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Review

Transcription factors involved in lens development from the preplacodal ectoderm

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ABSTRACT

Lens development is a stepwise process accompanied by the sequential activation of transcription factors. Transcription factor genes can be classified into three groups according to their functions: the first group comprises preplacodal genes, which are implicated in the formation of the preplacodal ectoderm that serves as a common primordium for cranial sensory tissues, including the lens. The second group comprises lens-specification genes, which establish the lens-field within the preplacodal ectoderm. The third group comprises lens-differentiation genes, which promote lens morphogenesis after the optic vesicle makes contact with the presumptive lens ectoderm. Analyses of the regulatory interactions between these genes have provided an overview of lens development, highlighting crucial roles for positive cross-regulation in fate specification and for feed-forward regulation in the execution of terminal differentiation. This overview also sheds light upon the mechanisms of how preplacodal gene activities lead to the activation of genes involved in lens-specification.

Introduction

The lens has been used as a very attractive model system for the study of tissue development in vertebrates. In the early 1900s, both Spemann and Lewis independently examined interactions between the retina and lens primordia in frog embryos. Using a hot needle, Spemann destroyed the presumptive retina region of the anterior neural plate in *Rana fusca* and found that the lens was missing on the manipulated side (Spemann, 1901). Thus, Spemann concluded that the retinal primordium is required for proper lens development. Lewis transplanted the retina primordium (optic vesicle) under the flank ectoderm in *Rana palustris* and found that an ectopic lens formed in the transplanted region (Lewis, 1904). Hence, Lewis proposed that stimuli from the optic vesicle were sufficient to convert the multipotent ectoderm into the lens. These studies are considered to be the first experimental documentation of embryonic induction, but it was not until the end of the 1980s that Grainger and colleagues showed that Lewis's conclusion provided an oversimplified view of events. Using a lineage tracing technique, they revealed that lenses formed ectopically on the flank in *R. palustris* and *Xenopus laevis* embryos were exclusively derived from contaminating donor cells carried along with the transplanted optic vesicles; thus, the optic vesicle was considered not sufficient to induce the lens (Grainger et al., 1988).

Grainger's group further investigated interactions between the retina and lens primordia in earlier stages of *Xenopus* development and found that the lens development is a successive process that begins in the ectoderm prior to its contact with the optic vesicle (Grainger, 1992; Henry and Grainger, 1987). According to their model, planar signals from the anterior margin of the developing neural plate and vertical signals from the anterior endomesoderm establish the lens-forming potential in the adjacent non-neural ectoderm by the neural plate stage. After neural tube formation, only the lateral part of this non-neural ectoderm makes contact with the developing optic vesicle and begins to differentiate into the lens. Interestingly, the anterior ectodermal region with lens-forming potential in Grainger's model appears to include the non-neural ectoderm known as the preplacodal ectoderm (PPE), which surrounds the anterior neural plate and includes the presumptive fields of lens, nasal, ear, adenohypophyseal, trigeminal and epibranchial placodes (Fig. 1A). Embryological studies have suggested that the PPE is formed as the pan-placodal ground state, which is then subdivided into the respective placodal tissues via local interactions with adjacent neural and mesodermal tissues (Baker and Bronner-Fraser, 2001; Jacobson, 1966).

After the classical embryological studies, molecular and genetic studies opened up a new era and significantly advanced our understanding of lens development. In 1991, a *paired* box gene, *Pax6*, was

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identified as the causal gene of the *Small eye* (*Sey*) mutation in mice; animals homozygous for the gene fail to develop lens placode (Hill et al., 1991; Hogan et al., 1986). *Pax6* was suggested to be implicated in the lens-forming potential identified in embryological studies, because of the *Sey* phenotype and *Pax6* expression that occurs in the presumptive lens ectoderm prior to contact with the optic vesicle in mice and chickens (Grindley et al., 1995; Li et al., 1994). The discovery of *Pax6* was followed by that of other transcription factor genes such as *Six1*, *Six3*, *FoxE3*, *Sox2*, *L-Maf*/*c-Maf* and *Prox1*, which are involved in PPE formation and/or subsequent lens differentiation (Brugmann and Moody, 2005; Cvekl and Duncan, 2007; Lang, 2004; Ogino and Yasuda, 2000; Schlosser, 2006; Streit, 2007). This review provides an overview of the sequential expression of these transcription factor genes, and then addresses their regulation and

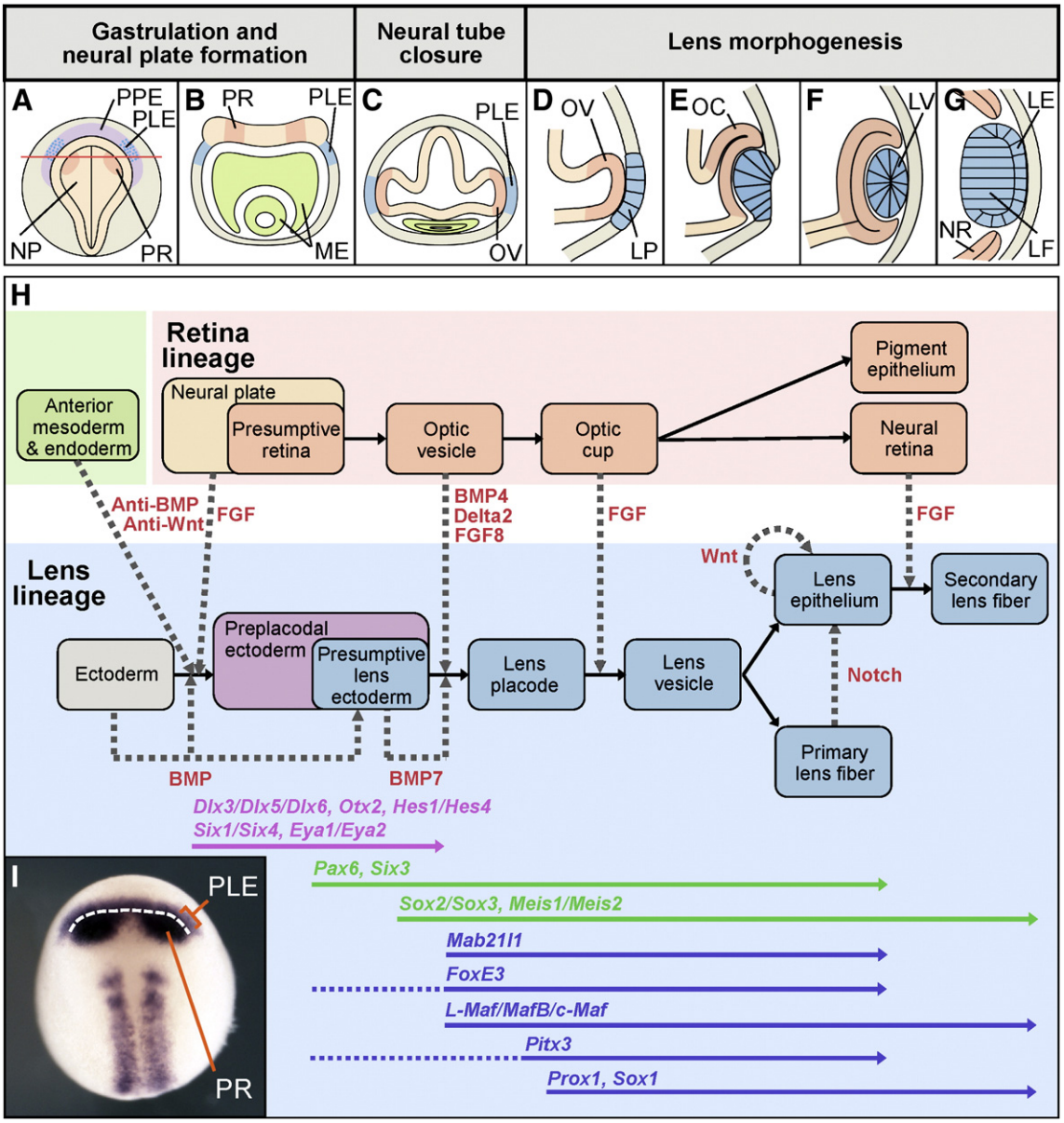

Fig. 1. Schematic illustration of vertebrate lens development, relationships between inductive interactions and sequential activation of transcription factors. (A–G) *Xenopus* was chosen as the vertebrate representative since its developmental lineages of lens, retina and other related tissues have been well studied (Eagleson and Harris, 1990; Eagleson et al., 1995). The developmental stages are indicated at the top of the figure. (A) Dorsal view of a neural plate-stage embryo (anterior at the top of the image) indicating the preplacodal ectoderm (PPE, purple), the presumptive lens ectoderm (PLE, dotted light blue), the neural plate (NP, light orange), and the presumptive retina field (PR, dark orange). (B) Transverse section of a neural plate-stage embryo through the PLE and PR. The red line in A indicates the plane of the section. Lens and retina lineages are shown in light blue and dark orange, respectively, in B-G, and mesoderm and endoderm (ME) are shown in light green. (C) Transverse section of a neural tube-stage embryo where the optic vesicle (OV), which develops from the PR, reaches the PLE. (D-F) Close-ups showing lens vesicle formation. The PLE overlying the OV becomes thickened to form the lens placode (LP), which subsequently separates from the head ectoderm to form the lens vesicle (LV). OC, optic cup. (G) Close-up of a maturating lens. LE, lens epithelium; LF, lens fiber; NR, neural retina. (H) Major signaling factors are indicated in red with dotted gray arrows. FGF signaling from the neural retina is responsible for the formation of the secondary lens fibers from the lens epithelium (Lovicu and McAvoy, 2005). The other signaling pathways are explained in the text. At the bottom, Expression profiles of preplacodal genes, lens-specification genes and lens-differentiation genes (pink, green, and blue arrows, respectively) studied in *Xenopus*, zebrafish, chicken and mouse embryos. The dotted blue lines of *FoxE3* and *Pitx3* represent species differences in initial expression profiles. Details and references are described in the text. (I) *In situ* hybridization analysis of *Pax6* expression in a neural plate-stage *Xenopus* embryo (dorsal view). The PLE and PR are indicated. The boundary between the neural plate and PPE is indicated by a white broken line. Note that preplacodal *Pax6* expression occurs broadly adjacent to the anterior margin of the neural plate.

functions in detail. Finally, the genetic pathways involved are discussed from an overall perspective, with emphasis on the transition from PPE to lens-field specification, and from lens specification to lens differentiation.

