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Review

The role of homeobox genes in retinal development and disease

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ABSTRACT

Homeobox genes are an evolutionarily conserved class of transcription factors that are critical for development of many organ systems, including the brain and eye. During retinogenesis, homeodomain-containing transcription factors, which are encoded by homeobox genes, play essential roles in the regionalization and patterning of the optic neuroepithelium, specification of retinal progenitors and differentiation of all seven of the retinal cell classes that derive from a common progenitor. Homeodomain transcription factors control retinal cell fate by regulating the expression of target genes required for retinal progenitor cell fate decisions and for terminal differentiation of specific retinal cell types. The essential role of homeobox genes during retinal development is demonstrated by the number of human eye diseases, including colobomas and anophthalmia, which are attributed to homeobox gene mutations. In the following review, we highlight the role of homeodomain transcription factors during retinogenesis and regulation of their gene targets. Understanding the complexities of vertebrate retina development will enhance our ability to drive differentiation of specific retinal cell types towards novel cell-based replacement therapies for retinal degenerative diseases.

Introduction

The retina is a remarkably complex, yet structurally simple laminar tissue made up of seven distinct retinal cell types (6 neuronal and 1 glial), that are born sequentially in a temporally ordered and overlapping manner (Young, 1985). These cells are organized into three cellular layers, which include the ganglion cell layer (GCL), the inner nuclear layer (INL) and the outer nuclear layer (ONL) (Fig. 1). As mouse embryonic retinal development proceeds, retinal ganglion cells (RGC) are generated first, followed by cone photoreceptors, horizontal cells and amacrine cells. During the postnatal period, rod photoreceptors, bipolar cells and Müller glia are specified and complete differentiation. Retinal cells arise from a common pool of multipotent retinal progenitor cells (RPC) (Livesey and Cepko, 2001; Turner and Cepko, 1987; Wetts and Fraser, 1988). RPC competence gradually becomes restricted to produce the correct retinal cell types within the appropriate temporal window. Initiation of intrinsic transcription factor expression in the RPC population plays a critical role in directing these multipotent cells to adopt specific retinal cell fates (Marquardt, 2003; Ohsawa and Kageyama, 2008). Transcription factors of the homeodomain, basic helix-loop-helix (bHLH) and forkhead box (FOX) families work in concert to direct retinal cell fate specification and differentiation. Homeobox genes encode transcription factors containing a 60 amino acid DNA-binding homeodomain. These homeodomains canonically bind to core tetranucelotide TAAT/ATTA motifs (Gehring et al., 1994). Unique adjacent consensus motifs impart specificity of binding of individual transcription factors (Gehring et al., 1994). Homeobox genes can be further classified based on the presence of additional conserved domains, such as the paired and LIM domains. Homeobox genes play a number of critical roles during vertebrate forebrain (Wigle and Eisenstat, 2008) and eye development including specification of the eye field and the optic stalk, progenitor cell fate determination, and retinal cell differentiation and survival. Due to these critical roles, mutations in human homeobox genes can lead to a number of ocular abnormalities, ranging from colobomas to anophthalmia (Table 1). We will focus

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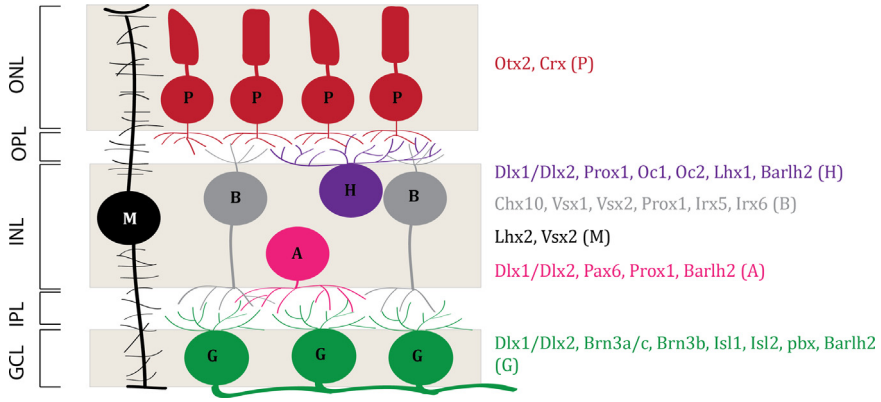

Fig. 1. Expression of homeobox genes in the mature vertebrate retina. Homeobox genes are required for specification and differentiation of retinal cells. RGCs (green, G) are located in the GCL which also contains displaced amacrine cells (not shown). The INL contains the cell bodies of the amacrine cells (pink, A), horizontal cells (purple, H), bipolar cells (gray, B) and the Müller glia (black, M). The ONL contains the light-sensitive rod and cone photoreceptors (red, P). Synaptic connections between the photoreceptors in the ONL and horizontal and bipolar cells are made in the OPL while connections between bipolar cells and horizontal cells are made with the RGC in the IPL. Homeobox genes are expressed in all retinal cell types, including RPC. Specific homeobox genes expressed in the cells of the vertebrate retina are shown on the right and are also color coordinated with the cell type in which they are expressed. In addition, the first letter corresponding to the retinal cell types is indicated next to the homeobox genes expressed in that specific cell type. [GCL, ganglion cell layer; INL, inner nuclear layer; IPL, inner plexiform layer; ONL, outer nuclear layer; OPL, outer plexiform layer; RGC, retinal ganglion cell; RPC, retinal progenitor cell].

our review on members of the homeobox gene family, and the critical role each gene plays in RPC fate specification, retinal cell type differentiation and inherited eye diseases.

Rax

Retina and anterior neural fold homeobox gene (*Rax*), previously referred to as the retinal homeobox gene (*Rx*), belongs to the paired-like (Prd-L) homeodomain (HD) family. In addition to the paired-like homeodomain, the RAX protein contains a conserved OAR domain in the C-terminus named for conservation of this region between *opt*, *aristaless* and *rax* (Furukawa et al., 1997). *Rax* genes have been identified in a number of model systems including mice, zebrafish, chick, *Xenopus*, and *Drosophila* with the number of *Rax* genes varying depending on the species. Both the structure and the expression pattern of the *Rax* genes are highly conserved across these species (Chuang and Raymond, 2001; Furukawa et al., 1997; Mathers et al., 1997; Muranishi et al., 2012). *Rax* is among the earliest genes expressed in the developing retina. During zebrafish gastrulation, *rx3* expression in the anterior forebrain permits specification of the eye field over telencephalic fate (Stigloher et al., 2006). Murine *Rax* is first expressed at E7.5 in the cephalic neural fold (Furukawa et al., 1997; Mathers et al., 1997). At E9.5, *Rax* expression initiates in the optic vesicle and is subsequently localized to the neural retina. *Rax* is expressed uniformly throughout the developing neuroblastic layer (NBL), with peak expression observed at E16.5. With the onset of retinal cell differentiation, *Rax* expression progressively decreases but is maintained in photoreceptors of the developing retina until the second postnatal week.

Rax is essential for early specification of eye development as demonstrated by the loss of eye structures in vertebrate models lacking *Rax* expression, including mice and zebrafish (Furukawa et al., 1997; Loosli et al., 2003; Mathers et al., 1997). In addition, *Rax* null mice have reduced brain structures, which can range from a lack of ventral forebrain or the complete absence of the forebrain. Conversely, *Rax* gain-of-function experiments in *Xenopus* and zebrafish result in ectopic retinal tissue (Mathers et al., 1997; Terada et al., 2006). In the absence of *Rax*, upregulation of *Pax6* expression is not induced in the ventral neuroectoderm, suggesting that *Rax* is upstream of *Pax6* and required to specify RPCs (Fig. 2) (Zhang et al., 2000). Similar to the loss of eye structures observed in different model organisms, human *RAX* mutations have been associated with both anophthalmia and microphthalmia (Table 1) (Abouzeid et al., 2012; Bardakjian and Schneider, 2011; Voronina et al., 2004). Mutations are typically located in either the DNA binding homeodomain or the OAR domain and result in impaired expression of RAX target genes (Lequeux et al., 2008).

