in normal animals (top, arrowhead). By contrast, in the mutants, several ectopic termination zones are seen (bottom, arrows), in addition to a normal lateral-posterior spot (bottom, arrowhead). The axes in the right superior colliculus are indicated: A, anterior; L, lateral; M, medial; P, posterior. (G–J) Coronal sections of embryonic eyes with dorsal towards top. In the mutants, the expression of dorsal markers *Efnb2* (G) and *Tbx5* (I) is lost, while transcripts of *Ephb2* (H) and *Vax2* (J), which are normally ventrally enriched, are now expanded throughout the developing retina (H,J). gcl, ganglion cell layer; inl, inner nuclear layer; le, lens; nr, neuroretina; prl, photo receptor layer. Scale bar: 500  $\mu$ m in A,G,H; 100  $\mu$ m in I,J.

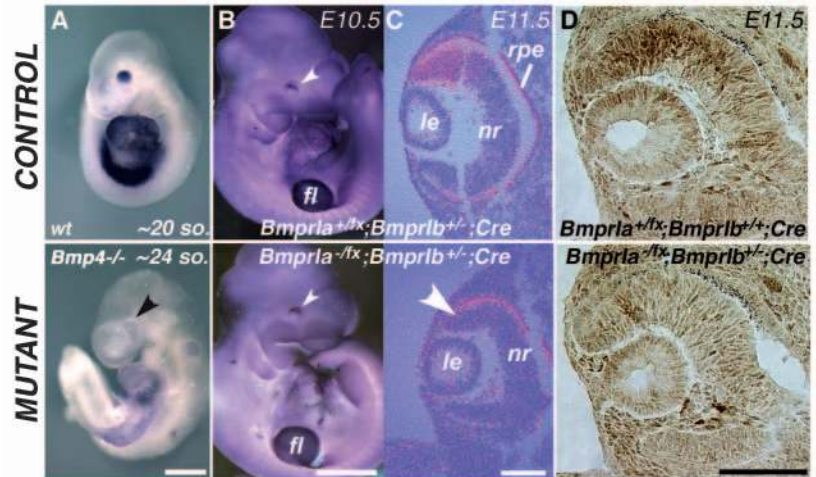
reflects the timing of loss of *Bmpr1a* function as a result of Cre-mediated recombination of the *Bmpr1a*<sup>f/x</sup> allele. This is further supported by the distribution of phosphorylated Smad1/5/8 protein (P-Smad) in the retina, which is a direct read-out of active Bmp signaling (Massague, 1998). High levels of P-Smad are detected in the dorsal retina of control animals but not in the mutant retina at E11.5 (Fig. 3D).

The retinoic acid signaling pathway is also implicated in the regulation of retinal dorsoventral patterning (Hyatt et al., 1996; Marsh-Armstrong et al., 1994; McCaffery et al., 1999). Genes coding for enzymes regulating retinoic acid synthesis, including *Rldh1a1* for Raldh1 and *Rldh1a3* for Raldh3, are differentially expressed along the dorsoventral axis of the retina (Fig. 4A,C). It has been suggested that the differential expression of these genes along this axis may be regulated by a *Bmp4*-*Tbx5* pathway (Mic et al., 2002). However, the expression of these genes appears unaffected in *Bmpr1a*<sup>-f/x</sup>;*Bmpr1b*<sup>+/-</sup>;*Cre* mutants (Fig. 4B,D).

### Bmp signaling is essential for retinal growth and neurogenesis

To address the absolute requirement of Bmp signaling in the retina, we also generated mutants that completely lack function of both *Bmpr1a* and *Bmpr1b* in the retina. The *Bmpr1a*<sup>-f/x</sup>;*Bmpr1b*<sup>-/-</sup>;*Cre* double mutants appear indistinguishable from the normal littermates until E10.5 (not shown). At birth, however, these double null mutants exhibit anophthalmia (Fig. 5A). Gross morphological abnormalities are apparent by E12.5, when double mutant embryos show smaller eyes with a rough margin of the retinal pigment epithelium and discontinuity of the pigment ventrally (Fig. 5B,C). Histological examination of double mutant retinas revealed that the abnormal retinal morphology first manifests as reduced retinal neuroectoderm thickness beginning at E11.25–11.50 (not shown), associated with excess apoptosis (Fig. 5D).

