Development 136, 3895-3906 (2009) doi:10.1242/dev.041202

Lhx2 links the intrinsic and extrinsic factors that control optic cup formation

Sanghee Yun[1,2], Yukio Saijoh[2,3], Karla E. Hirokawa[4], Daniel Kopinke[5], L. Charles Murtaugh[5] Edwin S. Monuki[4] and Edward M. Levine[1,2,*]

A crucial step in eye organogenesis is the transition of the optic vesicle into the optic cup. Several transcription factors and extracellular signals mediate this transition, but whether a single factor links them into a common genetic network is unclear. Here, we provide evidence that the LIM homeobox gene *Lhx2*, which is expressed in the optic neuroepithelium, fulfils such a role. In *Lhx2*$^{-/-}$ mouse embryos, eye field specification and optic vesicle morphogenesis occur, but development arrests prior to optic cup formation in both the optic neuroepithelium and lens ectoderm. This is accompanied by failure to maintain or initiate the expression patterns of optic-vesicle-patterning and lens-inducing determinants. Of the signaling pathways examined, only BMP signaling is noticeably altered and *Bmp4* and *Bmp7* mRNAs are undetectable. *Lhx2*$^{-/-}$ optic vesicles and lens ectoderm upregulate *Pax2*, *Fgf15* and *Sox2* in response to BMP treatments, and *Lhx2* genetic mosaics reveal that transcription factors, including *Vsx2* and *Mitf*, require *Lhx2* cell-autonomously for their expression. Our data indicate that *Lhx2* is required for optic vesicle patterning and lens formation in part by regulating BMP signaling in an autocrine manner in the optic neuroepithelium and in a paracrine manner in the lens ectoderm. We propose a model in which *Lhx2* is a central link in a genetic network that coordinates the multiple pathways leading to optic cup formation.

KEY WORDS: BMP, Anophthalmia, Eye field transcription factor, Lens, Optic vesicle, Retina

INTRODUCTION

Vertebrate early eye formation follows a conserved sequence of events (Fig. 1A). Soon after gastrulation begins, the eye field is specified in the anterior neural plate. The first morphological landmarks are bilateral indentations (optic sulci) in the eye field, followed by evagination of the lateral walls of the diencephalon, giving rise to the optic vesicles (OVs). Interaction between the OV and surface ectoderm (SE) induces the lens placode, and a concerted invagination of the lens placode and OV produces the optic cup (OC). Coincident with these events are lens morphogenesis, the establishment of dorsoventral polarity of the OV, and regional patterning of the optic neuroepithelium into neural retina, retinal pigment epithelium (RPE) and optic stalk. Disruptions in these early steps lead to severe congenital anomalies, including absent eyes (anophthalmia), small eyes (microphthalmia) and optic fissure closure defects (coloboma) (Fitzpatrick and van Heyningen, 2005).

The homeobox genes *Rx* (*Rax*), *Pax6*, *Six3* and *Optx2* (*Six6*), the T-box gene *ET*, the orphan nuclear receptor *Tll*, and the LIM homeobox gene *Lhx2* are expressed in dynamic and overlapping patterns in the *Xenopus* eye field and are collectively defined as eye field transcription factors (EFTFs) (Zuber et al., 2003). EFTF overexpression in toto induces ectopic eye fields that lead to well-formed eyes, and sufficiency experiments suggest that EFTFs participate in a network analogous to the retinal determination gene network in *Drosophila* (Pappu and Mardon, 2004; Silver and Rebay, 2005; Zuber et al., 2003). That EFTFs are required for early eye organogenesis is revealed by their loss-of-function mutations in human and several model systems (Bailey et al., 2004; Fitzpatrick and van Heyningen, 2005; Graw, 2003).

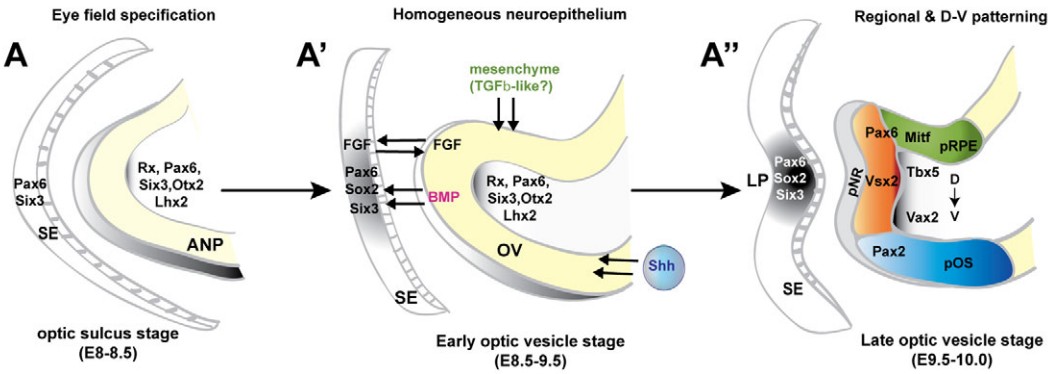
It is not clear, however, whether the factors that regulate the subsequent events of eye organogenesis, namely those occurring during the OV-to-OC transition, are linked into a common genetic network. These events, which include regional and axial patterning of the optic neuroepithelium and lens induction in the SE, are highly dependent on multiple signals, including sonic hedgehog (Shh) from the ventral midline, FGFs from the presumptive lens ectoderm and OV, TGFβ superfamily ligands from the mesenchyme, and BMPs from the OV (Fig. 1A) (Bharti et al., 2006; Chow and Lang, 2001; Martinez-Morales et al., 2004; Yang, 2004). An important outcome of these signals is the establishment of defined expression domains for several EFTFs and other transcription factors, including *Pax2*, *Vsx2* (*Chx10*), *Mitf*, *Tbx5*, *Vax2* and *Sox2* (Behesti et al., 2006; Fuhrmann et al., 2000; Furuta and Hogan, 1998; Gotoh et al., 2004; Hyer et al., 1998; Jensen, 2005; Kim and Lemke, 2006; Macdonald et al., 1995; Morcillo et al., 2006; Murali et al., 2005; Nguyen and Arnheiter, 2000).

Because multiple pathways function simultaneously during the OV-to-OC transition, their coordination seems likely. EFTFs, and *Pax6* and *Lhx2* in particular, are the best candidates for accomplishing this. In contrast to *Rx* and *Six3* mutants (Carl et al., 2002; Mathers et al., 1997), which arrest prior to OV formation, and *Six6* and *Tll* mutants, which progress past OC formation (Hollemann et al., 1998; Li et al., 2002), eye morphogenesis in *Pax6* and *Lhx2* mutants arrests at the OV stage (Hill et al., 1991; Porter et al., 1997). However, regionalization of the OV occurs in the *Pax6* mutant (Baumer et al., 2003), suggesting that *Pax6* does not act as a central coordinating factor. Whether *Lhx2* acts in this manner has not been addressed. Overexpression of EFTF combinations that do not include *Lhx2* but are still able to induce ectopic eyes always activates endogenous *Lhx2* expression in the ectopic eye fields, whereas combinations that are unable to induce

[1]Department of Ophthalmology and Visual Sciences, John A. Moran Eye Center, [2]Department of Neurobiology and Anatomy, [3]Program in Human Molecular Biology and Genetics, and [5]Department of Human Genetics, University of Utah, Salt Lake City, UT 84132, USA. [4]Department of Pathology, University of California, Irvine, CA 92697, USA.

*Author for correspondence (ed.levine@utah.edu)

Accepted 22 September 2009



B

Embryonic stage	Somite stage	Optic features to allow stage identification
E8.0-8.25	~ss8	Anterior neural plate
E8.25-8.5	ss8-14	Optic sulcus
E8.5-9.0	ss14-20	Early optic vesicle
E9.0-9.5	ss20-25	Early optic vesicle
E9.5-10.0	ss25-30	Late optic vesicle
E10-10.5	ss30-40	Optic cup

Fig. 1. Schematic representation of mouse early eye development. (**A-A″**) *Rx*, *Pax6*, *Six3*, *Otx2* and *Lhx2* are homogeneously expressed in the neuroepithelium at optic sulcus (A) and early optic vesicle (OV) (A′) stages. Signals drive regional and dorsoventral patterning in the OV (A′,A″). The lens-forming region of the surface ectoderm (SE) expresses *Pax6* and *Six3* at the optic sulcus stage (A). BMP and FGF signaling and *Sox2* expression drive lens specification (A′) and placode formation (A″). (**B**) Developmental stages and eye morphology relevant to this study [adapted from Theiler (Theiler, 1972)]. ANP, anterior neural plate; LP, lens placode; pOS, presumptive optic stalk; pNR, presumptive neural retina; pRPE, presumptive retinal pigment epithelium.

eyes also fail to activate *Lhx2* (Zuber et al., 2003). Where examined, expression of the vertebrate *Lhx2* orthologs initiates in the presumptive eye field and continues through early eye organogenesis (Seth et al., 2006; Tetreault et al., 2008; Viczian et al., 2006) (S.Y. and E.M.L., unpublished).

Although *Lhx2* is known to be essential for mouse OC morphogenesis, how it controls early eye development is unresolved. Zebrafish *lhx2* (*belladonna*) mutants do not have anophthalmia (Seth et al., 2006), probably because of redundancy with other LIM homeobox genes (Kikuchi et al., 1997). A recent study of the mouse *Lhx2* mutant suggested that there is a delay in eye field specification and identified *Six6* as a transcriptional target of *Lhx2* (Tetreault et al., 2008), but a mechanistic explanation of how *Lhx2* regulates early eye development was not forthcoming, especially as *Six6* mutants have microphthalmia and reduced retinal progenitor cell proliferation rather than anophthalmia (Li et al., 2002). Here, we provide evidence in mouse that the eye field and OV form on schedule in the absence of *Lhx2*, but patterning of the optic neuroepithelium arrests prior to regionalization and the establishment of dorsoventral polarity. We also elucidate that *Lhx2* is required for these processes and for lens formation by both cell-autonomous and non-cell-autonomous mechanisms in a manner that leads us to propose that *Lhx2* acts to link the multiple pathways needed for the OV-to-OC transition.

MATERIALS AND METHODS

Animals

Mouse strains are listed in Table 1. $Lhx2^{+/-}$ eyes are indistinguishable from $Lhx2^{+/+}$ eyes and were used as controls. $Hes1^{creERT2}$ mice were produced by homologous recombination of the *creERT2* cDNA into the *Hes1* locus (D.K. and L.C.M., unpublished). Primers used for PCR genotyping are listed in Table S1 in the supplementary material. Embryos were staged and matched for comparison by somite number. Somite stage (ss) and embryonic day (E) are indicated for all samples; ss is used in the text for samples up to ss22, which covers eye field specification and early OV stages, and E is used for samples ss23 and older (see Fig. 1B). Animal protocols were approved by the University of Utah IACUC.