An overview of lens development with sequential activation of transcription factors

Lens development from the PPE is accompanied by sequential activation of transcription factor genes. In this section, we give an overview of their expression profiles using data from zebrafish, *Xenopus*, chicken and mouse. However, we note that there are differences between species, which are discussed in the following sections and shown in Table 1.

At the end of gastrulation, the PPE that surrounds the developing anterior neural plate expresses "preplacodal genes", such as *Dlx3* (Akimenko et al., 1994; Feledy et al., 1999), *Dlx5* (Bhattacharyya et al., 2004; Luo et al., 2001; Yang et al., 1998), *Dlx6* (Quint et al., 2000), *Hes1* (Lee et al., 2005), *Hes4* (Murato and Hashimoto, 2009), *Six1* (Bessarab et al., 2004; Pandur and Moody, 2000; Sato et al., 2010), *Six4* (Esteve and Bovolenta, 1999; Ghanbari et al., 2001; Kobayashi et al., 2000), *Eya1* and *Eya2* (David et al., 2001; Ishihara et al., 2008; Sahly et al., 1999; Xu et al., 1997) (Fig. 1H, bottom panel). *Otx2* is also expressed in the anterior part of the PPE (Bovolenta et al., 1997; Martinez-Morales et al., 2001; Zygar et al., 1998). Fate map studies have shown that the PPE includes the presumptive lens ectoderm (PLE) at the periphery of the presumptive retina fields of the neural plate in chicken (Bhattacharyya et al., 2004) and *Xenopus* embryos (Eagleson et al., 1995; Zygar et al., 1998) (Figs. 1A, B).

After the expression of preplacodal genes, "lens-specification genes", *Pax6* (Grindley et al., 1995; Li et al., 1994; Nornes et al., 1998; Walther and Gruss, 1991; Zygar et al., 1998) and *Six3* (Bovolenta et al., 1998; Kobayashi et al., 1998; Murato and Hashimoto, 2009; Oliver et al., 1995a; Zhou et al., 2000), start to be expressed in the PLE at the neural plate stage (Fig. 1I). *Pax6* expression is initially broad in the part of the PPE that includes both the presumptive lens and nasal ectoderm, but eventually localizes to the PLE as development proceeds. After neural tube closure, the presumptive retina region protrudes from the forebrain to form the optic vesicle (Fig. 1C). *Sox2* (Kamachi et al., 1998), *Sox3* (Kamachi et al., 1998; Penzel et al., 1997; Zygar et al., 1998), *Meis1 and Meis2* (Zhang et al., 2002), which are also involved in lens specification, are activated in the PLE around the time when it makes contact with the optic vesicle.

After contact, the PLE cells begin to elongate and form the lens placode (Fig. 1D), which is associated with the activation of the "lens-differentiation genes", *Mab21l1* (Yamada et al., 2003), *FoxE3* (Blixt et al., 2000; Brownell et al., 2000; Kenyon et al., 1999; Shi et al., 2006), *Pitx3* (Pommereit et al., 2001; Semina et al., 2000; Zilinski et al., 2005), *L-Maf* (Ishibashi and Yasuda, 2001; Ogino and Yasuda, 1998), *MafB* (Ishibashi and Yasuda, 2001), *c-Maf* (Kajihara et al., 2001; Kawauchi et al., 1999; Reza and Yasuda, 2004a), *Sox1* (Kamachi et al., 1998; Nishiguchi et al., 1998) and *Prox1* (Duncan et al., 2002; Glasgow and Tomarev, 1998; Oliver et al., 1993; Tomarev et al., 1996). The preplacodal genes, such as *Dlx5* (Bhattacharyya et al., 2004), *Six1* (Bessarab et al., 2004; Pandur and Moody, 2000), *Six4* (Ghanbari et al., 2001; Kobayashi et al., 2000), *Eya1* and *Eya2* (David et al., 2001; Ishihara et al., 2008; Sahly et al., 1999) and *Otx2* (Bovolenta et al., 1997; Martinez-Morales et al., 2001; Zygar et al., 1998) are turned off in the PLE by the time of the lens placode formation. In chickens and mice, the thickened lens placode then invaginates toward the optic vesicle and pinches off from the head ectoderm to form a hollow structure, the lens vesicle. In *Xenopus* and zebrafish, the thickened lens placode simply delaminates from the head ectoderm to form the solid lens vesicle (Easter and Nicola, 1996; Ishibashi and Yasuda, 2001) (Figs. 1E, F).

The cells comprising the posterior wall of the lens vesicle elongate dramatically while differentiating into primary lens fibers (Fig. 1G). Cell cycle arrest, enucleation, and the strong up-regulation of crystallins (lens-specific structural proteins) are all associated with this differentiation process (McAvoy, 1980; Piatigorsky, 1981). Among the genes expressed in the lens placode, *Sox1*, *Sox2*, *Sox3* (Kamachi et al., 1998; Nishiguchi et al., 1998), *L-Maf* (Ishibashi and Yasuda, 2001; Ogino and Yasuda, 1998) and *c-Maf* (Kajihara et al., 2001; Kawauchi et al., 1999) are preferentially maintained in the lens fibers. The anterior cells in the lens vesicle form a monolayer of lens epithelial cells that surrounds the lens fibers. The lens epithelial cells continuously proliferate at the equatorial region and eventually differentiate into secondary lens fibers. In contrast to lens fibers, the lens epithelial cells preferentially maintain expression of *Hes1* (Lee et al., 2005), *Pax6* (Grindley et al., 1995; Li et al., 1994), *Six3* (Bovolenta et al., 1998; Oliver et al., 1995a), *Mab21l1* (Yamada et al., 2003), *FoxE3* (Blixt et al., 2000; Brownell et al., 2000; Kenyon et al., 1999; Shi et al., 2006), *Pitx3* (Pommereit et al., 2001; Semina et al., 2000; Zilinski et al., 2005) and *MafB* (Ishibashi and Yasuda, 2001; Kawauchi et al., 1999). Growth of the lens vesicle results from the continuous overlay of concentric layers of new secondary lens fibers around the older layers. During the formation of the lens vesicle, the optic vesicle also changes its shape to form the optic cup, which is associated with the neural retina and retinal pigment epithelium layers.

Regulation and functions of preplacodal genes

Dlx3/Dlx5/Dlx6 required for neural/non-neural boundary formation

The *Dlx3*, *Dlx5* and *Dlx6* genes are vertebrate homologues of *distal-less*, a homeobox gene that is essential for limb and antenna development in *Drosophila* (Cohen et al., 1989; Panganiban and Rubenstein, 2002). Zebrafish embryos express *Dlx3* in the non-neural ectoderm surrounding the anterior margin of the developing neural plate from the mid-gastrula stage (Akimenko et al., 1994; Dutta et al., 2005). In *Xenopus* embryos, *Dlx3*, *Dlx5* and *Dlx6* show expression patterns similar to those observed for zebrafish *Dlx3* (Feledy et al., 1999; Luo et al., 2001). Expression of these *Xenopus* genes, which covers a broader domain than the PPE, is induced by low level bone morphogenetic protein (BMP) signaling that is most likely generated by antagonistic interactions between BMP signaling from the ventral non-neural ectoderm and anti-BMP signaling from the involuting organizer that gives rise to the anterior endoderm and mesoderm (Fig. 2, pink panel). Misexpression experiments in *Xenopus* have shown that *Dlx3* inhibits neural plate formation whereas its dominant negative form causes expansion of the neural plate, which suggests that *Dlx3* positions the neural/non-neural boundary and reserves the non-neural region for PPE formation (Woda et al., 2003). Similar results were obtained in chicken embryos with *Dlx5*, where its ectopic expression suppressed neural gene expression and instead promoted expression of a preplacodal gene, *Six4* (McLarren et al., 2003). However, this ectopic expression was not sufficient to activate genes involved in the later development of placodal tissues, such as *Pax6*.

Mouse PPE expresses *Dlx5* and *Dlx6* (Quint et al., 2000) and it has been shown that *Dlx5*-knockout mice have multiple craniofacial defects, including the ears, nose, mandibles and calvaria, but have no obvious eye defects (Depew et al., 1999). Since genetic redundancy is expected between *Dlx5* and *Dlx6*, mice that lack both *Dlx5* and *Dlx6* were generated and these animals exhibited more severe craniofacial defects, including the complete loss of eye structures (Robledo et al., 2002). Although this study showed a requirement for *Dlx* genes, it is unclear whether their functions are directly involved in, or are an indirect prerequisite for lens development.

Table 1
Expression, regulation and functions of the genes involved in lens development from the PPE.