**Fig. 3.** High levels of Bmp signaling activity, principally mediated by Bmp4, tightly regulate *Tbx5* expression in the dorsal retina. (A,B) Whole-mount in situ hybridization for *Tbx5*. Lateral views of E9.0-9.5 embryos (A) and E10.5 embryos (B) probed for *Tbx5* transcripts. (A) Dorsal specific *Tbx5* expression is lost in the optic vesicle of an advanced *Bmp4*<sup>-/-</sup> mutant embryo (arrowhead). (B) *Tbx5* transcripts in the dorsal retina (arrowheads) are detected at comparable levels in both control (top) and *Bmpr1a*<sup>-flx</sup>;*Bmpr1b*<sup>+/-</sup>;*Cre* mutant (bottom) samples at E10.5. *Tbx5* transcripts are also strongly expressed in the forelimb buds (fl) in both control and mutant. (C) In situ hybridization on coronal sections of E11.5 embryos with dorsal towards the top. *Tbx5* expression is lost by E11.5 in the mutant retina (bottom, arrowhead). (D) Immunohistochemistry for P-Smad1/5/8 detects high levels of P-Smad protein in the dorsal retina of control embryos, defining regions receiving the highest Bmp signal (top). This pattern is lost in mutants (bottom). so, somites; fl, forelimb; le, lens; nr, neuroretina; rpe, retinal pigment epithelium. Scale bar: 500 μm in A; 1 mm in B; 100 μm in C,D.

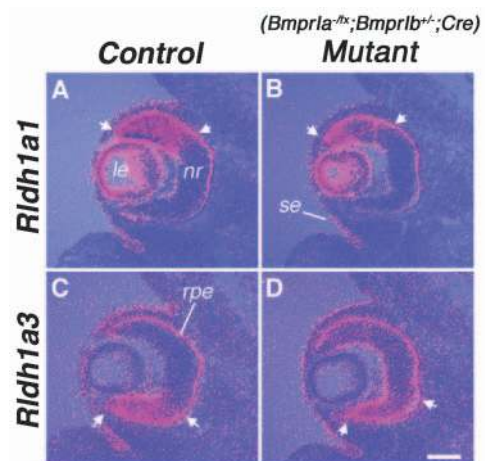


Compared with E11.5 wild-type retina, where apoptotic cells are observed in a central region of the retina around the future optic disc (Trousse et al., 2001) (Fig. 5D, top), high levels of apoptosis are found throughout the retina in the double mutants (Fig. 5D, bottom). In contrast to this elevated apoptosis in the mutant retina, the rate of cell proliferation appears unaffected in the majority of double mutant retinæ until around E11.5 (Fig. 5E,G). However, a drastic reduction in cell proliferation becomes evident soon thereafter (Fig. 5F,G).

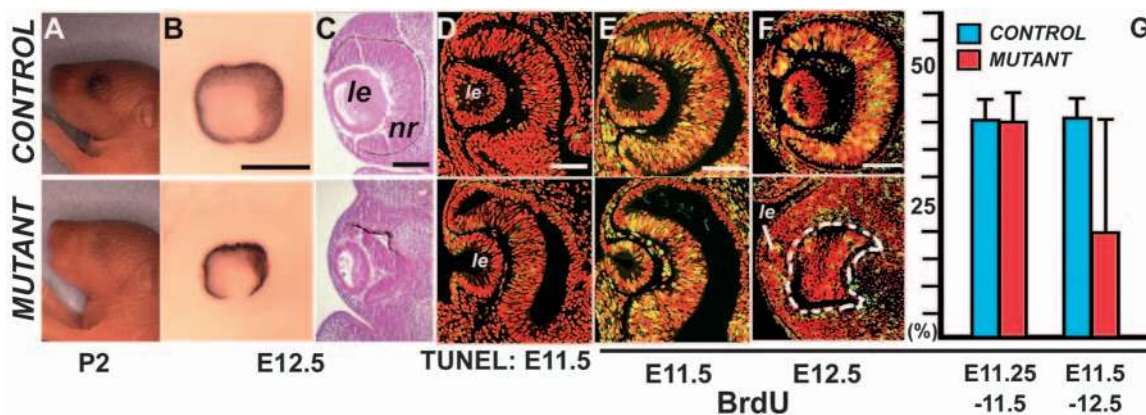
These cellular changes are associated with specific alterations in the expression of regulatory genes implicated in retinal growth. *Chx10* is a key regulator of cell number in the retinal progenitor population (Burmeister et al., 1996; Green et al., 2003). In *Bmpr1a*<sup>-flx</sup>;*Bmpr1b*<sup>+/-</sup>;*Cre* double mutants, expression of *Chx10* is attenuated by E11.5, thus preceding obvious morphological defects (Fig. 6A). Cyclin D1, a crucial G1-S phase cell cycle regulator, is strongly expressed in the wild-type retina (e.g. Fig. 6B, left) and its loss leads to defects in retinal development (Ma et al., 1998; Sicinski et al., 1995). In the double-null mutants, the expression of CyclinD1 is dramatically downregulated specifically in the retina (Fig. 6B, right), indicating its dependence on Bmp signaling. By contrast, the expression of retinal anterior homeobox gene *Rx* and LIM domain-containing transcription factor gene *Lhx2*, both of which are required for optic cup formation (Mathers et al., 1997; Porter et al., 1997), is maintained in the severely affected double mutant retina at E12.5 (Fig. 6C,D).