In addition to a role in early eye development, *Rax* regulates expression of genes required for photoreceptor development (Fig. 2). Knockdown of zebrafish and *Xenopus Rax* orthologs results in reduction of photoreceptor-specific gene expression (Nelson et al., 2009; Pan et al., 2010). Early *in vitro* studies demonstrated that RAX can directly bind to labeled oligonucleotide probes of the *arrestin* promoter (a photoreceptor specific gene) through the photoreceptor conserved element (PCE-1) *in vitro* and activate expression of *arrestin* in reporter gene assays (Kimura et al., 2000). More recently, the *Xenopus Rax* gene was demonstrated to occupy PCE-1 containing promoter elements of *rhodopsin* and *red cone opsin in vivo* (Pan et al., 2010). *Rax* has also been shown to regulate the expression of the murine homolog of *orthodenticle* (*Otx2*), a homeobox gene essential for determination of photoreceptor cell fate (Muranishi et al., 2011). A conserved enhancer upstream of the OTX2 initiation codon was identified and termed the embryonic enhancer locus for photoreceptor *Otx2* transcription (EELPOT). RAX was shown to bind to EELPOT *in vivo* by chromatin immunoprecipitation (ChIP), and significantly activates EELPOT-luciferase reporters *in vitro*. Additionally, *Otx2* expression is dramatically decreased with concomitant decrease in cone–rod homeobox (*Crx*) expression (a transcriptional target of OTX2) upon conditional knockout of *Rax* in RPC (Muranishi et al., 2011).

Pax2 and Pax6

The paired box (*Pax*) gene family encodes transcription factors that are important for many developmental processes. In total, nine mammalian *Pax* genes have been identified; all contain a paired domain (Chi and Epstein, 2002; Mansouri et al., 1999; Noll, 1993). The *Pax* gene family is further subdivided into four groups based on the presence or absence of additional structural domains, which include either partial or complete homeodomains. PAX3, PAX4, PAX6 and PAX7 are among the PAX proteins that contain complete homeodomains; PAX2 is unique within this transcription factor family since it does not contain a homeodomain.

Table 1
Retinal expression and mutant phenotypes of selected homeobox genes.

Gene	*Retinal expression*	*Retinal phenotype of knockout mice*	*Transcriptional targets*	*Human disease*
Rax	RPC	*Eyeless*	*Pax6*(+) *Otx2*(+), *β-arrestin*(+), *rhodopsin*(+)	Anophthalmia
*Pax2**	Ventral optic vesicle, optic fissure, optic stalk	Failure to close optic fissure, guidance defects of optic nerve	*Pax6*(−)	Coloboma syndrome and renal hypoplasia
Pax6	RPC, amacrine cells	*Small Eye* (heterozygotes); Anophthalmia in homozygotes	*Pax2*(−), *Atoh7*(+), *Ngn2*(+), *Crx*(−)	Aniridia, cataracts, Peter's anomaly, coloboma, optic nerve hypoplasia, foveal hypoplasia
Lhx1	Post-mitotic horizontal cells	Failure of proper horizontal cell lamination	ND	ND
Lhx2	Optic cup, optic stalk, MG	Anophthalmia, reactive gliosis in MG CKO	*Rax*(+), *Pax6*(+), *Six3*(+), *Six6*(+)	ND
Vsx2	RPC in early retinal development. Bipolar and MG in late development	Reduced RPC proliferation and failure of bipolar cell differentiation	$P27^{KIP1}$(−), *Crx*(−), *Vsx1*(−)	Microphthalmia
Meis1/Meis2	RPC	Microphthalmia due to impaired RPC proliferation, partial retina ventralization	*smad1*(+), *tbx5*(+), *vax2*(−), *Cyclin D1*(+), *C-myc*(+)	ND
Vsx1	Cone bipolar cells	Differentiation defects in type 7 bipolar cells	*Recoverin*(+), *Neto1*(+), *NK3R*(+), *CaB5*(+), *Vsx2*(−), *Cabp5*(−), *Irx6* (−)	ND
Vax2	Ventral retina	Ventral retina dorsalization, ventral RGC axon pathfinding defects; retina replaces optic nerve in the *Vax1*/*Vax2* double knockout	*Pax6*(−)	Coloboma
Pbx	RGC	No mouse phenotype is described. Zebrafish RGC axons fail to enter tectum. Dorsal/temporal patterning defects	*gdf6a*(+), *aldh1a2*(+), *tbx5*(+), *hmx*(+), *atoh7*(+)	ND
Dlx1/Dlx2	RGC, horizontal, amacrine	~30% loss of late-born RGC	*Brn3b*(+), *Crx*(−) *TrkB*(+), *Dlx56ie*(+), *Nrp-2*(−) (forebrain)	ND
Dlx5/Dlx6	RGCs, horizontal, amacrine	ND	ND	ND
Brn3b	RGCs	~60–80% loss of RGC	*Brn3a*(+), *Brn3c*(+) *Dlx1/Dlx2*(−), *Otx2*(−), *Crx*(−)	ND
Brn3a/*Brn3c*	RGC	Altered RGC dendritic stratification in *Brn3a* CKO; No retinal phenotypes in Brn3c knockouts	ND	ND
Isl1	RGC	Apoptosis of ~67% of RGCs	*Brn3a*(+), *Brn3b*(+)	ND
Isl2	RGC	Aberrant ipsilateral projection of RGC axons	*Zic2*(−), *EphB1*(−)	ND
Oc1	RGC (E12.5 – P16), horizontal cells	75% reduction in horizontal cells	*Lhx1*(+), *Prox1* (+)	ND
Oc2	RGC (E12.5 – P16) horizontal cells	ND	ND	ND
Prox1	Horizontal, bipolar, amacrine	Embryonic lethal, absence of horizontal cells, increase in rods and Müller glial cells	$p27^{KIP1}$(+), $p57^{KIP2}$(+)	ND
Irx5	Type 2 and Type 3(a/b) bipolar cells, MG	Defects in Type 2 and Type 3(a/b) bipolar cell development	*CaBP5*(+), *PMCA1*(+), *Recoverin*(+)	ND
Irx6	Type 2 and Type 3a bipolar cells, MG		*Vsx1*(−)	ND
Otx2	RPE, cones, rods, bipolar cells	Microphthalmia, increase of amacrine cells, decrease in photoreceptors (CKO); lack of head structures in conventional KO	*Crx*(+), *Rax*(+), *Blimp1*(+)	Microphthalmia
Crx	Cones, rods, bipolar	No gross abnormalities; photoreceptor degeneration postnatally	*Crx*(+), *Cone Opsin* (+), *rhodopsin*(+), *Nr3e*(+), *Otx2*(−)	Leber's Congenital Amaurosis, cone–rod dystrophy, retinitis pigmentosa

CKO, conditional knockout mouse; ND, not determined; (+) indicates positively regulated transcriptional targets; (−) indicates negative regulation of transcriptional target; Pax2* does not contain a full homeobox.

Both *Pax2* and *Pax6* are expressed in the developing eye with *Pax6* expression found in the neural retina, the retinal pigment epithelium (RPE) and the lens surface ectoderm. In contrast, *Pax2* expression is restricted to the optic stalk and optic cup. *Pax2* expression is observed in the optic stalk structures of both mice and zebrafish. During early embryonic development (E9.0–E11.0), *Pax2* expression is localized to ventral optic cup and optic stalk structures (Macdonald et al., 1997; Nornes et al., 1990; Torres et al., 1996). Sonic Hedgehog (Shh) signaling from the midline demarcates the division between optic stalk from neural retina structures by regulating expression of *Pax2* and *Pax6*, which are expressed in these domains (Cai et al., 2013; Macdonald et al., 1995). During axonal outgrowth from the neural retina, *Pax2* expression remains elevated in the optic disc and optic nerve, but is excluded from the neural retina. Loss of *Pax2* function in mice and the zebrafish results in the failure to close the choroid fissure, significant axonal pathfinding defects and coloboma (Macdonald et al., 1997; Torres et al., 1996). Similarly, mutations in human *PAX2* result in papillorenal syndrome where patients have both optic nerve colobomas and renal hypoplasia (Table 1) (Sanyanusin et al., 1995; Schimmenti et al., 1997). Recently, fibroblast growth factor (FGF) signaling has been observed to regulate *Pax2* expression in the optic disc and optic fissure (Cai et al., 2013). Mutation of FGF receptors, *Fgfr1* and *Fgfr2*, results in failure to close the optic fissure with significant down-regulation of *Pax2* expression. Expression of the RPE marker microphthalmia-associated transcription factor (*Mitf*) was upregulated in the open fissure, suggesting a cell fate switch from optic disc to RPE.