Gene name	Expression	Upstream genes and signals[a]	Gain-of-function phenotype[a]	Loss-of-function phenotype[a]	References
Preplacodal genes					
Dlx3	PPE (X, Z)	BMP, Anti-BMP (X)	Inhibition of neural plate formation (X)	Expansion of neural plate (X)	Feledy et al. (1999) Luo et al. (2001) Woda et al. (2003) Dutta et al. (2005)
Dlx5	PPE (X, C, M)	BMP, Anti-BMP (X) *Fgf8*, low level BMP (C)	Inhibition of neural plate and lens formation, ectopic *Six4* expression (C)	Severe craniofacial defects in *Dlx6* −/− background (M)	Yang et al. (1998) Quint et al. (2000) Luo et al. (2001) Robledo et al. (2002) McLarren et al. (2003) Bhattacharyya et al. (2004) Litsiou et al. (2005) Bailey et al. (2006) Sjodal et al. (2007)
Otx2	PPE (X, C) PLE (X, M)	ND	Ectopic ***FoxE3*** expression (in combination with Notch) (X)	Heterozygous mutants form lens-less, abnormal eyes (M)	Matsuo et al. (1995) Bovolenta et al. (1997) Zygar et al. (1998) Martinez-Morales et al. (2001) Ogino et al. (2008)
Hes1	PPE, LP (M)	ND	ND	Variable lens defects: small lens to complete loss of lens (M)	Lee et al. (2005)
Hes4	PPE (X)	ND	ND	Failure of LV formation, reduction of *Pax6*, *Six3* and *Notch2* expression (X)	Murato and Hashimoto (2009)
Six1	PPE (X, Z, C, M)	*Fgf8*, anti-BMP (X, C) Anti-Wnt, ***Dlx5*** (C)	Ectopic *Eya1* expression (X) Ectopic *Six4* expression (in combination with *Eya2*) (C)	Reduction of PPE (X, C) Reduction of *Pax6* expression (C) Defects in ear and nose, but not in eye (M)	Oliver et al. (1995b) Pandur and Moody (2000) Laclef et al. (2003) Brugmann et al. (2004) Bessarab et al. (2004) Ozaki et al. (2004) Ahrens and Schlosser (2005) Litsiou et al. (2005) Christophorou et al. (2009) Sato et al. (2010)
Six4	PPE (X, Z, C, M)	*Fgf8*, anti-BMP, anti-Wnt, *Dlx5*, *Six1*, *Eya2* (C)	ND	No lens defect in *Six1*−/− background (M)	Esteve and Bovolenta (1999) Kobayashi et al. (2000) Ghanbari et al. (2001) Ozaki et al. (2001) Grifone et al. (2005) Litsiou et al. (2005)
Eya1	PPE (Z, X, C) LP, LV, LE (M)	*Six1* (X)	ND	No lens defect (M)	Xu et al. (1997) Sahly et al. (1999) Xu et al. (1999a) David et al. (2001) Grifone et al. (2007) Ishihara et al. (2008)
Eya2	PPE (C)	*Fgf8*, anti-BMP, anti-Wnt (C)	Ectopic *Six4* expression (in combination with *Six1*) (C)	No lens defect in *Eya1*−/− background (M)	Litsiou et al. (2005) Ishihara et al. (2008) Grifone et al. (2007) Christophorou et al. (2009)
Lens-specification genes					
Pax6	PPE, PLE, LP, LV, LE, LF (X, Z, C, M)	*Hes4* (X) *Six1* (C) *Fgf8*, *Bmp7*, FGFR, anti-Wnt, *Six3*, ***Meis1*/*Meis***, ***Pax6***, ***Sox2***, ***Pou2f1***, ***Prep1*** (M)	Ectopic expression of *L-Maf* and ***δ-crystallin*** (in combination with *Sox2*) (C)	Failure of LP formation, loss of *L-Maf*, *Prox1* and ***δ-crystallin*** expression (C) Failure of LP formation, loss of *Sox2*, *Six3*, *Sfrp1*, *Sfrp2* and *FoxE3* expression (M)	Li et al. (1994) Grindley et al. (1995) Nornes et al. (1998) Zygar et al. (1998) Wawersik et al. (1999) Ashery-Padan et al. (2000) Faber et al. (2001) Kamachi et al. (2001) Goudreau et al. (2002) Reza et al. (2002) Zhang et al. (2002) Aota et al. (2003) Bailey et al. (2006) Liu et al. (2006) Donner et al. (2007) Murato and Hashimoto (2009) Smith et al. (2005) Christophorou et al. (2009) Smith et al. (2009) Machon et al. (2010) Rowan et al. (2010)

Table 1 (*continued*)

Gene name	Expression	Upstream genes and signals[a]	Gain-of-function phenotype[a]	Loss-of-function phenotype[a]	References
Six3	PLE, LP, LV, LE (X, Z, C, M)	*Pax6* (M)	Ectopic lens formation (non-cell autonomous) (Medaka)	Variable lens defects; small lens to complete loss of lens, reduction of *Pax6*, *Sox2*, *FoxE3* and *Prox1* expression (M)	Oliver et al. (1995a) Oliver et al. (1996) Bovolenta et al. (1998) Kobayashi et al. (1998) Zhou et al. (2000) Goudreau et al. (2002) Purcell et al. (2005) Liu et al. (2006) Murato and Hashimoto (2009)
Sox2	PLE, LP (C, M) LV, LE, LF (C)	***Pax6, Sox2*** (C) *Bmp4*, *Bmp7*, *Six3* (M)	Ectopic ***δ-crystallin*** and *L-Maf* expression (in combination with *Pax6*) (C)	Failure of LV formation, reduction of *β-crystallin* and *Prox1* expression (M)	Kamachi et al. (1995) Furuta and Hogan (1998) Kamachi et al. (1998) Wawersik et al. (1999) Ashery-Padan et al. (2000) Kamachi et al. (2001) Reza et al. (2002) Shimada et al. (2003) Liu et al. (2006) Inoue et al. (2007) Smith et al. (2009)
Sox3	PLE, LP, LV, LE, LF (X, C)	ND	ND	ND	Penzel et al. (1997) Kamachi et al. (1998) Zygar et al. (1998)
Lens-differentiation genes					
Sox1	LV, LE, LF (C, M)	ND	ND	Failure of LF differentiation, reduction of ***γ-crystallin*** expression (M)	Kamachi et al. (1998) Nishiguchi et al. (1998)
Mab21l1	PLE, LP, LV, LE (M)	*Pax6* (M)	ND	Failure of LP invagination, reduction of *FoxE3* expression (M)	Yamada et al. (2003)
FoxE3	PPE (X) PLE, LP, LV, LE (X, Z, M)	*Delta2*, ***Otx2, RBP-Jκ/Su(H)*** (X) *Pax6*, *Six3*, *Mab21l1*, *Pitx3*, *Bmpr1a*, *Acvr1*, *RBP-Jκ/Su(H)*, ***Sip1*** (M)	Inhibition of LF differentiation (X)	Failure of LE formation (Z, M) Reduction of *Pdgfra* expression (M)	Kenyon et al. (1999) Blixt et al. (2000) Brownell et al. (2000) Yamada et al. (2003) Yoshimoto et al. (2005) Liu et al. (2006) Shi et al. (2006) Ogino et al. (2008) Rowan et al. (2008) Ho et al. (2009) Medina-Martinez et al. (2009) Rajagopal et al. (2009)
Pitx3	PPE, PLE (Z) LP, LV, LE (X, M)	ND	ND	Failure of LE formation, reduction of *FoxE3*, *Pdgfra* and *αA-crystallin* expression (M)	Semina et al. (2000) Pommereit et al. (2001) Dutta et al. (2005) Zilinski et al. (2005) Ho et al. (2009) Medina-Martinez et al. (2009)
L-Maf	PLE, LP, LV (X, C) LE (M) LF (X, C, M)	*Pax6*, *Sox2*, *Fgf8* (C)	Ectopic lens cell formation, ectopic expression of ***α-, β- and δ-crystallins***, *Filensin*, *c-Maf*, *MafB*. *Prox1*, ***p27kip1*** and *MIP* (C) Ectopic *γ-crystallin* expression (X)	Failure of LP formation, loss of ***δ-crystallin*** and *Prox1* expression (C) No lens defect (M)	Ogino and Yasuda (1998) Ogino and Yasuda (2000) Vogel-Hopker et al. (2000) Ishibashi and Yasuda (2001) Reza et al. (2002) Ochi et al. (2003) Shimada et al. (2003) Reza et al. (2007a) Reza et al. (2007b) Takeuchi et al. (2009)
c-Maf	PLE (M) LP (C, M) LV (Z, C, M) LE (C) LF (Z, M)	*L-Maf* (C) *Fgfr1/2/3* (M)	Ectopic expression of *δ-crystallin*, *MafB* and *MIP* (C)	Failure of LV differentiation, reduction of ***α-, β- and γ-crystallins*** expression (M)	Kawauchi et al. (1999) Ring et al. (2000) Kajihara et al. (2001) Reza and Yasuda (2004a) Yang et al. (2006) Reza et al. (2007b) Zhao et al. (2008)
MafB	PLE, LP (X) LV (X, Z) LE (X, Z, C, M) LF (C)	*L-Maf*, *c-Maf* (C)	Ectopic expression of *δ-crystallin* and *MIP* (C) Ectopic *γ-crystallin* expression (X)	No lens defect (M)	Kawauchi et al. (1999) Ishibashi and Yasuda (2001) Kajihara et al. (2001) Reza et al. (2007b) Oliver et al. (1993)
Prox1	LP, LV, LE, LF (Z, C, M)	*L-Maf* (C) *Pax6*, *Six3*, *Fgfr1/2/3* (M)	ND	Failure of LF differentiation, reduction of *p27kip1*, *p57kip2* and *γ-crystallin* expression (M)	Tomarev et al. (1996) Glasgow and Tomarev (1998) Wigle et al. (1999) Duncan et al. (2002)

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Table 1 (*continued*)

Gene name	Expression	Upstream genes and signals[a]	Gain-of-function phenotype[a]	Loss-of-function phenotype[a]	References
					Reza et al. (2002) Ashery-Padan et al. (2000) Liu et al. (2006)

[a] Direct upstream and downstream genes of the genes indicated in the extreme left column are shown with bold letters. The model organisms used for expression and/or functional analyses are given in parenthesis: X, *Xenopus*; Z, zebrafish; C, chicken; M, mouse. Tissue names are abbreviated as in Fig. 1. ND, not determined.