During mouse embryonic development, retinal neurogenesis initiates at around E11.5, the time at which the abnormal phenotypes of the double mutant retina become first apparent. Initiation of neurogenesis is associated with the expression of *Math5*, a mouse homologue of the *Drosophila* pro-neural gene, *atonal*. *Math5* expression gradually expands from center to periphery to represent a wave of retinal ganglion cell differentiation (Brown et al., 1998; Brown et al., 2001). In *Bmpr1a*<sup>-flx</sup>;*Bmpr1b*<sup>+/-</sup>;*Cre* double mutants, *Math5* expression is not initiated in the retina at E11.5 (Fig. 6E). A potential target gene of *Math5* is *Brn3b*, which encodes a POU-homeodomain-containing transcription factor required for normal retinal ganglion cell differentiation (Liu et al., 2001;

Wang et al., 2001). Consistent with the loss of *Math5* expression in the double null mutants, expression of *Brn3b* is not initiated in the mutant retina (Fig. 6F). By contrast, *Pax6*, which is required for the expression of a number of neurogenic bHLH genes (including *Math5*) and in the regulation of retinal progenitor population (Marquardt et al., 2001), is expressed in the double mutant retina at E11.5 (Fig. 6G) and E12.5 (not shown). We next examined general neuronal markers to determine if these cells had any neural



**Fig. 4.** Asymmetric expression of Raldh genes in the retina is maintained in *Bmpr1a*<sup>-flx</sup>;*Bmpr1b*<sup>+/-</sup>;*Cre* mutants. In situ hybridization on coronal sections of E12.5 embryos with dorsal towards the top. Arrows indicate the domains of expression within the retina. (A,C) The transcripts for genes encoding retinoic acid synthesizing enzymes Raldh1 (*Raldh1a1*) (A) and Raldh3 (*Raldh1a3*) (C) are asymmetrically expressed in the retina with high levels dorsally and ventrally, respectively. In addition, they are also expressed at low levels in the surface ectoderm surrounding the eye region (A,C), the lens (A) and the dorsal RPE (C). (B,D) The expression pattern is not significantly altered in the *Bmpr1a*<sup>-flx</sup>;*Bmpr1b*<sup>+/-</sup>;*Cre* mutant embryos. le, lens; nr, neuroretina; rpe, retinal pigmented epithelium; se, surface ectoderm. Scale bar: 100 μm.



**Fig. 5.** Defects in retinal growth in *Bmpr1a<sup>defx</sup>;Bmpr1b<sup>-/-</sup>;Cre* double mutant mice. (A) Side views of neonates. The double null mutant has an anophthalmic phenotype (bottom). (B) Side views of the eyes of E12.5 embryos. Reduced eye size and incomplete closure of the retinal pigmented epithelium ventrally are seen in the double mutant (bottom). (C) Coronal sections of embryos shown in B. (D) Cell-death analyses at E11.5 indicate a significant increase in TUNEL-positive cells (green) in the mutant retina (counterstained with propidium iodide). (E-G) Although there is no significant change in the percentage of BrdU-positive cells up to E11.5 (green/yellow cells) (E,G), there is a rapid reduction of cell proliferation by E12.5 (F,G; broken line in F outlines the degenerating mutant retina; a relatively large error bar in the E11.5-12.5 mutant group reflects a spectrum of phenotypic severity during these stages). le, lens; nr, neuroretina. Scale bar: 500  $\mu$ m in B; 200  $\mu$ m in C; 100  $\mu$ m in D-F.