Pax6 plays numerous critical roles in development of the eye, including the retina and the lens. *Pax6* structure and function is

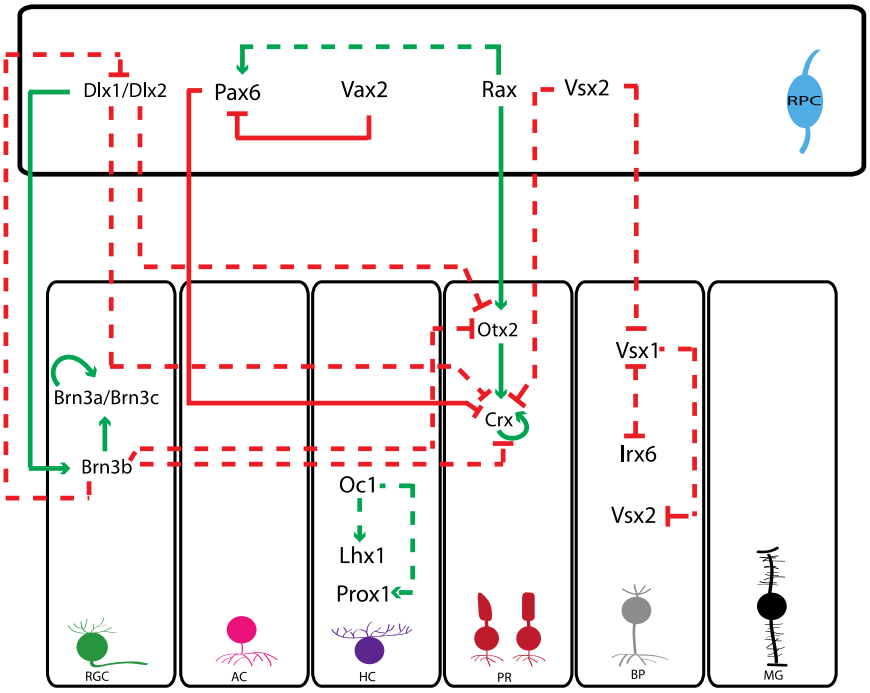

Fig. 2. Regulatory interactions of homeobox genes during retinogenesis. Throughout retinal development, homeodomain-containing transcription factors, along with bHLH and forkhead box transcription factors (not shown), control retinal cell fate and differentiation by regulating the expression of target homeobox genes. Here, we focus on the interactions between members of homeobox gene families in this process. Top panel: *Pax6*, *Rax*, *Vsx2* and *Vax2* are amongst the earliest expressed transcription factors (observed by ~E9.0) in the RPC, required for maintaining multipotency and specification of cell fate. *Pax6* is required to maintain multipotency of RPC and restrict *Crx* expression in peripheral RPC. In order to ventralize the retina, VAX2 represses *Pax6*. RAX activates *Pax6* in early RPC, while in later retinal development RAX plays a role in photoreceptor specification by activating expression of *Otx2* and additional photoreceptor target genes outside of the homeobox family. VSX2 represses expression of its family member, *Vsx1*. During bipolar cell development, Vsx1 is required for the genesis of type 7 ON bipolar cells by repressing *Vsx2* and other markers specific for OFF bipolar cells. Vsx1 also repressed *Irx6* and the bipolar cell type 3a program. Conversely, Irx6 restricts type 3a cells from adopting type 2 characteristics by repressing expression of *Vsx1*. In addition, VSX2 promotes bipolar fate by repressing photoreceptor gene expression. *Crx* is a predicted target of VSX2. *Dlx1/Dlx2* expression initiates by E11.5 and activates the expression of *Brn3b* promoting RGC fate. *Dlx1/Dlx2* is also predicted to repress expression of *Otx2* and *Crx*, restricting photoreceptor fate. In the RGC, BRN3b activates the expression of its family members, *Brn3a/c* which subsequently autoregulates its expression. BRN3b specifies early-born RGC by downregulating the expression of *Dlx1/Dlx2*, *Otx2* and *Crx* which are critical for late born RGC differentiation and photoreceptors development, respectively. *Oc1* is required for horizontal cell development and is located upstream of *Lhx1* and *Prox2* in horizontal cell genesis. In photoreceptor precursors, OTX2 drives expression of *Crx*, which is required for differentiation of photoreceptors. Up-regulation of *Crx* also positively regulates its own expression. [Solid lines indicate chromatin immunoprecipitation (ChIP)-verified direct regulation. Dashed lines represent predicted direct regulation. Transcriptional activation, →; transcriptional repression, —|; AC, amacrine cell; BP, bipolar cell; *Dlx*, gene; DLX, protein; HC, horizontal cell; MG, Müller glia; PR, photoreceptor; RGC, retinal ganglion cell; RPC, retinal progenitor cell].

highly conserved between vertebrates and *Drosophila* (Quiring et al., 1994). The *eyeless* (*ey*) mutant phenotype was first described in *Drosophila*, which as the name suggests, results in complete lack of eyes due to mutations in the *ey* gene, subsequently identified as the *Drosophila* homolog of the vertebrate gene *Pax6*. Similar mutations in the vertebrate *Pax6* homologs are observed in both humans and mice, leading to congenital aniridia and the semi-dominant "Small Eye" (*Sey*) mutation phenotype, respectively (Table 1) (Brown et al., 1998; Hill et al., 1991; Tzoulaki et al., 2005). Homozygous null mutations of *Pax6* are lethal and result in complete absence of eye structures in mice. Over-expression of *Pax6* also leads to abnormalities of the eye, including microphthalmia and retinal dysplasia (Manuel et al., 2008; Schedl et al., 1996). *Pax6* expression initiates at E8.0 in both the optic pits and a broad region of surface ectoderm (Grindley et al., 1995; Walther and Gruss, 1991). In the neural ectoderm, expression becomes progressively localized to the distal optic vesicles. From E9.5, *Pax6* is highly expressed in the optic cup and the developing lens placodes, followed by continued expression in the prospective neural retina and lens. *Pax6* is key to establishing the optic cup/optic stalk boundaries during ocular development through reciprocal control of *Pax2* expression (Schwarz et al., 2000). *Pax6* expression is expanded into the optic stalk in the absence of *Pax2*, where this tissue acquires neural retina fate. Conversely, in the absence of *Pax6*, ectopic *Pax2* expression is found in the optic cup margin prior to degeneration of the optic cup, demonstrating mutual repressive interactions between *Pax6* and *Pax2* during optic development. In addition, molecular interactions were observed between PAX2 and PAX6 and *Pax6* and *Pax2* enhancers, respectively *in vitro*. In the developing neural retina, *Pax6* is expressed in early RPC and required for maintenance of PRC multipotency. In the absence of *Pax6*, RPC competence is dramatically restricted, producing exclusively amacrine cells at the expense of all remaining retinal cell types (Marquardt et al., 2001). Loss of RPC competency in the absence of *Pax6* can be partially explained by failure to activate expression of proneural bHLH genes, such as *Atoh7* (formerly *Math5*) and neurogenin-2 (*Ngn2*), which are both direct targets of PAX6 regulation, and required for RGC development and retina neurogenesis, respectively (Marquardt et al., 2001; Riesenberg et al., 2009; Wang et al., 2001). *Pax6* plays dual roles in RPC fate specification, depending on the spatial location of the RPC. Cells located in the peripheral retina require *Pax6* for completion of neurogenesis and restriction of *Crx* expression (Fig. 2) while centrally localized RPC require *Pax6* to retain multipotency (Oron-Karni et al., 2008). Recently, *Pax6* was identified as being downstream of suppressor-of-fused (*Sufu*), a negative regulator of the Hedgehog signaling pathway (Cwinn et al., 2011; Svard et al., 2006; Varjosalo et al., 2006). Conditional loss of *Sufu* in the proximal retina results in *Pax6* down-regulation and phenocopies the *Pax6* conditional knockout described above

(Cwinn et al., 2011). In addition to its role in the neural retina, *Pax6* is required for lens development. Conditional knockout of *Pax6* in the surface ectoderm at the lens induction stage (~E9.5) results in failed lens placode formation, with a lack of surface ectoderm thickening and invagination, and the absence of lens placode-specific gene expression (Ashery-Padan et al., 2000). In addition to aniridia, mutations in human PAX6 contribute to several retina disorders including coloboma, optic nerve hypoplasia, foveal hypoplasia, and Peter's anomaly (Azuma et al., 1996, 2003; Hanson et al., 1994).

Lhx1 and Lhx2

Lhx1 (also referred to as *Lim1*) and *Lhx2* belong to the LIM-homeodomain (LIM-HD) subfamily of homeobox genes. In addition to a DNA-binding homeodomain, LIM-HD proteins contain two zinc finger LIM domains, which participate in protein–protein interactions (Hobert and Westphal, 2000; Porter et al., 1997; Sanchez-Garcia and Rabbitts, 1994). In the developing retina, *Lhx1* is expressed exclusively in postmitotic horizontal cells with expression initiating at E14.5 (Edqvist and Hallbook, 2004; Liu et al., 2000b). Thinning of the outer plexiform layer is observed in retinas with conditional mutations for *Lhx1* (Poche et al., 2007). Horizontal cells of the *Lhx1* null retina are properly specified, but fail to localize to the correct laminar position in the outer INL. In retinas lacking the Onecut transcription factor, *Oc1*, 80% of horizontal cells are lost with down-regulation of *Lhx1* expression, demonstrating upstream regulation of *Lhx1* by OC1 (Wu et al., 2013). An early role for *Lhx1* in optic vesicle development has also been demonstrated in chick (Kawaue et al., 2012). *Lhx1* expression is observed in the proximal optic vesicles. *Lhx1* overexpression in chick optic vesicle induces formation of ectopic neural retina tissue in the outer optic cup, demonstrated by increased expression of neural retina specific genes.