Otx2 and Hes1/Hes4 link PPE formation to lens-field specification

The gene *Otx2* encodes a bicoid-type homeodomain protein essential for rostral head development (Matsuo et al., 1995; Simeone et al., 1993). *Xenopus* embryos express *Otx2* from the late gastrula stage in the anterior non-neural ectoderm, including part of the PPE, as well as in the anterior neuroectoderm. After neural tube closure, this non-neural expression remains in the PLE overlying the optic vesicle until just before lens placode thickening (Zygar et al., 1998). In chicken embryos, not only *Otx2* but also its closely related gene, *Otx1*, is expressed in the anterior ectoderm including the PPE, and *Otx1* expression remains in the PLE overlying the optic vesicle (Bovolenta et al., 1997; Plouhinec et al., 2005). Zebrafish embryos also express *Otx1* in the PPE and maintain the expression in the lens epithelium (Mercier et al., 1995), but expression of zebrafish *Otx2* in the lens lineage is unclear. Mouse *Otx2* is expressed in the PLE overlying the optic vesicle, but its early expression in the PPE has not been reported yet (Martinez-Morales et al., 2001). Mice with a

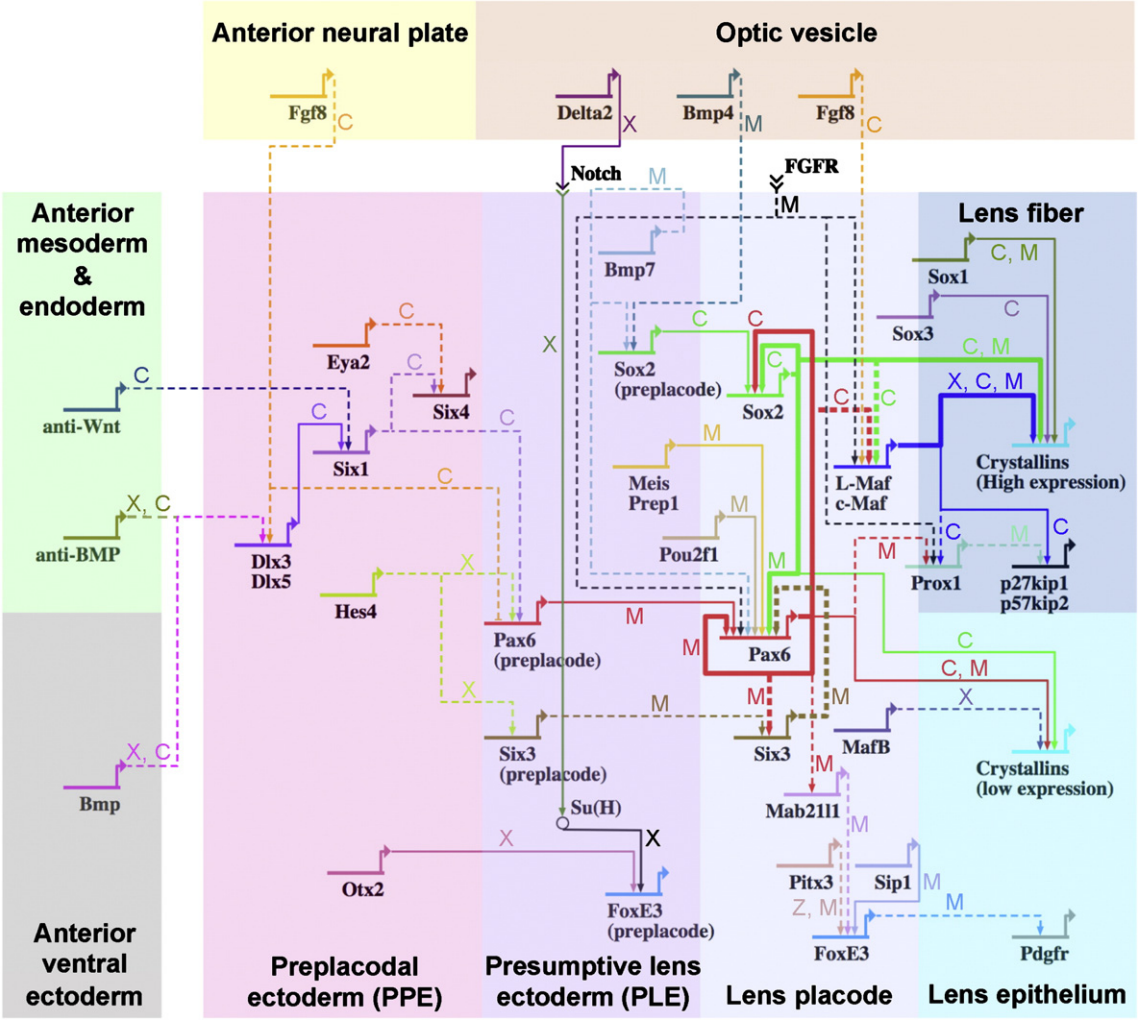

Fig. 2. Genetic pathways for the induction of lens differentiation from the PPE. The pathways were deduced from gene interactions characterized in *Xenopus*, zebrafish, chicken and mouse, and depicted using BioTapestry (Longabaugh et al., 2005). Each gene is indicated by a short horizontal line that represents its cis-regulatory region and a bent arrow that represents its transcription start site. Solid lines connecting genes indicate direct gene interactions revealed by *cis*-regulatory analyses, and dashed lines connecting genes indicate gene interactions characterized by loss-of-function and/or gain-of-function experiments. Of the lines indicating gene interactions, those with an arrow at the end and those with a short horizontal line at the end indicate activation and repression of downstream genes, respectively. The bold lines highlight cross-regulatory interactions between *Pax6*, *Sox2* and *Six3*, and feed-forward regulation involving *Maf* genes. Letters (X, Z, C, M) beside lines representing gene interactions indicate the model animals (*Xenopus*, zebrafish, chicken, and mouse) in which the interaction was identified, respectively. The funnel-like symbols and open circle indicate receptors and a downstream signal transducer, respectively. Tissues are identified using different background colors.

homozygous disruption of *Otx2* fail to develop all structures anterior to rhombomere 3, while heterozygous *Otx2*-mutant mice have severe craniofacial defects with abnormal eyes that lack the lens (Matsuo et al., 1995). This genetic study identifies a requirement for *Otx2*, but does not indicate its direct involvement in lens development. Evidence for the direct involvement of *Otx2* was provided by a study that identified the Otx2 protein as a regulator of the *FoxE3* enhancer in the PLE of *Xenopus* embryos (see the section for *FoxE3*). Further molecular and genetic studies are expected to investigate the direct involvement of *Otx2* in other species and to reveal other Otx2 target genes during lens development.

The *Hes4* gene encodes a basic-helix-loop-helix transcriptional repressor (Davis and Turner, 2001). *Xenopus Hes4*, also known as *Xhairy2*, is expressed from the mid-gastrula stage in the neural/non-neural border that includes both the PPE and neural crest regions (Murato and Hashimoto, 2009). This *Hes4* expression may involve activation by *Notch2*, a member of the *Notch* family expressed in the PPE (Ogino et al., 2008), given that *Hes4* is a direct target of Notch signaling in the developing somite (Davis et al., 2001). Knockdown of *Xenopus Hes4* reduces the preplacodal expression of *Pax6*, *Six3* and *Notch2*, but does not affect the pan-preplacodal expression of *Dlx5* or neural expression of *Pax6* and *Six3* at the neural plate stage (Fig. 2, pink panel) (Murato and Hashimoto, 2009). Later, the embryos fail to express the lens differentiation genes, *FoxE3* and *L-Maf*, at the time of lens placode formation, and lens development is arrested. The reduction of *FoxE3* expression in the *Hes4*-knockdown embryos may be because of the reduction of *Notch2* expression, given that Notch-activated Su(H)/RBP-Jκ protein directly activates the *FoxE3* enhancer in the PLE (see the section for *FoxE3*). Interestingly, preplacodal *Hes4* expression depends indirectly on *Rx*, a homeobox gene expressed in the adjacent retina-field of the neural plate (Andreazzoli et al., 2003). Misexpression of *Rx* expands the adjacent *Hes4* expression, whereas knockdown of *Rx* reduces *Hes4* expression at the periphery of the retina-field in *Xenopus* embryos. Given the essential role of *Hes4* in *Pax6* and *Six3* expression, this cell-nonautonomous, indirect interaction between *Rx* and *Hes4* may be critical for the specification of the lens-field in the immediate vicinity of the retina-field, though the mechanism mediating their interaction is unclear.

According to Metazome (http://www.metazome.net/), the comparative genomics database, both humans and *Xenopus* have *Hes4*, but mice do not. Expression of another member of the *Hes* family, *Hes1*, occurs in the mouse PPE prior to neural tube closure and this expression persists in the developing lens placode (Lee et al., 2005). *Hes1*-knockout mice exhibit defects that range from a reduced size to the complete absence of the lens, but *Pax6* expression is still retained in the preplacodal PLE. It is unclear whether the functional difference between *Xenopus Hes4* and mouse *Hes1* is due to species differences in terms of the requirement for *Hes* to activate *Pax6*, or that mice express another *Hes*-related gene(s) that acts upstream of *Pax6*. Further functional analysis of *Hes* genes may reveal molecular mechanisms that link PPE formation to lens specification.

Six1/Six4 and Eya1/Eya2 are essential for the development of non-lens, placodal tissues

The *Six1* and *Six4* genes are vertebrate homologues of *Drosophila sine oculis* (*so*) and *D-Six4*, respectively (Kawakami et al., 1996; Oliver et al., 1995b; Seo et al., 1999). *Eya1* and *Eya2* are vertebrate homologues of a *Drosophila* gene, *eyes absent* (*eya*) (Xu et al., 1997). So and Eya proteins directly interact to form a transcriptional regulatory complex; So binds to its target DNA and Eya binds to So and recruits co-activators (Pignoni et al., 1997). *so* and *eya* are both expressed in the fly eye imaginal disc under the control of *eyeless* (*ey*), a homologue of vertebrate *Pax6* (Desplan, 1997; Quiring et al., 1994). *ey* expression is also under the control of *so* and *eya*, forming a cross-regulatory circuit (Pignoni et al., 1997). As well as *ey*/*Pax6* (Halder et al., 1995), ectopic expression of *so* together with *eya* induces ectopic eye tissues in fly (Pignoni et al., 1997).

