Cell fate decisions, transcription factors and signaling during early retinal development

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Abstract

The development of the vertebrate eyes is a complex process starting from anterior-posterior and dorso-ventral patterning of the anterior neural tube, resulting in the formation of the eye field. Symmetrical separation of the eye field at the anterior neural plate is followed by two symmetrical evaginations to generate a pair of optic vesicles. Next, reciprocal invagination of the optic vesicles with surface ectoderm-derived lens placodes generates double-layered optic cups. The inner and outer layers of the optic cups develop into the neural retina and retinal pigment epithelium (RPE), respectively. In vitro produced retinal tissues, called retinal organoids, are formed from human pluripotent stem cells, mimicking major steps of retinal differentiation in vivo. This review article summarizes recent progress in our understanding of early eye development, focusing on the formation the eye field, optic vesicles, and early optic cups. Recent single-cell transcriptomic studies are integrated with classical in vivo genetic and functional studies to uncover a range of cellular mechanisms underlying early eye development. The functions of signal transduction pathways and lineage-specific DNA-binding transcription factors are dissected to explain cell-specific regulatory mechanisms underlying cell fate determination during early eye development. The functions of homeodomain (HD) transcription factors Otx2, Pax6, Lhx2, Six3 and Six6, which are required for early eye development, are discussed in detail. Comprehensive understanding of the mechanisms of early eye development provides insight into the molecular and cellular basis of developmental ocular anomalies, such as optic cup coloboma.
Lastly, modeling human development and inherited retinal diseases using stem cell-derived retinal organoids generates opportunities to discover novel therapies for retinal diseases.

Keywords
Cell determination; Ciliary marginal zone; Differentiation; Ectoderm; Homeodomain; Lhx2; Neuroectoderm; Optic cup; Otx2; Pax6; Retinal progenitor cells; Retinal pigmented epithelium; Six3; Six6

1. Introduction

Eye development is an excellent model to understand a series of precisely orchestrated and coordinated embryonic morphogenetic processes that result in the formation of highly complex three-dimensional (3D) biological structure, the camera-like eye (see Chow and Lang, 2001; Fuhrmann, 2010; Sinn and Wittbrodt, 2013; Miesfeld and Brown, 2019; Buono and Martinez-Morales, 2020). The initial processes that establish a general 3D structure of the eye, the optic cup and associated lens vesicle, occur during the final stages of gastrulation, including the formation of the anterior neural tube. Following a symmetric separation of the single retinal field within the anterior neural plate, a pair of symmetric lateral evaginations of the separated retinal field give rise to the optic vesicles. The optic cups are subsequently formed together with the lens vesicles from the surface ectoderm via a highly coordinated process of reciprocal invaginations (see Cvekl and Ashery-Padan, 2014; Cvekl and Zhang, 2017).

The major advances in the eye development were made first through the experimental embryology followed by cell, molecular biology and genetic studies of individual genes and their encoded proteins. These studies revealed major roles for sequence-specific DNA-binding transcription factors, growth factors and their natural inhibitors. Identification of genes regulating Drosophila compound eye morphogenesis, cloning of their vertebrate homologues and their loss-of-function studies, served as another driving force to dissect the cellular and molecular mechanisms of vertebrate eye development (see Desplan, 1997; Oliver and Gruss, 1997; Freund et al., 1996). Next, these pioneering studies stimulated more complex genetic studies of individual genes and their combinations as well as large-scale analyses of eye mutants in a range of model vertebrates. Conditional gene targeting in mice using the cre-loxP system allowed detailed studies of mammalian gene functions with remarkable temporal and spatial precision. Genetic data from various model organisms and studies of human congenital eye disorders were ultimately unified at the large genomic scale by the completion of the Human Genome Project in 2003. Human, mouse, rat and other mammalian genomes now facilitate comparative studies of gene function, analyses of evolutionarily conserved non-coding regions and their roles in gene control and generators of non-coding RNAs.

The establishment of multiple-OMICs as powerful unbiased methods increased the number of genes to be studied in individual tissues and organs and helps to prioritize follow up functional studies (Clark et al., 2018; Argelaguet et al., 2019; Pijuan-Sala et al., 2020). Further improvements of these high-throughput methods continuously expand the repertoire
of transformative molecular studies at the single cell levels to probe both chromatin dynamics and gene expression (Boix et al., 2021). Both individual mRNAs and proteins can be now analyzed at the single molecule level in fixed and living cells (see Tutucci et al., 2018). Implementation of high resolution microscopy and development of fluorescent proteins fused to any protein of interest advanced cell biology studies of complex tissues and organs to unprecedented levels. Finally, a toolbox of CRISPR-Cas9 based genome engineering methods drives complex genetic experiments in model organisms and offers a variety of cell labeling opportunities that ultimately raise our hopes to apply these methods towards postnatal correction of specific genetic defects (see Doudna and Charpentier, 2014).

The fundamental question is how the 3D body plan, individual tissues and organs are formed from ~230 different cell types originating from a single fertilized egg with identical DNA information using epigenetic mechanisms of gene control (see Felsenfeld, 2015; Henikoff and Greally, 2016; Moris et al., 2016). Organogenesis of complex organs, including brain and eye, share a common trajectory from pluripotent inner cell mass cells, sequential formation of three germ cell layers during gastrulation, formation of lineage-specific progenitors such as the neuroectoderm and pre-placodal ectoderm, emergence of committed progenitors such as the retinal progenitor cells (RPCs), and, finally, generation of terminally differentiated cells. These processes involve temporal and spatial orchestration of symmetric and asymmetric cell divisions generating early tissue primordia, such as the optic cup (see Adler and Canto-Soler, 2007; Casey et al., 2021).

Human and mouse genomes encode ~20,000 protein-coding genes, including >1800 DNA-binding transcription factors (Vaquerizas et al., 2009; Lambert et al., 2018) and even higher total number of non-coding RNAs (i.e. rRNAs, tRNAs, microRNAs, and other ncRNAs). The current numbers from GENCODE and FANTOM projects list 17,597 lncRNAs and 7569 small RNAs encoded in human genome (see Ali and Grote, 2020). The mammalian genomes code over 1000 genes required for normal eye function (Moore et al., 2018). The overall size of genomic regulatory sequences with elaborate cis-regulatory grammar (see Calo and Wysocka, 2013; Long et al., 2016; Jindal and Farley, 2021) significantly exceeds the size of protein coding- and ncRNA regions of the genomic DNA. Developmental studies of the eye across a wide range of vertebrate and invertebrate model organisms also inform general concepts of evolution of diverse visual systems (see Piatigorsky and Wistow, 1989; Gehring and Ikeo, 1999; Vopalensky and Kozmik, 2009; Swafford and Oakley, 2019; Koenig and Gross, 2020; Lamb, 2020).

During gastrulation and the earliest stages of tissue and organ formation, cell type identity is a transitional property. Cells acquire their final identity in parallel with their spatial localization and within a community of similar cells under the control of signaling centers as revealed by recent single cell transcriptomic analyses (Argelaguet et al., 2019; Peng et al., 2016; Peng et al., 2016; Pijuan-Sala et al., 2020; Qiu et al., 2022) and in vivo visualization of cell movements, such as those during the formation of optic vesicles (Rembold et al., 2006; England et al., 2006; Kwan et al., 2012). The molecular foundation governing these processes is tightly controlled expression of genes involved in extracellular signaling (e.g. cell surface receptors, secreted growth factors and their inhibitors), cell adhesion and polarity (e.g. cell adhesion and extracellular matrix proteins, ECMs), cell
migration (e.g. Rho family of small GTPases and regulators of actin cytoskeleton) and cell invaginations or evaginations (e.g. formation of filopodia and regulators of apical constriction). Evidence exists that these genes are under direct regulation by lineage-specific DNA-binding transcription factors and formation of transient gene regulatory networks (GRNs) (see Davidson, 2010). These GRNs, comprised from two or more lineage-restricted transcription factors, are thought to control individual stages of cell type specification and determination through direct binding to promoters and enhancers of genes encoding “morphogene tic” proteins described above. The morphogen-mediated patterning effects employ signaling gradients to initiate these GRNs (see Briscoe and Small, 2015). Recent studies of regional patterning of mouse cortical progenitors showed that following depletion of “primary” acting transcription factors disrupts regional expression of other transcription factors, involved in the same patterning processes (Ypsilanti et al., 2021).

Here we dissect the single eye field formation during gastrulation and subsequent morphogenetic movements culminating with the formation of two bilateral optic cups using the mouse as a leading mammalian model organism (Section 2). Chicken, frog, medaka and zebrafish models provide numerous invaluable insights into embryogenesis as their embryos are available in large numbers, size and are directly experimentally accessible. Section 3 is focused on five critical HD proteins Otx2, Pax6, Lhx2, Six3 and Six6, studied using mouse gene loss-of-function studies in our laboratories. Parallel roles of Sox2, Rax, Meis1, Meis2, Pax2, Vsx2, Mitf and other transcription factors during the anterior neural plate patterning and its transition into the optic cup are discussed as well. Section 4 covers human optic cup congenital defects known as optic cup coloboma, including animal models. Section 5 provides insights into recent transformative technologies of genome engineering, analyses of gene expression at single cell level, and expansion of computational biology and bioinformatics tools and their key roles towards comprehensive understanding of all mechanisms governing human eye development and relevance to congenital and multifactorial conditions leading to impaired vision.

2. **Formation of the anterior neuroectoderm, eye field and transition into bilateral optic vesicles and optic cups**

Within the neural plate, multiple morphogenetic processes occur in parallel resulting in anterior-posterior (A/P), dorso-ventral (D/V) and medio-lateral (M/L) patterning events leading to brain and eye formation. The transition from the neural plate into the neural tube represents actual “3D-morphogenesis” resulting in formation of a series of vesicles that develop at the anterior end of the neural tube, including a symmetric pair of optic vesicles, precursors of the optic cups (see Reichenbach and Pritz-Hohmeier, 1995; Wurst and Bally-Cuif, 2001). In mouse, these processes represent a developmental window between E8.0 and E12.5 embryos. Optic cup formation is morphologically similar between mouse and chick (see Adler and Canto-Soler, 2007). Fish and frog studies are discussed here to highlight numerous common modalities as well as a few specific features and their consequences for analyses of mammalian models. *In vitro* formation of optic cup-like organoid structures from pluripotent stem cells help illustrate the self-organization principles of early embryogenesis (see Rosado-Olivieri and Brivanlou, 2021).
2.1. Neuroectoderm formation

Gastrulation in mouse spans from E6.25 to E9.5 (see Bardot and Hadjantonakis, 2020). The anterior-posterior axis is established just prior the onset of gastrulation and dynamic cell fate maps (Fig. 1A) are supported by recent unbiased transcriptomic data from single cell studies (Peng et al., 2016, 2020; Peng et al., 2019; Argelaguet et al., 2019). These early cell fates are determined by the dynamically changing BMP, FGF, TGF-β/Nodal and Wnt signaling interpreted by proliferating cells (Stuckey et al., 2011). These signals are thought to control cell sorting events and hence drive topographical changes within all cell layers, as these cells migrate away from the primitive streak (see Stern, 2004; Liu and Niswander, 2005; Guillemot and Zimmer, 2011). The ectoderm is formed at E6.5 from the epiblast cells that do not pass through the primitive streak and remain on the anterior side of the epiblast. Mid-stages of mouse gastrulation (E7.0–E8.5) culminate with notochord formation (see Reichenbach and Pritz-Hohmeier, 1995; Balmer et al., 2016; Darnell and Gilbert, 2017). The notochord is a transitional rod along the head-to-tail axis originating from axial mesoderm and serves as a major signaling center for bilateral and D/V patterning of the embryo. Around E7.5, ectoderm proximal to the node generates neural plate tissue (Li et al., 2013). The anterior border region between the neuroectoderm and surface ectoderm has been defined in chicken, frog and zebrafish embryos and generates a pool of common progenitor cells, the anterior pre-placodal region, that gives rise to the lens, olfactory and adenohypophyseal progenitors (see Gunhaga, 2011; Saint-Jeannet and Moody, 2014; Cvekl and Zhang, 2017).

The formation of the ectodermal cell lineage has been probed using multi-omics profiling at single-cell resolution between E4.5 and E7.5 of the mouse embryogenesis. Through visualization of chromatin accessibility and analysis of DNA methylation, these epigenetic studies revealed that pluripotent epiblast cells are primed for an ectoderm fate starting as early as at E4.5 embryos (Argelaguet et al., 2019). Single cell RNA-seq studies covering E8.0–E9.5 (Peng et al., 2016; Peng et al., 2019; Pijuan-Sala et al., 2020) identified clusters of cells marked by expression of HD-containing transcription factors Dlx2, Dlx5, Pax6, Six1 and Six3 (Liu et al., 2006; Sato et al., 2010; Walther and Gruss, 1991; Dixit et al., 2013) consistent with the formation of a transient anterior pre-placodal region; however, additional studies focused on cell sorting, migration and epithelialization are needed. Importantly, these data support a “default” model, wherein primitive ectoderm is primed for the conversion into the neuroectoderm (Argelaguet et al., 2019).

2.2. Early patterning of the anterior neuroectoderm and eye field formation

2.2.1. Eye field formation—The neuroectoderm represents spatially organized populations of neural progenitor cells generated through patterning processes mediated by dorsaling factors (BMPs and Wnts) produced by the non-neural ectoderm and ventralizing factors (Shh) produced by the notochord and the floor plate (see Wurst and Bally-Cuif, 2001). The most anterior portion of the neural tube subsequently generates the forebrain (prosencephalon) and its major subregions, telencephalon, eye, diencephalon and hypothalamus. Recent studies revealed that mouse E7–E7.5 epiblast cells commit to a regional identity prior to acquiring their neural identity (Metzis et al., 2018). This process, called “primary regionalization”, allocates cells to anterior or posterior regions of the future.
nervous system (Metzis et al., 2018). Around E9.0, the mouse neural tube closes (see Nikolopoulou et al., 2017) and gastrulation culminates by morphogenetic resolution of the primitive streak at E9.5 (see Bardot and Hadjantonakis, 2020). As these transitional populations of lineage-restricted cells generate more complex and dynamically evolving 3D structures, the signaling environment undergoes parallel changes resulting in specification and determination of new cell types (see Table 1) and gradual formation of the bilateral optic cups (see Table 2).