Table 1. Mouse lines

Allele	Targeting region	Reference
Lhx2^{-}	Deletion in exon 2 and 3	(Porter et al., 1997)
Lhx2^{f}	*loxP* sites in intron 1 and 3	(Mangale et al., 2008)
Fgf8^{f}	*loxP* sites in intron 4 and 5; GFP insertion in 3′UTR	(Macatee et al., 2003)
Shh$^{GFP\text{-}cre}$	*GFPcre* knock-in	(Harfe et al., 2004)
Rosa26^{creER}	*creER* knock-in	(Badea et al., 2003)
Hes1^{creERT2}	*creERT2* knock-in	D.K. and L.C.M., unpublished
Tg(Le-cre)	*cre* is under the control of *Pax6* regulatory sequence (transgenic)	(Ashery-Padan et al., 2000)
Rosa26^{Z}	*loxP-neo pA; lacZ knock-in*	(Soriano, 1999)

Table 2. Primary antibodies

Antibody	Host	Dilution factor	Source
LHX2	Rabbit	50	Edwin Monuki
LHX2	Goat	1000	Santa Cruz (sc-19344)
VSX2	Sheep	300	Exalpha Biologicals (X1180P)
PAX6	Mouse	10	DSHB (P3U1)
PAX6	Rabbit	1500	Chemicon (AB2237)
SHH	Mouse	50	DSHB (5E1)
PAX2	Rabbit	100	Covance (PRB-276P)
OTX2/1	Rabbit	1000	Chemicon (AB9566)
SIX3	Rabbit	800	Guillermo Oliver
SOX2	Rabbit	400	Abcam (ab15830)
MITF	Mouse	400	Exalpha Biologicals (X1405M)
pSMAD1/5/8	Rabbit	500	Cell Signaling Technology (9511S)
pERK1/2	Rabbit	500	Cell Signaling Technology (9101S)
pHH3	Rabbit	500	Upstate Biotechnology (06-570)
β-gal	Rat	1000	Nadean Brown

Tamoxifen (TM) treatments

TM (Sigma T5648) was dissolved at 10 mg/ml in peanut oil (Sigma P2144). Pregnant *Rosa26*^{creER} mice were administered 0.1 mg TM per gram body weight (gbw) by oral gavage at E7.5 (Park et al., 2008); 0.01 mg TM per gbw was administered at E8.5 to pregnant *Hes1*^{creERT2} mice.

Immunohistochemistry

Embryos were dissected in HBSS and fixed in cold 4% paraformaldehyde (PFA) for 45 minutes. Frozen tissues were prepared as previously described (Clark et al., 2008) and cryosectioned at 10-12 μm in the coronal plane. For whole-mount immunohistochemistry, fixed embryos were washed in PTW (0.5% Triton X-100 in PBS) before serum blocking. Table 2 lists primary antibodies, which were detected with species-specific secondary antibodies conjugated with either Alexa Fluor 488 or 568 (Invitrogen, Eugene, OR, USA). Immunofluorescence images were obtained using epifluorescent illumination except where noted.

To detect phosphorylated SMAD1, 5 or 8 (pSMAD1/5/8), sections were subjected to antigen retrieval (in 0.18 mM citric acid, 77 μM sodium citrate, pH 6.0, at 80°C for 15 minutes), followed by 0.3% H_2O_2 (30 minutes, 25°C). Antibody staining was performed with the Vectastain ABC Kit (Vector Labs, Burlingame, CA, USA).

To detect phosphorylated ERK (pERK), embryos were fixed in 8% PFA overnight at 4°C. Following washes with PBS containing 0.5% NP40, embryos were dehydrated in cold methanol. Embryos were incubated in 5% H_2O_2 diluted in methanol (1 hour) and then rehydrated. Embryos were processed with the Vectastain ABC Kit.

Apoptosis was detected by TdT-mediated dUTP nick end labeling (TUNEL) using the In Situ Cell Death Detection Kit, POD (Roche).

RNA in situ hybridization

Whole-mount in situ hybridizations were performed as described (Nagy, 2003) with the following modifications: hybridization solution comprised 5× SSC, 0.1% SDS, 50% formamide; washing solution comprised 2× SSC, 1% SDS, 50% formamide. cDNA templates were obtained from other laboratories (see Acknowledgements), with the exception of the *Dbx1* template, which was generated by RT-PCR from pooled E14-P0 embryonic mouse brains. Primer sequences are listed in Table S1 in the supplementary material.

X-Gal staining

E9.0 embryos were fixed in 0.2% glutaraldehyde and 1% PFA in PBS containing 0.02% NP40 (PBN) for 15 minutes at room temperature. Following washes with PBN, embryos were placed in X-Gal staining solution (5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 2 mM $MgCl_2$, 1 mg/ml X-Gal, in PBS, pH 7.3). Staining was carried out overnight at room temperature. After staining, embryos were washed in PBN and post-fixed in 4% PFA overnight at 4°C. Embryos were rinsed in PBS and cleared in glycerol for bright-field microscopy and photography. Sections were prepared by mounting and freezing in OCT (Sakura Finetek, Torrance, CA, USA) and cut on a cryostat.

Organotypic head cultures

Heads from ss18-24 embryos were transected at the midbrain and washed in cold DMEM/10 mM HEPES, then placed into holes made in 1.5% agarose and cultured in DMEM supplemented with N-2 (Invitrogen) for 48 hours in the presence or absence of 1 μg/ml BMP7 (R&D Systems). BMP4 beads were prepared as previously described (Furuta et al., 1997). Briefly, Affi-Gel Blue beads (BioRad, 100-200 mesh, #153-7302) of 25-50 μm diameter were rinsed with water. One hundred beads were incubated overnight (4°C) in 5 μl of 1 μg/ml BMP4 (R&D Systems). Bovine serum albumin (1 μg/ml in PBS) served as a control. Beads were placed into dorsal mesenchyme. mRNA was detected by whole-mount in situ hybridization. Sections were prepared post-staining.

Quantification and statistical analysis

For quantification, comparable regions of optic neuroepithela in *Lhx2* mutants and controls were captured using a Fluoview 1000 confocal microscope for cell counting. The percentage of the total (DAPI-positive) cells that was pHH3-positive was quantified and statistical significance between mutants and controls determined by Student's *t*-test; error bars indicate s.d.

RESULTS

Eye field specification and OV identity are initiated, but not maintained, in the absence of *Lhx2*

As *Lhx2* is expressed from the earliest stages of eye formation, it is possible that specification of the eye field is altered in its absence. This was recently suggested to be the case (see Tetreault et al., 2009). However, OV formation is apparent in *Lhx2* mutants at ss18 (~E8.75; see Fig. S1A,B in the supplementary material), which suggests that the functional process of eye field specification still occurs. To address this more directly, we examined the expression of *Rx*, an EFTF that is essential for eye field specification and OV formation (Mathers et al., 1997; Medina-Martinez et al., 2009), in ss6 and ss12 mouse embryos (Fig. 2A-D′). ss6 corresponds to the anterior neural plate stage, when eye field specification occurs. Optic sulci are formed by ss12, and are the earliest morphological indications of OV formation. Although *Rx* mRNA may be slightly reduced in the mutant compared with the control at ss6 (Fig. 2A,B), it was still readily detected. At ss12, *Rx* expression was robust (Fig. 2C-D′). Furthermore, the Rx expression domain was bifurcated at the midline (arrowheads in Fig. 2C′,D′), suggesting that the anterior neural ectoderm responds appropriately to midline signals that split the eye field. At ss18, PAX6 expression (Fig. 2E,F) and the relative proportion of cells positive for phosphorylated histone H3 (pHH3) were similar in mutant and control (see Fig. S1A,B,G in the supplementary material). By E9 (ss20-22), however, expression of *Rx* (Fig. 2G,H) and PAX2 (Fig. 2I,J) was reduced in the *Lhx2*$^{-/-}$ OV, which failed to keep pace with the growth of the wild-type OV as indicated by its reduced size and the reduced proportion of pHH3-positive cells (see Fig. S1C-G in the supplementary material). These data suggest that eye field specification and initiation of OV morphogenesis are relatively normal in the absence of *Lhx2*, but once the OV has formed, *Lhx2* is required to maintain growth and gene expression in the optic neuroepithelium.

To better understand the extent of the molecular changes occurring in the optic neuroepithelium, we examined the expression patterns of EFTFs at later stages. At E9.5, PAX6 was expressed in the OV in a dorsoventral gradient, with highest expression dorsally (Fig. 2K). In the newly formed OC (E10.25), PAX6 was highly expressed throughout most of the retina and RPE and weakly in the optic stalk (Fig. 2M). In the E9.5 *Lhx2*$^{-/-}$ OV, PAX6 was highly expressed ventrally (Fig. 2L, arrowheads). The same pattern was

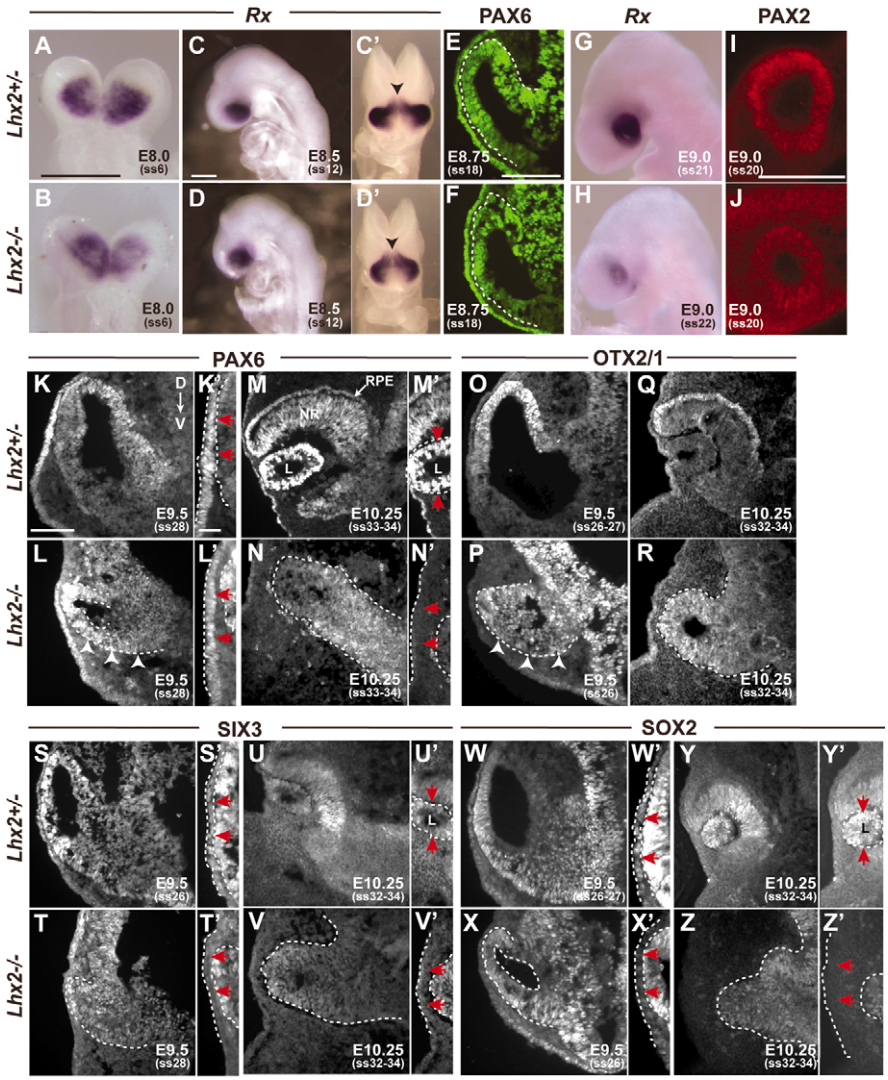


Fig. 2. Eye field specification initiates normally in the absence of *Lhx2*, but the patterns of the EFTFs are altered during optic vesicle maturation. (**A-D′**) *Rx* mRNA expression at ss6 and ss12. Arrowheads (C′,D′) indicate a lack of *Rx* expression at the midline. (A,B,C′,D′) Frontal views; (C,D) lateral views. The image in D is reflected on the vertical axis. (**E**,**F**) PAX6 protein expression at ss18. (**G**,**H**) *Rx* mRNA expression at ss21-22. (**I**,**J**) Single-scan confocal images of PAX2 expression at ss20. (**K-Z′**) PAX6 (K-N), OTX2/1 (O-R), SIX3 (S-V) and SOX2 (W-Z) protein expression at E9.5 and E10.25. Arrowheads (L,P) indicate higher level of PAX6 and OTX2/1 in E9.5 ventral *Lhx2*$^{-/-}$ OV compared with *Lhx2*$^{+/-}$ controls (K,O). Dashed line delineates the boundary of the OV. (K′-N′,S′-Z′) Magnified views of K-N and S-Z to better visualize SE and lens vesicle staining patterns (red arrows). NR, neural retina; RPE, retinal pigment epithelium; L, lens. Scale bars: 500 μm in A-D′,G,H; 100 μm in E,F,I-Z; 50 μm in K′-N′,S′-Z′.