Because *so* and *eya* play central roles in fly eye development, and the Six1 and Six4 proteins interact with Eya1 and Eya2 proteins as do So and Eya (Heanue et al., 1999; Ohto et al., 1999), *Six1*, *Six4*, *Eya1* and *Eya2* were initially predicted to function in vertebrate eye development (Wawersik and Maas, 2000). Expression analyses in *Xenopus* (Ahrens and Schlosser, 2005; Brugmann et al., 2004) and chicken embryos (Litsiou et al., 2005) have shown that *Six1*, *Six4* and *Eya2* are expressed in the PPE under the control of FGF8, anti-BMP and anti-Wnt signals which are derived from the adjacent anterior neural plate and endomesoderm. *Dlx5* appears to be involved in *Six1*/*Six4* activation by these signals, since a PPE enhancer of *Six1* is directly regulated by Dlx5 protein, and misexpression of *Dlx5* induces ectopic *Six4* expression in chicken embryos (Fig. 2, pink panel) (McLarren et al., 2003; Sato et al., 2010).

Misexpression of *Six1* was performed in *Xenopus* and chicken embryos to examine its activity for specifying the placodal fate. In *Xenopus*, misexpression induces expansion of preplacodal *Eya1* expression at the expense of epidermis and neural crest formation (Brugmann et al., 2004). In chickens, co-misexpression of *Six1* and *Eya2* induces ectopic expression of *Six4*, but does not activate genes involved in the later development of placodal tissues, such as *Pax6* in the lens and *Pax2* in the ear (Christophorou et al., 2009). However, targeted expression of a dominant negative form of *Six1* results in a decrease in the number of *Pax6*-expressing cells in the lens placode, *Pax2*-expressing cells in the ear placode, and *Pax3*-expressing cells in the trigeminal placode, suggesting a general requirement for *Six1* or *Six1*-related activity during placodal tissue development in chicken embryos (Christophorou et al., 2009).

In contrast to the studies in chickens, genetic studies in mice show that *Six1*, *Six4*, *Eya1* and *Eya2* are all dispensable for lens development. Homozygous disruption of *Six1* induces multiple defects in the kidney, skeletal muscle, thymus and placode-derived structures including the inner ear and nose, but their eyes appear normal (Laclef et al., 2003; Ozaki et al., 2004). Homozygous disruption of *Six4* also has no effect on eye morphology (Ozaki et al., 2001). Mice that lack both *Six1* and *Six4* show more severe craniofacial defects, including failure of trigeminal gangliogenesis, but their eye structures are mostly normal and have developed lenses (Grifone et al., 2005; Konishi et al., 2006). Neither *Eya1*-deficient mice nor mice with compound homozygous mutations in both *Eya1* and *Eya2* have any obvious defects in their eyes, though they have severe defects in ear, kidney and muscle (Grifone et al., 2007; Xu et al., 1999a). These studies in mice and chickens suggest that *Six1*/*Six4* activity is generally involved in the development of some non-lens placodal tissues, such as the ear and trigeminal ganglia. Their involvement in lens development appears to be species-dependent, although we cannot rule out possible functional compensation for *Six1*/*Six4* activity by another related gene(s) in mice.

Regulation and functions of lens-specification genes

Pax6 has multiple regulatory steps and functions as a hub gene

The *Pax6* gene encodes a transcription factor containing a paired domain and a homeodomain (Walther and Gruss, 1991). Genetic studies have demonstrated that the *Drosophila* homologue, *eyeless*, is necessary for compound eye formation and that its misexpression induces ectopic eye formation on the wing, legs and antennae (Halder et al., 1995). In addition to *ey*, *Drosophila* express two more *Pax6* homologues, *twin of eyeless* (*toy*) and *eye gone* (*eyg*), both of which are necessary for eye formation (Czerny et al., 1999; Jang et al., 2003). *toy* acts upstream of *ey*

and these two genes control eye specification (Czerny et al., 1999), whereas *eyg* is independently regulated by Notch signaling and controls eye growth (Dominguez et al., 2004).

As described in the "Introduction", mouse *Pax6* gene is responsible for the mutant, *Small eye* (*Sey*) (Hill et al., 1991; Hogan et al., 1986). In homozygous *Sey* embryos, the PLE fails to develop into the lens placode upon contact with the optic vesicle. Subsequently, the optic vesicle fails to develop into the optic cup and degenerates, which results in the absence of eyes (Grindley et al., 1995). After the discovery of the absolute requirement of *Pax6* for eye development, its multiple roles and regulation have been investigated in many elaborate studies (Hanson and Van Heyningen, 1995; van Heyningen and Williamson, 2002). In cultured lens epithelial cells, Pax6 protein activates the mouse *αA-crystallin* promoter but represses the chicken *βB1-crystallin* promoter (Cvekl et al., 1995; Duncan et al., 1998). Pax6 also acts as both an activator and a repressor of the chicken *δ-crystallin* enhancer (Fig. 3, lower panel) (Muta et al., 2002). This suggests that Pax6 can act either as an activator or as a repressor, depending upon the regulatory context. In chicken embryos, the requirement of *Pax6* activity was examined by the targeted expression of a dominant negative form of *Pax6* in the PLE overlying the optic vesicle (Reza et al., 2002). This dominant negative *Pax6* inhibits expression of *L-maf*, *Prox1* and *δ-crystallin*, but not *Sox2* or *Six3*, and then results in the failure of lens placode formation. This phenotype is rescued by the co-expression of *L-Maf*, which shows that *L-Maf* plays a crucial role in lens differentiation downstream of *Pax6* (Fig. 2, light blue panel). In mice, a floxed allele of *Pax6* was conditionally deleted at different stages of embryonic development. When it is deleted in the preplacodal PLE using an *AP2α* promoter-driving Cre recombinase expression, placodal thickening fails to occur (Smith et al., 2009). When it is deleted in the lens placode using a *Pax6* lens enhancer (EE)-*Cre*, the embryo loses the placodal *Six3* and *Prox1* expression and lens development is arrested (Ashery-Padan et al., 2000). In either case, *Sox2* expression is initially maintained but is then lost in the lens remnant. This indicates that the preplacodal expression of *Sox2* is *Pax6*-independent, but that after the lens placode formation, *Sox2* requires *Pax6*. This observation is consistent with the discovery of a *Sox2* enhancer regulated by *Pax6* (see the section describing *Sox2*).

The early preplacodal expression of *Pax6* at the neural plate stage has been extensively studied in chickens (Bhattacharyya et al., 2004). *Pax6* expression initially overlaps with *Dlx5* expression in both the lens and nasal progenitor cells of the PPE. Then, *Dlx5* expression is diminished in *Pax6*-expressing lens progenitor cells, whereas *Pax6* expression is down-regulated in *Dlx5*-expressing nasal progenitor cells. This cell fate segregation process is reminiscent of the separation of eye and antennal primordia from a common imaginal disc in *Drosophila*, where expression of the fly homologues of *Pax6* (*eyeless*) and *Dlx* (*dll*) initially overlaps but later localizes separately to the eye and antennal primordia, respectively (Curtiss et al., 2002). In the fly, epidermal growth factor receptor (EGFR) signaling suppresses *eyeless* expression and instead promotes the antenna fate. In chickens, the role of EGFR signaling appears to be substituted by fibroblast growth factor 8 (FGF8) signaling from the anterior neural ridge, which suppresses *Pax6* and instead promotes the nasal fate (Bailey et al., 2006). By contrast, another study showed that the long exposure of the PPE to BMP signals leads to its differentiation into lens, whereas a short exposure results in its differentiation into nasal epithelium (Sjodal et al., 2007). Future studies may reveal a relationship between FGF8 and BMP signaling in this fate segregation process.

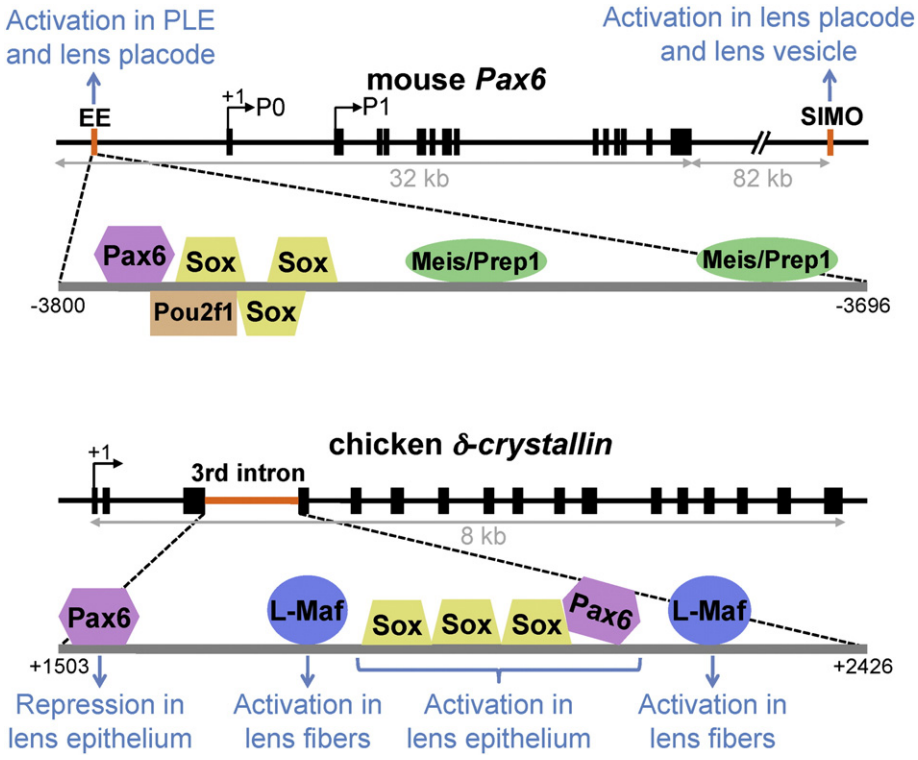

Fig. 3. Cis-regulation of mouse *Pax6* and chicken *δ-crystallin* genes. For *Pax6*, only two enhancers active in the lens lineage, EE and SIMO, are shown, although this gene has other enhancers for other tissues, such as retina and diencephalon. The SIMO element is located approximately 82 kb downstream of the last exon of *Pax6*. *Pax6* has two promoters, P0 and P1 (Xu et al., 1999b). In EE, the 5′-most Sox-binding site overlaps with both Pou2f1- and the 2nd Sox-binding sites, and the 2nd Sox-binding site overlaps with the 3′-most Sox-binding site. Meis1, Meis2 and Prep1 recognize the same binding sites. The lens enhancer of the *δ-crystallin* gene is located in the 3rd intron. This enhancer is activated in lens epithelial cells by Pax6-binding to the 3′ site, which depends on neighboring Sox2/Sox3-binding, as described in the main text. L-Maf-binding to the flanking sites strongly activate this enhancer in lens fiber cells (Ogino and Yasuda, 1998; Shimada et al., 2003), whereas the 5′-most Pax6-binding attenuates the enhancer activity (Muta et al., 2002).