Cell fate mapping experiments in frog (Eagleson and Harris, 1990; Saha and Grainger, 1992), zebrafish (Woo and Fraser, 1995; Bielen and Houart, 2012; Young et al., 2019) and chick (Fernández-Garre et al., 2002; Kobayashi et al., 2002) embryos have shown that the prospective retinal neuroectoderm, the “eye field”, is formed after neurogenesis in the medial portion of the anterior neural plate that gives rise to the ventral diencephalon.

Data on mouse are sparse due to in utero development; nevertheless, a study using the whole-embryo culture system and vital dye labeling system revealed similarities with other vertebrates (Inoue et al., 2000). Thus, current models are mostly based on comparative embryology and co-localized expression patterns of genes encoding individual transcription factors and signaling centers essential for individual stages of optic cup formation. To form the neural plate in E7.0 mouse embryos, inhibition of BMP signaling by noggin and other soluble factors such as chordin produced by the node is required (Fig. 1A, Tables 2 and 3). A schematic representation of the anterior neural plate of zebrafish, including signaling molecules and emergence of the eye field is shown in Fig. 1B.

The patterning events along the A/P, D/V and M/L axes that are controlled by concentration gradients of diffusible growth factors and other signaling mechanisms (Fig. 1B). Due to challenges in experimental manipulations, these processes remain poorly understood regarding the precise sequence of their downstream events to elicit novel gene expression patterns and subsequent intricate responses within communities of proximally and distally located cells. The A/P patterning studied in zebrafish requires inhibition of Wnt signaling at the anterior parts through secretion of Wnt inhibitors Cer1 (Cerberus), Dkk1 (Dickkopf1) and Frzb (frizzled related protein, also known as Sfrp3), produced by the underlying mesendoderm (Fig. 1B, Table 3, Glinka et al., 1998; Mukhopadhyay et al., 2001; Houart et al., 2002). In mouse, data exist showing that Dkk1 proteins are key antagonists to modulate Wnt3 activity during head morphogenesis (Lewis et al., 2008). Studies in zebrafish demonstrate that BMP signaling (Table 3) is required to separate the telencephalon from the eye field (Bielen and Houart, 2012). The neuroepithelial organization of the eye field in zebrafish requires extracellular matrix (ECM) protein Laminin1 (Lama 1) to establish the apicobasal orientation of the earliest RPCs (Ivanovitch et al., 2013).

From E7.5 of mouse embryonic development, rapid symmetric cell divisions within the neural plate result in its thickening which then begins to fold, elevate and converge at the midline (see Shparberg et al., 2019). The closing occurs in a bi-directional zipper-like manner initiating at the forebrain/midbrain level with closing at E8.5 and extending both anteriorly and posteriorly (Table 2). Hence the eye field as well as other parts of diencephalon adopt new transitional 3D shapes (see Wurst and Bally-Cuif, 2001). The key driver to symmetrically separate the eye field is Shh signaling (Table 3) which is expressed...
in the ventral forebrain and prechordal mesoderm (Fig. 1B). *Shh* gene loss-of-function in mouse results in a single eye, i.e. cyclopia (Chiang et al., 1996). Another important regulator of this process is transcription factor Six3 (see Section 3.4) which regulates *Shh* in the ventral midline of the diencephalon (Geng et al., 2008). The ~1 Mbp *Shh* locus (Anderson et al., 2014) is an excellent example of a critical gene under the control of at least 15 distal enhancers, including individual enhancers bound by Six3, Sox2 and Sox3 or Otx1 and Otx2 (Jeong et al., 2008).

### 2.2.2. Transcription factors and cell fate determination during eye field formation

At the cell fate decision level, the formation of the eye field follows the general concept of embryonic induction comprised of multiple steps along the competence → specification → determination pathway (Table 1). In the eye, these stages were first defined for lens induction using tissue transplantations in frogs (Spemann, 1901; Saha et al., 1989; Lemaire and Gurdon, 1994) and subsequently chicken models (Sullivan et al., 2004; Patthey and Gunhaga, 2014) and generalized towards mammalian models (see Gunhaga, 2011). The challenge for these studies is the absence of early morphological markers. Thus, analysis of temporal and spatial expression of DNA-binding transcription factors during the eye field formation and identification of transcriptomic signatures of individual subpopulations of cells via single cell RNA-seq provide critical insights into the initial stages of eye development.

The first systematic analysis of transcription factors in the presumptive eye field took advantage of accessible frog embryology (Kenyon et al., 2001), coupled with direct visualization of the dynamics of gene expression in the anterior neural plate integrated with earlier mouse genetic loss-of-function studies (Acampora et al., 1995; Grindley et al., 1995; Quinn et al., 1996; Mathers et al., 1997; Porter et al., 1997; Marquardt et al., 2001; Bäumer et al., 2002; Lagutin et al., 2003). These aggregate data led to a set of “eye field transcription factors” (EFTFs), including Lhx2, Nr2e1 (Tll), Otx2, Pax6, Rax, Six3, Six6 and Tbx3 (Zuber et al., 2003). The follow up studies provided additional insights into specific roles of individual “original” EFTFs, their mutual regulatory loops, and their parallel roles in early forebrain development and retinogenesis in frogs (Viczian et al., 2009; Motahari et al., 2016). Importantly, mouse studies using conditional gene targeting of Pax6 (Marquardt et al., 2001; Oron-Karni et al., 2008), Six3 and Six6 (Liu et al., 2010; Liu and Cvekl, 2017; Diacou et al., 2018) and Lhx2 (Yun et al., 2009; Gordon et al., 2013; Roy et al., 2013; Gueta et al., 2016) confirmed the general principles of the frog-based EFTF model. Cell fate maps of chick embryos also defined the prospective eye field cells within the anterior neural plate (Fernández-Garre et al., 2002; Sánchez-Arrones et al., 2009). These cells require prospective lens ectoderm-derived BMP4 (see Table 3) to maintain their eye field identity (Pandit et al., 2015).

Most recently, cell transplantation experiments using frog gastrula and early neurula stages examined both specification and determination of the retinal progenitor cells (Louie et al., 2020). Through analysis of transplanted cell fates and expression of transcription factors Sox2, Rax, Pax6 and Lhx2 and Wnt receptor Fzd5, the authors concluded that the early retinal specification occurred in cells that express Sox2 and initiate Rax expression. Determination (Table 1) was achieved 2 h later, marked by Sox2, Rax and Fzd5 expression.
while Pax6 and Lhx2 mRNAs were detected an hour later (Louie et al., 2020). As the cell cycle length is very rapid at these stages (50–90 min; see Heasman, 2006; Siefert et al., 2015), it is likely that these changes occur within one or two cell divisions that first elicit expression of the earliest EFTFs, followed by next cell division and onset of robust expression of Pax6 and Lhx2. Regarding the cell cycle, evidence exist that these cell fate processes occur during the early G1-phase of the cell cycle and during mitosis as the chromatin is being reprogrammed (see Ma et al., 2015).

We propose that a combinatorial action of two or more EFTFs elicit expression of novel cell surface molecules and receptors (see below), required for RPCs to reach the determination stage. For example, in zebrafish, ephrin receptor-ephrin signaling (Table 3) maintains segregation of the eye field from surrounding anterior neural plate domains (Cavodeassi et al., 2013). Additional examples include the inner nuclear membrane protein Nemp1 which is required for expression of multiple EFTFs in the frog model (Mamada et al., 2009) as well as Laminin1 forming the surrounding ECM described above in zebrafish (Ivanovitch et al., 2013). Recent progress with single cell RNA-seq applied to this problem provides unbiased insights into this cell fate decision trajectory in midstage mouse embryos (Cao et al., 2019; Argelaguet et al., 2019; He et al., 2020). These studies are primed to reveal a wide range of candidate genes encoding diverse proteins underlying these processes for detailed functional studies using suitable model organisms.

### 2.3. Optic cup morphogenesis: formation of the initial eye 3D-structure

Two symmetric domains of cells emanating from the separated eye field form a pair of optic vesicles. Their lateral evaginations lead to the formation of optic cups. Despite morphological differences in optic cup formation between various vertebrates described below, such as the size of the luminal space and delamination of the lens placode in zebrafish to form the lens without generation of the lens vesicle, the genetic programs underlying these processes employ homologous genes and signaling pathways (see Martinez-Morales et al., 2017).

#### 2.3.1. Optic cup formation in mouse and chick—

The bilateral evaginations of the prospective mouse forebrain generate multiple domains of cells including the optic sulci (E8.0) and optic vesicles (E8.5, Fig. 1C) with a large luminal space within the neural tube (Fig. 2A). In addition, electron microscopic studies in mouse embryos show that optic vesicle cells change their shape from columnar, into apically constricted wedge-shape, elongated and ultimately shortened as the C-shaped vesicle formations are completed (Svoboda and O’Shea, 1987).

Between E8.5-E9.0 of mouse embryonic development (Fig. 1C), the optic vesicles are formed while the neural tube closes around E8.75. At E9.0, the distal portion of the evaginating optic vesicle, the presumptive neuroretina, reaches the anterior pre-placodal ectoderm that thickens to generate the lens placode (E9.5) (see Grainger, 1992; Cvekl and Zhang, 2017). Through the reciprocal invaginations between the lens placode and optic vesicles, the 3D-structure of the early optic cup and the polarized lens vesicle are formed between E9.5-E11 of the mouse development (see Cvekl and Ashery-Padan, 2014;
In addition to optic vesicle and surface ectoderm, the neural crest derived periocular mesenchymal cells fill up the adjacent 3D space (Fig. 2) serving as the source of additional morphogenetic signals, e.g. retinoic acid (Table 3) studied in mouse (Mic et al., 2004) and TGF-β studied in chicken (Grocott et al., 2011).

The evaginating optic vesicles are patterned along their proximal-distal (P/D) axis (Fig. 2). Mouse data suggest that the distal portion of the optic vesicle in contact with surface ectoderm is slated to become neural retina while the proximal portion differentiates into the RPE and both outside proximal regions will form the dorsal and ventral portions of the optic stalk (Mui et al., 2005). The boundary between the future neural retina and RPE is called “optic cup margin” or “ciliary marginal zone” (CMZ). This CMZ is formed by E12.5 (Fig. 2) and marked by expression of transcription factors Meis1, Meis2, Msx1, Nr2f2, Otx1, and Pax6 (Martínez-Morales et al., 2001; Fujimura et al., 2009; Matsushima et al., 2011; Dixit et al., 2013; Dupacova et al., 2021). Roles for transcription factors Six3, Six6, Otx1 and Wnt/β-catenin signaling within the CMZ (Diacou et al., 2018) are described in subsection 3.6. The cells from CMZ give rise both to the ciliary body (see Beebe, 1986) and iris epithelia (see Davis-Silberman and Ashery-Padan, 2008). In mammalian models, evidence exist that a subset of CMZ progenitors contributes to neural retinogenesis (Marcucci et al., 2016; Bélanger et al., 2017).

To form the C-shape curved optic vesicles at E9.0 mouse embryos, the optic vesicles bend at multiple locations (see Fig. 2A). These epithelial bends are thought to originate from the adjacent surface ectoderm and periocular mesenchymal cells and later by invaginating lens placode and/or be established as boundaries between differentially patterned cells that adopt their definitive 3D shape to minimize the energy requirements through physical forces governing cell adhesions. The traditional approaches to investigate these morphogenetic movements included analyses of cell shape and polarity, cell-to-cell contacts across the epithelium and between each distinct layer of cells through filopodia (Chauhan et al., 2009). Other insights are obtained through studies of the apical belt tension, causing the tissue invagination (Chauhan et al., 2011) and depletion of ECM protein fibronectin 1 (Fn1) from the lens placode (Huang et al., 2011).

Recent studies analyzed five stages of mouse embryogenesis (12, 16, 20, 24 and 26-somites) covering the E8.5-E9.5 developmental window (Table 2) using scRNA-seq and microdissected eye tissues, including the optic vesicles, surface ectoderm and surrounding mesenchyme (Yamada et al., 2021b). Fourteen major clusters were identified, including seven clusters expressing high levels of EFTFs Lhx2, Pax6, Rax, and Six3. Four of these clusters (ov1-ov4) were directly linked to the optic vesicle formation (Yamada et al., 2021b). Both global (Cao et al., 2019; Pijuan-Sala et al., 2020; Qiu et al., 2022) and “microdissected” (Yamada et al., 2021b) scRNA-seq data provide rich resources of data to reveal critical genes encoding molecular components governing eye field, optic vesicle and optic cup morphogenesis.

Genetic loss-of-function studies, including elaborate single and double conditional knockouts, usually affect the patterning events and less frequently per se retinal tissue folding. For example, somatic loss-of-function of mouse Rax lack optic vesicles and lens
primordia (Mathers et al., 1997). In Lhx2 null embryos, optic vesicles are formed at E9.5; however, transformation into optic cups is blocked (Porter et al., 1997) (see section 3.5. for details). Notably, a recent study of mouse Arl13b gene encoding ADP ribosylation factor like GTPase13B expressed in cilia inverted the optic cup orientation with RPE cells facing the lens while retaining the normal basal cell polarity (Fiore et al., 2020). These unique patterning defects and expansion of ventral fates were shown through the disruption of Shh signaling (Fiore et al., 2020). Another patterning reverse was found in transcription factor AP-2α (Tfap2a) optic vesicle mutants leading to replacement of the RPE by neural retinal and optic stalk-like tissue (Bassett et al., 2010). These are significant findings that pave the road for additional studies of genes and proteins in the Arl13b, AP-2α and other pathways using mouse genetics.

Another feature of the optic vesicle and cup formation is that there is a transient ventral cleft along the entire P/D axis of the optic cup called “optic” or “choroid” fissure (Fig. 2B). This transitional morphology results from asymmetrical invagination of the optic vesicle (see Patel and Sowden, 2019; Yoon et al., 2020; Chan et al., 2021). From this stage, the ventral optic fissure gradually closes (see Section 4) and the fully formed bilayered optic cup comprised of the neuroretina and RPE is formed by E12.5 (see Table 2).

2.3.2. Optic cup formation in zebrafish and medaka—Non-canonical Wnt signaling promotes cell adhesion which is required for zebrafish eye field unity (Cavodeassi et al., 2005). Depletion of Wnt11 or eye field specific receptor Fz5 disrupts forebrain morphogenesis and delays eye field evagination (Cavodeassi et al., 2005; England et al., 2006). Additional signals coordinating these earliest RPCs migrations include co-regulation of Wnt and Eph/EphrinB1 signaling shown in Xenopus (Lee et al., 2006). The diencephalic progenitors migrate through the zebrafish eye field (Varga et al., 1999; England et al., 2006). Hence, a physical split is accomplished resulting in two bilateral eye fields with a narrow luminal space located in the center of the prospective forebrain (see Cavodeassi, 2018; Giger and Houart, 2018).