observed in E10.25 *Lhx2*$^{-/-}$ optic neuroepithelium, except at the distal tip (Fig. 2N). The normal pattern of OTX2 and OTX1 (OTX2/1) expression also exhibited a dorsoventral gradient (Fig. 2O,Q), and, similar to the change in PAX6, OTX2/1 staining was high in the ventral *Lhx2*$^{-/-}$ optic neuroepithelium (Fig. 2P,R, arrowheads). By contrast, the distribution of SIX3 and of SOX2 was similar in the *Lhx2*$^{-/-}$ and control optic neuroepithelia at E9.5 (Fig. 2S,T,W,X), although their expression levels appeared reduced in the mutant at E10.25 (Fig. 2U,V,Y,Z). These data indicate that *Lhx2* inactivation does not eliminate the expression of all transcription factors important for eye development, but is required to maintain their proper spatial distribution or expression levels.

Pax6, *Six3* and *Sox2* are also expressed in the SE and are essential for lens development (Ashery-Padan et al., 2000; Furuta and Hogan, 1998; Liu et al., 2006). Whereas PAX6 was abundantly expressed in the *Lhx2*$^{-/-}$ SE at E9.5, its expression was downregulated by E10.25 (Fig. 2K′-N′, red arrows). SIX3 and SOX2 were weakly expressed or absent at both stages (Fig. 2S′-Z′, red arrows). Since *Lhx2* is not detected in the lens ectoderm, and OC morphogenesis is unaffected by conditional deletion of *Lhx2* in this region (see Fig. S2 in the supplementary material), the changes in lens development in the germline *Lhx2* mutant indicate that *Lhx2* is upstream of neuroepithelium-derived signals required for lens induction.

Lhx2 is required for regionalization and dorsoventral patterning of the optic neuroepithelium

Regionalization of the optic neuroepithelium slightly precedes the OV-to-OC transition (Ashery-Padan and Gruss, 2001; Chow and Lang, 2001; Martinez-Morales et al., 2004). *Vsx2* expression is activated by E9.5 and marks the presumptive neural retina. *Mitf* and *Pax2*, which are expressed broadly in the early OV (~E8.5), become restricted by the late OV stage to domains that correspond to RPE (*Mitf*) and optic stalk and ventral neural retina (*Pax2*). In addition to marking these domains, these factors are essential for maintaining regional identity (Baumer et al., 2003; Martinez-Morales et al., 2004; Horsford et al., 2005; Rowan et al., 2004). Interestingly, VSX2 and MITF expression failed to initiate in the *Lhx2*$^{-/-}$ OV (Fig. 3A-H), and PAX2, although expressed initially (Fig. 2J), was downregulated by E9.5 and largely absent by E10.25 (Fig. 3I-L). These observations suggest that the absence of *Lhx2* causes an arrest in eye development prior to regionalization.

Dorsoventral patterning also initiates during OV stages (Chow and Lang, 2001; Furimsky and Wallace, 2006). Inversion of dorsoventral polarity leads to disruption of OV development and failure of OC formation in chick embryos (Uemonsa et al., 2002). The failure of OC formation combined with the expansion of PAX6 and loss of PAX2 in the ventral optic neuroepithelium imply defects

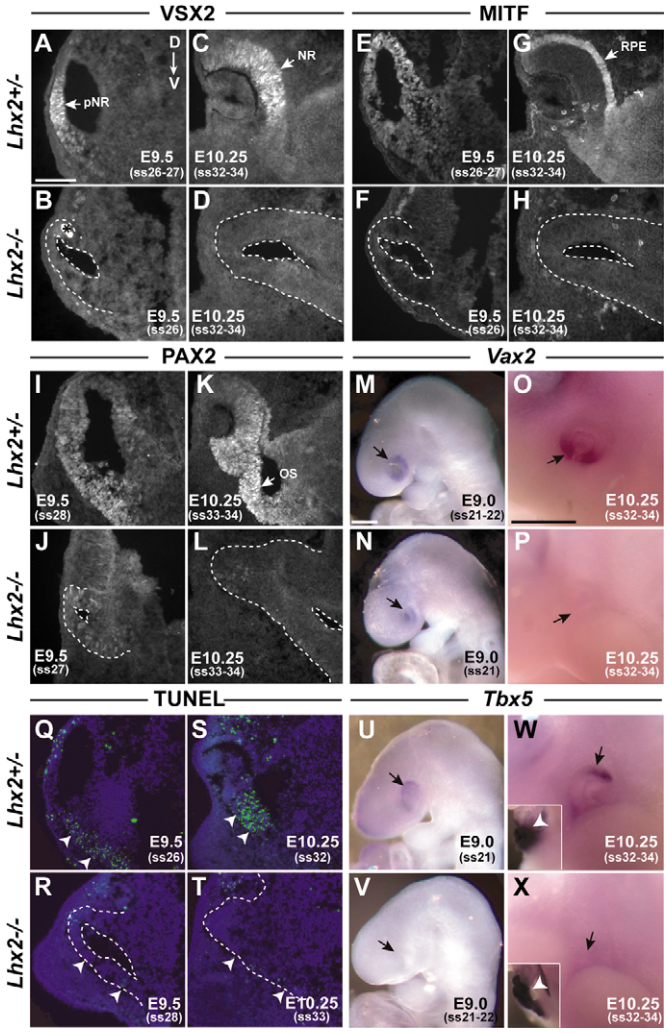


Fig. 3. *Lhx2* is required for domain determination of OV and dorsoventral patterning. Marker expression in control (*Lhx2*$^{+/-}$) and *Lhx2*$^{-/-}$ optic neuroepithelia. (**A-D**) VSX2 protein expression at E9.5 and E10.25. (**E-H**) MITF protein expression at E9.5 and E10.25. (**I-L**) PAX2 protein expression at E9.5 and E10.25. The arrow in K indicates the optic stalk. (**M-P**) *Vax2* mRNA expression at E9.5 and E10.25. (**Q-T**) Apoptosis. Arrowheads indicate ventral OV and the optic stalk region. (**U-X**) *Tbx5* mRNA expression. Arrows indicate OV and OC. Arrowheads in insets indicate *Tbx5* in the embryonic heart. The images in M and V are reflected on the vertical axis. Scale bars: 100 μm in A-L; 500 μm in M,N,U,V; 250 μm in O,P,W,X.

in dorsoventral patterning. To address this, we examined the expression pattern of *Vax2*, a homeobox gene required for ventral identity and for repression of *Pax6* expression in the ventral OV (Barbieri et al., 1999; Chow and Lang, 2001; Mui et al., 2005). Like *Pax2*, *Vax2* was expressed in the entire OV at early stages and became restricted to the ventral OV and OC (Fig. 3M,O). In the *Lhx2*$^{-/-}$ OV, *Vax2* was expressed at E9.0, but not at E10.25 (Fig. 3N,P). The extensive apoptosis that occurs in the wild-type optic stalk (Fig. 3Q,S) (Laemle et al., 1999; Silver and Hughes, 1974) was reduced in the absence of *Lhx2* (Fig. 3R,T), further indicating problems with dorsoventral patterning.

The ventral expansion of PAX6 and OTX2/1 in the *Lhx2*$^{-/-}$ OV suggests two possible explanations for the fate of the optic neuroepithelium: (1) that the ventral OV domain has acquired dorsal OV identity; or (2) that the entire OV has acquired an identity more akin to the dorsal diencephalon. If the former possibility were true, we would expect the dorsal marker *Tbx5* to expand ventrally. However, *Tbx5* mRNA was not detected at all in the *Lhx2*$^{-/-}$ OV (Fig. 3U-X). If the latter possibility were true, we would expect ectopic expression of genes that mark the dorsal diencephalon, such as *Wnt3a*, *Axin2* and *Dbx1*, but this was also not observed (see Fig. S3A-L in the supplementary material). These observations indicate that the optic neuroepithelium is not respecified. Rather, they suggest a developmental arrest and that *Lhx2* is required to link eye field specification and OV formation with lens induction and OV patterning.

Hedgehog and FGF signaling pathways are active in the *Lhx2*$^{-/-}$ OV and LHX2 expression is not dependent on *Shh* or *Fgf8*

Signals emanating from the forebrain, extraocular mesenchyme, SE and OV arbitrate the regionalization and dorsoventral polarity of the optic neuroepithelium (Ashery-Padan and Gruss, 2001; Chow and Lang, 2001; Fuhrmann et al., 2000; Martinez-Morales et al., 2004). The expansion of PAX6 and loss of PAX2 in the *Lhx2*$^{-/-}$ optic neuroepithelium are similar to that observed in zebrafish when Hedgehog (Hh) signaling is perturbed and in *Shh* mutant mice (Chiang et al., 1996; Ekker et al., 1995; Macdonald et al., 1995). We therefore sought to determine whether a regulatory relationship exists between Hh signaling and *Lhx2*. SHH expression was still observed in the *Lhx2*$^{-/-}$ ventral diencephalon (Fig. 4A,B), and *Gli1*, a read-out of Hh signaling (Sigulinsky et al., 2008), was also expressed (Fig. 4C,D, arrows and arrowheads). LHX2 was also expressed in the OV of *Shh*$^{GFP\text{-}cre/GFP\text{-}cre}$ mice (Fig. 4E,F), which lack midline Hh signaling, resulting in cyclopia and eye patterning defects (Harfe et al., 2004). These findings indicate that Hh pathway activation and *Lhx2* expression are not directly dependent on each other.

In the optic stalk of zebrafish *belladonna* mutants, the expression of *fgf8*, *pax2* and *vax*2 is diminished, and blocking FGF signaling reduces *lhx2* expression (Seth et al., 2006). These data suggest a mechanism of reciprocal regulation between FGF8 signaling and *Lhx2* expression. However, *Fgf8*, *Erm* [*Etv5*; a downstream target of FGF8 signaling (Roehl and Nusslein-Volhard, 2001)] and phosphorylated ERK1/2 [(MAPK3/1) pERK; downstream effectors of FGF-MAPK signaling (Pan et al., 2006)] were expressed in the mutant OV and SE in a manner similar to that observed in controls (Fig. 4G-P). To address the possibility that *Fgf8* regulates expression of *Lhx2*, we examined *Lhx2* expression in *Rosa26*$^{creER/+}$*; Fgf8* $^{f/f}$ mice, in which *Fgf8* begins to be ablated at E7.5 and the ablation is complete by E9.0 (Fig. 4Q,R). Although *Rosa26*$^{creER/+}$*; Fgf8* $^{f/f}$ mutants showed morphological defects in the telencephalon and OV, LHX2 was still expressed in the OV, as was PAX2 (Fig. 4S-V). These data indicate that FGF signaling is not *Lhx2* dependent and argue against *Lhx2* and *Fgf8* regulating each other's expression.