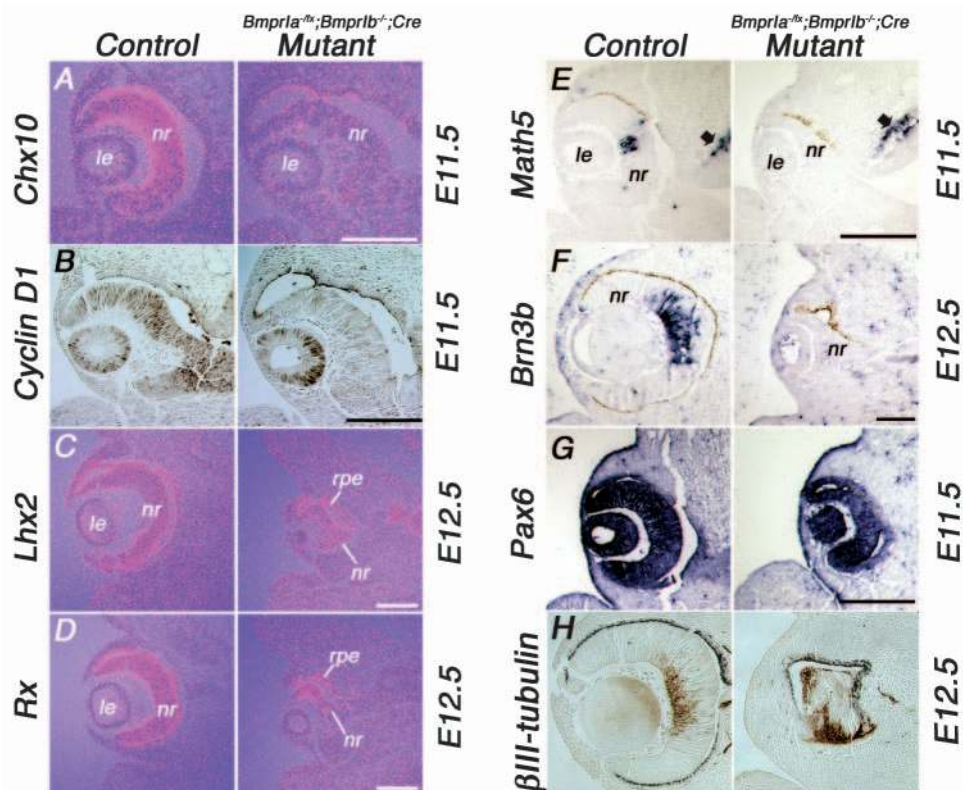
character. Some  $\beta$ III-tubulin-positive cells were detected in the mutant retina (Fig. 6H), although they were not restricted to a central domain.

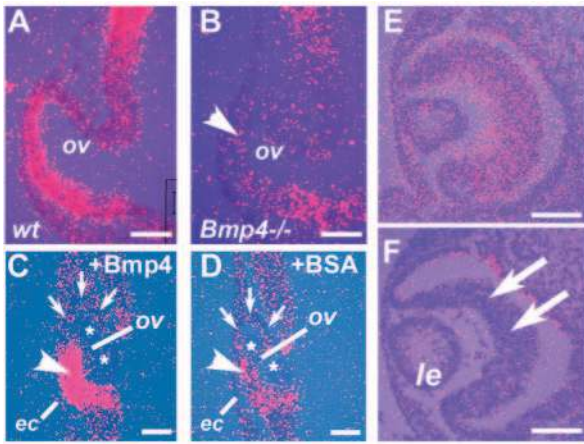
### Bmp signaling is required for the expression of Fgf15 in the developing retina

Fibroblast growth factors constitute another class of secreted

signaling molecules that have potential functions in retinal development (see Discussion). These include the regulation of cell survival and proliferation, as well as retinal neurogenesis. As these processes are affected in the Bmp receptor mutants, it is possible that Fgfs act as mediators of Bmp signaling in this regard. One of the Fgf family members expressed specifically within the optic vesicle is *Fgf15* (McWhirter et al.,

**Fig. 6.** *Bmpr1a<sup>defx</sup>;Bmpr1b<sup>-/-</sup>;Cre* double null mutants display a range of defects in the expression of retinal marker genes. Analyses of gene and protein expression in coronal sections of embryonic retina at the indicated stages by radioactive in situ hybridization (A,C,D), DIG in situ hybridization (E,F,G) or immunostaining (B,H). (A) At E11.5, expression of *Chx10* is attenuated in the mutant retina (right) compared with control. (B) CyclinD1 is specifically absent in the mutant retina (right), whereas protein expression in the adjacent lens tissue is maintained. (C,D) *Lhx2* and *Rx*, two early pan-retinal markers, are expressed in the mutant retina at comparable levels with the control, despite the apparent degenerative phenotypes at this stage. (E) The mutant retina fails to initiate retinal neurogenesis at E11.5 as indicated by the loss of *Math5* expression. Arrows indicate comparable *Math5* expression both in the diencephalons of both control and mutant. This is not simply a developmental delay, as *Math5* is absent even at E12.5 (not shown). (F) *Brn3b*, a downstream target of *Math5* expressed in the newly born ganglion cells in the central retina, fails to be induced in the mutants. (G) Expression of *Pax6* is maintained in the mutant. (H) Neuronal tubulin expression is detected in the mutant retina. le, lens; nr, neuroretina; rpe, retinal pigment epithelium. Scale bar: 100  $\mu$ m.