Live cell recordings of optic vesicle formation are available in zebrafish (England et al., 2006) and medaka fish (Rembold et al., 2006). The migration trajectories of individual cells through their extended lamellipodia and filopodia and gross migration patterns forming the eye field in parallel with prospective forebrain cells were examined using fluorescently tagged proteins and confocal in vivo time-lapse microscopy (Rembold et al., 2006). Modulated convergence of ventromedial (earliest) RPCs results in formation of the eye field. The cells of the lateral eye field move first towards the midline and next towards the forming the epithelium through complex cell-to-cell adhesion machinery and subcellular polarization within both symmetric optic sulci. The evagination of the eye field is driven by single-cell migration to enlarge the volume of these transient structures even though cell proliferation is very limited (Rembold et al., 2006). Interestingly, the enlargement of optic vesicles involves “softening” of the epithelialized neural tube cells within the precise domain of cells undergoing the evagination (see Sinn and Wittbrodt, 2013). The immigrating individual cells to this area intercalate and epithelialize to assure the enlargement of the optic vesicle. Following the evagination stage, cell movements through the subsequent stages of optic cup formation were analyzed by 4D time-lapse imaging and cell tracking in zebrafish (Kwan et
al., 2012). The key findings include extended period of optic vesicle evagination, elongation of the optic vesicle driven by rapid movement of cells in a coordinated pinwheel pattern, concurrent movement of lens progenitor cells at the surface, and movement of cells around both anterior and posterior rims of the invaginating optic vesicle (Kwan et al., 2012). Importantly, despite differences in chick and fish cell fate maps, cell movements found in zebrafish were also observed in the chick embryos (Kwan et al., 2012).

Early studies demonstrated that Fgf8 signaling contributes to the nasal-temporal patterning during the onset of zebrafish optic vesicle evagination (Picker and Brand, 2005). The follow up studies extended this model to a combinatorial action of Fgf8, Fgf3 and Fgf24 through expression of fgt8 and fgf3 genes in the dorsal forebrain and fgt24 in the olfactory placode (Picker et al., 2009). These dorsal sources of FGFs confine expression of two forkhead transcription factor genes, foxg1 and foxd1, to the dorsal and ventral half of the evaginating optic vesicles (Picker et al., 2009). The initial nasal-temporal cell positional identity within the optic vesicle is parallel to the D/V axis of the neural tube and subsequent cell compaction and their directed movements establish the final nasal-temporal cell subdivision of the late optic cup (Picker et al., 2009).

The asymmetry in the optic vesicle invagination requires a coordinated flow of epithelial cells around the distal rims of the optic cup (Fig. 2B) (Heermann et al., 2015). A massive increase of cells was found in zebrafish using transgenic line expressing membrane-coupled green fluorescent protein in RPCs. The origin of the cells is the lens-averted neuroepithelium that functions as a reservoir for cells migrating to their destination in the CMZ (Heermann et al., 2015). Pharmacological inhibition of mitosis via hydroxyurea and aphidicolin result in changes in cell number and their density as well as organ volume; nevertheless, mitosis is dispensable for the basic optic cup morphogenesis analyzed by 4D time-lapse imaging and cell tracking (Kwan et al., 2012). A similar conclusion regarding mitosis is applicable for expansion of the zebrafish presumptive RPE cells (Cechmanek and McFarlane, 2017). Finally, cell numbers within the optic vesicle and early optic cup were not reduced by cell death in zebrafish (Li et al., 2000).

Regarding optic fissure closure, several well characterized zebrafish models exist (see Chan et al., 2021; Yoon et al., 2020) as further described in Section 4. Like in higher vertebrates, zebrafish CMZ is established at the periphery of retina (see Reh and Fischer, 2006; Fischer et al., 2013). Live cell imaging for zebrafish CMZ (Wan et al., 2016) confirm the presence of retinal stem cells at the extreme periphery of the CMZ (Xue and Harris, 2012). Early frog studies have shown that CMZ subsequently evolves into four zones/compartments (Perron et al., 1998) marked by differential gene expression and variable rates of cell proliferation with major impact on retinal formation following the end of embryogenesis and retinal regeneration (Wan et al., 2016).

### 2.4. Optic cup formation in vitro: mechanical forces, self-organization and retinal organoids

Studies conducted in the last decade using pluripotent cell differentiation demonstrate that self-organization, i.e., spontaneous production of a highly ordered structure or pattern from cells with minimal prepattern and presence of spatially homogenous signaling cues, plays a
major role in production of 3D retinal and other organoids (see Eiraku et al., 2012; Sasai et al., 2012; Gritti et al., 2021). These studies suggest that self-organization is a general process of normal embryonic development (see Lancaster and Knoblich, 2014; Simon and Hadjantonakis, 2018).

The embryonic stem (ES) cell-derived optic cups do not require the presence of the lens in the aperture (Eiraku et al., 2011; Nakano et al., 2012) even though both normally positioned (Hayashi et al., 2016) or misplaced lenses are generated during various pluripotent cell-based retinal differentiation protocols (Mellough et al., 2015). Amongst these procedures, an earlier pioneering study found formation of the “cornea-lens-retina” primitive eye-like structures derived from cultured mouse ES cells (Hirano et al., 2003) demonstrating a range of options for pluripotent cell differentiation towards the eye and its individual tissues. Another recent experimental model generates brain-optic cup human organoids (Gabriel et al., 2021). Parallel studies of pluripotent cells from fish show that optic vesicle formation is a “hard-wired” feature (Zilova et al., 2021). The simplest explanation is that cup-like eyes without any lens evolved prior the functional “upgrade” in vision to generate the lens (see Vopalensky and Kozmik, 2009). We thus propose that the coordination of the in vivo reciprocal invagination between the lens placode and optic vesicle (Chauhan et al. 2009, 2011; Plageman et al., 2010; Muccioli et al., 2016; Houssin et al., 2020; Magalhães et al., 2021) is a logical step that evolved to assure robustness of the eye morphogenesis.

Morphogenesis requires the precise coordination of mechanical forces to produce shapes of individual tissues and organs to reach energetically sustainable mechanical equilibrium (see Hannezo and Heisenberg, 2019). The theoretical framework to describe 3D organization of epithelial sheets such as those forming the optic and lens vesicles and optic cups, have emerged in recent years (Hannezo et al., 2014; Saisas et al., 2015; Latorre et al., 2018). The epithelial apical-basal cell polarity (see Fig. 2A, E9.0 stage embryos) is determined by composition of the membranes and oriented interactions with the ECM (see Martin-Belmonte and Mostov, 2008). The cell shape is determined through a combination of adhesion and cytoskeleton contractile forces mediated by the Rho family GTPases (see Lecuit and Lenne, 2007; Montell, 2008), such as Rac1 and RhoA through the local increase of apical belt tension causing mouse lens placode invagination (Chauhan et al., 2011). Cdc42 functions via formation of F-actin-rich basal filopodia that physically tether the lens epithelial and neuroretina cells (Chauhan et al., 2009). These constriction mechanisms indeed depend on ECM attachment as shown by studies of γ1-laminin (lanc1) in zebrafish (Nicolás-Pérez et al., 2016). The external forces and mechanical equilibrium applied towards epithelial sheets can generate two adaptive responses: to buckle to relieve the stress (Trushko et al., 2020) or cell confinement through cell compression to increase their height, i.e. transition from cuboidal into columnar cell shape (Hannezo et al., 2014).

The formation of optic cups in 3D Matrigel ECM does not require external forces normally imposed by surrounding tissues, i.e. lens, periocular mesenchyme and surface ectoderm, as described above (Fig. 2) (Eiraku et al., 2011; Nakano et al., 2012). Three processes have been thus proposed to drive this self-organization: a) Increased flexibility of the distal portion of the optic vesicle, the prospective neuroretina, through local reduction of phospho-myosin light chain 2 (pMLC), b) a strong apical constriction of the boundary...
domain between the prospective neuroretina and RPE curved in an acute angle (hinge) and c) proliferation of retinal neuroepithelium and its tangential expansion to produce apically-convex invagination (Eiraku et al., 2011; Sasai et al., 2012). The apical constrictions of cells are detected by visualization of cell morphology at the hinge regions (Eiraku et al., 2011). Experimental studies are consistent with these models as pMLC levels are regulated by Rho-dependent coiled-coil kinase (ROCK) and its activity can be pharmacologically modulated by Y27632 and blebbistatin depending on the precise time when these inhibitors are used (Eiraku et al., 2011), further elaborated by our studies (Lowe et al., 2016). Finally, treatments with mitotic inhibitor aphidicolin blocks the final stage of optic cup organoids described above (Eiraku et al., 2011). In addition, computer simulation further supports this “relaxation-expansion” model of optic cup morphogenesis (Eiraku et al., 2011; Okuda et al., 2018). Taken together, optic cup formation in vivo is a product of coevolution of self-organization needed to produce ancestral cup-like primitive eyes in the absence of any lens. Evolution of camera-like eye required addition of the optical components, including the lens and cornea (see Jonasova and Kozmik, 2008). Hence, complex optic cup morphogenesis in vivo involves coordination with other proximal ocular cells such as the prospective lens ectoderm and periocular mesenchymal cells.

3. **Otx2, Pax6, Lhx2, Six3 and Six6: Model transcription factors and their roles in early retinal development**

The formation of eye field and subsequent stages of optic cup formation require a coordinated action of multiple sequence-specific DNA-binding transcription factors such as Lhx2, Otx2, Pax6, Rax, Six3, Six6, Sox2 and Tbx3. Vsx2 (formerly Chx10) is considered as the earliest specific marker of RPCs (Liu et al., 1994; Rowan et al., 2004). Herein we focus on Otx2, Pax6, Lhx2, Six3 and Six6 as model transcription factors examined by loss-of-function genetic studies throughout multiple stages of retinal development. Roles of additional transcription factors, such as Meis1, Meis2, Rax, Nr2e1 (Tlx, Tll), Nr2f1 (COUP-TFI), Sox2, Vax1, Vax2, Vsx2, Tbx3 and Tfap2a (AP-2α) as well as signal regulated transcription factors, such as Gli2 (Shh), Rbpj (Notch) and Smads (BMP/TGF-β signaling) are also discussed. The early progenitors employ factors expressed in many related cell types while in terminally differentiated retinal cells, the amacrine cells, bipolar cells, cone and rod photoreceptors, horizontal cells, Müller glial cells, retinal ganglion cells and retinal pigmented epithelium (RPE), “late onset” cell type-specific factors such as Crx and Nrl are employed by rod and cone photoreceptors only, respectively, as reviewed elsewhere (see Livesey and Cepko, 2001; Harada et al., 2007; Hennig et al., 2008; Byerly and Blackshaw, 2009; Fuhrmann, 2010; Heavner and Pevny, 2012; Fujimura, 2016; Amram et al., 2017; Miesfeld and Brown, 2019; Seritrakul and Gross, 2019; Raeisossadati et al., 2021). Importantly, recent studies revealed novel molecular mechanisms of eye regulatory transcription factors, namely their dynamic and stable interactions with chromatin as discussed below.
3.1. Transcription factors, generation of new cell lineages and formation of cell type memory

To activate expression of new target genes that are required for formation of novel cellular phenotype, individual lineage-specific DNA-binding factors need to bind to either transcriptionally “poised” or “bivalent” chromatin, marked by a combination of active and repressive histone posttranslational modifications (H3K4me3 and H3K27me3) or even compacted and transcriptionally silent “closed” heterochromatin (marked by H3K27me3 and H3K9me3) (Boyer et al., 2005). Studies of chromatin “opening” by transcription factors FOXA1 and GATA4, key regulators of albumin gene expression in early liver cells, coined term “pioneering” transcription factors (Cirillo et al., 2002) to indicate their ability to bind nucleosomal DNA in the absence of any other similar protein and chromatin remodeling complex (see Zaret, 2020). Pioneering transcription factors thus play a primary role in establishing competence (see Table 1) for new gene expression patterns (see Iwafuchi-Doi, 2019).

Follow up studies revealed additional structurally distinct transcription factors such as paired domain (PD) and HD PAX7 (Budry et al., 2012) and ETS domain PU.1 (Minderjahn et al., 2020) with in vivo pioneering activities. However, in the eye and retina, pioneering transcription factors remain mostly unknown and require experimental studies. A team of SOX2 and catalytically independent PARP-1 pioneering activities conquers specific intractable genomic loci (Liu and Kraus, 2017) (Fig. 3). The pioneering activity of early factor Otx2 (see section 3.3) is highly context dependent (Buecker et al., 2014). Another pioneering factor RBPJ (Fernandez Garcia et al., 2019) regulates retinal development after optic cup formation (Rowan et al., 2008). The pioneering activity of Gata3 (Tanaka et al., 2020) and TFAP2A (Fernandez Garcia et al., 2019) established in other systems could play roles in lens development; however, loss-of-functions of these genes do not affect the earliest formation of lens precursor cells (Maeda et al., 2009; Martynova et al., 2019; Pontoriero et al., 2008). Thus, additional data are needed to determine occupancy of pioneering transcription factors within the chromatin landscape together with visualization of accessible domains using cells from midstage embryos.

Current studies show that structurally distinct transcription factors employ different strategies to bind to nucleosomal DNA (Zhu et al., 2018). For example, Sox2 locally distorts nucleosomal DNA, facilitates detachment of DNA from nucleosome and affects conformation of N-terminal histone H4 tail required for nucleosome stacking (Dodonova et al., 2020). Following pioneering transcription factors binding to DNA (Fig. 3), recruitment of BRG1-containing chromatin remodeling BAF complexes is a logical next step to elicit changes in local chromatin structures within the promoters and distal enhancers that are required for specific gene activation (see Hoffman et al., 2018).

Establishment and maintenance of cell type memory is the fundamental epigenetic regulatory mechanism (see Kadauke and Blobel, 2013; Palozola et al., 2019). Two general mechanisms have been proposed. First, an increasing number of transcription factors has been shown to stably associate with chromatin during mitosis through “mitotic bookmarking” (see Kadauke and Blobel, 2013; Bellec et al., 2018; Palozola et al., 2019). Two ubiquitously expressed and universal sequence-specific transcription factors TBP, a
TATA-box binding subunit of general transcriptional factor TFIID and CTCF, a global regulator 3D chromatin organization and DNA looping, share this property (Christova and Oelgeschlaeger, 2002; Chen et al., 2002; Teves et al., 2016; Owens et al., 2019).