Lhx2 is required for BMP signaling

BMP signaling mediated by *Bmp4* and *Bmp7* is essential for several aspects of eye development, including lens induction and dorsoventral patterning of the optic neuroepithelium (Chow and Lang, 2001; Furuta and Hogan, 1998; Wawersik et al., 1999; Yang, 2004). *Bmp4* is first detected in the distal OV and SE between E8.5 and E9.0, and its expression is maintained at a high level in the dorsal OV and OC (Furuta and Hogan, 1998). *Bmp7* is widely expressed in the OV and is progressively restricted to the ventral region of the late OV and to the optic stalk after OC

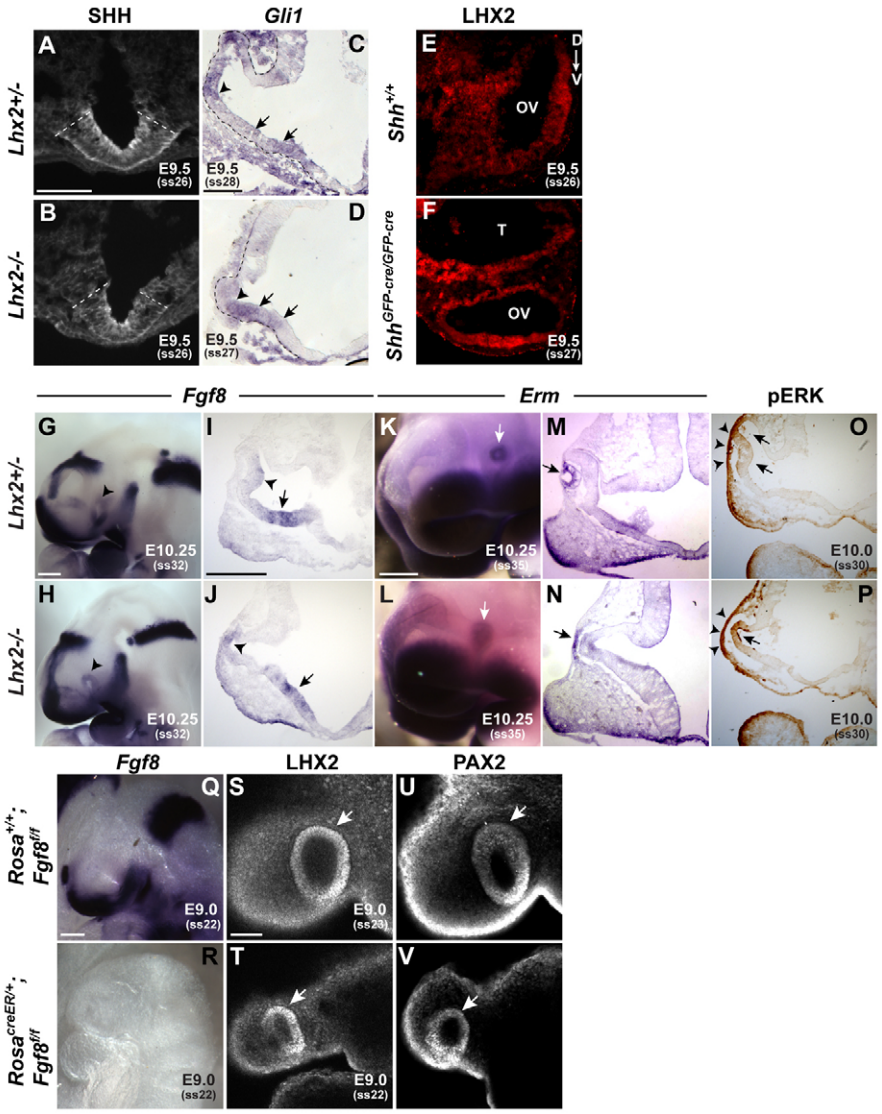


Fig. 4. Hh and FGF signaling are not dependent on *Lhx2*, and *Lhx2* expression is not dependent on *Shh* or *Fgf8*. (**A**,**B**) SHH protein expression at the ventral midline (between dashed lines) in E9.5 control and *Lhx2*$^{-/-}$ mouse embryos. (**C**,**D**) *Gli1* mRNA expression in ventral diencephalon (arrows) and OV (arrowheads) at E9.5. Dashed lines indicate the boundary of the neuroepithelium. (**E**,**F**) LHX2 protein expression in *Shh*$^{+/+}$ and *Shh*$^{GFP\text{-}cre/GFP\text{-}cre}$ OVs. (**G-J**) *Fgf8* mRNA expression in comparable regions of control and *Lhx2*$^{-/-}$ optic neuroepithelium (arrowheads) and ventral diencephalon (arrows). (**K-N**) *Erm* mRNA expression in comparable regions of control lens placode and *Lhx2*$^{-/-}$ SE (arrows). (**O**,**P**) pERK detection in control and *Lhx2*$^{-/-}$ SE (arrowheads) and distal OV (arrows). (**Q**,**R**) *Fgf8* mRNA expression in *Rosa*$^{+/+}$*; Fgf8*$^{f/f}$ and *Rosa*$^{creER/+}$*; Fgf8*$^{f/f}$ embryos at E9.0 after tamoxifen treatment at E7.5. (**S-V**) Single-scan confocal images of LHX2 and PAX2 expression in OVs from control (S,U) and *Fgf8*-inactivated (T,V) embryos. Arrows indicate OVs. T, telencephalon. Scale bars: 100 μm in A-F,Q-V; 500 μm in G,H,K,L; 50 μm in I-J,M-P.

formation (Morcillo et al., 2006). Although *Bmp7*$^{-/-}$ mice exhibit a range of eye anomalies, from microphthalmia to anophthalmia, the anophthalmic phenotype of *Bmp7*$^{-/-}$ mice is morphologically similar to that of *Lhx2*$^{-/-}$ mice (Dudley et al., 1995; Luo et al., 1995). Interestingly, we found that *Bmp7* and *Bmp4* mRNA expression levels are downregulated in the *Lhx2*$^{-/-}$ OV (Fig. 5A-J). To assess whether BMP signaling is affected, we examined the phosphorylation status of SMAD1, 5 and 8 (pSMAD1/5/8), a read-out of BMP signaling (Massague, 1998; Murali et al., 2005). Whereas pSMAD1/5/8 was present in the optic neuroepithelium in controls at E9.5 and E10.25 (Fig. 5K,M), it was not detectable in the *Lhx2*$^{-/-}$ optic neuroepithelium (Fig. 5L,N), indicating disrupted BMP signaling. In the lens-forming region of the SE, pSMAD1/5/8 was detected in controls at both stages and in the *Lhx2*$^{-/-}$ embryo at E9.5, but was absent in the mutant at E10.25 (Fig. 5O-R, regions between dashed lines are SE or lens placode). The persistence of *Bmp4* in the nasal placode (Fig. 5I,J, arrowheads) and of pSMAD1/5/8 in the extraocular mesenchyme (Mes; Fig. 5K-R) during these stages reveal that BMP signaling in the optic neuroepithelium and in the E10.25 lens ectoderm are dependent on *Lhx2*.

Lhx2 utilizes non-cell-autonomous and cell-autonomous mechanisms to regulate OV patterning

To determine whether the patterning defects in the *Lhx2*$^{-/-}$ OV are attributable to disrupted BMP signaling, we treated E9.0 heads with combinations of BMP7 and BMP4 for 2 days (Fig. 6A). As *Bmp7* is widely expressed in the OV, BMP7 was added directly to the medium. BMP7-treated wild-type OVs exhibited strong expression of *Pax2* (n=11/11), *Vsx2* and *Mitf* (Fig. 6B,D; data not shown). In the *Lhx2*$^{-/-}$ OV, BMP7 treatment restored *Pax2* expression (n=8/8), although at reduced levels compared with the wild type (Fig. 6C,E). However, it was not sufficient to initiate expression of *Vsx2* or *Mitf* (data not shown).

Fgf15 is a potential downstream target of BMP4-mediated signaling in the neural retina (Murali et al., 2005). As *Fgf15* is downregulated in the *Lhx2*$^{-/-}$ optic neuroepithelium (see Fig. S4 in the supplementary material), we assessed whether implantation of BMP4-coated beads is sufficient to restore its expression. Whereas *Fgf15* expression was enhanced in wild-type optic neuroepithelium cultured with BMP4 (n=5/5; Fig. 6F,H,J), we did not detect *Fgf15* in *Lhx2*$^{-/-}$ OVs (n=0/3; Fig. 6G,I,K). These

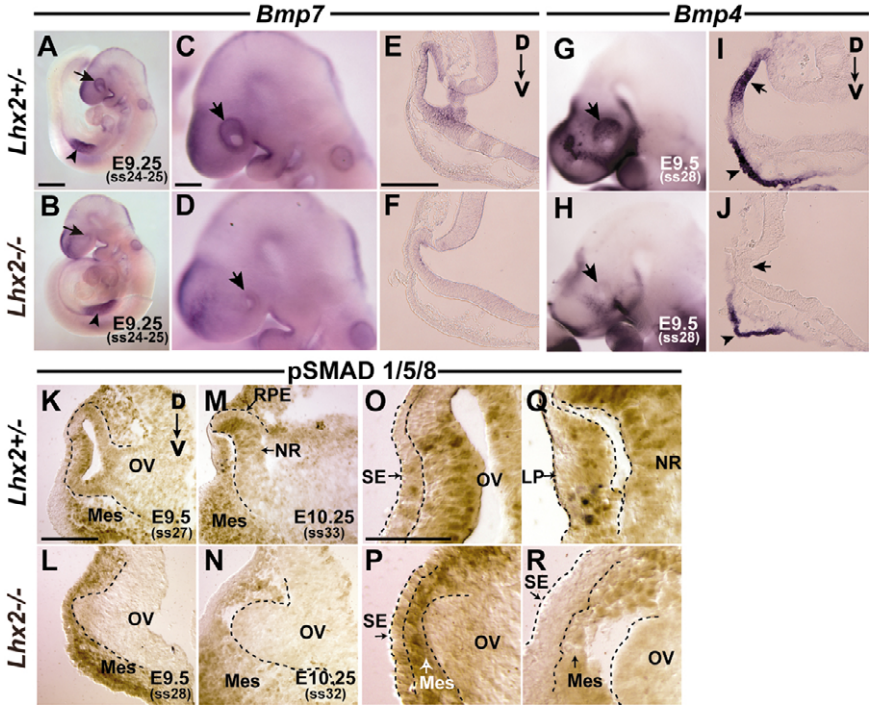


Fig. 5. BMP signaling is altered in the absence of *Lhx2*. (**A-F**) *Bmp7* mRNA expression at E9.25 in control and *Lhx2*$^{-/-}$ embryos. Arrows (A-D) indicate OVs and arrowheads (A,B) indicate hindlimb expression. C and D are magnified views of heads in A and B. E and F are coronal sections of the same samples as shown in A-D. The image in A is reflected on the vertical axis. (**G-J**) *Bmp4* mRNA expression at E9.5 in control and *Lhx2*$^{-/-}$ embryos. Arrows indicate OVs. I and J are coronal sections of the heads from G and H. Arrowheads (I,J) indicate *Bmp4* expression in nasal placode, which is unaffected by *Lhx2* inactivation. (**K-R**) pSMAD1/5/8 immunohistochemistry at E9.5 and E10.25 in control and *Lhx2*$^{-/-}$ optic neuroepithelia, SE and mesenchyme. Note the strong staining in the mesenchyme in all samples. (O-R) Magnified views of the lens-forming region of SE. Mes, mesenchyme. Scale bars: 1 mm in A,B; 500 μm in C,D,G,H; 50 μm in E,F,I-N; 25 μm in O-R.

observations suggest that there are defects in BMP4-mediated signaling in addition to those induced by the absence of *Bmp4* expression.