Expression analysis of *Pax6* in homozygous *Pax6*$^{Sey\text{-}1Neu}$ mice has suggested that *Pax6* switches its regulation during lens development (Grindley et al., 1995). *Pax6*$^{Sey\text{-}1Neu}$ is a point mutation that still permits gene transcription but does not allow production of a functional Pax6 protein. The homozygous *Pax6*$^{Sey\text{-}1Neu}$ mice initially express *Pax6* mRNA in the preplacodal PLE, but expression is lost by the lens placode stage, which suggests that *Pax6* expression becomes dependent on positive feedback regulation around the time of lens placode formation. The lens placode expression of *Pax6* not only requires this positive feedback but also requires BMP7 signaling (Fig. 2, purple and light blue panels). Mouse *Bmp7* expression occurs broadly in the head ectoderm, including the PLE, by the time of optic vesicle apposition, and subsequently persists in the lens placode and the surrounding ectoderm (Wawersik et al., 1999). *Bmp7*-knockout mice initially express *Pax6* in the preplacodal PLE but subsequently lose *Pax6* expression and fail to form the lens placode. In addition to BMP7, *Pax6* expression requires FGFR signaling, as shown by the targeted expression of a dominant negative form of FGFR1 in the mouse lens placode (Faber et al., 2001).

The regulatory interactions between *Pax6* and Wnt/β-catenin signaling are somewhat complex. *Pax6* expression in the mouse lens placode requires suppression of Wnt/β-catenin signaling, as suggested by the targeted expression of a constitutive active form of β-catenin in the PLE that reduces the *Pax6* expression and results in the failure of lens placode formation (Machon et al., 2010; Smith et al., 2005). This suppression of Wnt/β-catenin signaling requires earlier *Pax6* expression, possibly in the PLE and/or the optic vesicle, since *Sey* mutant embryos activate a Wnt/β-catenin signaling reporter (BAT-gal) in the PLE, and fail to express the secreted Wnt inhibitors, *Sfrp1* and *Sfrp2*, in the PLE and optic vesicle (Machon et al., 2010; Wawersik et al., 1999). Also, the elimination of *β-catenin* expression results in ectopic lens formation in the periocular ectoderm (Smith et al., 2005) and partially restores lens formation in *Rx*-deficient mouse embryos that completely lack retinal tissues (Swindell et al., 2008), suggesting that the roles for retinal tissue in lens induction involve the suppression of Wnt/β-catenin signaling in the PLE. This observation in mice seems to be consistent with the finding in chickens that the optic vesicle inhibits activation of *Wnt2b* in the PLE in an indirect manner (Grocott et al., 2011). *Wnt2b* is broadly activated in the head ectoderm by TGF-β signaling from the migrating neural crest cells that invade most areas of the head. However, since the developing optic vesicle physically prevents invasion of the neural crest cells near the PLE, the PLE is reserved as a *Wnt2b*-negative area. In contrast to the PLE and placode stages, the later morphogenesis of lens requires Wnt/β-catenin signaling, as shown by disruption of β-catenin or a Wnt coreceptor *Lrp6* in mice that causes a failure in lens epithelium formation in the developing lens vesicle (Smith et al., 2005; Stump et al., 2003).

Pax6 expression is controlled by a number of evolutionarily-conserved *cis*-regulatory elements, which are distributed over more than 180 kb of the mouse genome (Kleinjan et al., 2006). One of these elements is the *Pax6* ectodermal enhancer, or EE, which has been widely used for conditional gene disruption and targeted misexpression in the lens placode as described above (Fig. 3, upper panel) (Kammandel et al., 1999; Williams et al., 1998). Targeted disruption of the EE reduces, but does not eliminate *Pax6* expression in the lens placode, indicating the presence of at least one other enhancer for *Pax6* expression in the lens placode (Dimanlig et al., 2001). The search for transcription factors that directly regulate the mouse EE element has identified Meis1, Meis2 (Zhang et al., 2002), Pax6 itself, Sox2 (Aota et al., 2003), Pou2f1 (Donner et al., 2007) and Prep1 proteins (Rowan et al., 2010). *Meis1* and *Meis2* are TALE-family homeobox genes that are expressed in the PLE overlying the optic vesicle in a *Pax6*-independent manner. *Meis1* null mice have small lenses that fail to detach from the surface ectoderm as occurs in EE-knockout mice (Dimanlig et al., 2001; Hisa et al., 2004). The Pax6 protein interacts directly with the Sox2 protein on the EE to stimulate transcription synergistically (see the Sox2 section for details). Pou2f1, a member of POU family of transcription factors, also interacts with the Sox2 protein on the EE to stimulate transcription (Donner et al., 2007). Prep1 is another member of the TALE-family, and modulates *Pax6* expression timing and levels (Rowan et al., 2010). *Pax6* has another lens enhancer, designated as SIMO, which was discovered in *Aniridia* patients as a conserved region separated from the *PAX6* coding region by a chromosomal translocation (Fig. 3, upper panel) (Kleinjan et al., 2001). Transgenesis with a 420 kb yeast artificial chromosome (YAC) clone containing the EE, all *PAX6* coding regions and the 3′ non-coding region, including SIMO, rescues the homozygous *Sey* phenotype, whereas another YAC clone terminating just 5′ of the SIMO cannot. This indicates an essential role for the 3′ region, including SIMO, in *Pax6* function.

Six3 marks the PLE in a Pax6-independent manner

The *Six3* gene encodes a transcription factor containing a Six domain and a homeodomain (Oliver et al., 1995a). *Six3* was initially considered as a vertebrate homologue of *Drosophila sine oculis* (*so*), because of its eye-specific expression. However, phylogenetic comparisons of genomic structures and amino acid sequences have suggested that *Six3* is a homologue of *optix*/*D-Six3*, another member of the *Six* family in *Drosophila* (Boucher et al., 2000). As well as *so*, the *optix* gene is expressed in the eye primordium and is capable of inducing ectopic eye formation. However, in contrast to *so*, *optix* is independent from the cross-regulatory network involving *eyeless*, *so* and *eya* (Seimiya and Gehring, 2000). While the So protein recruits its specific co-activator, Eya, to activate transcription, the Six3 protein does not interact strongly with Eya (Ohto et al., 1999). Instead, Six3 interacts with the Groucho family of co-repressors (Zhu et al., 2002).

As described in the "overview" section, *Six3* has an expression pattern very similar to that of *Pax6*. Involvement of *Six3* in lens formation was first demonstrated by its misexpression in medaka fish embryos, which led to ectopic lens formation in the presumptive otic region in a cell non-autonomous manner (Oliver et al., 1996). Analysis of homozygous *Sey* embryos has shown that expression of the mouse Six3 protein is initially *Pax6*-independent in the PLE, but later both its expression and nuclear localization become *Pax6*-dependent in the lens placode (Fig. 2, purple and light blue panels) (Purcell et al., 2005). This observation is consistent with the loss of *Six3* expression by conditional disruption of *Pax6* in the mouse lens placode (Ashery-Padan et al., 2000).

Since the simple targeted disruption of mouse *Six3* results in the truncation of the forebrain, including the eye primordia (Lagutin et al., 2003), roles for *Six3* in lens development were examined by conditional disruption of *Six3* in the lens placode (Liu et al., 2006). The resulting lens phenotypes ranged from small lenses to complete absence of the lens. In severe cases, the lens placode failed to invaginate to form the lens vesicle, where *Pax6* expression was reduced but not eliminated, and *Sox2*, *FoxE3* and *Prox1* expression was lost. The role of *Six3* in the maintenance of *Pax6* expression was also shown by the rescue of the haploinsufficient lens phenotype of *Pax6* by targeted *Six3* expression (Goudreau et al., 2002).

Sox2/Sox3 are regulatory partners of Pax6

The *Sox2* and *Sox3* genes encode Sry-related transcription factors containing a high-mobility group (HMG) DNA-binding domain (Denny et al., 1992). Chicken embryos show activation of both *Sox2* and *Sox3* in the PLE, and later activation of their close relative, *Sox1*, after lens vesicle formation, whereas mouse embryos lack *Sox3* expression in the lens lineage (Kamachi et al., 1998). Involvement of the *Sox* genes in lens development was revealed by a search for

enhancer binding factors of the chicken δ-*crystallin* gene (Kamachi et al., 1995, 1998). The Sox proteins form a complex with the Pax6 protein on the δ-*crystallin* enhancer to stimulate transcription synergistically (Fig. 3, lower panel) (Kamachi et al., 2001). Pax6 alone binds very poorly to this enhancer because it has a limited similarity to the optimum binding sequence of Pax6; however, Sox-binding to the enhancer stabilizes neighboring Pax6-binding. The Sox2–Pax6 combination also regulates the lens enhancers of chicken *Sox2* (N-3) and mouse *Pax6* (EE) (Aota et al., 2003; Inoue et al., 2007), which suggests the mutual activation of *Pax6* and *Sox2* (Fig. 2, light blue panel). Co-misexpression of *Pax6* and *Sox2* induces ectopic δ-*crystallin* expression in the head ectoderm of chicken embryos, whereas misexpression of either *Pax6* or *Sox2* alone cannot (Kamachi et al., 2001). It has also been shown that the co-misexpression of *Pax6* and *Sox2* induces ectopic expression of *L-Maf*, whose misexpression alone induced δ-*crystallin* expression in the head ectoderm (Reza et al., 2002).