In early eye development, the prototypic mitotic bookmarking factor is SOX2 (Teves et al., 2016) (see sections 3.3. and 3.5). Recently, mitotic bookmarking of Pax6 has been demonstrated in non-ocular cultured cells (Lan et al., 2021). RBPJ, the major transcriptional effector of Notch signaling, is another important bookmarking factor (Lake et al., 2014). Studies of Drosophila HD transcription factors Prospero (vertebrate homologue: Prox1) have also shown that this transcription factors is mitotically implanted and regulated via liquid-liquid phase separation (Liu et al., 2020); nevertheless, Prox1 is not required for retinal progenitor cell formation but their proliferation (Dyer et al., 2003). Histone variants H3.3 (Ng and Gurdon, 2008; Gehre et al., 2020) and macroH2A (Pasque et al., 2011) maintain active and repressed states, respectively, through their binding within transcriptionally active genes (see Nowak and Corces, 2004) and retargeting into the same heterochromatic regions in G1 phase of the cell cycle (Sato et al., 2019). Second, autoregulation of lineage-specific transcription factors assures that their expression is minimally disrupted following cell division (see Hobert, 2011; Ptashne, 2013a, 2013b). Autoregulation of early retinal transcription factors Otx2 and Pax6 is discussed below.

Taken together, these two molecular mechanisms are independently linked to specific transcription factors and their unique roles in earliest stages of development and to establish and propagate cell type memory as the fundamental epigenetic process throughout the organogenesis and final tissue maintenance.

3.2. Cis-regulatory grammar of temporally and spatially controlled transcription

Specific combinations of typically between three to five DNA-binding transcription factors execute the cis-regulatory grammar of any tissue-specific promoters and enhancers and lead to the formation of promoter-enhancer loops (Fig. 4) and ultimately assembly of over 100 proteins/enzymes involved in transcriptional initiation and elongation (see Berger, 2007; Roeder, 2019; Hamamoto and Fukaya, 2022).

Individual promoters contain a single or a cluster of adjacent transcriptional start sites that are defined by a presence of a small set of “core” promoter cis-regulatory sites such the TATA-box, initiator, and downstream core promoter element, across approximately 70–80 bp (see Kim and Shiekhattar, 2015; Vo Ngoc et al., 2017). These sites are recognized by TATA-box binding protein (TBP) within the TFIID complex, TAF1/TAFII-250 and TAF2/ TAFII-150, and TAF1 largest subunits of the TFIID complex, respectively (Louder et al., 2016). The core promoters expand in both 5′- and 3′-directions into 150–250 bp promoter regions that include additional cis-regulatory sites recognized by sequence-specific DNA-binding transcription factors and that may regulate tissue-specificity and/or regulation by growth factor signaling. The hallmark of promoter cis-regulatory grammar is the precision in spacing between individual cis-sites (Fig. 4B) since even changes at single base pair (bp) level perturb spatial positions of individual transcription factors with respect to the 10.5 bp helical periodicity of DNA in B-conformation (see Vo Ngoc et al., 2017).
The distal enhancers (commonly 200–500 bp of DNA) are comprised from concentrated clusters of cis-sites recognized by a combination of lineage-specific and signal-regulated transcription factors (see Barolo and Posakony, 2002; Long et al., 2016). A prototypic enhancer is comprised from a cluster 3–6 individual cis-binding sites with both flexible and rigid spacing. Two or three similar DNA sequences (in either orientation) that are recognized by the same transcription factor are often present. Additional mechanisms such as synergistic binding between two adjacent transcription factors, e.g. Pax6 and Sox2 (see below), and possibilities to change local DNA shape (see Calo and Wysocka, 2013; Spitz and Furlong, 2012; Jindal and Farley, 2021) are also involved (Fig. 4B). Many enhancers can function with different promoters although there are some notable exceptions described in our studies of the mouse αA-crystallin locus (Yang et al., 2006). As a result of the promoter-enhancer looping (Fig. 4C), enhancers act from both proximal and far distal positions in each 5′ or 3′-promoter position (often over 100 kbp and sometimes even over 1 Mbp from the promoter) and independent of their orientation (see Dekker et al., 2017).

A number of active enhancers generate short bidirectional noncoding RNAs, called enhancer RNAs (eRNAs, Fig. 4A) (see Lam et al., 2014; Arnold et al., 2020). Presence of eRNAs detected by RNA-seq together with H3K4me1/H3K27ac enrichments can be used to understand gene control of complex loci with dozens of complex tissue-specific enhancers such as those found in the Pax6 locus (see 3.4). Three general mechanisms of eRNA have been proposed: a) Strengthening of promoter-enhancer looping through additional eRNA:protein interactions (e.g. with Mediator complex, cohesin and transcription factor YY1), b) eRNA-dependent increase of histone acetylation catalyzed by CBP/p300, and c) eRNAs promote release of RNA polymerase II from the promoter-proximal pause into the elongation phase (see Hou and Kraus, 2021).

Some loci, especially those encoding genes that define cell type identity, contain clusters of enhancers that occupy 5–10 kbp of DNA and are called “super-enhancers” (Whyte et al., 2013). They are visualized as regions marked by robust H3K27ac modified histones and presence of co-factors such as Mediator and BRD4. The Mediator complex of over 31 individual subunits interacts with both individual transcription factors and RNA polymerase II (see Fig. 4A). Recent studies of cell fate determination point to both additive and synergistic actions of multiple enhancers activated by lineage-specific transcription factors that can be predicted from eRNA data coupled with active H3K4me1 chromatin domains (Choi et al., 2021).

### 3.3. Otx2: early on stage and more than meets the eye

A bicoid-class homeobox gene Otx2, together with its structurally similar Otx1 and more divergent Crx, are three mammalian homologues of Drosophila ocelliless (oc, known earlier as orthodenticle, otd), a key regulator of fly anterior brain and photoreceptor differentiation (see Simeone, 1998). Otx2 (Fig. 5A) is a multifunctional transcription factor which is expressed earlier than any other eye field marker gene in the anterior neuroectoderm.

Otx2 (OTX2) gene is localized on syntenic regions of mouse and human chromosomes 14. The coding sequences occupy 10 kbp while the entire locus spreads over 300 kbp of genomic DNA, including poorly understood Otx2os1 IncRNA (Alfano et al., 2005).
optimal DNA-binding site contains the 5′-TAAT-3′ sequence (Fig. 5A) that is recognized by many other HD-containing TFs. Nevertheless, many unique nucleotides flank this core tetranucleotide to improve the specificity (Berger et al., 2008; Jolma et al., 2015).

The protein-interacting partners of Otx2 relevant to eye morphogenesis include basic helix-turn-helix (bHLH) E-box binding transcription factor Mitf in chicken RPE cells (Martínez-Morales et al., 2003). Other partners identified in chicken include HD transcription factors Meis2 (Agoston and Schulte, 2009) and transcriptional corepressor Tle4 (also known as Grg4), a WD40 containing motif protein and negative regulator of Wnt signaling (Puelles et al., 2004). The posttranslational modifications of Otx2 remain poorly understood (Fig. 5A). It has been shown in frogs that interaction between Otx2 and its ubiquitously expressed corepressor Tle1 (also known as Grg1) is regulated by its phosphorylation (Satou et al., 2018). Nevertheless, precise roles of these posttranslational modifications and protein-protein interactions remain to be determined.

To form specific cis-regulatory grammar, both the Otx2-binding sites and Otx2 proteins require additional properties. There are both monomeric and dimeric binding sites and cooperating structurally similar DNA-binding partners (Yasuoka et al., 2014), a general property of other classes of transcription factors (Jolma et al., 2015). Using frog gastrulas, ChIP-seq studies have shown that Otx2 binding next to Lim1 or Gsc HD-proteins activates or represses expression of batteries of target genes, such as those encoding signaling proteins chordin (chrd) and wnt8a, respectively (Yasuoka et al., 2014). Otx2 in vivo binding sites have also been studied using ChIP-seq in 4–5 week old retinas dissected into the neural retina and RPE (Samuel et al., 2014). It is noteworthy that cis-regulatory grammar of similar transcription factors Crx contain multiple binding sites with different orientation and spacing to regulate both shared as well as cone- and rod-specific genes (Corbo et al., 2010). This principle is likely to be applicable for cis-regulatory grammar of Otx2-regulated promoters and enhancers in other cell types. In any case, identification of genes directly regulated by Otx2 between E7.0–E9.5 as well as the prospective RPE cells will require implementation of CUT&RUN and/or CUT&Tag chromatin landscape analysis methods that require much smaller number of cells compared to traditional ChIP-seq studies (Meers et al., 2019; Kaya-Okur et al., 2020).

In mouse embryos, Otx2 is initially widely expressed in the epiblast and other parts of the pre-streak embryos (Simeone et al., 1993). After the onset of gastrulation, Otx2 is expressed in the entire embryonic ectoderm (see Table 2). In midstreak embryos (E7.0–7.5), Otx2 is expressed in the anterior ectoderm domain corresponding to presumptive forebrain and midbrain regions (Simeone et al., 1993). In E8.0 mouse embryos this pattern continues, including the prospective eye field (Table 2). From E9.5–E10, the prospective RPE cells of the optic vesicle primarily express Otx2 followed by its reduction in the RPCs (Martínez-Morales et al., 2001). Subsequently, Otx2 protein is detected and required for formation of both precursors of photoreceptors (Nishida et al., 2003; Béby et al., 2010; Emerson et al., 2013; Housset et al., 2013), bipolar cells (see Livesey and Cepko, 2001; Fuhrmann, 2010; Miesfeld and Brown, 2019; Seritrakul and Gross, 2019) and RPE (Martínez-Morales et al., 2003). Somatic depletion of Otx2 in mouse embryos disrupts prospective forebrain and midbrain development from E6.5 and demonstrates its critical role.
for anterior neuroectoderm specification (Acampora et al., 1995). At E17.5 mouse embryos, heterozygous Otx2 mutations display a range of eye abnormalities including anophthalmia and microphthalmia (Matsuo et al., 1995) in agreement with human genetic studies (see Section 5).

Another Otx2 mechanism is both cell autonomous regulation of genes encoding cell adhesion proteins, including retinal R-cadherin (Cdh4) and ephrin-A2 (Efna2), and non-cell autonomous regulation of genes encoding HD-proteins En2 and Six3 in mouse E8.5-E9.5 embryos (Rhinn et al., 1999). Otx2 also regulates cell-autonomously expression of homebox-gene Hex1 and Wnt1 in mouse neuroectoderm (Rhinn et al., 1999). In Xenopus, Otx2 and Sox2 regulate together expression of Rax (Danno et al., 2008). Mouse Rax is a critical HD EFTF required for optic vesicle development and is expressed both in the eye field and prospective hypothalamus (Mathers et al., 1997; Lu et al., 2013). From mouse chimera studies using wild type and Rax null cells, it has been proposed that Rax regulates expression of cell-surface molecules involved in cell sorting events in the eye field (Medina-Martinez et al., 2009). This mechanism is further supported by studies of Cxcr4a gene encoding seven transmembrane regions chemokine receptor (human homologue: CXCR4/CD184) that controls boundary formation through its expression in the zebrafish eye field to prevent cell mixing (Bielen and Houart, 2012).

Transcriptional regulation of the Otx2 locus is controlled by multiple distal evolutionarily conserved enhancers (Kurokawa et al. 2004a, 2004b, 2014; Wilken et al., 2015; Sakurai et al., 2010; Westenskow et al., 2009; Takasaki et al., 2007; Bhansali et al., 2020; Kaufman et al., 2021). The main principle of Otx2 gene control is its repression by Gbx2 HD transcription factor through a forebrain/midbrain FM enhancer (Inoue et al., 2012) and its activation by Rax using EELPOT (15 kbp) enhancer in the mouse neuroretina (Muranishi et al., 2011). In addition, mouse DHS-4D enhancer is regulated by bHLH factors Ascl1 and Neurog2 either redundantly or using a compensatory mechanism (Kaufman et al., 2021). Another important mechanism is Otx2 autoregulation in mouse (see 3.1.) via multiple enhancers (Westenskow et al., 2009; Bhansali et al., 2020). Collectively, at least seven retinal-enhancers have been identified in the Otx2 loci with different spatial and temporal expression properties (Emerson and Cepko, 2011; Muranishi et al., 2011; Bhansali et al., 2020; Kaufman et al., 2021).

Additional studies will require systematic deletion of individual enhancers and their combinations in genomic DNA followed by analyses of mouse embryos as well studies of the Otx2 locus by Hi-C and 4C-seq using chromatin between E7.5-E10.5 to identify additional candidate enhancers through the identification of DNA looping patterns as we described elsewhere (Bhansali et al., 2020). The activities of individual enhancers will then be probed to examine how they particularly respond to BMP, FGF, Notch, Shh, TGF-β and Wnt signaling.

### 3.4. Pax6: indispensable multitasker

Pax6 is arguably the most important transcription factor for eye development as it is expressed in all ocular cell progenitors, including neuroectoderm (Walther and Gruss, 1991; Grindley et al., 1995; Marquardt et al., 2001), surface ectoderm (Walther and Gruss, 1991;
Grindley et al., 1995; Ashery-Padan et al., 2000) and periorcular mesenchyme (Baulmann et al., 2002) and in a range of differentiated ocular cell types (see Shaham et al., 2013). Pax6 is a PD and HD containing EFTF (Fig. 6) first discovered within a group of vertebrate homologues of Drosophila Paired (Prd) gene (Walther and Gruss, 1991) with a follow up isolation and characterization of four entirely novel Drosophila genes, eyeless (ey), eye gone (eyg), twin of eyeless (toy) and twin of eyegone (tog) (see Shaham et al., 2013; Cvekl and Callaerts, 2017). Eyeless is the closest homologue of vertebrate Pax6 genes. Human PAX6 (Ton et al., 1991) is located on chromosome 11p13. Mouse Pax6 (Hill et al., 1991) is located on a syntenic region of chromosome 2. Their loci occupy over 400 kbp of genomic DNA and their protein sequences are identical (see Hanson and Van Heyningen, 1995; Shaham et al., 2013; Cvekl and Callaerts, 2017).