As both *Bmp4* and *Bmp7* are downregulated in the *Lhx2*$^{-/-}$ OV, we tested the effect of adding BMP4 and BMP7 together to the cultures. Surprisingly, this condition elicited a strong enhancement of *Fgf15* expression in both wild-type (n=7/7) and *Lhx2*$^{-/-}$ (n=2/3) optic neuroepithelia (Fig. 6L-Q). Although we cannot rule out the possibility that adding both ligands simply raises the BMP concentration over a threshold, functional redundancy of BMP7 and BMP4 signaling in eye development is unlikely as a *Bmp4* knock-in allele at the *Bmp7* locus only partially rescues the eye phenotype in *Bmp7*$^{-/-}$ mice (Oxburgh et al., 2005). Furthermore, expression of *Tbx5*, *Vsx2* and *Mitf* was not induced in any of the BMP treatment paradigms we tried (data not shown).

As BMP signaling is needed for lens induction (Furuta and Hogan, 1998; Wawersik et al., 1999), we asked whether BMP treatment restores *Sox2* expression in the SE of the *Lhx2* mutant. Whereas *Sox2* expression in the wild-type OV culture was not dependent on BMP supplementation (n=3/3; Fig. 6R,T,V,X), it was detected in the mutant only when BMP7 (n=2/2), or BMP4 and BMP7 (n=2/2), were added (Fig. 6S,U,W,Y). These data reveal that BMP7, or BMP4 and BMP7 added together, restore some, but not all, of the determinants needed for OV development and lens induction in *Lhx2* mutants.

The failure of BMP treatment to activate genes such as *Vsx2* and *Mitf* led us to suspect that some determinants of OV patterning require *Lhx2* in a cell-autonomous manner for their expression. We generated genetic mosaics in which wild-type cells surround *Lhx2* mutant cells and assessed whether *Vsx2* and *Mitf* are expressed in the mutant cells. We utilized a newly generated tamoxifen-inducible Cre driver in which *creERT2* is knocked into the *Hes1* locus. Under low-dose tamoxifen administration at E8.5, rare cells in the OC express a recombination reporter by E10.5 (see Fig. S5 in the supplementary material), indicating that this approach is suitable for generating *Lhx2* mutant cells at low frequency. As predicted, we found that *Lhx2* mutant cells in the neural retina do not express VSX2 (Fig. 7A-D; n=17/17), nor do they express MITF in the RPE (Fig. 7E-H; n=23/23). We also examined PAX2 expression (Fig. 7I-P) as its response to BMP treatment was relatively weak, and observed that most *Lhx2* mutant cells were also PAX2 negative (Fig. 7I-L; n=48/50), which suggests that *Lhx2* utilizes both cell-autonomous and non-cell-autonomous mechanisms to regulate *Pax2* expression. By contrast, PAX6 was expressed in all *Lhx2* mutant cells analyzed (Fig. 7M-P; n=33/33), consistent with our data and the work of others indicating that *Pax6* expression is independent of *Lhx2* function.

DISCUSSION

In addition to the morphological changes occurring during the OV-to-OC transition, complex patterning and inductive interactions are executed during this period. In this study, we found that *Lhx2* has a unique role in mediating this transition. Even though other EFTFs continue to be expressed in the *Lhx2*$^{-/-}$ OV, loss of *Lhx2* results in a failure of the optic neuroepithelium to become regionalized and patterned along the dorsoventral axis. In addition, the interaction between the optic neuroepithelium and SE that leads to lens formation is severely compromised. We propose that *Lhx2* acts as a central factor in eye organogenesis by coordinating several of the crucial events that occur during the transition of the OV to OC, in part through its role in establishing a BMP signaling center in the optic neuroepithelium.

Lhx2 links eye field specification to OV patterning

Our analysis of eye development from ss6 to ss18 suggests that eye field specification in the anterior neural plate and initiation of OV morphogenesis occur on, or close to, schedule in the absence of *Lhx2*. Our conclusion differs from that of Tetreault et al., who concluded that *Lhx2* is required for eye field specification and for the correct timing of EFTF expression, which was based on their observations of weak or absent expression of *Rx*, *Six3* and *Pax6* in the presumptive eye field of *Lhx2* mutant embryos from E8.25 and

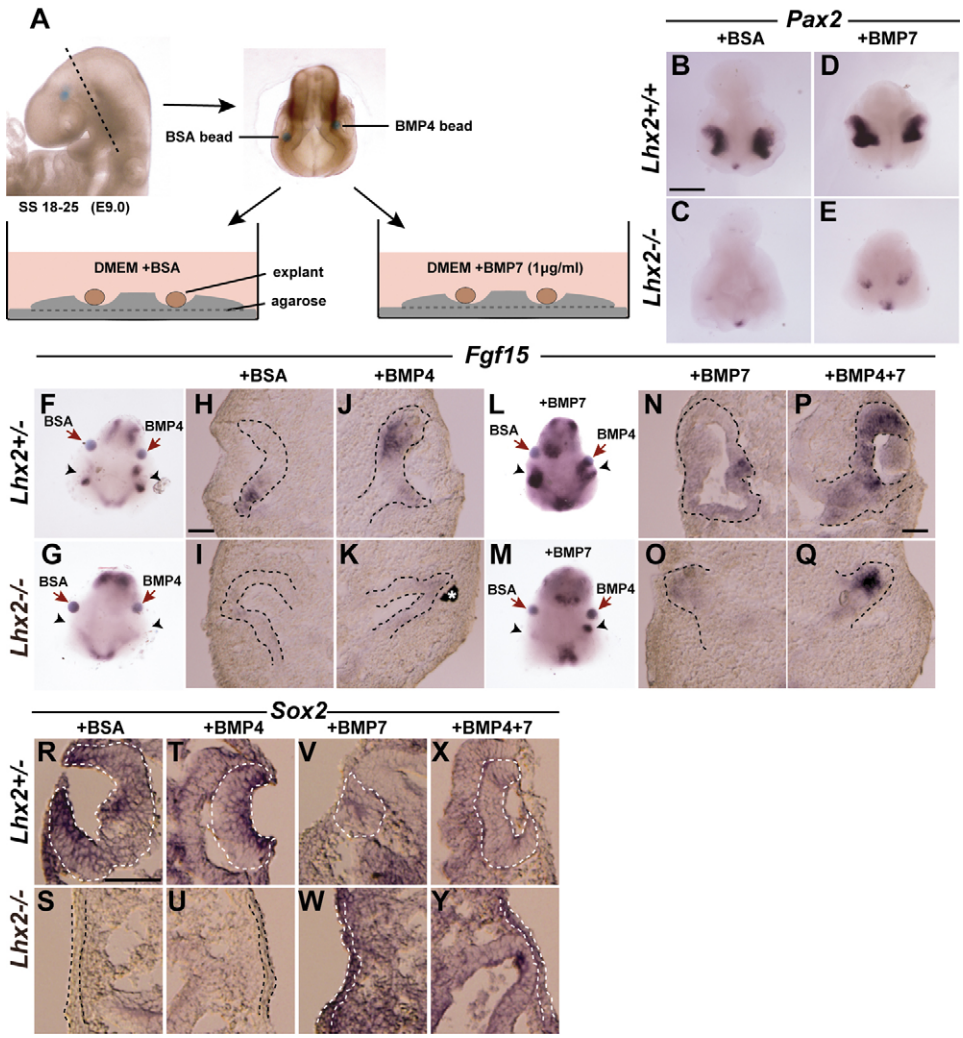


Fig. 6. BMP treatments restore the expression of optic-vesicle-patterning and lens-specification genes. (**A**) Organotypic culture method. Dashed line indicates approximate position at which heads were transected. Heads containing BSA- (control) and BMP4-soaked beads were cultured in medium containing BSA or BMP7. Wild-type (*Lhx2*$^{+/+}$ or *Lhx2*$^{+/-}$) and *Lhx2*$^{-/-}$ heads were cultured for 48 hours. (**B-E**) *Pax2* response to BMP treatments. (**F-Q**) *Fgf15* response to BMP treatments. Arrowheads (F,G,L,M) point to *Fgf15* expression. Red arrows indicate BSA- and BMP4-coated beads. Dashed line delineates the optic neuroepithelium. Asterisk in K indicates an artifact. (**R-Y**) *Sox2* response to BMP treatments. SE is located between the dashed lines. Coronal sections, except frontal views in B-G,L,M. Scale bars: 500 μm in B-G,L,M; 50 μm in H-K,N-Y.

E8.5 litters (Tetreault et al., 2009). The lack of *Rx* and *Six3* expression at E8.25 was surprising to us because the absence of either factor prevents OV morphogenesis (Carl et al., 2002; Mathers et al., 1997), whereas OV morphogenesis clearly occurs in the *Lhx2* mutant, and embryo chimeras show that *Rx* mutant cells are excluded from the optic neuroepithelium as early as E8.0, demonstrating its cell-autonomous requirement (Medina-Martinez et al., 2009). We determined that *Rx* is expressed in ss6 and ss12 embryos. The discrepancy in *Rx* expression between the two studies is probably not due to genetic differences because we observed similar staining in two different genetic backgrounds (*Lhx2*$^{+/-}$ were backcrossed two generations with 129sv or Black Swiss mice) and both studies analyzed the same allele. One possibility is that *Lhx2* is required for stable EFTF expression in the eye field and that their expression levels fluctuate in its absence. We consider this unlikely, however, as we observed robust *Rx* expression in every mutant embryo analyzed between ss4 and ss12 (n=5). Another possible explanation lies in how the mutant and control embryos were matched for comparison. Whereas Tetreault and colleagues reported the staging of embryos according to the time of vaginal plug (i.e. gestational age), our embryos were staged by counting somites and matched for comparison by equivalent somite stage. As embryogenesis proceeds rapidly during this period, comparing embryos at the same somite stage controls for the normal, but sometimes considerable, variation in developmental progression that occurs among such embryos (including among littermates). It also controls for the possibility of general developmental delays that might occur in *Lhx2* mutants. Although we do not exclude the possibility that variability in EFTF expression might occur in the anterior neural plate of *Lhx2* mutants, our analysis of somite-stage-matched embryos suggest that the initiation of *Rx* expression is not significantly delayed in the *Lhx2*$^{-/-}$ anterior neural plate. Furthermore, because OV morphology, PAX6 expression and the proportion of pHH3-positive cells are similar to those in the wild type as late as ss18, we propose that the functional output of eye field specification is operating in the absence of *Lhx2*. Rather, the marked differences in morphology and marker expression that occur from ss20 onward indicate that the absence of *Lhx2* leads to a developmental arrest after OV formation, but prior to the patterning events that precede OC formation.