As well as Pax6, the Maf family of proteins provides crucial partners for the Sox proteins for lens-specific transcription. The chicken δ-*crystallin* enhancer contains two Maf-binding sites in addition to the binding site for the Sox–Pax6 complex (Fig. 3, lower panel) (Muta et al., 2002; Ogino and Yasuda, 1998, 2000). *Sox2* stimulates the activity of *L-Maf* to induce ectopic δ-*crystallin* expression in the head ectoderm of chicken embryos (Shimada et al., 2003). The mouse γ*F-crystallin* promoter also contains both Maf- and Sox-binding sites (Ogino and Yasuda, 2000). Disruption of mouse *Sox1* results in a severe reduction in γ-*crystallins* expression and defective lens fiber differentiation (Nishiguchi et al., 1998). Conditional disruption of mouse *Sox2* at either the preplacode stage or the placode stage results in the failure of lens vesicle formation, where β-*crystallin* and *Prox1* expression is reduced but *Pax6* expression remains (Smith et al., 2009).

As described in the section dealing with *Pax6*, expression of mouse *Sox2* in the lens placode requires *Pax6*, whereas the preplacodal expression of *Sox2* is *Pax6*-independent and requires broad *Bmp7* expression in the head ectoderm (Wawersik et al., 1999). In addition to *Bmp7*, the preplacodal expression of mouse *Sox2* requires *Bmp4* expression, which occurs in the optic vesicle and in a broad domain of the head ectoderm, including the PLE (Furuta and Hogan, 1998). Targeted disruption of mouse *Bmp4* results in the loss of *Sox2* expression without affecting *Pax6* expression in the PLE, and this is followed by the failure of lens placode formation. This phenotype can be rescued in explant cultures of the eye primordium (i.e., the PLE and optic vesicle) by supplementing the BMP4 protein. However, the phenotype cannot be rescued if the mutant optic vesicle is substituted with BMP4-carrying beads, which suggests that BMP4 activates downstream genes in the optic vesicle and/or serves as one of the multiple inductive signals from the optic vesicle for *Sox2* expression and subsequent lens formation.

Regulation and functions of lens differentiation genes

Mab21l1 is involved in lens vesicle formation downstream of Pax6

Mab21l1 is a vertebrate homologue of *mab-21*, a gene that is essential for sensory ray development in *Caenorhabditis elegans* (Mariani et al., 1998). A genetic study has shown that *mab-21* lies on a common genetic pathway with *mab-18*, a *C. elegans* homologue of *Pax6* (Chow and Emmons, 1994). The Mab21l1 protein is enriched in nuclei (Mariani et al., 1999), although there is no clear evidence for its function as a DNA-binding transcription factor.

In mice, *Mab21l1* lies genetically downstream of *Pax6*, since the expression of *Mab21l1* in the PLE and underlying optic vesicle is reduced significantly in homozygous *Sey* embryos (Yamada et al., 2003). *Mab21l1*-knockout mice form the thickened lens placode, which subsequently fails to invaginate due to deficient cell proliferation (Yamada et al., 2003). Chimera analysis has suggested that the failure of lens invagination is not a secondary effect caused by defects in the optic vesicle but is due to cell-autonomous direct effects in the lens placode. Expression of *FoxE3*, but not *Pax6*, *Six3*, *Sox2*, or *c-Maf*, is significantly reduced in the lens placode, which indicates that *Mab21l1* lies genetically upstream of *FoxE3*.

FoxE3 and Pitx3 maintain lens epithelial cells

The *FoxE3* gene encodes a transcription factor containing a winged-helix DNA-binding domain (Blixt et al., 2000; Kenyon et al., 1999). While the expression of mouse *FoxE3* first occurs in the PLE around the time of optic vesicle apposition (Yamada et al., 2003), expression of *Xenopus FoxE3*, which is also known as *Xlens1*, commences earlier in the PPE at the neural plate stage and continues in the lens placode and subsequently in the lens epithelium (Fig. 1H, bottom panel) (Kenyon et al., 1999).

Misexpression of *FoxE3* in *Xenopus* embryos results in the overthickening of the PLE and inhibition of lens fiber differentiation, which suggests a role for *FoxE3* in the proliferation and maintenance of the specified but undifferentiated lens progenitor cells (Kenyon et al., 1999). This observation is consistent with the phenotype of a mouse missense mutant of *FoxE3*, *dysgenetic lens* (*dyl*) (Blixt et al., 2000; Brownell et al., 2000). In this mutant, the lens vesicle fails to close and does not separate from the surface ectoderm. The anterior lens epithelial cells fail to proliferate and are eliminated by premature fiber differentiation and apoptosis. Expression of *Pdgfrα*, which localizes to the epithelium in the wild-type lens, is severely reduced. Expression of *Prox1*, which is normally evident in the equatorial lens fiber cells, is instead expanded to the anterior region, which corresponds to the lens epithelium in wild-type embryos. The defects observed in *dyl* mice resemble the clinical manifestations of human anterior segment dysgenesis and, more particularly, Peter's anomaly (Ormestad et al., 2002). A search of patients associated with these defects has identified a frameshift mutation in *FOXE3* (Semina et al., 2001).

As described in the section dealing with *Mab21l1*, *Pax6* and *Mab21l1* function genetically upstream of *FoxE3* in mice (Fig. 2, light blue panel). *Sip1*, a member of the ZFHX1 family of transcription factor genes, and the BMP receptor genes, *Bmpr1a* and *Acvr1*, also lie upstream, since their targeted disruptions in the lens placode reduce *FoxE3* expression (Rajagopal et al., 2009; Yoshimoto et al., 2005). In *Xenopus*, a combination of the Otx2 protein and Su(H)/RBP-Jκ, a nuclear signal transducer of Notch signaling, directly activates a *FoxE3* enhancer in the PLE overlying the optic vesicle (Ogino et al., 2008). Notch signaling appears to involve *Notch2* expression in the PLE, as well as the expression of a Notch ligand, *Delta2*, in the optic vesicle adjacent to the PLE. This study shows how the activity of a preplacodal gene (*Otx2*) and local signaling from the optic vesicle (Delta-Notch) are integrated in the PLE for lens-field formation. The later role of Notch signaling was examined in mice by the conditional disruption of *Su(H)/RBP-Jκ* in the lens placode (Rowan et al., 2008). The mutant mice form very small lenses with defective cell proliferation, a significant reduction in the expression of lens epithelial marker genes, including *FoxE3*, and expanded expression of lens fiber markers.

The *Pitx3* gene is a member of the *Pitx* family of *bicoid*-type homeobox genes (Semina et al., 1997). While the expression of *Xenopus* and mouse *Pitx3* first occurs in the lens placode (Medina-Martinez et al., 2009; Pommereit et al., 2001), the expression of zebrafish *Pitx3* commences at an earlier stage in the PPE adjacent to the anterior margin of the neural plate and remains in the lens lineage (Fig. 1H, bottom panel) (Dutta et al., 2005).

The *aphakia* (*ak*) gene is a natural mutant of mouse *Pitx3*, with a 652 bp deletion 2.5 kb upstream of the putative *Pitx3* transcription start site and a larger deletion that eliminates its putative promoter

region together with the non-coding exon 1 and part of intron 1 (Rieger et al., 2001). In *ak* mice, the developing lens vesicle fails to form the lens epithelium and lens fibers, and eventually degenerates (Grimm et al., 1998; Medina-Martinez et al., 2009). A similar but not identical phenotype was observed in mice whose *Pitx3*-coding sequence was mostly replaced with GFP-coding sequence (Ho et al., 2009). In these *Pitx3*-GFP knockin mice, the expression of lens epithelial marker genes, such as *FoxE3*, *Pdgfrα* and *E-cadherin*, is reduced as in the *ak*/*ak* mice, but the expression of a lens fiber marker, *Prox1*, is expanded anteriorly with precocious expression of *β*- and *γ-crystallin*s, which indicates that *Pitx3* is essential for maintenance of the lens epithelial cell character (Fig. 2, light blue and light cyan panels). The loss of *FoxE3* expression was also observed in zebrafish morphants of *Pitx3* (Shi et al., 2006). Mutations in human *PITX3* have been identified in families with autosomal-dominant cataracts and anterior segment mesenchymal dysgenesis (ASMD) (Semina et al., 1998).

L-Maf/*c-Maf*/*MafB* for pan-*crystallin* regulation

L-Maf, *MafB* and *c-Maf* encode transcription factors that have a basic-leucine zipper (bZIP) domain for DNA binding and dimerization (Ogino and Yasuda, 1998; Reza and Yasuda, 2004b). The name "*MafA*" was proposed as another name for *L-Maf* when quail *L-Maf* was isolated following the discovery of chicken *L-Maf* because their orthologous relationship was not clear at that time (Benkhelifa et al., 1998). In chickens, *L-Maf* is the first gene to be expressed in the PLE immediately after its contact with the optic vesicle, and its expression remains restricted to the lens placode (Ogino and Yasuda, 1998), where *c-Maf* is turned on (Reza and Yasuda, 2004a). In the developing lens vesicle, *L-Maf* is predominantly expressed in the lens fibers, which is complementary to the lens epithelium-preferred expression of *c-Maf*. Expression of *MafB* occurs after lens vesicle formation in both the lens epithelium and lens fibers (Reza and Yasuda, 2004b; Reza et al., 2007b).

The involvement of the *Maf* genes in lens differentiation was discovered during a search for the transcription factor that binds to a *cis*-regulatory motif shared by the *crystallin* genes (Ogino and Yasuda, 1998). The striking ability of *L-Maf* to induce lens cell differentiation was demonstrated by ectopic expression experiments. Its misexpression in the head ectoderm of chicken embryos and cultured neural retina cells induces ectopic expression of all lens-specific genes tested up until now, including all classes of *crystallin*s, *c-Maf*, *MafB*, *Prox1*, a cyclin-dependent kinase inhibitor, *p27kip1*, and the *MIP* gene encoding a lens-fiber–specific water channel protein (Fig. 2, dark blue panel) (Ogino and Yasuda, 1998; Reza et al., 2007a, 2007b). The genes, *c-Maf* and *MafB*, also possess the ability to induce the expression of lens-specific genes, although they are not as potent as *L-Maf* (Reza et al., 2007b).