The major form of Pax6 is a protein with N-terminal PD, comprised of PAI and RED subdomains that independently bind to DNA (Xu et al., 1999a; Epstein et al., 1994a, 1994b), internal HD and C-terminal intrinsically disordered transcriptional activation domain (Fig. 6A,D). Alternate splicing generates Pax6 (5a) proteins with a 14 amino acid insertion in the PAI subdomain that no longer binds DNA (Epstein et al., 1994b; Kozmik et al., 1997). Thus, its DNA-binding is mediated by the RED subdomain and/or the HD (Epstein et al., 1994b; Xie and Cvekl, 2011). The third form is paired domain-less protein Pax6(HD), also known as Pax6 (APD) or Pax6 p32/p33, that binds to DNA as homodimer (Czerny and Busslinger, 1995; Wilson et al., 1996). Binding of Pax6 and Pax6 (5a) to DNA was examined both in vitro (Epstein et al., 1994a, 1994b; Kozmik et al., 1997; Xie and Cvekl, 2011) and in vivo (Sun et al., 2015, 2016; Swisa et al., 2017) and the “optimal” binding sites that emerged from these studies are shown in Fig. 6C.

The Pax6-interacting DNA-binding transcription factors include Sox2 (Kamachi et al., 2001; Hu et al., 2017), TBP and pRb (Cvekl et al., 1999), and various HD-containing transcription factors, such as Lhx2, Prox1, Rax, Six3 and Vsx2 (Mikkola et al., 2001); nevertheless, their natural joint target cis-sites and genes remain understudied (Matsushima et al., 2011). Likewise, chromatin remodeling complexes associated with Pax6, such as BAF (via Brg1/Smarca4), ISWI (via Snf2h/Smarca5), Mll1, Mll2, p300 and CBP were only systematically studied in lens cells (Sun et al., 2016). Regarding transcriptional repression, NuRD complexes with histone deacetylases Hdac1 and Hdac2 and ATP-dependent subunits Chd4 and Chd8 were also found in lens cells (Sun et al., 2016). In all mouse tissues examined by global transcriptomic analyses, Pax6 functions as both transcriptional activator and repressor (Wolf et al., 2009; Huang et al., 2011; Shaham et al., 2013; Farhy et al., 2013; Walcher et al., 2013; Sun et al., 2015); however, the underlying molecular mechanisms remain unknown. We propose that these dual activities are regulated by Pax6 posttranslational modifications that affect interactions with chromatin remodelers described above and precise cis-acting regulatory grammar and interactions with adjacent transcription factors, also appropriately modiOed by their posttranslational modifications at the Pax6-regulated promoters and enhancers. Pax6 proteins are phosphorylated by Erk1/2, MAPK p38 and homeodomain-interacting protein kinase HIPK2 (Fig. 6A, Mikkola et al., 1999; Kim et al., 2006) and dephosphorylated by protein phosphatase 1 (Yan et al., 2007). Sumoylation of the Pax6(HD) was also detected (Yan et al., 2010). However, Pax6 posttranslational modifications catalyzed by these enzymes have never been probed during
the early eye development. In contrast, studies in *Xenopus* eye focused on optic stalk formation revealed that hedgehog-dependent E3-ligase Midline1 (Mid1) regulates ubiquitin-mediated proteasomal degradation of Pax6 (Pfirrmann et al., 2016). Interestingly, our data show that expression of Mid1 in mouse retina is also under the Pax6 control (Farhy et al., 2013).

Pax6 expression is first detected by *in situ* hybridization in the prospective forebrain-hindbrain of E8.0 mouse embryos (Walther and Gruss, 1991), including the eye field (see above); however, recent scRNA-seq studies found expression of Pax6 mRNAs already in a variety of cells in the E7.5 embryos, including the ectoderm (Peng et al., 2016; Peng et al., 2019). The latter findings suggest earlier role in neural specification corresponding with a report that PAX6 is essential for neural ectoderm cell fate in human system (Zhang et al., 2010).

Following the onset of Pax6 expression in the mouse anterior neural plate described above, bilateral expression of Pax6 is detected in the optic sulci at E8.5 and strong expression propagates in all cells of the forming optic vesicles and cups (Table 2). Pax6 is expressed both in proximal and distal cells of the optic vesicle and cup as well in the surface ectoderm giving rise to the prospective lens ectoderm, lens placode and lens vesicle (Walther and Gruss, 1991). Following lens vesicle formation and its separation from the surrounding surface ectoderm, expression of Pax6 continues within the “restored” surface ectoderm and its subsequent differentiation into the corneal epithelium (Walther and Gruss, 1991). Pax6 is also expressed in the perioculcular mesenchymal cells (Fig. 2), cells that migrate into the eye and form other eye structures, i.e., corneal endothelium and stroma, part of the iris and trabecular meshwork (Baulmann et al., 2002) as well as in the lacrimal gland (Kammandel et al., 1999; Makarenkova et al., 2000).

Loss-of-function studies of *Pax6* in mice were conducted using both somatic (Hogan et al., 1986; Hill et al., 1991; Grindley et al., 1995; Quinn et al., 1996) and conditional mutants using different cre-lines (Marquardt et al., 2001; Oron-Karni et al., 2008; Farhy et al., 2013; Klimova and Kozmik, 2014). In addition, compound heterozygous and homozygous mutants with other functionally related genes such as Pax2 (Bäumer et al., 2003) and Sox2 (Matsushima et al., 2011) have been analyzed. Mouse chimeras, containing mixture of wild type and Pax6-deficient cells were also used for analysis of formation of individual ocular tissues (Collinson et al. 2000, 2001).

The key initial findings were that *Small eye (Sey)* mouse homozygous model *Sey/Sey* lacks the eyes (Hogan et al., 1986) and that the causative mutation G194X in the Pax6 coding sequence generates premature stop codon (Hill et al., 1991). However, as Pax6 expression includes all eye tissues, it is unclear how Pax6 mutations cause all these interconnected eye developmental defects as each tissue express Pax6 proteins with variable temporally and spatially regulated intensities and the defects found can be caused by both cell autonomous and non-cell autonomous functions of Pax6 proteins. Thus, conditional inactivation of Pax6 and analyses of chimeric mice were required to address these issues.
Using α-cre line active from E10.5 and driven by retinal α-Pax6 enhancer, our data demonstrate that Pax6 is required for multipotency of RPCs (Marquardt et al., 2001) and their proliferation (Oron-Karni et al., 2008). Both dysregulation of cyclin D1 and aberrant accumulation of negative regulator of cell proliferation Cdkn1c (p57Kip2) were found using mRax-Cre at E10.5 mouse embryos/advanced optic cup stage (Klimova and Kozmik, 2014). Others have found reduced expression of a set of cell cycle regulators Cdkn1a (p21), Cdknb (p27Kip1), Cdknc and Cdkna encoding two distinct Ink4a/p16 and Arf/p19 proteins in cultured E9.5 optic vesicles from Pax6 null mouse embryos (Duparc et al., 2007).

The neural competence in the retina requires a precise expression levels of Pax6 and Sox2 in the optic cup progenitor cells (Matsushima et al., 2011). Importantly, Sox2 is required for proliferation of RPCs within the optic cup and depletion of Sox2 initiates gradual cell fate conversion from retinal to ciliary body progenitors (Matsushima et al., 2011). Expression of proneurogenic bHLH transcription factors, i.e., Atoh7 (Math5), Mash1, Neurod1, Ngn2 and Neurod4 (Math3), is regulated by Pax6 in mouse RPCs (Oron-Karni et al., 2008; Klimova and Kozmik, 2014). Current data show that Pax6 directly regulates Atoh7 through the retinal enhancer (Hufnagel et al., 2007; Riesenberg et al., 2009). ChIP-seq data show that Pax6 directly binds both Neurod1 and Ngn2 loci in mouse E12.5 forebrain (Sun et al., 2015) raising the possibility of similar mechanisms in the RPCs. In the mouse optic vesicles, Pax6 has also been shown to directly regulate expression of Ctnnd1 gene encoding a large cytoskeletal δ-catenin protein (Duparc et al., 2006). Segregation of wild type and Pax6 null cells in chimeric mouse embryos was evident through disrupted P/D patterning of the optic vesicle and was linked to cell adhesion defects of mutated cells (Collinson et al., 2000). Further studies are required to determine the entire spectrum of directly regulated genes by Pax6 in the RPCs.

Pax2, a PD transcription factor lacking the HD, is also expressed in the eye as both Pax2 and Pax6 are required for specification of pigmented epithelium lineage of the eye through joint regulation of Mitf, a key transcription factor controlling the pigmentation (Baumer et al., 2003). In the neural progenitors of the optic vesicle, Pax6 is required for inhibition of premature expression of “late” differentiation genes, including bHLH Ascl1 (Mash1) (Philips et al., 2005) and HD-containing transcription factor Crx (Oron-Karni et al., 2008; Klimova and Kozmik, 2014). Once the bilayered optic cups form, the expression of Pax6 and Pax2 do not overlap and inhibitory interaction between these transcription factors is thought to maintain the optic stalk and optic cup progenitor cell territories (Schwarz et al., 2000). Pax6 proteins are subsequently degraded in the optic stalk by Mid1 as described above (Pfirrmann et al., 2016). Mutations in both human PAX2 (Sanyanusin et al., 1995) and PAX6 (Azuma et al., 2003) cause optic cup coloboma (see below). Recent studies have shown that mouse cells forming the optic nerve head are tripotential towards neural retina, RPE and optic stalk fates (Bosze et al., 2021).

Regulation of Pax6 transcription in various progenitor and differentiated ocular cells is a complex process due to numerous evolutionarily conserved non-coding regions, with a significant portion of them acting as distal enhancers (see Shaham et al., 2012). Ocular expression of Pax6 originates from an upstream promoter P0 in lens while retinal and brain
transcription originates from the downstream promoter P1 (Xu et al., 1999b). The first enhancer known to be active in retina is “α-enhancer” (Plaza et al., 1995; Kammandel et al., 1999). This highly evolutionarily conserved 530 bp intronic enhancer appears to be required for the distal\textsuperscript{high}-proximal\textsuperscript{low} gradient of Pax6 expression and is used to generate the α-cre line discussed above (Marquardt et al., 2001) and is autoregulated (Bäumer et al., 2002). Our studies confirmed this gradient of Pax6 expression in human retinal organoids where VSX2\textsuperscript{+} cells appear first in the lower PAX6\textsuperscript{+} expressing cells (Lowe et al., 2016). The high levels of Pax6 in the peripheral optic cup, mediated by α-enhancer, seems to be important for establishing the iris progenitors and eventual iris growth, which is hampered in human PAX6 heterozygous mutations leading to aniridia (Davis-Silberman et al., 2005). Mouse studies have shown that this enhancer proved useful for conditional gene targeting in the peripheral optic cup and revealed Pax6 role for multipotency of the retinal progenitors (Marquardt et al., 2001) as well as roles of Six3 and Six6 described in subsection 3.6. Meis1 and Meis2 transcription factors also bind the mouse Pax6 α-enhancer in vivo (Dupacova et al., 2021). In the postnatal mouse retina, activity of this enhancer is inhibited by HD and LIM domain transcription factors Lhx3 and Isl1 that interact with transforming growth factor β\textsuperscript{1}-induced transcript 1 protein (Tgfbl1l, also known as ARA55 or Hic-5) (Kim et al., 2017), a transcriptional co-activator containing four LIM zinc-binding domains (Wang et al., 2005). Additional enhancers active in the retina remain poorly understood (Coutinho et al., 2011; Bhatia et al., 2014) as their genomic deletions have not been systematically evaluated and use of shadow enhancers in the mouse Pax6 locus may go well beyond those active during both lens (Antosova et al., 2016) and retinal development (Dupacova et al., 2021).

It remains unclear how Pax6 expression is regulated in a spatiotemporal manner. For example, in Rax null mouse embryos, expression of Pax6, Otx2 and Six3 was abolished in the mutated optic vesicles and lenses were not formed; however, expression of these factors was normal in the anterior neural plate (Zhang et al., 2000). Interestingly, lens absence in the Rax\textsuperscript{−/−} embryos can be restored through the depletion of β-catenin proteins in the head surface ectoderm (Swindell et al., 2008). The DNA-binding and transcriptomic studies of Lhx2, Otx2, Pax6, Rax and Sox2 in the early mouse eye structures remain to be performed to further dissect their regulatory circuits and how they control Pax6 locus expression in the eye field and subsequent stages of eye development.

Pax6 expression in the retina is regulated by multiple signaling pathways, i.e. BMP, Shh and TGF-β signaling (see Table 3). Their respective signaling molecules and their agonists and antagonists are generated by surrounding tissues as well as within the optic vesicle and cup itself (Table 3). Expression of their receptors is also spatially and temporally regulated. A concept based on two diffusible growth factors, the Turing network, can be used to explain general morphogenesis (see Green and Sharpe, 2015). Recent studies have shown Turing network, comprised of Pax6, TGF-β and follistatin (Fig. 7), as a possible spontaneous initiator of retinal development in chick embryos (Grocott et al., 2020). In human ES cells, TGF-β/Smad2/3 signaling promotes new cell fate only during the early G1 phase of the cell cycle (Pauklin and Vallier, 2013). It has been shown in Xenopus that noggin, an inhibitor of BMP signaling, induces Pax6 expression in the anterior neural plate, including the eye field (Motahari et al., 2016). BMP4 expression is repressed by EFTF Tbx3 and Pax6 together with Tbx3 control early retinal formation (Motahari et al., 2016). Moreover, Pax6 directly
and indirectly regulates various components of Ephrin, FGF, Notch, RA, TGF-β and Wnt signaling (see Table 3) in a plethora of ocular tissues (see Cvekl and Callaerts, 2017).

Taken together, while several mechanistic insights into the formation of eye field and subsequent stages of optic cup formation by Pax6 proteins already exist, a large body of work lies ahead to define gene control of Pax6 during the earliest stages of eye development, identification of its direct target genes, and elucidation of protein-protein interactions that determine Pax6 roles as transcriptional activator or repressor.

3.5. Lhx2 and the partnership with Ldb1/2: retinal-cortical parallels

Lhx2 is another evolutionarily conserved HD transcription factor (Fig. 5B) that is essential for both early retinal and hypothalamus development (see Byerly and Blackshaw, 2009). Lhx2 belongs to large family of LIM-homeodomain (LIM-HD) and LIM-only (LMO) proteins. The LIM domain, which is the hallmark of this family, consists of two N-terminal zinc-finger-like motifs that mediate protein-protein interaction (see Kadrmas and Beckerle, 2004). LHX2/Lhx2 genes are located on syntenic regions of human and mouse chromosomes 9 and 2, respectively. Lhx2 is the mammalian ortholog of the fly Apatours (Ap) gene (Rincón-Limas et al., 1999). Ap was the first LIM-homeobox gene isolated in Drosophila and was termed dorsal selector gene for controlling the dorsoventral identity in the wing disc (Cohen et al., 1992; Diaz-Benjumea and Cohen, 1993; Blair et al., 1994).