That the nature of the *Lhx2* developmental arrest is unique is best exemplified by comparing it with the *Pax6* mutant, which exhibits a remarkably similar morphological, but molecularly distinct, phenotype. In the absence of *Pax6*, the eye field is established and maintained through the OV stage as indicated by the persistent expression of *Rx*, *Lhx2*, *Six3*, *Otx2* and *Pax2* (Baumer et al., 2003; Bernier et al., 2001). Furthermore, regionalization of the optic neuroepithelium, an event that occurs late in the OV, still occurs, as

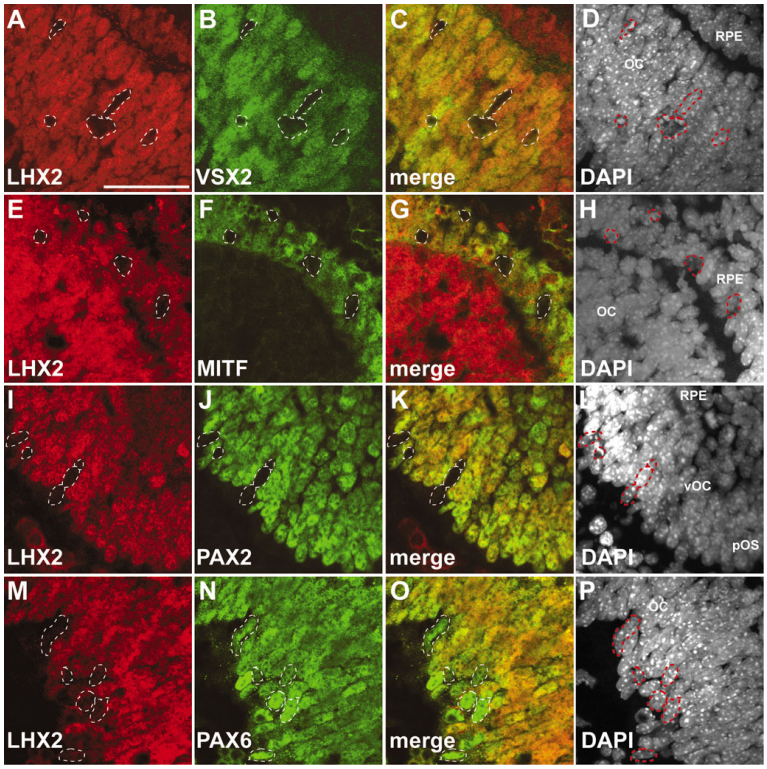


Fig. 7. Cell-autonomous regulation of VSX2, MITF and PAX2 expression by *Lhx2*. (**A-P**) Single-scan confocal images of LHX2, VSX2, MITF, PAX2 and PAX6 expression in mouse E10.5 optic neuroepithelium mosaic for *Lhx2*. LHX2-negative cells are outlined (dashed line). Images in each row are from the same section and the merged panels show the overlap of LHX2 with each marker. DAPI panels show that areas lacking LHX2 expression do contain cells. In contrast to VSX2, MITF and PAX2, PAX6 expression persisted in LHX2-negative cells. Scale bar: 40 μm.

indicated by the activation of *Vsx2* and *Mitf* expression and by their segregation into distinct domains (Baumer et al., 2003). By contrast, *Rx* expression in the *Lhx2*$^{-/-}$ OV drops by E9.5, and expression of *Vsx2*, *Mitf*, *Tbx5* and *Pax2* fails to initiate or persist, indicating a failure of the neuroepithelium to maintain its 'optic' character and to initiate regionalization. Another notable difference is that whereas ventral retinal identity is not only maintained but also expanded at the expense of dorsal identity in the *Pax6* mutant (Baumer et al., 2002), both dorsal and ventral retinal identity fail to be established in the *Lhx2* mutant (see below). It appears, then, that the OV patterning progresses further in the *Pax6* mutant, suggesting that there is a more profound and possibly earlier requirement for *Lhx2* than for *Pax6* in the maturation of the optic neuroepithelium during the OV stage.

The role of *Lhx2* in OV patterning

In addition to failing to maintain PAX2 expression, other indicators of ventral identity, such as *Vax2* expression and a high level of apoptosis in the optic stalk region, are diminished in the *Lhx2* mutant. These findings, along with our observation that PAX6 expression is expanded ventrally, led us to suspect that the OV is dorsalized, as these types of changes correlate with dorsalization in other models [in *Vax1; Vax2* double-knockout mice, *BF-1* (*FoxG1*) mutant mice, and zebrafish with perturbed Hh signaling] and in OV cultures treated with BMP4 (Behesti et al., 2006; Ekker et al., 1995; Huh et al., 1999; Macdonald et al., 1995; Mui et al., 2005; Yang, 2004). Surprisingly, *Tbx5* and *Bmp4*, which are markers for dorsal retina identity, are not expressed in the *Lhx2*$^{-/-}$ OV, indicating a lack of dorsal identity as well. This outcome is markedly different from that which results from perturbing the expression or activation of other cell-intrinsic factors (*Pax6*, *Pax2*, *Vax1*, *Vax2* and *Tbx5*) that regulate dorsoventral axis formation, which tends to cause expansion of one side of the axis at the expense of the other (Behesti et al., 2006; Huh et al., 1999; Macdonald et al., 1995; Mui et al., 2005). That dorsal and ventral determinants are lost or never activated in the *Lhx2*$^{-/-}$ OV supports a model by which *Lhx2* contributes to the formation of the entire axis by regulating key determinants on both sides.

Although the role of *Lhx2* in regulating dorsoventral polarity is likely to involve several mechanisms, our data reveal a requirement for *Lhx2* in extracellular signaling, which is crucial on both sides of the axis (Adler and Canto-Soler, 2007; Chow and Lang, 2001; Yang, 2004). On the ventral side, *Lhx2* might couple signaling pathways with their context-dependent targets. For example, Shh signaling emanating from the ventral diencephalon is active in the *Lhx2* mutant, and yet, *Vax2* and *Pax2*, which are genetically downstream of Shh signaling (Ekker et al., 1995; Macdonald et al., 1995; Take-uchi et al., 2003), are not expressed. Similarly, *Fgf8*, which is predicted to be an important signal for ventral identity in zebrafish and *Xenopus* (Lupo et al., 2005; Shanmugalingam et al., 2000), is expressed in the *Lhx2* mutant. Since *Shh* and *Fgf8* do not regulate *Lhx2* expression, *Lhx2* seems to provide a key factor needed by these signaling pathways to promote ventral identity. Interestingly, reintroduction of BMP7 into *Lhx2*$^{-/-}$ OV reactivates *Pax2* expression, although weakly. Not only does this reveal that the optic neuroepithelium retains some degree of competence to express *Pax2*, but also indicates that the control by *Lhx2* of ventral identity involves BMP signaling, which is consistent with the recent finding that *Bmp7* is required for ventral expression of *Pax2* and apoptosis in the late OV/early OC (Morcillo et al., 2006).

Several studies have revealed the importance of BMP signaling in establishing dorsal polarity and the neural retina domain in the optic neuroepithelium. BMP signaling in the dorsal OV is required for the expression of *Tbx5*, *Vsx2* and *Fgf15* (Behesti et al., 2006; Murali et al., 2005), and *Bmp4* misexpression in the ventral OC in chick induces ectopic *Tbx5* expression (Koshiba-Takeuchi et al., 2000). Although the absence of detectable *Bmp4* expression and signaling combined with the absence of *Tbx5*, *Vsx2* and *Fgf15* in the

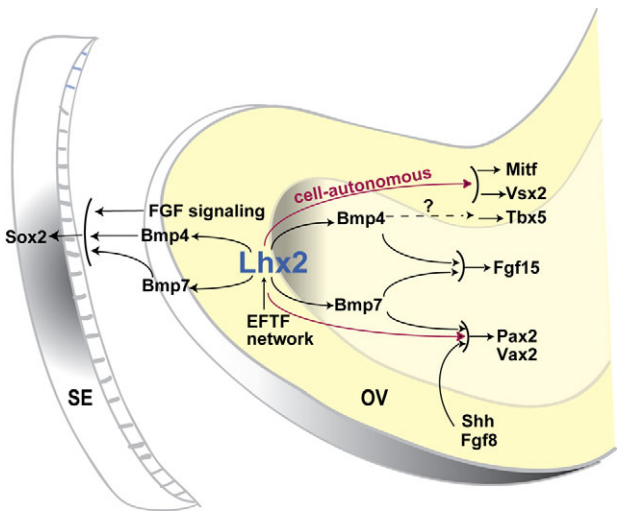


Fig. 8. Model of *Lhx2* function during mouse early eye organogenesis. *Lhx2*, under the control of the EFTF network, links lens specification and optic vesicle patterning through the regulation of BMP signaling (black arrows). *Lhx2* also promotes optic vesicle patterning by cell-autonomous mechanisms (red arrows). Why Bmp4 fails to upregulate *Tbx5* expression is not resolved (dashed line). The timing of action and influence of *Lhx2* on several pathways suggest that it acts to coordinate the multiple patterning events necessary for optic cup formation.

Lhx2$^{-/-}$ OV indicates a problem with dorsal polarity and retina domain determination, reintroduction of BMP4 in culture did not stimulate their expression. This was not due to problems with the source of BMP4 protein because treatment enhanced *Fgf15* expression in wild-type cultures. Rather, in addition to regulating BMP signaling through its effects on ligand expression, *Lhx2* might also regulate other factors required for *Bmp4*-mediated signaling. Interestingly, a robust increase in *Fgf15* expression occurred when BMP4 and BMP7 were added simultaneously. These findings suggest that *Lhx2*, acting through *Bmp7*, could influence BMP4 signaling. Since *Bmp7* expression overlaps with that of *Bmp4*, it is expressed at the right time and place to play such a role (Morcillo et al., 2006). Whether the enhanced effect of combining BMP7 and BMP4 in culture is due to extracellular interactions (i.e. overcoming inhibition by BMPR antagonists), or whether BMP7 signaling provides a cell-intrinsic component needed for BMP4 signaling, is an open question. Regardless, our data suggest that *Lhx2* acts by more than one mechanism to influence the expression of a battery of genes required for development of the optic neuroepithelium. This is further exemplified by our genetic mosaic analysis, which indicates that *Vsx2*, *Mitf* and *Pax2* (in many cells) require *Lhx2* in a cell-autonomous manner. This could reflect a requirement for *Lhx2* in BMP signaling at the intracellular level or a requirement for *Lhx2* in other mechanisms, such as direct transcriptional regulation, as proposed for *Six6* (Tetreault et al., 2008). Resolving these issues requires further study.

Lhx2 in the optic neuroepithelium is required for lens formation

Emergence of the lens from the SE occurs in a series of discrete steps termed competence, bias, specification and differentiation, initiating in the anterior neural plate and extending through OC formation (Donner et al., 2006; Henry and Grainger, 1987; Lang, 2004; Servetnick and Grainger, 1991). *Pax6* is expressed in the lens-forming region throughout this process, but its expression is regulated in two temporal phases: preplacodal and placodal (Grindley et al., 1995; Lang, 2004). These phases are demarcated by the appearance of the lens placode, which follows the activation of *Sox2* expression, a cell-autonomous factor required for lens specification (Kamachi et al., 1998). Our finding that *Pax6* is expressed in the *Lhx2*$^{-/-}$ SE at stages prior to placode formation, but is not maintained at a time when the lens placode normally forms, suggests that the SE arrests at, or just prior to, specification. This is strongly supported by our observation that *Sox2* is not detected in the *Lhx2*$^{-/-}$ SE.