LHX2 can function either as a transcriptional activator or repressor by interacting through its LIM domain with co-activators and co-repressors (Agulnick et al., 1996; Tetreault et al., 2009). *Lhx2* plays a key role in a number of developmental processes including erythropoiesis, forebrain and eye development (Porter et al., 1997). *Lhx2* belongs to a group of transcription factors collectively referred to as the eye field transcription factors (EFTFs), which are critical for early specification of the eye field (Zuber et al., 2003). *Lhx2* expression is first observed in the prospective eye field of the neural anterior plate (Porter et al., 1997; Tetreault et al., 2009; Yun et al., 2009) and by E10, *Lhx2* expression is observed throughout the optic neuroectoderm where it is localized to the optic stalk and most, if not all RPC (Gordon et al., 2013). In the mature neural retina, *Lhx2* is expressed in a small number of amacrine cells as well the Müller glia localized to the medial INL (Fig. 1) (de Melo et al., 2012; Gordon et al., 2013). Eye development is halted at the optic vesicle stage in *Lhx2* mutant embryos prior to formation of the optic cup, resulting in anophthalmia (Porter et al., 1997; Tetreault et al., 2009; Yun et al., 2009). However, mutations in the zebrafish *Lhx2* ortholog *lhx2b* do not result in anophthalmia, likely due to functional redundancy with *lhx2a* (Seth et al., 2006). Despite the anophthalmic phenotype observed in *Lhx2* knockout mouse models, LHX2 mutations have not yet been identified in human cases of anophthalmia (Desmaison et al., 2010).

Lhx2 has been shown to play an important role in early optic development by initiating the expression of a number of important EFTFs, including *Rax*, *Pax6* and *Six3* (Tetreault et al., 2009). As retinal development progresses from the optic vesicle to optic cup stages, *Lhx2* is required for regionalization and patterning of the optic neuroepithelium (Yun et al., 2009). In the optic vesicle, *Vsx2* expression demarcates the presumptive neural retina while *Mitf* and *Pax2* become localized to the RPE, and the optic stalk and neural retina, respectively. In $Lhx2^{-/-}$ optic vesicles, initiation of VSX2 and MITF expression fails, while expression of PAX2 does not persist past E9.5. These results demonstrate that in the absence of *Lhx2*, there is failed regionalization of the optic vesicle and eye development is arrested prior to regional specification (Yun et al., 2009). Dorsoventral patterning in *Lhx2* mutant optic vesicles is also disrupted. *Vsx2* is a target of *Lhx2* and its expression is required to maintain ventral identity and restrict *Pax6* expression to the ventral optic vesicle (Horsford et al., 2005; Rowan et al., 2004). $Lhx2^{-/-}$ optic vesicles are dorsalized since they lack *Vsx2* expression and consequently, *Pax6* expression expands into the ventral optic vesicle (Yun et al., 2009). Lhx2 was recently demonstrated to be required continuously for optic identity from vesicle stages to neural retinal development, by shutting down alternative thalamic fates (Roy et al., 2013).

Roles for *Lhx2* outside of the optic vesicle to optic cup transition have recently been identified. Conditional knockout of *Lhx2* function demonstrates that *Lhx2* is required for RPC maintenance (Gordon et al., 2013). Conditional *Lhx2* mutant retinas have significantly decreased RPC populations with a consequent increase in neurogenesis. *Lhx2* loss in early RPC populations results in excess RGC production while late removal of *Lhx2* in RPC results in supernumerary rod photoreceptor formation. Production of these cells occurs at the expense of all other retinal cell types.

Recently, *Lhx2* was found to be critical in maintaining Müller glia in a non-reactive state (de Melo et al., 2012). Conditional loss of *Lhx2* in mature Müller glial cells induces reactive gliosis as demonstrated by increased expression of the intermediate filament glial fibrillary acidic protein (GFAP) and glial hypertrophy. *Lhx2* null Müller glia are compromised in their ability to up-regulate neuroprotective factors in response to light damage.

Vsx2 and Vsx1

Similar to *Pax6* and *Rax*, *Vsx2* (formerly *Chx10*), is a member of Prd-L HD transcription factor family, and plays major roles in eye development. In addition to the paired homeodomain, VSX2 contains an additional conserved region known as the CVC domain, located immediately adjacent to the C-terminus of the HD, and named after the identification of *Chx10* and three other genes: *Vsx1*/*Vsx2* and *Ceh-10*, which are the *Chx10* homologs in goldfish and *Caenorhabditis elegans*, respectively (Levine et al., 1994; Liu et al., 1994). The CVC domain was reported to be required for the homeodomain of VSX2 to bind to DNA with high affinity and specificity (Zou and Levine, 2012). The HD and CVC domains are both required for VSX2 mediated repression (Dorval et al., 2005). The Prd-L HD:CVC proteins can be further classified into two groups, depending on the presence of a RV region, which is exclusive to *Vsx1* group, or the OAR region specific for *Vsx2* group (Chow et al., 2001). *Vsx2* and *Ceh-10* belong to the *Vsx2* group, which is highly conserved. However, the *Vsx1* group is considered to be a rapidly evolving gene and shares only 71% identity with murine *Vsx1* and human *VSX1* orthologs.

Vsx2 is expressed in the interneurons of the retina, hindbrain and spinal cord and expression is initiated in RPCs by E9.5 (Liu et al., 1994). During optic cup formation, contact between the optic vesicle and presumptive lens ectoderm is hypothesized to induce *Vsx2* expression (Nguyen and Arnheiter, 2000). *Vsx2* is down-regulated when the RPCs exit the cell cycle and differentiate (Green et al., 2003). During late retinal development and in mature retinas, *Vsx2* expression is restricted to the INL, where it is predominantly expressed in bipolar interneurons and a subset of Müller glia (Fig. 1) (Liu et al., 1994; Rowan and Cepko, 2004).

Consistent with its expression pattern, two major phenotypes are observed in *Vsx2* mutant mouse models: defects in RPC proliferation

and bipolar cell differentiation (Burmeister et al., 1996). In the mature mouse retina there are 12 bipolar cell sub-types: 11 cone bipolar and one rod bipolar (Wassle et al., 2009). The ocular retardation (*or^{J}*) mouse model carries a spontaneous mutation with a premature stop codon at the midpoint of *Vsx2*, and therefore no VSX2 is detected in the *Chx10*^{orJ} homozygote (*Chx10*$^{orJ/orJ}$) retina (Burmeister et al., 1996). *Chx10*$^{orJ/orJ}$ retinas demonstrate significant reduction of RPC proliferation (up to 83% loss of bromodeoxyuridine (BrdU) incorporating RPCs in the peripheral retina). Studies on *Vsx2* bacterial artificial chromosome (BAC) reporter mice have linked the reduction of RPC proliferation to RPE trans-differentiation, with increased expression of *Mitf*, a gene associated with onset and maintenance of pigmentation (Rowan et al., 2004). The severe RPC loss observed in *Chx10*$^{orJ/orJ}$ can also be attributed to de-repression of the cyclin-dependent kinase inhibitor 1b (*Cdkn1b*), also known as p27(KIP1), which subsequently silences cyclin D1 (Green et al., 2003). Genetic deletion of *Cdkn1b* on a *Vsx2* null background is able to partially rescue RPC proliferation. Complete loss of bipolar cells is observed in the postnatal *Chx10*$^{orJ/orJ}$ retina (Burmeister et al., 1996). In contrast to the role of *Vsx2* in early retinal development, *Vsx2* is dispensable for RPC proliferation in later development and instead, plays a critical role in bipolar cell fate determination (Livne-Bar et al., 2006). This role is supported by the observation that no bipolar cells are detected in the *Chx10*$^{orJ/orJ}$: *Cdkn1b*$^{-/-}$ double mutant retina, where the progenitor cell numbers are partially increased (Green et al., 2003). In addition, shRNA knockdown of *Vsx2* in postnatal retinas dramatically reduces bipolar cell production, without influencing RPC proliferation (Livne-Bar et al., 2006). Bipolar cells and rod photoreceptors are born in a temporally overlapping window during postnatal murine retinal development. *Vsx2* promotes a bipolar fate over a rod fate by repressing photoreceptor specific genes, while conversely, transcription factors critical to photoreceptor development repress *Vsx2* to promote rod genesis (Fig. 2) (Brzezinski et al., 2010; Dorval et al., 2005; Katoh et al., 2010). For example, conditional loss of *Blimp1*, a zinc-finger transcription factor, results in increased expression of *Vsx2* and increased production of bipolar cells with a concomitant reduction in photoreceptor numbers (Brzezinski et al., 2010; Katoh et al., 2010). Other phenotypes observed with *Vsx2* mutation include microphthalmia in both human and mice, and lack of optic nerves (Table 1) (Burmeister et al., 1996; Ferda Percin et al., 2000).