In mouse E14 and P2 fluorescently sorted RPCs, DNA-binding of Lhx2 was examined using ChIP-seq. Two closely related motifs, both with 5′-(A/C)ATTA-3′ core sequences, were generated (Zibetti et al., 2019). These motifs are nearly identical to the earlier Lhx2-binding motif identified by ChIP-seq in mouse olfactory neurons, see Fig. 5B (Monahan et al., 2017). Thus far, no posttranslational modifications of Lhx2 proteins have been reported.

In addition to Ldb1/2, three Lhx2 interacting partners were identified using educated guesses, including HD transcription factor Msx1 (Bendall et al., 1998), transcriptional coregulator Cited2 (Glenn and Maurer, 1999) and Pax6 (Mikkola et al., 2001). In cortical neurons, Lhx2 binds the NuRD chromatin remodeling complex (Muralidharan et al., 2017), comprised of ATPases such as Chd4 and Chd7 (see section 4.) and histone deacetylases (HDacs1/2) (see Lai and Wade, 2011). Large scale unbiased analysis of human proteome confirmed CITED2, CHD7, LDB1, MSX1 as well as multiple subunits of the BAF ATP-dependent chromatin remodeling complex, including SMARCA2/BRM, SMARCA4/BRG1, SMARCC1/BAF155 and SMARCC2/BAF170 as LHX2-binding partners (Luck et al., 2020).

The studies in the fly further revealed that Chip (dLdb) is the obligatory co-factor that interacts with the LIM domain of Ap and other LIM domain proteins (Morcillo et al., 1997). Its vertebrate counterparts are the limb domain binding proteins, LDBs/NLI (Agulnick et al., 1996). The combination of LIM-HD, LMO and the LDB proteins (Milán and Cohen, 1999) will eventually determine the cell specific transcriptional output and thus is pivotal for multiple developmental process from patterning to differentiation (see Dawid et al., 1998).
Mammals possess two LDB-containing proteins: Ldb1 and Ldb2. The LDB1/Ldb1 genes are located on syntenic regions of human and mouse chromosomes 10 and 19, respectively. Much larger genomic loci of LDB2/Ldb2 genes are located on syntenic regions of human and mouse chromosomes 4 and 5, respectively. Ldb2-null mice are fertile with only a few notable phenotypes, and thus its function is redundant to that of Ldb1 in most tissues (Mukhopadhyay et al., 2003; Narkis et al., 2012). In contrast, Ldb1-null embryos die at early stages of embryogenesis (E9.5-E10.0). The Ldb1 adaptor protein has been documented to play essential roles in multiple developmental processes, including the patterning and differentiation of central nervous system (CNS) lineages, specification and maintenance of hematopoietic stem cells, erythrocyte differentiation, limb, and heart development (Mukhopadhyay et al., 2003; Li et al., 2011; Narkis et al., 2012; Caputo et al., 2015).

Among the LIM-HD proteins, Lhx2 has been extensively studied for its roles in mouse CNS development and the mammalian Lhx2 has been termed “cortex selector gene” because it is pivotal for the formation of the cortical primordium through the patterning of the telencephalon (Porter et al., 1997; Monuki et al., 2001; Mangale et al., 2008). Lhx2 activity changes during development and thus the early role of inhibiting the formation the medial and lateral organizers of the cortical primordium (hem and anithem) is replaced by a function in cell proliferation in the cortical primordium (Mangale et al., 2008). During neural differentiation, Lhx2 is playing role in formation of the major axonal tracts required for somatosensory functions as well as for differentiation of specific CNS lineages including the hypothalamic tanyctyes (a radial glia like cell type) and Müller glia of the retina (see Chou and Tole, 2019).

Both Lhx2 and obligatory co-factors Ldb1/2 show stage-dependent functions in retinal development. In the Lhx2-deficient mice, eye development is arrested at the optic vesicle stage (E9.5) with a failure to initiate expression of Mitf and Vsx2, the earliest markers for the ocular pigmented and neural progenitors, respectively (Porter et al., 1997; Yun et al., 2009; Roy et al., 2013). This in turn inhibits specification of RPCs (Tables 1 and 3). At the same time the failure in optic vesicle development in the Lhx2 knockout embryos could be due to misexpression of thalamic and hypothalamic genes within the optic vesicle (Mangale et al., 2008; Roy et al., 2013). Subsequently, in the optic cup, Lhx2 is required to both maintain the proliferation and multipotency of RPCs, by sustaining Notch signaling and repressing the generation of early-born retinal ganglion cells. Lhx2 controls both the RPCs pool and the critical balance between neurogenesis and gliogenesis in the developing mouse retina (Gordon et al., 2013; de Melo et al. 2016, 2018; Gueta et al., 2016). Thus, Lhx2 expression is maintained in adult Müller glia and in the RPE. In the Müller glia, it acts to repress reactive gliosis in the absence of injury and is also required for injury-dependent induction of glial-derived neuroprotective factors (de Melo et al., 2016).

3.6. Six3: a cell-fate determinant essential for early forebrain and eye development

Six3 is a member of sine oculis (SIX) subfamily of vertebrate homeobox genes (Fig. 5) (Oliver et al., 1995). Six3 was identified based on its similarity with Drosophila sine oculis (so) gene, the founding member of genes encoding the SIX subfamily of HD-containing transcription factors (Cheyette et al., 1994). Additional mammalian SIX gene members

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include Six1, Six2, Six4, Six5 and Six6 (see Bürglin and Affolter, 2016). Sequence analysis indicates that Six3 and Six6 genes are closely related paralogs (Jean et al., 1999). Six3, Six6, and later found Drosophila Optix form a subgroup in the SIX subfamily (Jean et al., 1999; Seo et al., 1999). In mice, Six3 and Six2 are located next to each other at chromosome 17, and the syntenic relationship is conserved in human chromosome 2p21. Similarly, a cluster of Six6, Six1 and Six4 genes is located on mouse chromosome 12, and a similar SIX6, SIX1 and SIX4 gene cluster is located on human chromosome 14.

Mouse Six3 (Oliver et al., 1995) and Six6 (Jean et al., 1999) are 353 and 246 amino acid long proteins, respectively (Fig. 5C and D). Both Six3 and Six6 are marked by nearly identical C-terminal HDs that differ in a single amino acid residue: p. S220 in Six3 (Q62233.2) is changed to p. H141 in Six6 (Q9QZ28.2) (Jean et al., 1999). This subtle difference in their HDs is unlikely to affect the specificity of DNA binding based on a current model (https://www.umassmed.edu/wolfe-lab/homeodomains/). Studies of Six3-binding sites in vitro identified a core sequence 5′-ATTA-3′, which is also found in the motifs bound by many other HD transcription factors (Fig. 5C and D) (Zhu et al., 2002). The Six3-binding motif was validated in the cis-acting elements of Six3 target genes in vivo (Liu et al. 2006, 2010). The DNA-binding motif of Six6 in public databases is poorly defined and needs further investigations. Ongoing experiments to determine the chromatin landscape of Six3 and Six6 in embryonic eye tissues using chromatin immunoprecipitation and/or CUT&RUN will reveal both their in vivo binding motif(s) and direct target genes during eye development.

Six3 and Six6 proteins also share an evolutionarily conserved 126 amino acid SIX/SO domains (Fig. 5C and D) that mediate regulatory protein-protein interactions. Like Otx2, both Six3 and Six6 interact in vitro with Groucho family of co-repressors Tle4 and Tle5 (Zhu et al., 2002; Ravasi et al., 2010) and Tle1 (López-Ríos et al., 2003). Six6 also binds Otx2 and Vax2 found by a large scale two-hybrid protein interaction study using human and mouse cells (Ravasi et al., 2010). In Drosophila, So and Eya proteins form a functional complex that regulates multiple stages of compound eye development (Pignoni et al., 1997). In mice, Eya2 is expressed in the RPCs of the early optic cup (Xu et al., 1997); however, Eya2 null embryos appear normal (Grifone et al., 2007). Subsequently, a complex between Six3 and Eya1 was identified (Purcell et al., 2005). A prototypic structural model of SIX1-EY A2 complex is available (Patrick et al., 2013). Nevertheless, although Six2, Six4 and Six5 translocated Eya1/2/3 proteins from the cytoplasm into the nucleus, Six3 did not exhibit this activity in COS7 cells (Ohto et al., 1999). Moreover, Eya3 does not enhance in vitro Six6 DNA-binding activity (Hu et al., 2008). These findings challenge the earlier concept of functional interactions between Six3/Six6 and Eya during mouse eye development. Collectively, Six3-and Six6-interacting proteins during mouse eye development are largely unknown. Likewise, posttranslational modifications of both Six3 and Six6 remain to be determined. Studies using immunoprecipitation of protein extracts from eye tissues followed by mass spectrometry will provide additional novel insight into how Six3 and Six6 execute their functions during mammalian eye development.

Both Six3 and Six6 genes exhibit highly orchestrated temporal and spatial expression during mouse eye development with overlapping expression domains (Oliver et al., 1995; Jean et
Gene expression studies demonstrate that Six3 is one of the key markers that define the anterior neuroectoderm and eye field in early mouse embryos. Six3 mRNA is detected in the anterior neuroectoderm starting at E7.0-E7.5 and at high levels in the anterior neural ridge and eye field at E8.0-E8.5 (Lagutin et al. 2001, 2003; Liu et al., 2010) (see Table 2). From E8.5, Six3 mRNA expression becomes restricted in several tissues that are derived from the anterior neuroectoderm, anterior pre-placodal ectoderm, and eye field: ventral forebrain, optic vesicles, the inner layer of optic cups, prospective lens ectoderm, lens placode, the anterior portion of lens vesicles, and pituitary (Oliver et al., 1995; Liu et al. 2006, 2010; Liu and Cvekl, 2017). Six6 is also expressed in optic vesicles; however, its expression is delayed compared to Six3. Six6 expression commences in the optic vesicles at E8.5-E9.0 and mostly co-localizes with Six3 (Jean et al., 1999; Liu and Cvekl, 2017).

Functional studies in mice demonstrate that Six3 is an essential upstream regulator for early forebrain and eye development at multiple stages (Table 2). Germline homozygous inactivation of Six3 blocks the formation of the rostral forebrain and the eye field. Six3-mediated repression of Wnt signaling in the anterior neuroectoderm is essential for early forebrain and eye development (Lagutin et al., 2003). Conditional inactivation of Six3 at the prospective mouse lens ectoderm by Pax6 Le-Cre (Ashery-Padan et al., 2000) disrupts lens placode formation and its invagination (Liu et al., 2006). Importantly, Six3 directly regulates the expression of both Pax6 and Sox2 in this process (Liu et al., 2006). In the prospective retina, conditional inactivation of Six3 by Rax-cre disrupts optic-cup formation and neuroretinal specification but not RPE specification (Liu et al., 2010). Notably, we demonstrated that Six3 suppresses Wnt8b expression to promote neuroretinal specification (Liu et al., 2010). Collectively, our functional studies demonstrate that murine Six3 is essential for the specification of the forebrain and eye fields, lens cell placode, and neuroretinal progenitor cells.

Inactivation of both Six3 and Six6 demonstrates that Six3 and Six6 are jointly required for the maintenance of neuroretinal progenitor cells in mice (Fig. 8). At stages after neuroretinal specification, conditional inactivation of Six3 using Pax6 α-Cre does not overtly affect retinal development (Diacou et al., 2018). In parallel, germline inactivation of Six6 leads to variable phenotypes that are dependent on the genetic background (Li et al., 2002). In the mixed genetic background, Six6 inactivation does not cause any major phenotypes in either embryonic or adult mouse retinas (Liu and Cvekl, 2017; Diacou et al., 2018). However, Six3 depletion using Pax6 α-Cre (see above) in conjunction with Six6 germline inactivation causes drastic spatially-specific retinal phenotypes: retinal cells at the far peripheral regions lost neuroretinal identity and gained ciliary margin cell fate, whereas retinal cells at the mid peripheral regions maintained neuroretinal identity but lost multipotency, differentiating into a subset of amacrine cells without stratification (Diacou et al., 2018). In the Six3 and Six6 compound null retinas, Sox2 expression was drastically reduced while ciliary margin marker Otxl was ectopically upregulated (Diacou et al., 2018). Transcriptome profiling of Six3 and Six6 compound null retinas revealed that Wnt3a and a Wnt receptor Fzd1 were upregulated, and these findings were confirmed by in situ hybridizations. Explant cultures of E12.5 mouse eye cups demonstrated that Wnt3a is sufficient to promote ciliary margin fate at the distal optic cup (Diacou et al., 2018). Therefore, Six3 and Six6 are jointly required to...
maintain multipotent neuroretinal progenitor cells through activating retinogenic genes and suppressing Wnt/β-catenin signaling (Diacou et al., 2018).

Based on our genetic studies, we propose that co-expression of Six3 and Six6 in optic vesicles and optic cups evolved as a double safeguard mechanism to protect retinal differentiation. The close similarity in their HDs likely reflects the ability of Six3 to functionally compensate for Six6 and vice versa. Our model supports the notion that co-expression of duplicated genes is an evolutionarily mechanism to secure the formation of complex biological structures. Further experiments, such as replacing Six3 by Six6 via CRISPR-Cas9 mediated genome engineering, will determine whether Six6 fully compensates for Six3 during embryonic development.

Our data also demonstrate that suppression of multiple Wnt signaling molecules by Six3 is a common theme during early mouse forebrain (Lagutin et al., 2003) and retinal development (Liu et al., 2010; Diacou et al., 2018). For example, Wnt1, Wnt5b, and Wnt3a were ectopically expressed following Six3 inactivation at three different stages of embryonic development. Thus, Six3 is essential for the specification and maintenance of neuroretinal progenitor cells at least through suppressing Wnt/β-catenin signaling. In addition, Six3 is a cell-fate determinant that promote one cell fate against the other, and the cell fates suppressed by Six3 change over time.