Although the role of the optic neuroepithelium in lens development has been debated, the prevailing model is that signals from the OV are required at around the time of specification (Furuta and Hogan, 1998). Since conditional deletion of *Lhx2* in the SE has no obvious effect on eye development and *Lhx2* is expressed in the optic neuroepithelium, we can conclude that *Lhx2* regulates lens specification through a non-cell-autonomous mechanism, providing further support for the prevailing model. This distinguishes *Lhx2* from other EFTFs, such as *Six3*, *Pax6* and *Sox2*, which regulate lens formation cell-autonomously (Ashery-Padan et al., 2000; Furuta and Hogan, 1998; Lang, 2004; Liu et al., 2006; Ogino et al., 2008).

FGF-MAPK and BMP signaling, both of which depend on ligands produced in the OV, are required, but are not sufficient on their own, for lens specification (Faber et al., 2001; Furuta and Hogan, 1998; Gotoh et al., 2004). Whereas FGF-MAPK signaling is active in *Lhx2*$^{-/-}$ SE, BMP signaling is not by E10.25, consistent with the absence of *Bmp4* and *Bmp7* expression in the optic neuroepithelium. Interestingly, BMP7, or the combination of BMP4 and BMP7, was sufficient to induce *Sox2* expression in the SE in *Lhx2*$^{-/-}$ head cultures, revealing that the SE of the mutant is competent to express *Sox2*, and that *Sox2* expression in the SE depends on *Lhx2* through its regulation of *Bmp4* and/or *Bmp7* expression and signaling. This proposed pathway is supported by the following findings: (1) *Lhx2* is expressed in both *Bmp7*$^{-/-}$ and *Bmpr1a; Bmpr1b* double-knockout mice, the latter of which lack detectable BMP signaling in the optic neuroepithelium and SE; and (2) *Sox2* is not expressed in the SE of *Bmp4*$^{-/-}$ or *Bmp7*$^{-/-}$ mice (Furuta and Hogan, 1998; Murali et al., 2005; Wawersik et al., 1999). Thus, the intersection of *Lhx2* and BMP signaling extends to the formation of the lens, and our findings lend further support to the model that an interaction with the OV is essential for advancing the SE into the specification step and beyond.

Model for *Lhx2* function in early eye development

We propose a model for how *Lhx2* fits into the scheme of early eye organogenesis (Fig. 8). Although *Lhx2* is activated during eye field specification, its requirement for advancing eye organogenesis does not manifest until after OV formation. Mechanistically, activation of *Lhx2* expression is dependent on the EFTF network (Zuber et al., 2003). The competence of the optic neuroepithelium to respond in a context-specific manner (i.e. the expression of tissue-specific gene targets) to the various signaling pathways (BMP, Hh, FGF) is mediated by *Lhx2* at the early OV stage. *Lhx2* is required to induce or maintain the expression of genes required at the late OV stage for regionalization (*Mitf*, *Vsx2*, *Pax2*), establishment of retinal dorsoventral polarity (*Tbx5* and *Vax2*), retinal progenitor cell properties (*Fgf15*) and lens specification (*Sox2*). *Lhx2* regulates *Vsx2* and *Mitf* cell-autonomously, whereas *Pax2* is regulated by *Lhx2* by cell-autonomous and non-cell-autonomous mechanisms. Several of these genes (*Pax2*, *Fgf15*, *Sox2*) are also linked to *Lhx2*

through its regulation of BMP signaling. This model places *Lhx2* firmly at the center of a network that coordinates the development of the OV and SE to ensure the proper development of the OC.

Acknowledgements

We thank the following for mice and reagents: Heiner Westphal (*Lhx2* knockout mice and *Lhx2* cDNA); Anne Moon (*Fgf8*^{floxed} mice and *Fgf8* cDNA); Ruth Ashery-Padan and David Beebe (*Le-cre* mice); Nadean Brown (β-gal antibody); Guillermo Oliver (SIX3 antibody); Peter Mathers (*Rx* cDNA); Anna Barbieri (*Vax2* cDNA); Deborah Chapman (*Tbx5* cDNA); Valerie Wallace (*Gli1* cDNA); Deborah Guris (*Erm* cDNA); Brigid Hogan (*Bmp4* and *Bmp7* cDNAs); Ahmed Mansouri (*Pax2* cDNA); Elizabeth Robertson (*Sox2* cDNA); Cornelis Murre (*Fgf15* cDNA); Andrew McMahon (*Wnt3a* cDNA); and Frank Constantini (*Axin2* cDNA). We thank Sabine Fuhrmann, her laboratory, and the Levine laboratory for their input and encouragement; Amy Kircher, DeAndre' Reeve and Anna Clark for technical assistance; and Sabine Fuhrmann and Richard Dorsky for their insightful comments on the manuscript. Funding sources were as follows. E.M.L., Sybil Harrington Research Scholar Award from Research to Prevent Blindness, NEI (R01-EY013760) and NEI Core Vision Research Grant (P30-EY014800). Y.S., March of Dimes. L.C.M., Searle Scholars Program (06-B-116). D.K., Boehringer Ingelheim Fonds. Deposited in PMC for release after 12 months.

Supplementary material

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/136/23/3895/DC1

References

Adler, R. and Canto-Soler, M. V. (2007). Molecular mechanisms of optic vesicle development: complexities, ambiguities and controversies. *Dev. Biol.* **305**, 1-13.

Ashery-Padan, R. and Gruss, P. (2001). Pax6 lights-up the way for eye development. *Curr. Opin. Cell Biol.* **13**, 706-714.

Ashery-Padan, R., Marquardt, T., Zhou, X. and Gruss, P. (2000). Pax6 activity in the lens primordium is required for lens formation and for correct placement of a single retina in the eye. *Genes Dev.* **14**, 2701-2711.

Badea, T. C., Wang, Y. and Nathans, J. (2003). A noninvasive genetic/pharmacologic strategy for visualizing cell morphology and clonal relationships in the mouse. *J. Neurosci.* **23**, 2314-2322.

Bailey, T. J., El-Hodiri, H., Zhang, L., Shah, R., Mathers, P. H. and Jamrich, M. (2004). Regulation of vertebrate eye development by Rx genes. *Int. J. Dev. Biol.* **48**, 761-770.

Barbieri, A. M., Lupo, G., Bulfone, A., Andreazzoli, M., Mariani, M., Fougerousse, F., Consalez, G. G., Borsani, G., Beckmann, J. S., Barsacchi, G. et al. (1999). A homeobox gene, vax2, controls the patterning of the eye dorsoventral axis. *Proc. Natl. Acad. Sci. USA* **96**, 10729-10734.

Baumer, N., Marquardt, T., Stoykova, A., Ashery-Padan, R., Chowdhury, K. and Gruss, P. (2002). Pax6 is required for establishing naso-temporal and dorsal characteristics of the optic vesicle. *Development* **129**, 4535-4545.

Baumer, N., Marquardt, T., Stoykova, A., Spieler, D., Treichel, D., Ashery-Padan, R. and Gruss, P. (2003). Retinal pigmented epithelium determination requires the redundant activities of Pax2 and Pax6. *Development* **130**, 2903-2915.

Behesti, H., Holt, J. K. and Sowden, J. C. (2006). The level of BMP4 signaling is critical for the regulation of distinct T-box gene expression domains and growth along the dorso-ventral axis of the optic cup. *BMC Dev. Biol.* **6**, 62.

Bernier, G., Vukovich, W., Neidhardt, L., Herrmann, B. G. and Gruss, P. (2001). Isolation and characterization of a downstream target of Pax6 in the mammalian retinal primordium. *Development* **128**, 3987-3994.

Bharti, K., Nguyen, M. T., Skuntz, S., Bertuzzi, S. and Arnheiter, H. (2006). The other pigment cell: specification and development of the pigmented epithelium of the vertebrate eye. *Pigment Cell Res.* **19**, 380-394.

Carl, M., Loosli, F. and Wittbrodt, J. (2002). Six3 inactivation reveals its essential role for the formation and patterning of the vertebrate eye. *Development* **129**, 4057-4063.

Chiang, C., Litingtung, Y., Lee, E., Young, K. E., Corden, J. L., Westphal, H. and Beachy, P. A. (1996). Cyclopia and defective axial patterning in mice lacking Sonic hedgehog gene function. *Nature* **383**, 407-413.

Chow, R. L. and Lang, R. A. (2001). Early eye development in vertebrates. *Annu. Rev. Cell Dev. Biol.* **17**, 255-296.

Clark, A. M., Yun, S., Veien, E. S., Wu, Y. Y., Chow, R. L., Dorsky, R. I. and Levine, E. M. (2008). Negative regulation of Vsx1 by its paralog Chx10/Vsx2 is conserved in the vertebrate retina. *Brain Res.* **1192**, 99-113.

Donner, A. L., Lachke, S. A. and Maas, R. L. (2006). Lens induction in vertebrates: variations on a conserved theme of signaling events. *Semin. Cell Dev. Biol.* **17**, 676-685.

Dudley, A. T., Lyons, K. M. and Robertson, E. J. (1995). A requirement for bone morphogenetic protein-7 during development of the mammalian kidney and eye. *Genes Dev.* **9**, 2795-2807.

Ekker, S. C., Ungar, A. R., Greenstein, P., von Kessler, D. P., Porter, J. A., Moon, R. T. and Beachy, P. A. (1995). Patterning activities of vertebrate hedgehog proteins in the developing eye and brain. *Curr. Biol.* **5**, 944-955.

Faber, S. C., Dimanlig, P., Makarenkova, H. P., Shirke, S., Ko, K. and Lang, R. A. (2001). Fgf receptor signaling plays a role in lens induction. *Development* **128**, 4425-4438.

Fitzpatrick, D. R. and van Heyningen, V. (2005). Developmental eye disorders. *Curr. Opin. Genet. Dev.* **15**, 348-353.

Fuhrmann, S., Levine, E. M. and Reh, T. A. (2000). Extraocular mesenchyme patterns the optic vesicle during early eye development in the embryonic chick. *Development* **127**, 4599-4609.

Furimsky, M. and Wallace, V. A. (2006). Complementary Gli activity mediates early patterning of the mouse visual system. *Dev. Dyn.* **235**, 594-605.

Furuta, Y. and Hogan, B. L. (1998). BMP4 is essential for lens induction in the mouse embryo. *Genes Dev.* **12**, 3764-3775.

Furuta, Y., Piston, D. W. and Hogan, B. L. (1997). Bone morphogenetic proteins (BMPs) as regulators of dorsal forebrain development. *Development* **124**, 2203-2212.

Gotoh, N., Ito, M., Yamamoto, S., Yoshino, I., Song, N., Wang, Y., Lax, I., Schlessinger, J., Shibuya, M. and Lang, R. A. (2004). Tyrosine phosphorylation sites on FRS2alpha responsible for Shp2 recruitment are critical for induction of lens and retina. *Proc. Natl. Acad. Sci. USA* **101**, 17144-17149.

Graw, J. (2003). The genetic and molecular basis of congenital eye defects. *Nat. Rev. Genet.* **4**, 876-888.

Grindley, J. C., Davidson, D. R. and Hill, R. E. (1995). The role of Pax-6 in eye and nasal development. *Development* **121**, 1433-1442.

Harfe, B. D., Scherz, P. J., Nissim, S., Tian, H., McMahon, A. P. and Tabin, C. J. (2004). Evidence for an expansion-based temporal Shh gradient in specifying vertebrate digit identities. *Cell* **118**, 517-528.

Henry, J. J. and Grainger, R. M. (1987). Inductive interactions in the spatial and temporal restriction of lens-forming potential in embryonic ectoderm of Xenopus laevis. *Dev. Biol.* **124**, 200-214.