**Fig. 7.** *Fgf15* is a potential downstream target of Bmp signaling in the retina. (A) *Fgf15* is strongly expressed in the distal optic vesicle at E9.0-9.5 in a wild-type embryo. (B) The expression of *Fgf15* is absent in the eye (arrowhead) of an advanced *Bmp4*<sup>-/-</sup> mutant embryo with equivalent number of somites to the wild-type embryo shown in A. (C,D) Application of BMP4-soaked beads (asterisks) restores the expression of *Fgf15* in the distal optic vesicle of *Bmp4*<sup>-/-</sup> by 18 hours in culture (C, arrowhead). Under the same condition, BSA fails to restore the expression of *Fgf15* (D, arrowhead). Arrows indicate the future retinal pigmented epithelium slightly displaced anteriorly during explant culture. (E) *Fgf15* expression persists through later stages as shown for an E11.5 normal retina (*Bmpr1a*<sup>+/*fx*</sup>; *Bmpr1b*<sup>+/-</sup>; *Cre* control). (F) In *Bmpr1a*<sup>-/*fx*</sup>; *Bmpr1b*<sup>-/-</sup>; *Cre* double mutant, the expression of *Fgf15* is absent (arrows). le, lens; ov, optic vesicle. Scale bar: 50  $\mu$ m in A-D; 100  $\mu$ m in E,F.

1997). In wild-type embryos, the expression of *Fgf15* initiates at around the 15-somite stage (not shown), a few hours following the onset of *Bmp4* expression in the prospective retinal neuroectoderm. *Fgf15* expression is upregulated subsequently, and persists throughout embryonic retinal development (not shown). First, to determine whether the *Fgf15* expression is dependent on Bmp signaling, we examined *Bmp4*<sup>-/-</sup> mutant embryos for *Fgf15* transcripts. In the absence of *Bmp4*, *Fgf15* expression is drastically reduced or absent in the optic vesicle (Fig. 7B). Furthermore, in explant cultures, the expression of *Fgf15* can be restored by exposure of the optic vesicle of *Bmp4*<sup>-/-</sup> mutants to Bmp4 protein carried by beads (Fig. 7C). Consistent with these observations, the expression of *Fgf15* is diminished in the retina of *Bmpr1a*<sup>-/*fx*</sup>; *Bmpr1b*<sup>-/-</sup>; *Cre* double mutants at E11.5 (Fig. 7E,F). Thus, Bmp signaling largely mediated by Bmp4 is essential to induce the expression of *Fgf15* in the early developing retina. Bmp4 cannot induce *Fgf15* expression in the retinal pigmented epithelium, suggesting that this regulation may be permissive rather than instructive.

## Discussion

Using conditional inactivation of Bmp type I receptor function to disrupt Bmp signaling in a cell-autonomous manner, our analyses have revealed the *in vivo* role for Bmp signaling in mouse retinal patterning, thus validating previous gain-of-function studies in other vertebrate systems. In addition, they have uncovered a unique role of Bmp signaling in maintaining

growth and differentiation of the developing retina in the mouse.

## Bmpr1a and Bmpr1b play redundant roles during retinal development

Our studies provide the first direct genetic evidence for functional redundancy between the two Bmp type I receptors, Bmpr1a and Bmpr1b. These two receptors share a high degree of sequence similarity and are both capable of binding to Bmp2, Bmp4 and Bmp7 ligands, albeit with varying affinities (Mishina, 2003). However, previous studies indicate that they may possess distinct biological functions. A study using forced expression of constitutively active Bmpr1a and Bmpr1b receptors, both in transgenic mice and in neural stem cells, suggested a role for Bmpr1a in regulating proliferation in the developing central nervous system and for Bmpr1b in regulating apoptosis and terminal differentiation (Panchision et al., 2001). Similarly, in chick limb bud development, ectopic expression of constitutively active and dominant-negative forms of the two receptors revealed a role for Bmpr1b in mediating formation of the initial cartilaginous skeleton, and for Bmpr1a in controlling the later differentiation process (Zou et al., 1997). However, during chick neural tube patterning, expression of constitutively active forms of the two receptors revealed no significant qualitative differences in the cellular responses (Timmer et al., 2002). The apparent discrepancies in these studies may be attributable, in part, to differences in the experimental models and tissue or cell types examined. With respect to the phenotypes described here, the lack of retinal abnormalities in the conditional *Bmpr1a* null mutants or *Bmpr1b* mutants in comparison with the *Bmpr1a/Bmpr1b* double null mutant retina indicates the functional redundancy between the two receptors *in vivo*. One broad implication of the current study is that *Bmpr1a* and *Bmpr1b* may also play redundant roles in other tissues. For example, analysis of *Bmpr1a* function in the embryonic telencephalon revealed its requirement only in the dorsal midline, a domain where *Bmpr1b* is not expressed, unlike the more lateral telencephalic regions where the expression of *Bmpr1a* and *Bmpr1b* overlap (Hebert et al., 2002). Thus, it is likely that disruption of both receptors in the telencephalon may reveal a more extensive requirement for Bmp signaling. Furthermore, this general principle may be extended to receptors for other subgroups of the TGF $\beta$  superfamily ligands. For example, canonical TGF $\beta$  ligands can bind to both Tgfr1 (Alk5) and Acvr1 (Alk1), and activins can bind to Acvr1b (Alk4) and Acvr1 (Alk2). We do note that the degree of functional overlap between *Bmpr1a* and *Bmpr1b* may vary depending on the developmental context. For example, within the eye itself, optic nerve head axonal guidance may require only *Bmpr1b* function (Liu et al., 2003). Dorsoroventral patterning defects are present in *Bmpr1a*<sup>-/*fx*</sup>; *Bmpr1b*<sup>+/-</sup>; *Cre*, but not in *Bmpr1a*<sup>+/*fx*</sup>; *Bmpr1b*<sup>-/-</sup>; *Cre* mutants, suggesting that the two receptors are not completely equivalent. Such differences could arise from varying temporal/spatial expression domains, levels of expression and/or biochemical properties.