In contrast to the early embryonic expression of *Vsx2* in RPCs, *Vsx1* is expressed predominantly in the postnatal retina (Chow et al., 2001, 2004). *In situ* hybridization and reporter gene studies revealed that *Vsx1* expression is initiated within the INL no earlier than P5. By P12, *Vsx1* expression is restricted to the outer INL, where it is expressed in a subset of cone bipolar cells, but not in Müller glia or rod bipolar, amacrine, and horizontal cells (Fig. 1) (Chow et al., 2001). *Vsx1* is also a transcriptional target of *Vsx2* (Fig. 2) (Clark et al., 2008). In *Vsx2* deficient RPCs in both mice and zebrafish, *Vsx1* mRNA is up-regulated. In addition, *Vsx2* represses luciferase expression driven by the *Vsx1* promoter (Clark et al., 2008). Interestingly, the transcriptional role of *Vsx1* varies depending on the subtype of bipolar cell in which *Vsx1* is expressed (Chow et al., 2004; Shi et al., 2011). *Vsx1* is required to activate expression of genes required for differentiation and function of OFF bipolar cells, including *recoverin*, *Neto1*, *NK3R* and *CaB5* (Chow et al., 2004). Conversely, *Vsx1* negatively regulates expression of *Vsx2* and *Cabp5* in type 7 ON bipolar cells (Shi et al., 2011). Unlike *Vsx2*, *Vsx1* is not altered in human microphthalmic phenotypes.

Meis Genes

The Meis family proteins are the vertebrate homologs of Homothorax (Hth) in *Drosophila*. In the invertebrate retina, Hth promotes proliferation of RPC along with the *Pax6* homolog (*Ey*) and an additional transcription factor, *Tsh* (Bessa et al., 2002). Resembling its role in invertebrates, the vertebrate Meis family is involved in RPC proliferation. The Meis family consists of three members, which includes *Meis1*, *Meis2* and *Meis3*. Of these three factors, *Meis1* and *Meis2* expression is observed in the vertebrate retina. In mouse, chick and zebrafish, *Meis1/2* expression is observed in proliferating RPC and is downregulated upon the onset of neurogenesis (Bessa et al., 2008; Heine et al., 2008). Microphthalmic eyes are observed with *Meis1/2* knockdown (Bessa et al., 2008; Heine et al., 2008). This phenotype is the result of reduced cell numbers due to the downregulation of cell cycle regulatory proteins, including *cyclin D1* and *c-myc*.

Meis1 also plays a key role in retinal patterning. *meis1* knockdown in zebrafish embryos lead to decreased dorsal *tbx5* expression in the developing retina, with a concomitant increase in the ventral *vax* expression domain (Erickson et al., 2010). This patterning defect in the *meis1* mutant is due to the downregulation of *smad1*, a component of BMP signaling which is required for dorsal retinal patterning.

Vax genes

Vax genes are a family of homeodomain subfamily closely related to the *Emx* and *Not* genes, sharing sequence homology, similar chromosomal location and expression patterns (Hallonet et al., 1998). *Vax* genes are named for their highly specialized expression pattern during early neurulation (Ventral anterior homeobox-containing gene). In the mouse, *Vax1* mRNA is first detected at E8.0, in the anterior neural ridge and adjacent ectoderm. During embryogenesis, *Vax1* expression is restricted ventrally to the derivatives of these regions, including the basal forebrain, ventral optic vesicle, optic disk, optic stalk and optic chiasm (Hallonet et al., 1999, 1998). Mice targeted for deletion of *Vax1* show defects in RGC axonogenesis and axonal-glial associations, without influencing expression of *Pax2* or *BF1*. In addition, *Vax1*$^{-/-}$ axons fail to fasciculate and extend toward the midline of the hypothalamus, leading to an absence of the optic chiasm. These RGC axon pathfinding defects are partially due to the loss of important axon guidance cues, including *Netrin-1* and *EphB3*, but not *Slit1* (Bertuzzi et al., 1999). *Vax1* mutant mice also have coloboma due to failed choroid fissure closure. *Pax6* and *Rax* are ectopically expressed in the *Vax1* mutant optic nerve, whereas *Pax2* expression is unchanged.

VAX2 shares an identical homeodomain with VAX1 (Hanson et al., 1994). By E9.0, *Vax2* transcripts are detected in the ventral optic vesicle, with lower expression in the optic nerve and stalk (Barbieri et al., 1999). Within a short time, *Vax1* and *Vax2* expression overlaps in the ventral retina and optic stalk (Hanson et al., 1994). By E12.0, *Vax2* expression is restricted to the ventral neural retina in all retinal neuroblasts while during late embryonic retinal development, *Vax2* is only detected in the ventral RGCs (Barbieri et al., 2002; Mui et al., 2002). *Vax2* is not expressed in the adult retina.

Consistent with its expression pattern, *Vax2* plays a major role in ventralizing the embryonic retina. Mis-expression of *Vax2* in the dorsal retina alters the expression of putative dorsal–ventral (D/V) markers, including up-regulation of *EphB2*/*EphB3*, *Pax2* and *Vax2* itself, and down-regulation of the dorsally restricted transcription factor *Tbx5* (Barbieri et al., 1999; Schulte et al., 1999). In addition, ectopic *Vax2* expression in dorsal retina induces profound axonal pathfinding defects in dorsal RGCs (Schulte et al., 1999). In agreement with *Vax2* gain-of-function studies, ventral RGCs from *Vax2* null mice show complete dorsalization (Barbieri et al., 2002; Mui et al., 2002). The RGC axons from *Vax2*$^{-/-}$ ventral retina aberrantly project into the lateral rostral edge of the superior colliculus (SC) together with all the dorsal RGC axons, instead of into the medial rostral SC which are innervated by ventral RGC

axons. The ventral expression domain of *EphB2*/*EphB3* is absent in *Vax2*$^{-/-}$ ventral retina. However, in contrast to *Vax2* gain-of-function studies, the loss of *Vax2* function failed to alter the expression of early regulators of D/V polarization, such as *Pax2* and *Tbx5* (Barbieri et al., 2002; Mui et al., 2002). Interestingly, while ipsilateral projecting axons originate primarily from the ventral temporal retina, complete loss of ipsilateral projecting axons was observed in only one of two studies to examine *Vax2* mutant RGC axons (Barbieri et al., 2002). The coloboma phenotype is mild in *Vax2* mutants (Barbieri et al., 2002; Mui et al., 2005).

Despite their complementary expression pattern, *Vax1* and *Vax2* interact and function in concert in retina and optic nerve formation (Mui et al., 2005). *Vax1*/*Vax2* double knockout mice have more severe retinal colobomas when compared to the single mutants, and the optic nerve is transformed into a differentiated and laminated retina. By directly acting on the α-enhancer of *Pax6*, VAX1 and VAX2 negatively regulate *Pax6* expression in the optic neuroepithelium (Fig. 2). Of significance, the repression of *Pax6* transcripts in retinal development is closely related to the subcellular location of VAX2. Phosphorylation of Serine-170, which is C-terminal to the VAX2 homeodomain, sequesters VAX2 into the cytoplasm after E12.5, and in turn disables VAX2 repression of *Pax6* expression (Kim and Lemke, 2006). By antagonizing Serine-170 phosphorylation, Shh promotes VAX2 nuclear localization.

Pbx genes

With the Meis homeobox genes, the Pre-B cell leukemia homeobox (Pbx), homeobox transcription factors belong to the TALE class of homeobox genes. Pbx genes are critical in specifying and patterning the midbrain and hindbrain by cooperating with Engrailed, and in complex with Hox and Meinox, respectively (Erickson et al., 2007; Waskiewicz et al., 2001, 2002). The role of Pbx genes in vertebrate retinal development has largely been studied in zebrafish. Pbx gene expression is observed throughout retinal development with *pbx2* and *pbx4* expression initiated during early zebrafish optic cup development, while *pbx1* and *pbx3b* expression is observed shortly thereafter (French et al., 2007). In the developing retina, *pbx2* and *pbx4* play a role in RGC axon outgrowth. RGC axons from embryos lacking *pbx2* and *pbx4* project toward but fail to enter the optic tectum. This phenotype is thought to result from mis-regulation of pbx target genes required for dorsal retina (*aldh1a2*, *tbx5* and *hmx4*) and tectal patterning (*efna2*, *fabp7a* and *nat10*) (French et al., 2007). In addition, *atoh7* expression is downregulated in the dorsal retina of *pbx2*/*pbx4* mutants, which may also contribute to the observed pathfinding defects due to altered RGC differentiation. *Pbx* also plays a role in dorsal/temporal retina patterning. *gdf6a*, a BMP family growth factor, regulates dorsal/temporal expression of *aldh1a2* and *tbx5* in the zebrafish retina. In *pbx2*/*pbx4* mutants, *gdf6a* and its targets are strongly downregulated (French et al., 2007).