Hill, R. E., Favor, J., Hogan, B. L., Ton, C. C., Saunders, G. F., Hanson, I. M., Prosser, J., Jordan, T., Hastie, N. D. and van Heyningen, V. (1991). Mouse small eye results from mutations in a paired-like homeobox-containing gene. *Nature* **354**, 522-525.

Hollemann, T., Bellefroid, E. and Pieler, T. (1998). The Xenopus homologue of the Drosophila gene tailless has a function in early eye development. *Development* **125**, 2425-2432.

Horsford, D. J., Nguyen, M. T., Sellar, G. C., Kothary, R., Arnheiter, H. and McInnes, R. R. (2005). Chx10 repression of Mitf is required for the maintenance of mammalian neuroretinal identity. *Development* **132**, 177-187.

Huh, S., Hatini, V., Marcus, R. C., Li, S. C. and Lai, E. (1999). Dorsal-ventral patterning defects in the eye of BF-1-deficient mice associated with a restricted loss of shh expression. *Dev. Biol.* **211**, 53-63.

Hyer, J., Mima, T. and Mikawa, T. (1998). FGF1 patterns the optic vesicle by directing the placement of the neural retina domain. *Development* **125**, 869-877.

Jensen, A. M. (2005). Potential roles for BMP and Pax genes in the development of iris smooth muscle. *Dev. Dyn.* **232**, 385-392.

Kamachi, Y., Uchikawa, M., Collignon, J., Lovell-Badge, R. and Kondoh, H. (1998). Involvement of Sox1, 2 and 3 in the early and subsequent molecular events of lens induction. *Development* **125**, 2521-2532.

Kikuchi, Y., Segawa, H., Tokumoto, M., Tsubokawa, T., Hotta, Y., Uyemura, K. and Okamoto, H. (1997). Ocular and cerebellar defects in zebrafish induced by overexpression of the LIM domains of the islet-3 LIM/homeodomain protein. *Neuron* **18**, 369-382.

Kim, J. W. and Lemke, G. (2006). Hedgehog-regulated localization of Vax2 controls eye development. *Genes Dev.* **20**, 2833-2847.

Koshiba-Takeuchi, K., Takeuchi, J. K., Matsumoto, K., Momose, T., Uno, K., Hoepker, V., Ogura, K., Takahashi, N., Nakamura, H., Yasuda, K. et al. (2000). Tbx5 and the retinotectum projection. *Science* **287**, 134-137.

Laemle, L. K., Puszkarczuk, M. and Feinberg, R. N. (1999). Apoptosis in early ocular morphogenesis in the mouse. *Brain Res. Dev. Brain Res.* **112**, 129-133.

Lang, R. A. (2004). Pathways regulating lens induction in the mouse. *Int. J. Dev. Biol.* **48**, 783-791.

Li, X., Perissi, V., Liu, F., Rose, D. W. and Rosenfeld, M. G. (2002). Tissue-specific regulation of retinal and pituitary precursor cell proliferation. *Science* **297**, 1180-1183.

Liu, W., Lagutin, O. V., Mende, M., Streit, A. and Oliver, G. (2006). Six3 activation of Pax6 expression is essential for mammalian lens induction and specification. *EMBO J.* **25**, 5383-5395.

Luo, G., Hofmann, C., Bronckers, A. L., Sohocki, M., Bradley, A. and Karsenty, G. (1995). BMP-7 is an inducer of nephrogenesis, and is also required for eye development and skeletal patterning. *Genes Dev.* **9**, 2808-2820.

Lupo, G., Liu, Y., Qiu, R., Chandraratna, R. A., Barsacchi, G., He, R. Q. and Harris, W. A. (2005). Dorsoventral patterning of the Xenopus eye: a collaboration of Retinoid, Hedgehog and FGF receptor signaling. *Development* **132**, 1737-1748.

Macatee, T. L., Hammond, B. P., Arenkiel, B. R., Francis, L., Frank, D. U. and Moon, A. M. (2003). Ablation of specific expression domains reveals discrete functions of ectoderm- and endoderm-derived FGF8 during cardiovascular and pharyngeal development. *Development* **130**, 6361-6374.
Macdonald, R., Barth, K. A., Xu, Q., Holder, N., Mikkola, I. and Wilson, S. W. (1995). Midline signalling is required for Pax gene regulation and patterning of the eyes. *Development* **121**, 3267-3278.
Mangale, V. S., Hirokawa, K. E., Satyaki, P. R., Gokulchandran, N., Chikbire, S., Subramanian, L., Shetty, A. S., Martynoga, B., Paul, J., Mai, M. V. et al. (2008). Lhx2 selector activity specifies cortical identity and suppresses hippocampal organizer fate. *Science* **319**, 304-309.
Martinez-Morales, J. R., Rodrigo, I. and Bovolenta, P. (2004). Eye development: a view from the retina pigmented epithelium. *BioEssays* **26**, 766-777.
Massague, J. (1998). TGF-beta signal transduction. *Annu. Rev. Biochem.* **67**, 753-791.
Mathers, P. H., Grinberg, A., Mahon, K. A. and Jamrich, M. (1997). The Rx homeobox gene is essential for vertebrate eye development. *Nature* **387**, 603-607.
Medina-Martinez, O., Amaya-Manzanares, F., Liu, C., Mendoza, M., Shah, R., Zhang, L., Behringer, R. R., Mahon, K. A. and Jamrich, M. (2009). Cell-autonomous requirement for rx function in the mammalian retina and posterior pituitary. *PLoS One* **4**, e4513.
Morcillo, J., Martinez-Morales, J. R., Trousse, F., Fermin, Y., Sowden, J. C. and Bovolenta, P. (2006). Proper patterning of the optic fissure requires the sequential activity of BMP7 and SHH. *Development* **133**, 3179-3190.
Mui, S. H., Kim, J. W., Lemke, G. and Bertuzzi, S. (2005). Vax genes ventralize the embryonic eye. *Genes Dev.* **19**, 1249-1259.
Murali, D., Yoshikawa, S., Corrigan, R. R., Plas, D. J., Crair, M. C., Oliver, G., Lyons, K. M., Mishina, Y. and Furuta, Y. (2005). Distinct developmental programs require different levels of Bmp signaling during mouse retinal development. *Development* **132**, 913-923.
Nagy, A. (2003). *Manipulating the Mouse Embryo: a Laboratory Manual*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.
Nguyen, M. and Arnheiter, H. (2000). Signaling and transcriptional regulation in early mammalian eye development: a link between FGF and MITF. *Development* **127**, 3581-3591.
Ogino, H., Fisher, M. and Grainger, R. M. (2008). Convergence of a head-field selector Otx2 and Notch signaling: a mechanism for lens specification. *Development* **135**, 249-258.
Oxburgh, L., Dudley, A. T., Godin, R. E., Koonce, C. H., Islam, A., Anderson, D. C., Bikoff, E. K. and Robertson, E. J. (2005). BMP4 substitutes for loss of BMP7 during kidney development. *Dev. Biol.* **286**, 637-646.
Pan, Y., Woodbury, A., Esko, J. D., Grobe, K. and Zhang, X. (2006). Heparan sulfate biosynthetic gene Ndst1 is required for FGF signaling in early lens development. *Development* **133**, 4933-4944.
Pappu, K. S. and Mardon, G. (2004). Genetic control of retinal specification and determination in Drosophila. *Int. J. Dev. Biol.* **48**, 913-924.
Park, E. J., Sun, X., Nichol, P., Saijoh, Y., Martin, J. F. and Moon, A. M. (2008). System for tamoxifen-inducible expression of cre-recombinase from the Foxa2 locus in mice. *Dev. Dyn.* **237**, 447-453.
Porter, F. D., Drago, J., Xu, Y., Cheema, S. S., Wassif, C., Huang, S. P., Lee, E., Grinberg, A., Massalas, J. S., Bodine, D. et al. (1997). Lhx2, a LIM homeobox gene, is required for eye, forebrain, and definitive erythrocyte development. *Development* **124**, 2935-2944.
Roehl, H. and Nusslein-Volhard, C. (2001). Zebrafish pea3 and erm are general targets of FGF8 signaling. *Curr. Biol.* **11**, 503-507.
Rowan, S., Chen, C. M., Young, T. L., Fisher, D. E. and Cepko, C. L. (2004). Transdifferentiation of the retina into pigmented cells in ocular retardation mice defines a new function of the homeodomain gene Chx10. *Development* **131**, 5139-5152.
Servetnick, M. and Grainger, R. M. (1991). Changes in neural and lens competence in Xenopus ectoderm: evidence for an autonomous developmental timer. *Development* **112**, 177-188.
Seth, A., Culverwell, J., Walkowicz, M., Toro, S., Rick, J. M., Neuhauss, S. C., Varga, Z. M. and Karlstrom, R. O. (2006). belladonna/(lhx2) is required for neural patterning and midline axon guidance in the zebrafish forebrain. *Development* **133**, 725-735.
Shanmugalingam, S., Houart, C., Picker, A., Reifers, F., Macdonald, R., Barth, A., Griffin, K., Brand, M. and Wilson, S. W. (2000). Ace/Fgf8 is required for forebrain commissure formation and patterning of the telencephalon. *Development* **127**, 2549-2561.
Sigulinsky, C. L., Green, E. S., Clark, A. M. and Levine, E. M. (2008). Vsx2/Chx10 ensures the correct timing and magnitude of Hedgehog signaling in the mouse retina. *Dev. Biol.* **317**, 560-575.
Silver, J. and Hughes, A. F. (1974). The relationship between morphogenetic cell death and the development of congenital anophthalmia. *J. Comp. Neurol.* **157**, 281-301.
Silver, S. J. and Rebay, I. (2005). Signaling circuitries in development: insights from the retinal determination gene network. *Development* **132**, 3-13.
Soriano, P. (1999). Generalized lacZ expression with the ROSA26 Cre reporter strain. *Nat. Genet.* **21**, 70-71.
Take-uchi, M., Clarke, J. D. and Wilson, S. W. (2003). Hedgehog signalling maintains the optic stalk-retinal interface through the regulation of Vax gene activity. *Development* **130**, 955-968.
Tetreault, N., Champagne, M. P. and Bernier, G. (2008). The LIM homeobox transcription factor Lhx2 is required to specify the retina field and synergistically cooperates with Pax6 for Six6 trans-activation. *Dev. Biol*. **327**, 541-550.
Theiler, K. (1972). *The House Mouse: Development and Normal Stages from Fertilization to 4 Weeks of Age*. Berlin: Springer-Verlag.
Uemonsa, T., Sakagami, K., Yasuda, K. and Araki, M. (2002). Development of dorsal-ventral polarity in the optic vesicle and its presumptive role in eye morphogenesis as shown by embryonic transplantation and in ovo explant culturing. *Dev. Biol.* **248**, 319-330.
Viczian, A. S., Bang, A. G., Harris, W. A. and Zuber, M. E. (2006). Expression of Xenopus laevis Lhx2 during eye development and evidence for divergent expression among vertebrates. *Dev. Dyn.* **235**, 1133-1141.
Wawersik, S., Purcell, P., Rauchman, M., Dudley, A. T., Robertson, E. J. and Maas, R. (1999). BMP7 acts in murine lens placode development. *Dev. Biol.* **207**, 176-188.
Yang, X. J. (2004). Roles of cell-extrinsic growth factors in vertebrate eye pattern formation and retinogenesis. *Semin. Cell Dev. Biol.* **15**, 91-103.
Zuber, M. E., Gestri, G., Viczian, A. S., Barsacchi, G. and Harris, W. A. (2003). Specification of the vertebrate eye by a network of eye field transcription factors. *Development* **130**, 5155-5167.