## Bmp signaling is necessary for the patterning of the retinal dorsoventral axis

The requirement for Bmp signaling in dorsal retina specification is analogous to its role in dorsal neural tube patterning and dorsal forebrain differentiation in vertebrates

(Furuta et al., 1997; Hebert et al., 2002; Timmer et al., 2002), suggesting that this function is conserved in different parts of the developing nervous system. One potential implication is a conservation of the underlying genetic mechanisms, such as interactions with the Shh-mediated patterning activity that occurs in the developing neural tube. In the chick optic vesicle, reducing endogenous Shh activity results in an expansion of *Bmp4* expression (Zhang and Yang, 2001), suggesting opposing roles of Bmp and Shh signaling in retinal dorsoventral patterning.

Our study has identified the bona fide receptors that mediate the Bmp signaling in retinal dorsoventral patterning. The genetic evidence presented in this study indicates that the dorsal Bmp signal intersects with the ventral pathway at the level of or upstream of *Vax2* expression (Fig. 8D). In *Vax2* mutants, *Tbx5* expression, a downstream readout of Bmp signaling, is unaffected (Barbieri et al., 2002; Mui et al., 2002), whereas loss of *Tbx5* expression in *Bmpr1a<sup>-flx</sup>;Bmpr1b<sup>+/-</sup>;Cre* mutant animals results in expansion of the *Vax2*-positive domain. In the Bmp receptor mutant animals, asymmetric expression patterns of *Bmp4* and *Bmpr1b* appear unaffected (data not shown), suggesting that Bmp signaling is one of the most upstream events in dorsal retinal cell fate specification. Taken together, one possible model is that the ventral program is a default state, and Bmp signaling provides the instruction to initiate the dorsal program. Although the mechanisms underlying the gradient expression patterns of Eph-Ephrin molecules remain unclear, the *Bmpr1a<sup>-flx</sup>;Bmpr1b<sup>+/-</sup>;Cre* mutant animals, as well as *Vax2*-null mice (Barbieri et al., 2002; Mui et al., 2002), will provide useful genetic tools for future investigations of topographic map formation.

Some aspects of dorsoventral asymmetry are maintained in the *Bmpr1a<sup>-flx</sup>;Bmpr1b<sup>+/-</sup>;Cre*. Although retinoic acid activity

in the *Bmpr1a<sup>-flx</sup>;Bmpr1b<sup>+/-</sup>;Cre* retina has not been directly analyzed, the differential expression of *Raldh1* and *Raldh3* along the dorsoventral axis at least, is independent of Bmp-mediated patterning within the retina. Our results thus favor a model wherein the asymmetric retinoic acid activity in the early optic vesicle acts upstream of dorsal *Bmp4* expression in the retina. Alternatively, these two pathways may function independent of each other. It is not currently clear whether the potential retinoic acid activity gradient is translated into any aspect of dorsoventral patterning in the retina (McLaughlin et al., 2003).

### Bmp signaling regulates cell survival during retinal growth

Bmps have been implicated in the regulation of cell proliferation and cell death in various developmental contexts, including the developing retina (Trousse et al., 2001). The current genetic studies have uncovered potential downstream effectors of Bmp signaling in these processes. In the *Bmpr1a<sup>-flx</sup>;Bmpr1b<sup>-/-</sup>;Cre* double homozygous mutants retinal structures are specified initially, but cell proliferation and cell death are severely affected during mid-gestation. A previous study has shown that *Chx10* and cyclin D1, two genes whose expression is affected in the double mutants, may have a regulatory relationship in the retina (Chang et al., 2003). The phenotypes of individual homozygous mutants for *Chx10* and cyclin D1 include microphthalmia and photoreceptor degeneration, respectively (Burmeister et al., 1996; Sicinski et al., 1995); however, most retinal neurons are present in the eye of these mutants. The *Bmpr1a<sup>-flx</sup>;Bmpr1b<sup>-/-</sup>;Cre* double null mutant phenotype is more severe possibly due to the combinatorial loss of *Chx10* and cyclin D1.