Dlx genes

Distal-less (*Dll*) is required for *Drosophila* limb development. *Dlx* genes are the vertebrate orthologs of *Dll*. There are six murine *Dlx* genes, which are arranged into three bigenic clusters (*Dlx1*/*Dlx2*, *Dlx5*/*Dlx6*, and *Dlx3*/*Dlx7*), and are localized to mouse chromosomes 2, 6 and 11, respectively (Ghanem et al., 2003). Within the intergenic regions of *Dlx1*/*Dlx2* and *Dlx5*/*Dlx6*, several *cis*-acting regulators have been characterized, including I12a and I12b within *Dlx1* and *Dlx2* genes, and I56i and I56ii separating *Dlx5* and *Dlx6* genes (Poitras et al., 2007). Two conserved enhancer elements, URE1 and URE2, have also been found in the 5′ flanking region of *Dlx1* (Hamilton et al., 2005). These *cis*-acting elements are important for cross-regulatory interactions between the *Dlx* gene family members. For instance, DLX1 and DLX2 activate *Dlx5*/*Dlx6* expression by directly binding to I56i (Zhou et al., 2004). Of the six *Dlx* genes, four (*Dlx1*, *Dlx2*, *Dlx5* and *Dlx6*) are expressed in the developing forebrain, in differing, but temporally and spatially overlapping patterns.

Dlx1 and *Dlx2* were initially detected in the retinal neuroepithelium at E12.5 (Eisenstat et al., 1999). Our group has detected DLX2 expression by immunostaining in E11.5 retina, where DLX2 is expressed in a clear high-dorsal to low-ventral gradient (de Melo et al., 2008). At E13.5, both DLX1 and DLX2 are expressed throughout the retina. Almost 25% of all retinal cells express DLX2 at E13.5. The proportion of DLX2 expressing cells in the total retinal population declines during development. By E18.5, DLX1 and DLX2 expression is restricted to the GCL and the inner NBL, where they are co-expressed with markers for RGC, amacrine and horizontal cells (Fig. 1). DLX1 expression resembles that of DLX2 in embryonic retinas, but decreases dramatically after birth (de Melo et al., 2003). DLX1 is not detected in the adult retina. However, DLX2 expression is maintained in the GCL and the INL throughout adulthood.

Homozygous deletion of *Dlx1* and *Dlx2* is perinatally lethal, and leads to a 33% reduction of RGC numbers, in part due to enhanced apoptosis of late-born RGCs (de Melo et al., 2005). *TrkB*, a neurotrophin receptor family member, is a downstream target of DLX2 during murine retinal development and may contribute to *Dlx1*/*Dlx2* dependent survival of RGCs (de Melo et al., 2008). Similar to the *Dlx1*/*Dlx2* double mutant, mice lacking *Dlx1* or *Dlx2* die at birth, but no retinal phenotype has been reported in the single gene knockouts (Panganiban and Rubenstein, 2002). Although DLX2 is co-expressed with GAD65, GAD67 and GABA in the developing retina, we have not discovered any defects in GABAergic interneuron differentiation in the *Dlx1*/*Dlx2* null retinas (Zhang et al., in revision).

The role of *Dlx5* and *Dlx6* in retinogenesis remains undetermined. *In situ* hybridization revealed *Dlx5* mRNA expression in retina by E16.5 (Zhou et al., 2004). In the P0 and adult retinas, *Dlx5* mRNA is co-expressed with DLX2 in the GCL and INL. The *Dlx5*/*Dlx6* intergenic enhancer (MI56) co-expresses with DLX1, DLX2 and DLX5 in the RGCs, amacrine and horizontal cells.

The regulatory network of homeobox genes during retinogenesis is complex. *Dlx1*/*Dlx2* and *Brn3b* function in parallel but cross-regulatory pathways to determine RGC cell fate. While the *Atoh7*-*Brn3b* RGC pathway is critical for RGC specification and differentiation, another pathway exists whereby *Dlx1*/*Dlx2* cross-regulates expression of *Brn3b* for differentiation and survival of late-born retinal ganglion cells (Zhang et al., in revision, Fig. 2). DLX2 may function as a transcriptional repressor of *Crx* during photoreceptor differentiation, since in the *Dlx1*/*Dlx2* double mutant retinas there is increased *Crx* expression in the outer NBL and ectopic *Crx* expression in the GCL (Fig. 2) (de Melo et al., 2005). To date, no human eye diseases have been linked to mutations in the *Dlx* gene family.

Brn-3 genes

The POU-domain transcription factors were named for the identification of three mammalian homeodomain coding genes, *Pit1*, *Oct1*/*Oct2*, and one similar *C. elegans* gene *Unc-86*. The POU-domain is a bipartite DNA-binding protein domain, which contains a POU-specific region and a POU-homeodomain region. The class IV POU-domain proteins, BRN3a, BRN3b and BRN3c (*POU4f1*, *POU4f2* and *POU4f3*, respectively) are the homologs of *Unc-86* in *C. elegans*. *Brn-3* genes are expressed in the embryonic and adult central nervous system, and are required for the sensorineural development and survival.

Each of the *Brn-3* POU-homeodomain genes is expressed specifically in postmitotic RGCs (Fig. 1) (Xiang et al., 1995). In the

mouse, *Brn3b* expression is first detected in the RGCs at E11.5 in the central inner retina, followed by *Brn3a* and *Brn3c* expression two days later (Pan et al., 2005; Xiang et al., 1995). Through E15.5 to adulthood, *Brn3a* and *Brn3b* expression partially overlaps in 80% of RGCs. Although *Brn3a* and *Brn3b* share a similar global expression pattern, their expression is distinct, particularly in the postnatal retina. In P5 RGCs, *Brn3a* is predominately expressed with few RGCs expressing *Brn3b* (Quina et al., 2005). Only ~15% of RGCs express *Brn3c* (Xiang et al., 1995).

The overlapping expression pattern and a similar specific DNA-binding site [(A/G)CTCATTAA(T/C)] of the *Brn-3* proteins suggest a potential for functional redundancy in retinogenesis. However, targeted mutations of *Brn-3* genes show distinct developmental defects. Only the *Brn3b*$^{-/-}$ mouse shows an obvious retinal phenotype. Targeted deletion of *Brn3b* results in a 60–80% loss of RGCs in adult retinas, depending on the genetic mouse strain (Erkman et al., 1996; Gan et al., 1996). RGC loss in this model is due to enhanced apoptosis after E15.5. Initial cell fate specification and migration of RGCs in *Brn3b* mutant retinas is not affected. *Brn3b* is also required for RGC axon pathfinding and fasciculation (Erkman et al., 2000). *Brn3a*-null mutants die at birth, with loss of dorsal root and trigeminal neurons (Erkman et al., 1996; Xiang et al., 1996). *Brn3c*$^{-/-}$ mice display balance deficits and complete deafness, due to loss of vestibular and auditory hair cells (Erkman et al., 1996; Xiang et al., 1997). Neither *Brn3a* nor the *Brn3c* mutant mice display obvious retinal defects.

Brn3b is downstream of *Atoh7*, a bHLH transcription factor that is required for RGC development (Brown et al., 2001; Wang et al., 2001). The *Atoh7-Brn3b* regulation pathway promotes RGC differentiation by repressing a group of retinal development genes, including *Dlx1*/*Dlx2*, *Otx2* and *Crx* (Qiu et al., 2008). Recent work has shown that under the regulation of ATOH7, *Isl1*, a LIM-HD TF, defines a distinct but overlapping sub-population of RGCs expressing *Brn3b* (Mu et al., 2008; Pan et al., 2008). *Brn3a* and *Brn3c* are downstream of *Brn3b* as *Brn3a* expression is reduced in *Brn3b* mutant retinas (Fig. 2) (Erkman et al., 1996). Despite the essential requirement for *Brn3b* in RGC development, several independent research groups have reported that all *Brn-3* genes are functionally equivalent during retinogenesis. Over-expression of *Brn3a*, *Brn3b* or *Brn3c* in chick RPC has similar effects in promoting RGCs differentiation (Liu et al., 2000a). Knocking-in the *Brn3a* coding sequence into a *Brn3b* null background rescues RGCs from apoptosis and restores the proper RGC axon pathfinding process (Pan et al., 2005). Conditional deletion of *Brn3a* alters RGC dendritic stratification without influencing RGC axon central projections, while *Brn3b* conditional knockouts have reduced RGC number, loss of axon projections to medial terminal nuclei (MTN), lateral terminal nuclei (LTN) and corresponding visual sensory defects (Badea et al., 2009).