Critical to the understanding of the individual roles of Six3 and Six6 is to identify their key spatiotemporal upstream regulators. Although earlier studies examined human SIX3 (Lengler and Graw, 2001) and zebrafish Six3.1 promoters (Wargelius et al., 2003) and identified their regulation by Pax6 and established autoregulation in zebrafish (Suh et al., 2010), analyses of distal elements as well as expression of nearby Six3os1/SIX3-AS1 remain in their infancy. Most recently, identification of poised enhancers and analysis of topologically associated domains (TADs) including orphan CpG islands indicate intriguing complexity of both Six3 and Six2 gene control during early mouse embryogenesis (Pachano et al., 2021). Six3 is also a transcriptional target of Sox2 in the mouse forebrain, through a distal enhancer (Lee et al., 2013). Enhancers of Six6 in Xenopus were previously identified (Ledford et al., 2017). Earlier mouse studies showed that Lhx2 is required for Six6 expression and both Lhx2 and Pax6 transcription factors cooperate in direct regulation of Six6 expression (Tétrault et al., 2009). In medaka fish, NeuroD and Six6 reciprocal regulation via NeuroD-binding enhancer to regulate retinal expression of Six6 (Conte et al., 2010). In mouse forebrain, Soxb1, Sox2 and Sox3, control Six6 through binding of remote forebrain enhancer to restrict Six6 expression in this part of mouse CNS (Lee et al., 2012).

In conclusion, Six3 is expressed in the forebrain and eye primordia starting at early stages, and is later restricted to the lens and neuroretina in the eye at later stages. Six3 is required for the specification of the forebrain primordium, lens placode, and neuroretinal progenitor cells; and together with Six6, Six3 is required for the maintenance of neuroretinal progenitor cells (Fig. 8). These findings firmly establish Six3 as an essential upstream regulator of both forebrain and eye development.
4. Human genetic abnormalities and animal models with perturbed optic cup formation

A wide range of congenital abnormalities in human eyes affect both individual and multiple ocular tissues. Most of these early defects are called the microphthalmia-anophthalmia-colooboma (MAC) spectrum of genetic abnormalities (see Gregory-Evans et al., 2004; Hever et al., 2006; Williamson and FitzPatrick, 2014; Reis and Semina, 2015; Harding and Moosajee, 2019; ALSomiry et al., 2019; George et al., 2020). The microphthalmia and anophthalmia portion refers to the eyes of reduced size, microphthalmia, and no eye formation at all, respectively. As expected, individual EFTFs (i.e. OTX2, PAX6 and RAX) (Fantes et al., 2003; Ragge et al., 2005; Gonzalez-Rodriguez et al., 2010; Gerth-Kahlert et al., 2013; Deml et al., 2016) and other early expressed transcription factors in the lens (FOXE3) (Plaisancié et al., 2018), optic cup (ATOH7, SALL2, VAX1, VSX2) (Ferda Percin et al., 2000; Reis et al., 2011; Khan et al., 2012; Prasov et al., 2012; Kelberman et al., 2014; Slavotinek et al., 2012) and both tissues (SOX2 and YAP1 – regulator of Hippo-Yap signaling, Williamson et al., 2014) as well as regulatory proteins of retinoic acid (ALDH1A3, RARB, RBP4, and STRA6) (Pasutto et al., 2007; Gerth-Kahlert et al., 2013; Srour et al., 2013; Fares-Taie et al., 2013; Chou et al., 2015) and BMP (BMP4, BMP7, MAB21L1 and MAB21L2) (Wyatt et al., 2010; Reis et al., 2011; Deml et al., 2015; Seese et al., 2021) signaling are responsible for these rare abnormalities (see Patel and Sowden, 2019).

Choroid fissure defects known as optic cup coloboma are more common and account for approximately 10% of cases of pediatric blindness as they affect between 2.5 and 7.5/10,000 children (Onwochei et al., 2000). Optic cup coloboma is caused by mutations in genes linked to TGF-β signaling (GDF3, GDF6 and SMOC1) (Asai-Coakwell et al., 2009; Ye et al., 2010; Abouzeid et al., 2011; Gerth-Kahlert et al., 2013), DNA-binding transcription factors GLI2, MITF, PAX2, PAX6, SIX3, SIX6, and TFAP2A (Sanyanusin et al., 1995; Wallis et al., 1999; Gallardo et al., 2004; Bertolacini et al., 2012; Goolam et al., 2018; George et al., 2016; Kalaskar et al., 2020), transcriptional co-activator YAP1 (Williamson et al., 2014), chromatin remodelling ATPases CHD7 (Matias-Pérez et al., 2018; Kalaskar et al., 2020) and SMCHD1 (Shaw et al., 2017), and other genes such as PTCH1, SMO, TENM3, and TMEM67, that encode functionally diverse transmembrane proteins (Chassaing et al., 2016b; Lee et al., 2017) with both PTCH1 (Chassaing et al., 2016a) and SMO (Twigg et al., 2016) involved in Shh signaling. The precise molecular mechanisms of individual coding and non-coding mutations within the human MAC spectrum of genes remain mostly unknown; nevertheless, recent progress using both human and model organisms, including mouse, chicken and zebrafish, shed new light into the optic fissure closure (see Chan et al., 2021; Yoon et al., 2020).

The optic cup formation (Fig. 2) is initiated by distal invagination of the optic vesicle, and, consequently the ventral portion contains a fissure that provides both exit from the eye for retinal axons and an entry for the hyaloid artery required for blood supply into the retina (see Saint-Geniez and D’Amore, 2004). An important process is a circumferential growth/cell proliferation at the ventral portion of the optic cup (see Lamb et al., 2007) culminating
with two epithelial cell fusions. In contrast to prevailing epithelial fusions from their apical aspects, the cells here approach each other from the basal sides (Patel et al., 2020). Hence, the fusion requires active modulation of basement membrane dynamics (see Chan et al., 2021).

The cellular processes underlying choroid fissure closure were recently investigated in parallel studies including chicken, mouse and zebrafish models and identified two novel mechanisms called “fusion” and “intercalation” (Bernstein et al., 2018). The earliest stages include re-orientation of ventrally facing bilateral leading epithelial edges into another configuration to face each and reach mutually close symmetric apposition. The breakdown of the basement membranes occurs along the choroid fissure margin. Next, the closure is achieved by two distinct mechanisms, including simple tissue-fusion or intercalation of cells into the inter-fissure space (Bernstein et al., 2018).

Analysis of ocular embryonic tissues from ~5 to 7 weeks human gestation coupled with gene expression profiling in human and mouse microdissected cells revealed additional insights into the optic fissure closure (Patel et al., 2020). It was found that the human epithelial cells at the fissure margin undergo a transient epithelial-to-mesenchymal-like transition. The pericocular mesenchyme cells are cleared from the fissure before basement membrane breakdown while their presence is required to maintain the basement membrane as shown earlier in mouse kidney and lung models (Hirai et al., 1992; Ekblom et al., 1994).

At the molecular level, transcriptomic studies were performed using microdissected human and mouse optic cups (Patel et al., 2020) and in mouse coupled with functional studies in zebrafish (Cao et al., 2018). Following laser capture microdissection of human and mouse optic cups to prepare cells from the fissure margins and “control” dorsal optic cup cells, RNA-seq data were generated (Patel et al., 2020). Expression of nearly all known human coloboma-causing genes was found as well as a strong signature of the epithelial-to-mesenchymal transition (see above). Mouse data using E11.5 and E12.5 embryos further confirmed these findings. Microarray data were also generated from E11.5 mouse embryos to analyze optic cup cells in the center nasal and temporal retina (Cao et al., 2018). These transcriptomes consisted of known differential expression of coloboma genes and revealed novel interesting candidates. Amongst them, Afp112 encodes an actin filament associated adaptor protein located in plasma membrane and cytosol. In functional studies, expression of zebrafish afap112 regulates expression of pax2a (Cao et al., 2018). Earlier studies have shown that mutations in human PAX2 cause coloboma (Sanyanusin et al., 1995).

Loss-of-function studies of these genes in model organisms add mechanistic details into the optic cup fissure closure. Two MAC spectrum genes, MAB21L1 and MAB21L2, encode enigmatic nuclear proteins with nucleotidyltransferase activity (de Oliviera Mann et al., 2016) and mouse studies have shown that Mab21l1 regulates early lens development (Yamada et al., 2021a). Zebrafish mab21l2 null mutants fail to break the basement membrane required for the choroid fissure closure (Gath and Gross, 2019). Studies of Mab21l2 by RNA interference in chick model show control of cell proliferation and cell cycle exit in the optic cup (Sghari and Gunhaga, 2018). Biallelic mutations in transcription factor MITF cause coloboma and microphthalmia as well as a range of non-ocular
syndromes (George et al., 2016). Studies of zebrafish mitfa and tfec, members of the Mitf family of genes, have shown that they cause coloboma through the periocular mesenchymal/ neural crest cells located nearby the closure (Sinagoga et al., 2020). The chromo-domain helicase DNA-binding protein CHD7 is a catalytic subunit of chromatin remodeling PBAF complexes and heterozygous mutations are found in human CHARGE syndrome, with multiple abnormalities including coloboma. Chd7 is also expressed in mouse neural crest cells and regulates cell identity genes sox9 and twist1 as tested in Xenopus (Bajpai et al., 2010). Taken together, these nuclear proteins are likely to work together and will provide further insights into their downstream targets and their molecular and cellular mechanisms of action.

Localized mechanical and physical forces, regulated by Hippo-YAP signaling (Table 3) are thought to control cell-shape changes during optic cup fissure closure (see Chan et al., 2021). Mutation of coactivator YAP1 cause isolated coloboma in humans (Williamson et al., 2014) and zebrafish (Miesfeld et al., 2015). Two examples of closure executors are ECM protein nidogen (Carrara et al., 2019) and basement membrane protein Netrin-1 (Hardy et al., 2019). Zebrafish studies have shown that nidogen expression is downregulated upon initiation of fusion and is removed first from the basement membrane followed by laminins (Carrara et al., 2019). In contrast, Netrin-1 is expressed in chicken, human, mouse, and zebrafish models in the fissure margin during the fusion followed by its downregulation after the fusion (Hardy et al., 2019).

Recent progress in generation of human retinal organoids of various complexity using induced pluripotent stem (iPS) cells carrying a wide spectrum of individual mutations in the MAC genes provide novel research opportunities to gain insights into the pathological processes and provide a basis for the development of therapies to manage these diseases as well as other human inherited eye diseases. It will be important to both generate patient-specific iPS cell followed by corrections of the underlying mutations and to generate series of isogenic iPS cells carrying both heterozygous (i.e. typical human mutations) and homozygous (to increase the deleterious effects) mutations in representative genes. Generation of mutants in genes operating with the same presumptive pathway will provide additional advantages to probe details of the molecular mechanisms of individual transcription factors, signaling molecules, and proteins involved in the basement membrane breakdown and role of surrounding tissues, including the periocular mesenchymal cells.

5. Conclusions and perspectives

In this review article, we provide a comprehensive analysis of the earliest stages of in vivo mammalian optic cup morphogenesis as a model system that illuminates the basic principles of the initial 3D-tissue formation in concert with the neural tube morphogenesis and early brain patterning. Critical insights into these processes from chick, fish and frog models are also discussed. These early developmental processes culminate during the highly coordinated lens placode formation and subsequent formation of transient optic cup/lens vesicle structures to generate the future eye. The precise shape of the eye cup is critical for subsequent morphogenetic processes including formation of the cornea and other portions of the mammalian anterior segment to assure appropriate light path from the
cornea, through the lens and towards the photoreceptors. Studies of critical DNA-binding transcription factors and their molecular mechanisms, including identification of their upstream regulators, direct target genes and associated chromatin dynamics, are pivotal for our understanding of the epigenetic regulation of cell type specification and determination.

A major body of work lies ahead to understand transcriptional regulation of genes encoding the entire set of broadly defined EFTFs and identification of their direct downstream target genes at different stages of eye field, optic vesicle, and optic cup formation. In general, some of these factors are expressed prior the formation of eye field (e.g., Otx2 and Sox2) and they are also expressed outside of the presumptive eye field (e.g., Lhx2, Pax6 and Sox2). Sox2 plays global roles in the pluripotency of inner cell mass cells and formation of neuroectoderm. These factors are also known to form functional protein-protein complexes (e.g., Pax6-Sox2 and Lhx2-Ldb1). In addition, their expression domains and functions change following separation of the eye field, throughout the formation of the optic vesicles and cups, and subsequent cell fate decisions that generate all mature retinal cells. Evidence exist that EFTFs mutually control their expression (e.g., Rax regulates both Six3 and Six6, Six3 and Six6 jointly regulate Sox2, and Rax regulates Lhx2 and Pax6). Systematic studies of these GRNs remain in their infancy. The upstream signaling pathways that control expression of these EFTFs and their posttranslational modifications also remain poorly understood due to the intricate dynamic complexity of their underlying GRNs as well as redundancies that ensure robustness of the system (Fig. 7A and B). Many loci, including Bmp4, Otx2, Pax6, Shh, and Sox2, discussed above, span across more than 100 kbp and possesses multiple enhancers active at different stages of embryonic development (Fig. 4C). The prediction of distal enhancers active in particular cell type/developmental stage (see Klemm et al., 2019) is now boosted by 4C-seq and Hi-C studies of chromatin (see Dekker and Mirny, 2016). These candidate enhancers are then tested using transgenic reporters and or via their genomic deletions. It turns out that many loci contain “shadow” enhancers, i.e. two or more enhancers showing similar activities in transgenic assays and only simultaneous deletions of two or more regions ultimately impact \textit{in vivo} gene expression (see Kvon et al. 2021).

Genes directly regulated by both EFTFs and other transcription factors expressed later in the optic cup also remain largely unknown. The major challenge is that traditional ChIP-seq methods require analysis of millions of cells that are difficult to obtain from mouse embryos. These issues are currently being resolved through CUT&RUN and CUT&Tag methods coupled with chromatin studies at single cell levels, such as scATAC-seq and scNMT-seq (Clark et al., 2018; Sanguinetti et al., 2018). \textit{In vivo} mapping of genomic locations of other retinal transcription factors and signal regulated transcription factors will need systematic efforts and data already exist for Lhx2 (Zibetti et al., 2019), Meis1 and Meis2 proteins (Dupacova et al., 2021) in E14.5 neural retinas. Analysis of \textit{cis}-regulatory grammar of promoters and enhancers (Fig. 4B) of all critical genes/loci discussed in this review is essential as there is a major gap in our understanding of functional interactions between individual transcription factors and signal regulated transcription factors on DNA, their posttranslational modifications (see Weidemüller et al., 2021), and their coordinated recruitment of chromatin remodeling complexes (see Berger, 2007; Pulice and Kadoch, 2017; Raeisossadati et al., 2021).
Both the human and mouse retinal organoids also offer additional opportunities to probe chromatin landscape and its dynamic changes as these systems can produce enough cells and/or are suitable for genome engineering to aid cell sorting. Identification of their targets is required to understand the individual steps that lead to cell fate specification and determination (Table 1) and underlying changes in chromatin and 3D nuclear organization as shown by pioneering studies analyzing chromatin of E14.5 retinas (Norrie et al., 2019) and Lhx2 mutants (Zibetti et al., 2019). A major question is whether there are any deterministic single signals for these steps, or are early progenitors exposed to multiple signals and new cell fates are determined stochastically at the single cell level (see Moris et al., 2016). Nevertheless, the committed precursors are ultimately generated at the right numbers and positions due to quantitative limits of the available extracellular signals and their combinatorial effects and possible existence of the “default” differentiation states comparable to the earlier neuroectoderm formation (Argelaguet et al., 2019).