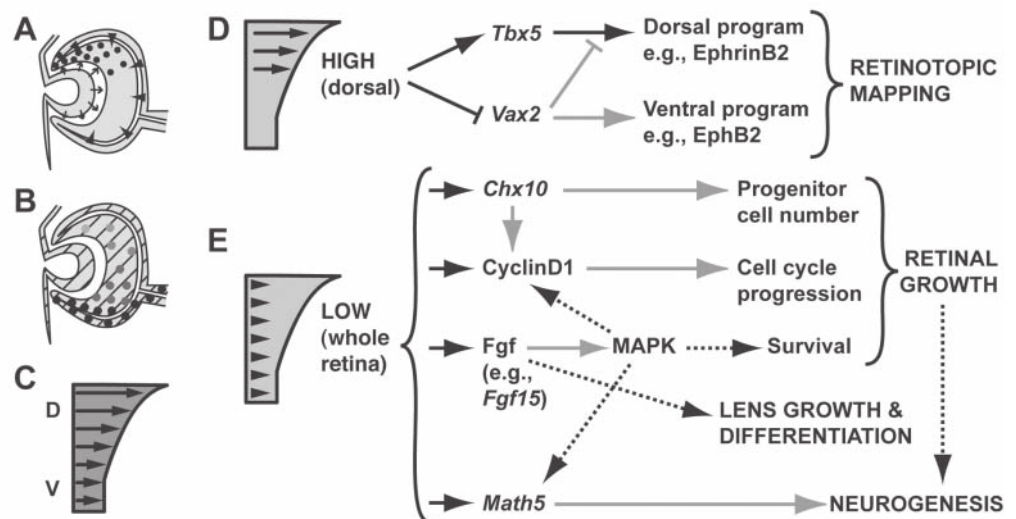
The *Bmpr1a<sup>-flx</sup>;Bmpr1b<sup>-/-</sup>;Cre* double mutants show

**Fig. 8.** A model for the function of Bmp signaling through *Bmpr1a/Bmpr1b* receptors in the retina.

(A) Schematic representation of an early embryonic eye, indicating the sources of Bmp ligands. Black dots represent dorsally localized *Bmp4* transcripts (Furuta and Hogan, 1998), arrows indicate Bmp7 from the lens ectoderm (Wawersik et al., 1999) and arrowheads indicate Bmp2 signaling from the retinal pigmented epithelium (Dudley and Robertson, 1997). (B) Expression of the corresponding Bmp receptors in the eye. *Bmpr1a* is ubiquitously expressed (hatching), whereas *Bmpr1b* shows a graded expression within the retina with highest levels

ventrally (dots). (C) A relative distribution Bmp signaling activity along the retinal dorsoventral (DV) axis, based on P-Smad1/5/8 immunostaining (Fig. 3D).

(D,E) Black and gray arrows indicate functions for genes/pathways deduced from the current study and those from previously published studies, respectively. Broken arrows indicate potential interactions. The potential relationships shown here are not intended to imply direct or linear pathways. (D) Relatively high levels of Bmp signaling are required to specify the dorsal program, ultimately leading to proper retinal topographic mapping. Loss of dorsal specification in *Bmpr1a<sup>-flx</sup>;Bmpr1b<sup>-/-</sup>;Cre* mice indicates that signaling activity falls below the threshold required for DV patterning in these mutants. For simplicity, only interactions suggested by genetic evidence are indicated. (E) Lower levels of Bmp signaling are required throughout the retina to maintain normal growth and to initiate neurogenesis. Candidate downstream targets of Bmp signaling include *Chx10* and cyclin D1, as well as *Fgf15*. Potential regulation of *Math5* expression by Mapk is, in part, postulated based on studies in *Drosophila* implying Egfr-Raf-Mapk signaling in the induction of the pro-neural gene *atonal*.





increased levels of apoptosis from E11.25, possibly owing to a lack of survival signals to the retinal progenitor cells. Although Bmps themselves may act as survival factors in the eye, fibroblast growth factors are also candidate mediators of Bmp signaling in this regard. Within the eye, they are expressed in the surface ectoderm (*Fgf1*, *Fgf2*), the neuroretina (*Fgf15*, *Fgf9*) (Nguyen and Arnheiter, 2000; Zhao et al., 2001) and the central retina-optic stalk region (*Fgf8*) (Chow and Lang, 2001). Several studies have implicated Fgfs in the specification and survival of the neuroretina (Lillien and Cepko, 1992; Park and Hollenberg, 1989; Pittack et al., 1997). Fgf signal transduction per se may operate through the Ras/Mapk or the PI-3 kinase/Akt pathway, which, in turn, are known to regulate cell survival by transcription-dependent and -independent inhibition of apoptosis (Brunet et al., 2001; Powers et al., 2000). Thus, the loss of Fgf15 signaling may, in part, contribute to retinal degeneration resulting from apoptosis in the *Bmpr1a<sup>-fx</sup>;Bmpr1b<sup>-/-</sup>;Cre* double mutants.