Islet1 and Islet2

Named for their role in binding to an enhancer of the insulin gene (Tanizawa et al., 1994), the Islet transcription factors belong to the LIM homeodomain family, and include Islet1 (*Isl1*) and *Isl2*. *Isl2* is required for the correct lateral projection of RGC axons (Pak et al., 2004). *Isl2* expression in the retina is first observed at E13.5 and becomes localized to contralaterally projecting RGCs. *Isl2* down regulates *Zic2* and *EphB1* expression, which are factors required for ipsilateral projection. Consistent with this observation, *Isl2* loss increases ipsilateral projections and upregulates *Zic2* and *EphB1* expression in *Isl2* null RGCs.

Isl1 plays a critical role in vertebrate neurogenesis. *Isl1* is required for the generation of motoneurons and its loss results in early embryonic lethality (Pfaff et al., 1996). *Isl1* expression is localized to post-mitotic RGC during early retinogenesis and maintained into adulthood (Mu et al., 2008; Pan et al., 2008). In addition, cholinergic amacrine cell and bipolar cells express *Isl1* postnatally (Elshatory et al., 2007). Similar to *Brn3b*, *Isl1* is key for differentiation and survival of RGCs, but dispensable for initial RGC generation (Mu et al., 2008; Pan et al., 2008). Prior to RGC apoptosis, axon pathfinding is disrupted in *Isl1* null retinas as RGC axons fail to reach the midline (Pan et al., 2008). ISL1 and BRN3B expression co-localizes in postmitotic RGCs and are regulated in parallel by *Atoh7* (Pan et al., 2008). As described for *Brn3b* genes above, *Isl1* regulates distinct downstream RGC targets and shares targets with Brn3b *in vivo* (Mu et al., 2008; Pan et al., 2008). Recently, ISL1 and BRN3B were shown to form complexes with each other that can bind to and regulate RGC specific gene expression (Li et al., 2014).

Barhl2

The BarH-like homeobox genes, *Barhl1* and *Barhl2*, are homologs of the *Drosophila* BarH genes, which play a role in the development of the compound eye (Higashijima et al., 1992). Vertebrate BarH transcription factors contain a homeodomain and one or two FIL domains, named for the presence of phenylalanine, isoleucine and leucine, and are required for *in vivo* transcription repression (Reig et al., 2007; Smith and Jaynes, 1996). Barhl1 and Barhl2 each contain two FIL domains.

Barhl2 expression is first observed in the vertebrate retina in the inner NBL at E14.5 and is maintained in adulthood with expression localized to both the GCL and the INL, where it is expressed in a subpopulation of postmitotic RGCs, amacrine cells and horizontal cells (Mo et al., 2004) (Fig. 1). *Barhl1* is not expressed in the developing or mature retina. In *Barhl2* null mice, a decrease in RGCs is observed while initial RGC specification is unaffected (Ding et al., 2009). Decreases in *Barhl2* expression in *Atoh7* and *Brn3b* null retinas demonstrate *Barhl2* functions downstream of the *Atoh7-Brn3b* RGC developmental pathway. Barhl2 also plays a key role in amacrine cell subtype specification. Loss of *Barhl2* results in significant reduction of both glycinergic and GABAergic amacrine cells and concomitant increase in cholinergic amacrine cells. Using morpholino knockdown, *ptf1a* was shown to be functionally upstream of *barlh2* in specification of zebrafish amacrine cells (Jusuf et al., 2012).

Onecut1 and Onecut2

The *Onecut* (*Oc*) class of transcription factors have recently been demonstrated to play a role in vertebrate retinal development. Of the three *Oc* family members (*Oc1*, *Oc2* and *Oc3*), *Oc1* and *Oc2* are highly expressed in the developing retina (Wu et al., 2012). In addition to an atypical homeodomain, unique in amino acid composition compared to traditional homeodomains, *Oc* transcription factors contain an additional DNA binding domain known as the CUT domain (Jacquemin et al., 1999; Lemaigre et al., 1996; Vanhorenbeeck et al., 2002). From E12.5 to P0, *Oc1* and *Oc2* are highly expressed in RGC (Wu et al., 2012). *Oc1* and *Oc2* expression is also observed in RPCs during prenatal stages of retina development. Postnatally (P5–P16), *Oc1* and *Oc2* expression is observed in horizontal cells (Fig. 1). Despite the high level of expression of *Oc1* in RGC, RGC development in the *Oc1*$^{-/-}$ retina is unaffected (Wu et al., 2013). However, *Oc2* expression levels increase in the GCL of *Oc1*$^{-/-}$ mutants, suggesting that *Oc2* and *Oc1* may be functionally redundant for RCG development. Loss of *Oc1* significantly affects horizontal cell genesis with an 80% reduction in horizontal cells in the mature retina. *Prox1* and *Lhx1* expression is also reduced, demonstrating a genetic pathway whereby *Oc1* acts upstream of *Prox1* and *Lhx1* during horizontal cell development (Fig. 2). OC1 has also been shown to cooperate with OTX2 in RPC to specify

cone and horizontal cell fates through co-regulation of thyroid receptor hormone beta (*Thrb*) (Emerson et al., 2013). Cone precursors subsequently reduce *Oc1* while maintaining *Otx2* expression, whereas horizontal cells decrease *Otx2* and maintain *Oc1* expression.

Prox1

Prox1 is the vertebrate ortholog of the *Drosophila* gene *prospero*. PROX1 contains a sequence-divergent homeodomain adjacent to a carboxyl terminal Prospero domain, which facilitates proper alignment of PROX1 binding to DNA. Though PROX1 is a homeodomain-containing transcription factor, the homeodomain and the prospero domain of PROX1 combine to form a unique single-structural unit referred to as the Homeo-Prospero domain through which it binds to DNA (Elsir et al., 2012; Ryter et al., 2002; Yousef and Matthews, 2005). *Prox1* expression is first observed in the developing eye at E9.5 in the lens placode, followed by expression in the lens vesicle and the lens fibers at E10.0 and E12.5 (Wigle et al., 1999). *Prox1* expression is observed in the neural retina by E14.5 where it is expressed in RPC and newly postmitotic horizontal cells (Dyer et al., 2003). In the adult retina, *Prox1* is expressed in horizontal, amacrine, and to a lesser extent, bipolar cells (Fig. 1). *Prox1* knockout mice die at mid-gestation and have a number of developmental ocular defects. In the lens of the *Prox1*$^{-/-}$ mice, abnormal proliferation and down-regulation of cell cycle inhibitors, p27^{KIP1} and p57^{KIP2} results in failed elongation and polarization of the lens (Wigle et al., 1999). In the neural retina, *Prox1* loss results in significant horizontal cell loss and failure of early RPC to exit the cell cycle (Dyer et al., 2003). Failed cell cycle exit in early RPC produces a lower proportion of early-born cell types and an increased proportion of late-born cell types. Conversely, ectopic *Prox1* induces horizontal cell production and premature cell cycle exit, producing smaller clones. Two transcription factors have been identified upstream of *Prox1* in horizontal cell production: *Foxn4* and *Onecut1* (*Oc1*) (Li et al., 2004; Wu et al., 2013). Knockouts of *Foxn4* and *Oc1* both demonstrate significant reduction in *Prox1* expression and horizontal cell production. Direct regulation of *Prox1* expression by these transcription factors has yet to be established.

Irx genes

The Iroquois (Irx) genes were first discovered in *Drosophila* where they control bristle patterning on the dorsal mesothorax by regulating proneural *achaete-scute* genes (Leyns et al., 1996). Irx genes belong to the TALE superclass of homeodomain transcription factors and also contain an Iro box specific to the Irx family. Irx genes in *Drosophila*, mice and humans are organized into trigene clusters. *Drosophila* has one *Irx* cluster, which includes the genes *araucan*, *caupolican* and *mirror*. Mice and humans possess two clusters of *Irx* genes: Cluster A (IrxA) containing *Irx1*, *Irx2* and *Irx4* and Cluster B [IrxB] containing *Irx3*, *Irx5* and *Irx6* (Cavodeassi et al., 2001; Gomez-Skarmeta and Modolell, 2002; Peters et al., 2000). Zebrafish contain 11 *Irx* orthologous that are grouped according to their similarity to mammalian vertebrate *Irx* genes (Dildrop and Ruther, 2004; Feijoo et al., 2004).