The targeted expression of a dominant negative form of *L-Maf* in the preplacodal PLE of chicken embryos completely blocks lens placode formation and inhibits *δ-crystallin* expression without affecting *Pax6* or *Sox2* expression (Fig. 2, light blue panel) (Reza et al., 2002). This indicates that *L-Maf* activity is essential downstream of *Pax6* and *Sox2*. *L-Maf* is actually under the control of *Pax6* and *Sox2*, since their co-expression induces ectopic *L-Maf* expression in the head ectoderm, and targeted expression of a dominant negative form of *Pax6* inhibits *L-Maf* expression in the PLE. FGF8 signaling is also responsible for *L-Maf* expression because *Fgf8* is expressed in the optic vesicle, and the transplantation of FGF8-coated beads or misexpression of *Fgf8* induces ectopic *L-Maf* expression in the head ectoderm (Vogel-Hopker et al., 2000). Interestingly, FGF signaling negatively regulates the stability of the L-Maf protein at the post-translational level; FGF-activated extracellular-signal regulated kinase (ERK) phosphorylates L-Maf and recruits it to the proteasome-mediated degradation pathway (Ochi et al., 2003). These findings imply that FGF signaling induces the expression of *L-Maf* mRNA but inhibits the accumulation of the L-Maf protein for tight control of the timing of differentiation into lens fibers.

Expression of the *Maf* genes in *Xenopus* lens development is different from chicken (Ishibashi and Yasuda, 2001). *MafB* is the first gene to be expressed in the PLE around the time when the optic vesicle makes contact. *L-Maf* expression follows a little later, just before the lens placode thickening. During lens vesicle formation, the expression of *MafB* and *L-Maf* becomes segregated into the lens epithelium and lens fibers, respectively. Misexpression experiments have shown that both *MafB* and *L-Maf* induce ectopic *γ-crystallin* expression in the ectoderm of *Xenopus* embryos (Ishibashi and Yasuda, 2001). The relationships between their expression and optic vesicle signaling were examined by surgically removing the presumptive retina region from the neural plate (Ishibashi and Yasuda, 2001). Manipulated embryos develop a small lens expressing *MafB*, but not *L-Maf*, in the absence of the optic vesicle. This observation implies the possibility that "free lens" formation, an idea which has been discussed for over a century as evidence against the lens induction model of Spemann and Lewis (Grainger, 1992), may be due to the induction of *crystallin* expression by the optic vesicle-independent expression of *MafB*.

In mice, the role performed by *L-Maf* in chicken lens development appears to be undertaken by *c-Maf*. As in the case of chicken *L-Maf*, expression of mouse *c-Maf* occurs in the PLE overlying the optic vesicle and predominates in lens fibers of the developing lens vesicle (Kawauchi et al., 1999). The maintenance of this *c-Maf* expression requires FGFR-signaling, since a lens vesicle lacking three FGF receptor genes (*Fgfr1-3*) revealed a significant reduction in *c-Maf* expression but maintained *Pax6*, *Six3* and *Sox1* expression (Zhao et al., 2008). Targeted disruptions of mouse *Maf* genes have shown that *c-Maf* is indispensable for lens differentiation (Kawauchi et al., 1999; Ring et al., 2000), whereas the functions of *L-Maf* and *MafB* can be compensated by *c-Maf* (Takeuchi et al., 2009). The *c-Maf* knockout mice initially form the lens vesicle, but its development is soon arrested and it is left as a small hollow structure. The posterior cells of the hollow structure fail to form elongated lens fibers and continue to proliferate inappropriately with strong *Pax6* expression. Fiber-specific *γ-crystallin* expression (*γA*, *γB*, *γC*, *γD*, *γE* and *γF*) is completely abolished. The expression of other classes of *crystallin*s (*α* and *β*), unaffected in *Prox1*-knockout mice (Wigle et al., 1999), is severely reduced, whereas *Prox1* expression is unaffected. This observation agrees with the finding that high-level expression of *αA-crystallin* in mouse lens fibers is associated with c-Maf recruitment to the *αA-crystallin* promoter and chromatin remodeling, whereas *Pax6* weakly activates the *αA-crystallin* promoter in the lens epithelium (Yang et al., 2006).

The close relationship between *c-Maf* and congenital eye disease has been investigated in mice and humans. The mapping of dominant and semi-dominant mouse mutations that cause cataracts has led to the identification of base substitution mutations with amino acid changes in the regions encoding the c-Maf DNA-binding domain and transactivation domain, respectively (Lyon et al., 2003; Perveen et al., 2007). A mutation with an amino acid change in the DNA-binding domain was also identified in a human family with cataract, microcornea and iris coloboma (Jamieson et al., 2002).

Prox1 controls the cell cycle and cell elongation for lens fiber differentiation

Prox1 is a vertebrate homologue of the *Drosophila* homeobox gene, *prospero* (Oliver et al., 1993). The *prospero* gene is expressed in lens-secreting cone cells of the compound eye and central nervous system in flies. In the nervous system, the asymmetric distribution of the *prospero* transcript and protein from neuroblasts to their daughter cells is crucial for the formation of distinct cell lineages (Hirata et al., 1995). As with the fly Prospero protein, the Prox1 protein changes

its subcellular localization during mouse lens development (Duncan et al., 2002). Prox1 initially localizes to the cytoplasm of the lens placode cells and then becomes localized to the nuclei of the posterior cells of the invaginating lens placode that give rise to primary lens fibers. By contrast, the lens epithelium progenitors in the anterior portion of the lens placode maintain Prox1 in both the cytoplasm and nuclei. In the mature lens, Prox1 exhibits strong nuclear localization in the newly differentiating lens fiber cells at the equatorial region, whereas lens epithelial cells maintain Prox1 predominantly in the cytoplasm.

In mice, *Prox1* expression requires *Pax6* and *Six3* in the lens placode (see sections for *Pax6* and *Six3*) and later FGFR signaling (*Fgfr1-3*) in the lens vesicle (Fig. 2, light blue and dark blue panels) (Zhao et al., 2008). *Prox1*-knockout mice exhibit defects in lens fiber formation (Wigle et al., 1999). Cell elongation is not observed, and, instead, abnormal cellular proliferation is evident in the posterior region of the mutant lens vesicle. The fiber-specific expression of *p27kip1* and *p57kip2* and expression of some of the *γ-crystallins* (*γB* and *γD*) are abolished, whereas the lens epithelial expression of *E-cadherin* is expanded to the posterior cells. However, the expression of other *crystallins* remains normal.

Conclusions

The expression, regulation and functions of the genes described in the preceding sections are summarized in Table 1. Their genetic interactions, which are summarized in Fig. 2, highlight some interesting features of the tissue determination process. Firstly, there is a crucial role for a cross-regulatory circuit in lens-field specification. The lens-specification genes, *Pax6*, *Six3* and *Sox2*, are initially regulated independently of each other in the PLE, but subsequently, they form a positive cross-regulation network to stabilize their expression around the time when the optic vesicle makes contact. The cross-regulation is mediated, in part, by their auto-regulation mechanisms; *Sox2* stimulates the auto-regulation of *Pax6*, and *Pax6* stimulates the auto-regulation of *Sox2*. BMP and FGFR signaling appears to be involved in this cross-regulation. Secondly, feed-forward regulation is involved in the execution of lens differentiation. The combination of *Pax6* and *Sox2* leads not only to the weak activation of *crystallins* but also to the activation of *L-Maf* and *c-Maf*, which are powerful regulators of the *crystallins* and of lens fiber differentiation. FGF/FGFR signaling from the optic vesicle/optic cup appears to be involved in the activation of the *Maf* genes by *Pax6* and *Sox2*. Since the discovery of the logical interactions in these regulatory networks has depended significantly on the *cis*-regulatory analyses of the *crystallins*, *Pax6* and *Sox2*, *cis*-regulatory analysis of other lens genes may reveal additional interesting features of the gene regulatory circuits, as demonstrated by the comprehensive analysis of the *cis*-regulatory network involved in sea urchin endomesoderm formation (Davidson et al., 2002).

Fig. 2 also reveals questions remaining to be answered; for example, the pathways that link the preplacodal genes to the lens-specification genes are still unclear. The *Dlx* genes are required for the formation of the PPE, which includes the PLE, from the non-neural ectoderm; however, at present, there is no evidence to indicate that they are directly involved in the regulation of *Pax6*, *Six3* or *Sox2*. *Six1* and *Six4* are downstream targets of the *Dlx* gene products and it was believed that they played central roles in PPE formation (Brugmann and Moody, 2005; Schlosser, 2006; Streit, 2007); however, genetic studies in mice have shown that they are essential for nasal epithelium and inner ear development, but dispensable for lens development (see the section for *Six1/Six4 and Eya1/Eya2*). This suggests that the pathway controlling lens formation branches off from the pathway for other placodal tissues at a fairly early stage in mouse development. *Otx2* directly activates *FoxE3*, but its involvement in the regulation of other lens genes has not yet been reported. The broad expression of *Bmp4*/*Bmp7* is not sufficient to explain localized *Sox2* expression. *Hes4* is required for *Pax6* and *Six3* expression, but it encodes a transcriptional repressor that cannot directly activate gene expression. However, the loss of *Notch2* expression in *Hes4*-knockdown embryos is reminiscent of the essential role for Notch signaling in the activation of *eyeless* in the fly eye imaginal disc. Future analyses that focus on the initial activation of lens-specification genes will help to elucidate the fundamental mechanisms controlling the emergence of the different cranial sensory tissues, such as lens, nasal epithelium and inner ear, from their common developmental denominator, PPE.

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