### A novel role for Bmp signaling in retinal neurogenesis

Despite well-characterized roles of signaling molecules, including hedgehog (Hh) and Dpp, in *Drosophila* retinal neurogenesis, the signaling events upstream of mouse retinal neurogenesis remain elusive. In particular, the requirement of Hh in retinal ganglion cell specification (i.e. induction of *atonal* homologs) is unclear (Stenkamp and Frey, 2003). Analysis of *Bmpr1a<sup>-fx</sup>;Bmpr1b<sup>-/-</sup>;Cre* double mutants has revealed a novel function for Bmp signaling in retinal neurogenesis. Although we observe abnormal neuronal tubulin expression in the E12.5 double mutant retina, these labeled cells are not positive for early pro-neural genes such as *Math5* (Fig. 6) and *Neurod1* (data not shown), or for neurofilament (data not shown). The rapid degeneration of the double mutant retina subsequent to E12.5 precludes analysis of later-born neurons. We therefore conclude that Bmp signaling is required for early neurogenesis in the mouse retina. This is in contrast to the observation that initiation of neurogenesis and expression of *atonal* in the *Drosophila* eye can occur in the absence of Dpp signaling (Fu and Baker, 2003). Given that *Pax6* expression is maintained in the absence of neurogenesis, it is likely that Bmp signaling intersects with the Pax6-mediated pathway to initiate differentiation of retinal progenitor cells. In this context too, the effects of Bmp signaling on pro-neural gene expression may involve an Fgf receptor-Ras/Mapk signal. Exogenous FGFs are reported to be capable of inducing ganglion cell markers in retinal explants (Guillemot and Cepko, 1992). This model is also analogous to that in *Drosophila* where the Egr-Raf-Mapk pathway has been implicated in the induction of *atonal* (Greenwood and Struhl, 1999; Kumar and Moses, 2001). There also remains the possibility that this abnormality in retinal differentiation is tightly coupled to the decreased growth in the mutant retina (Fig. 8E). The possible causative regulatory relationships between these multiple developmental processes are the subject for future studies.

### Different threshold requirements for Bmp signaling in various aspects of retinal development

Our in vivo genetic analysis has generated novel insights regarding the mechanisms by which Bmp signaling regulates

various aspects of retinal development. Given the range of Bmp ligands and receptors expressed in the developing retina (Fig. 8A,B), in principle, potentially distinct signaling properties of individual ligands and receptors may underlie different cellular responses. Our data are more consistent with an alternative model wherein different aspects of retinal development, such as patterning and morphogenesis, are regulated at distinct threshold levels of Bmp signaling input (Fig. 8D,E). Our results strongly indicate the existence of at least two threshold levels of Bmp signaling. First, specification of the dorsal retina requires relatively high levels of Bmp signaling that cannot be maintained by only one functional allele of *Bmpr1b* (Fig. 8D). The observations that the wild-type dorsal retina expresses high levels of both Bmp4 ligand (Furuta and Hogan, 1998) and P-smad protein (Fig. 3D) corroborate this idea. Second, complete elimination of the function of both *Bmpr1a* and *Bmpr1b* receptors in double null mutants leads to catastrophic effects in the developing retina, including defects in retinal proliferation and retinal neurogenesis. These data suggest the presence of a lower threshold of signaling throughout the retina that can maintain overtly normal retinal morphogenesis and differentiation in the *Bmpr1a<sup>-fx</sup>;Bmpr1b<sup>+/-</sup>;Cre* or *Bmpr1a<sup>+fx</sup>;Bmpr1b<sup>-/-</sup>;Cre* mutants. A recent study using Noggin overexpression in chick has shown a requirement for Bmp signaling in the development of the ventral optic cup (Adler and Belecky-Adams, 2002). In these experiments, inhibition of Bmp signaling resulted in a range of abnormalities, including microphthalmia, ventral retinal colobomas, altered expression of dorsoventral markers and optic nerve pathfinding defects similar to those reported for the *Bmpr1b<sup>-/-</sup>* mutants (Liu et al., 2003). These ventral retina defects may represent a phenotype intermediate between the dorsoventral patterning defects and the retinal degeneration described in our system, indicating possible existence of a third threshold of Bmp signaling during retinal development. The mechanisms by which individual cells divide the overall receptor-mediated signaling into graded units of activities and assign them to elicit qualitatively distinct genetic programs may have implications for other growth factor signaling systems.

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### Note added in proof

During the review of our revised manuscript, a study revealing redundant roles of *Bmpr1a* and *Bmpr1b* in the developing mouse neural tube was published (Wine-Lee et al., 2004).

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