Expression of all vertebrate Irx genes is first observed in the GCL during early retinal development (Cohen et al., 2000). Postnatally, subsets of Irx transcription factors are required for development of specific bipolar cell subtypes. In addition to GCL expression, *Irx5* expression is observed in the INL at P14 (Cheng et al., 2005) (Fig. 1). In mature retinas, Irx5 expression is found in Müller glia and type 2 OFF and type 3 OFF cone bipolar cells. *Irx5* null retinas lack expression of a number of markers expressed in type 2 OFF (recoverin) and type 3 OFF (CaBP5 and PMCA1) cone bipolar cells, respectively, indicating that *Irx5* plays a role in development of these bipolar cell subtypes.

Irx6 also plays a role in the development of bipolar cell subtypes (Star et al., 2012). Co-localization of *Irx6* expressing cells with bipolar subtype specific markers demonstrates *Irx6* is localized to both type 2 and type 3a bipolar cells (Fig. 1). Loss of *Irx6* in the murine retina results in loss of type 3a bipolar cells and axon stratification defects in remaining 3a bipolar cells in the IPL. Resembling *Irx5*, *Irx6* expression is also observed in the GCL, but development of this cell type is unaffected in mutants. Misregulation of *Vsx1* and *Bhlhb5* is also observed in the absence of *Irx6* (Fig. 2). *Vsx1* is ectopically expressed in the type 3a bipolar cells while *Bhlhb5* expression was reduced in both type 2 and 3a bipolar cells, demonstrating a requirement for Irx6 mediated repression of *Vsx1* in type 3a cells and activation of *Bhlhb5* in type 2 and 3a bipolar cells.

Otx2

Otx genes, the mammalian orthologs of the *Drosophila* gene orthodenticle (*Otd*) family, belong to the paired-class of homeodomain proteins which are required for proper anterior–posterior patterning during embryogenesis. *Otx2* belongs to a family of transcription factors, which includes *Otx1*, *Otx2* and the *Otx*-like protein, *Crx*. The homeodomain of *Otx2* differs from that of *Otd* by only two amino acids (Simeone et al., 1993). *Otx2* plays a critical role in specification of the rostral central nervous system, as *Otx2* deletions result in embryonic lethality due to a lack of anterior head structures, including the forebrain, midbrain and rostral hindbrain (Acampora et al., 1995; Gonzalez-Rodriguez et al., 2010; Matsuo et al., 1995). During development of the murine retina, *Otx2* expression initiates at E11.5 where it is strongly expressed in the RPE and weakly expressed in the NBL of the neural retina (Martinez-Morales et al., 2003; Martinez-Morales et al., 2001; Nishida et al., 2003). At E12.5, *Otx2* expression increases in the neural retina. During late embryonic retinal development, *Otx2* is localized to the outer NBL, which includes the prospective photoreceptor layer. During adulthood, *Otx2* expression significantly decreases in both the neural retina and RPE (Fig. 1).

Otx2 plays critical roles in both photoreceptor cell fate determination and bipolar cell development (Koike et al., 2007; Nishida et al., 2003). In the absence of *Otx2* expression in the developing retina, a cell fate switch from photoreceptors to amacrine cells is observed. Microarray analysis of the *Otx2* CKO retina compared to WT controls also demonstrated decreases in a number of transcription factors specific to photoreceptor development, including *Crx*, *Nr2e3 and Nrl* (Omori et al., 2011). *Otx2* is reported to be genetically upstream of *Crx* based on reporter gene assays (Fig. 2) (Nishida et al., 2003). However, transcriptional regulation by OTX2 of the *Crx* promoter *in vivo* has yet to be identified. Conditional knockout of *Otx2* in postnatal bipolar cells results in decreased expression of the mature bipolar cell marker, protein kinase C (PKC), demonstrating a requirement for *Otx2* in bipolar cell development (Koike et al., 2007). Consistent with this observation, increases in bipolar specific genes, including *Vsx2* and *Bhlhb4*, are observed in *Otx2* CKO retinas (Omori et al., 2011).

In addition to its role in photoreceptor and bipolar cell development, *Otx2* is also required for proper development of the RPE (Lane and Lister, 2012; Martinez-Morales et al., 2003, 2001). Interestingly, in mice both *Otx2* and *Mitf* are required for normal development of the RPE, whereas zebrafish only require expression of *otx1a* and *otx2* (Lane and Lister, 2012).

Mutations in human *OTX2* result in a number of ocular disorders including microphthalmia, optic nerve hypoplasia, optic nerve aplasia, colobomas and anophthalmia (Bardakjian and Schneider, 2011; Beby and Lamonerie, 2013; Ragge et al., 2005;

Tajima et al., 2009; Verma and Fitzpatrick, 2007; Wyatt et al., 2008). OTX2 mutations are autosomal dominant and account from 3% of all (bilateral) microphthalmia and anophthalmia cases (Bardakjian and Schneider, 2011; Verma and Fitzpatrick, 2007). In addition to ocular diseases, OTX2 plays a role in the malignant childhood brain tumor, medulloblastoma, where its expression is amplified in both cell lines and primary medulloblastoma tumors (Boon et al., 2005; de Haas et al., 2006).

Crx

The cone–rod homeobox-containing gene (*Crx*) also belongs to the orthodenticle family of homeobox genes. The CRX homeodomain shares 88% and 86% homology with the homedomains of OTX1 and OTX2, respectively, whereas the overall protein homology between CRX and OTX1 and OTX2 is 40% and 44%, respectively (Freund et al., 1997). *Crx* is specifically expressed in both photoreceptors and pinealocytes (Furukawa et al., 1999). *Crx* is first expressed at E12.5 in the outer NBL, corresponding to birth of cone photoreceptors. *Crx* expression peaks at postnatal day 6 (P6) of retinal development, which coincides with birth and maturation of rod photoreceptors. *Crx* expression is maintained in adulthood in the mature ONL (Fig. 1). Knockout mouse models of *Crx* fail to form photoreceptor outer segments from P14 when these structures are established and lack rod and cone electroretinogram (ERG) activity. Contrary to *Otx2*, *Crx* is not required for specification or photoreceptor cells fate, but rather for development and maintenance of photoreceptors by controlling the expression of photoreceptor specific genes, including rhodopsin, cone opsins, *Nr2e3*, *Nrl* and *Trβ2* (Corbo et al., 2010; Furukawa et al., 1999; Hennig et al., 2008). In addition to binding the above gene promoters, *Crx* binds its own promoter *in vivo* and autoregulates its expression in a dose-dependent manner (Fig. 2) (Furukawa et al., 2002; Hennig et al., 2008). *Crx* also binds to the promoter of *Otx2*, which as described above, lies genetically upstream of *Crx* (Hennig et al., 2008; Nishida et al., 2003). This interaction results in repression of *Otx2* expression, as demonstrated by a greater than two-fold increase in *Otx2* expression in $Crx^{-/-}$ mouse models (Hennig et al., 2008). The genetic interactions of *Crx* suggest a regulatory mechanism for development of photoreceptors whereby *Otx2* is first required for photoreceptor cell fate specification, subsequently leading to the downstream activation of *Crx* (Hennig et al., 2008). Expression of *Crx* increases in committed photoreceptor precursors and auto-regulates its expression while concomitantly repressing the expression of *Otx2*. *Crx* then activates expression of photoreceptor-specific genes required for the terminal differentiation and survival of rod and cone photoreceptors (Blackshaw et al., 2001; Hennig et al., 2008; Swaroop et al., 2010).

Consistent with *Crx* regulating a number of photoreceptor specific gene targets, CRX mutations have been implicated in a number or retinal diseases including retinitis pigmentosa, cone–rod dystrophy and Leber congenital amaurosis (LCA) (Freund et al., 1997; Rivolta et al., 2001; Sohocki et al., 1998).

Summary

Homeobox genes are critical for many aspects of retinal development including specification of the eye field, and retinal cell fate specification and differentiation. This review highlights a number of homeobox transcription factors and their contribution to retinal development through regulation of downstream target gene expression. Due to the critical contributions of these transcription factors to the proper development of the vertebrate retina, mutations in homeobox genes have been shown to result in a wide range of human ocular disorders. With the emergence of cell-based therapies as a potential treatment for some degenerative retinal disorders, furthering our understanding of how homeodomain-containing transcription factors direct specific retinal cell fates from stem cells or committed progenitor pools will be critical for the generation of the desired replacement retinal cells.

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