Another major gap in this field is general lack of data for chromatin remodeling complexes and enzymes that interact with individual EFTFs and other transcription factors and how they control transcriptional activation and repression in proliferating and differentiating cells. Most of the present data are on ATP-dependent chromatin remodeling by Brg1-containing BAF complexes originating from early zebrafish (Gregg et al., 2003) and mouse (Aldiri et al., 2015; Holdhof et al., 2021) studies. For example, mutations in SMARCA4/BRG1 cause retinal dystrophy (Cappuccio et al., 2019) and functionally similar ATPase CHD7 of the NuRD complex belongs to the MAC family of genes (see Section 4).

Multidisciplinary understanding of the optic cup formation also requires insights into the physical forces and tensions generated by epithelial sheets of cells and their dynamic changes and modulation as direct results of their proliferation, changes on their cell shape, cell-to-cell interactions, and mechanical properties of the surrounding ECM; all involved in the epithelial evaginations and invagination (see Casey et al., 2021). Advanced models exist to calculate tensions and their consequences for both gross anatomical and local shapes during the neural tube formation. The work ahead is to identify critical cell surface receptors, cell adhesion molecules and their interactions with ECM proteins driving the initial epithelialization processes, tissue bending, and tissue fusions in mouse and human optic vesicle/cup morphogenesis. In teleosts (zebrafish and medaka), both cell movements and reepithelization can be analyzed using novel mutants in candidate genes described above. The current chicken model proposes that the underlying forces that shape optic cup formation originate by differential rates of early progenitor cell proliferation, actomyosin contraction, and regional programmed cell death (see Hosseini and Taber, 2018). The adjacent tissues and ECM proteins provide external physical constraints (see Hosseini and Taber, 2018). It is likely that multiple redundant mechanisms evolved to control these processes to assure that possible perturbation of these processes can be compensated by other morphogenetic mechanisms (see Hosseini and Taber, 2018).

The advantage for these future studies is an availability of diverse in vitro systems to produce optic cups and other types of retinal organoids (see Sinha et al., 2016; O’Hara-Wright and Gonzalez-Cordero, 2020; Guy et al., 2021) and power of tissue engineering using 3D scaffolds. These settings will allow researchers to generate local
sources of signaling molecules that mimic the presence of surrounding lens and periocular mesenchymal cells and determine the roles of individual ECM proteins. Another approach is to model concentration gradients of growth factors (Martinez-Morales et al., 2005) and to examine their effects on morphogenetic processes in vitro (see Kwan, 2014; Casey et al., 2021). Cell sorting processes can be visualized through genome engineering coupled with use of multiple fluorescent proteins for live cell imaging and tracking (Lusk et al., 2021). When the critical molecules are identified, their functions will be tested using a range of mutations, including complete to partial loss-of-functions, gain-of-function and parallel analyses of control and mutated cells including chimeric embryos used to study cell autonomous and non-cell autonomous processes.

The cellular composition of the RPCs in the neuroretina along the central to peripheral axis deserves further attention (see Venters et al., 2015). The unresolved question is the likely presence of two subpopulations of cells, central RPCs committed towards the neuroretina and more peripheral cells capable to generate both RPCs and RPE. The challenge is to harness their potential to regenerate human retina through comparative mouse, chicken, and zebrafish studies (Lu et al., 2020). These studies will require precise mapping of cell fate decisions along the central to peripheral axis and define multipotency of the optic cup margin cells towards reaching both the earlier states of common RPCs, “normal” differentiation into mature retinal neurons, and differentiation into ciliary body and muscle cells of the iris.

Eye regeneration has been reported in a range of model organisms (see Fischer, 2005; Lamba et al., 2008; Barbosa-Sabanero et al., 2012; Wan and Goldman, 2016; Stern et al., 2018). Two main mechanisms involved are transdifferentiation and activation of stem/progenitor cells such as transdifferentiation of RPE cells into the retina in chicken and other model organisms (Luz-Madrigal et al., 2014). It means that cells within tissues can sense absence of adjacent tissues, re-enter the cell cycle and transiently dedifferentiate prior activation of EFTFs together with pluripotency genes such as c-Myc, Sox2 and Klf4 (Luz-Madrigal et al., 2014) and Tet3-driven DNA demethylation (Luz-Madrigal et al., 2020). These and other findings require better understanding of cellular composition within specific niches of the peripheral regions of the optic cups at single cell level in model organisms as well as human.

In conclusion, generation of the eye field, followed by formation of RPCs and early optic cup morphogenesis is a fascinating system to probe the fundamental questions of 3D tissue morphogenesis, cell fate specification and determination coupled with underlying changes in the chromatin landscape, tissue self-organization, and hidden potential for tissue self-repair in mammalian systems. Recent advances of various –OMICs, particularly at single cell levels, imaging and quantification, chromatin and nuclear organization offer exciting opportunities to address the outstanding questions. Genome engineering via CRISPR-Cas9 technologies to produce single or complex mutations, to generate fluorescent fusion proteins, and conditional degrons to control protein expression are already being explored. Finally, but not last, computational biology applied to 3D-tissue modeling during optic cup morphogenesis (Hilfer and Hilfer, 1983; Oltean et al., 2016) adds quantitative strengths to experimental cell biology, developmental biology, and genetic studies.
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Data availability

No data was used for the research described in the article.

Abbreviations:

| Abbreviation | Description                  |
|--------------|------------------------------|
| A/P          | Anterior-posterior           |
| bHLH         | basic helix-loop-helix       |
| BMP          | bone morphogenetic protein   |
| bp           | base pair                    |
| CNS          | central nervous system       |
| D/V          | dorsal/ventral               |
| ECM          | extracellular matrix         |
| CMZ          | ciliary marginal zone        |
| E            | mouse embryonic day          |
| ES           | embryonic stem               |
| eRNA         | enhancer RNA                 |
| EFTF         | eye field transcription factor|
| GRN          | gene regulatory network      |
| FGF          | fibroblast growth factor     |
| HD           | homeodomain                  |
| iPS          | induced pluripotent stem cell|
| LDB          | LIM domain binding           |
| LIM-HD       | LIM-homeodomain              |
| LMO          | LIM-only                     |
| MAC          | microphthalmia-anophthalmia-coloboma |
| M/L          | medio-lateral                |
| PD           | paired domain                |
proximal-distal
retinal progenitor cells
retinal pigmented epithelium
retinoic acid
sonic hedgehog
TATA-box binding protein
three-dimensional

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**Fig. 1.**
The earliest stages of neuroectoderm formation and eye field formation. A) Cell fate maps of the mouse E7.0-E8.5 gastrula (Peng et al., 2016). B) Anterior neural plate (blue) patterning in zebrafish prior and during the formation of the eye field (purple) (see Giger and Houart, 2018). Two opposite gradients of Sfrps (anterior-high/posterior-low) and Wnts (anterior-low/posterior-high) control regionalization of the anterior neural plate (see Cavodeassi et al., 2013). C) 3D-sketches of the E8.0 and E8.5 mouse embryos showing folding of the neural tube (prospective diencephalon, blue), location and bilateral split of the single eye field (purple). At E8.5, the observable optic vesicle is marked (purple). Abbreviations: Embryonic mesoderm, EMB-MES; endoderm, ENDO; extraembryonic mesoderm, EXM; prospective forebrain, FB; prospective midbrain and hindbrain, M/HB; neuroectoderm, NE; primitive
streak, PS; spinal cord, SC; surface ectoderm, SE. Crossed arrows indicate three embryonic axes.
**Fig. 2.**

3D-formation of the optic cup (mouse E9.0-E12.5). A) Diagrammatic visualization of the mouse E9.0 embryo showing formation of the optic vesicle. B) 3D-formation of the optic cup. Note that ventral retinal blood vessels leaving the fissure are not shown for simplicity. Abbreviations: ciliary margin zone, CMZ; dorsal/ventral optic stalk, dOS/vOS; neuroretina, NE; optic fissure, OF; optic nerve, ON; optic stalk, OS; prospective lens ectoderm, PLE; prospective neuroretina, PNR; periocular mesenchyme, POM; prospective RPE, PRPE; surface ectoderm, SE.
Fig. 3.
Chromatin opening by pioneering transcription factors. Pioneering transcription factors such as SOX2 move through the chromatin and scan potential target sites on nucleosomal DNA (Dodonova et al., 2020). When sites are located, chromatin opening requires recruitment of chromatin remodelers, eviction and/or remodeling of one or a few nucleosomes and may include displacement of linker histone H1. The initial opening allows other DNA-binding transcription factors such as Pax6 to join the pioneering transcription factors and recruit additional transcription factors, such as transcription factors such as signal regulated transcription factors (SRTFs) and other enzymes and complexes to facilitate transcription.
Fig. 4.
Summary models of tissue-specific transcriptional control. A) Regulation of transcription by promoter-enhancer looping mediated by CTCF and cohesin. The physical proximity of the distal enhancer is brought together by protein-protein interactions involving enhancer-bound transcriptional factors (TFs), their associated chromatin remodelers (BAF) and the Mediator (Med) complex with the promoter-bound TFs. Transcriptional activity is illustrated by RNA polymerase II at the core promoter (see Carter and Zhao, 2021). Nucleosomes (not shown for simplicity) are decorated by specific combinations of histone PTMs, such as H3K4me1 and H3K27ac (enhancer) and H3K4me3 and H3K27ac (promoter) (Ernst et al., 2011; Rada-Iglesias et al., 2011). Nascent bidirectional formation of two eRNAs is also shown. B) Cis-regulatory grammar (positioning) of model enhancers. Three different motifs (arrows)
are shown with variable affinity (color gradients) to their cognate transcription factors (TFs). Motif number, orientation and order are shown by eight representative examples. Spacing between individual sites is based on 10.5 bp per DNA turn. If two centers of adjacent 6–10 bp long binding sites are separated by 10–11 bp, both TF1 and TF2 are on the same side of DNA and probability of their cooperative action due to permissive steric requirements is high (cooperative binding). In contrast, other alignments are functionally suboptimal (non-cooperative binding) (see Long et al., 2016 for additional details). C) 3D-organization of a representative topologically associated domains (TADs) showing formation and dynamics of multiple sub-TAD loops. During cellular differentiation, different enhancers and employed and topologically associated domains organization changes (see Atlasi and Stunnenberg, 2017; Hansen et al., 2018).
Fig. 5.
Molecular structures and DNA-binding of Otx2, Lhx2, Six3 and Six6. A) Otx2 (mouse: 289 amino acids, 31.6 kDa), phosphorylation sites: Y18, S28, and Y31; ubiquitylation K59; N-terminal HD (amino acids: 38–98). DNA-binding logo from ChIP-seq data (Samuel et al., 2014). B) Lhx2 (mouse: 406 amino acids; 44.4 kDa), internal HD (amino acids: 266–325), N-terminal LIM1 domain 1 (LD1, amino acids: 53–105) and LIM2 domain 2 (LD2, amino acids 115–168). C) Six3 (mouse 333 amino acids, 35.6 kDa), HD (amino acids: 207–266), Six domain (SD), mutation F88E (Q domain) disrupts interaction with Tle4 and Tle5 (Zhu et al., 2002). N-terminal portion of the HD (Hu et al., 2008) does not interact with DNA as it lacks basic Arg residues. The DNA-binding logo (weblogo.berkeley.edu/logo.cgi) was generated using known sites (Liu et al., 2006, 2010). D) Six6 (mouse 246 amino acids, 27.7 kDa). Phosphorylation sites: T212, S221, S225, S227 and S228. DNA-binding logo was obtained from ISMARA (https://www.ismara.unibas.ch/ISMARA/scratch/NHBE_SC2/ismara_report/pages/SIX6.html). Structural modeling: We first obtained crystal structure of OTX2 from PDB id 2dms and structurally aligned it with Aristaless HD which is in complex with DNA (PDB id:3lnq). The final model obtained shows OTX2 bound to linear DNA with important residues K74, R90, and Q97 highlighted. We next generated a homology model for human SIX3 sequence, using the M4T modeling server (Fernandez-Fuentes et al., 2007). We then performed structural alignment of human SIX3 homology model and the crystal structure of HoxA9 and Pbx1 HDs bound to DNA (PDB id: 1PUF). The HD domain of
SIX3 is shown in yellow and residues R282, S283, and L284 are highlighted. Finally, we generated structural alignment of the homology model of SIX6 and the protein component of 1B72 structure using the same strategy as described for SIX3. The resulting model shows SIX6 bound to linear DNA with N-terminal residues R140, H141, and L142 highlighted. The HD domain of SIX6 is shown in yellow.
Fig. 6.
Molecular structure and interactions of Pax6 with DNA and chromatin. A) Mouse/human Pax6, 422 amino acids, 46 kDa; Pax6 (5a), 436 amino acids, 48 kDa. Phosphorylation: S413 by ERK1/2 and p38 (Mikkola et al., 1999); Y281, Y304 and Y373 by HIPK2 (Kim et al., 2006); sumoylation: various lysine residues such as K53 and K89 of Pax6 (5a) (Yu et al., 2010; Yan et al., 2010). Our studies show functional interactions between Pax6 and Pax6 (5a) regulation of crystallin promoters (Chauhan et al., 2004). B) Model of Pax6 interaction with the nucleosome. PAX6 PAI domain (blue), RED domain (red), and HD domain (yellow) bound to the DNA that is wrapped around the nucleosome. Using the crystal structure of PAX6 (6PAX) (Xu et al., 1999a), we first identified nucleotide binding motifs along the DNA. We next performed nucleotide motif alignment of 6PAX (PAI-linker region) and the corresponding motif of 6S01. The PAX6 HD domain with its binding motif was placed along the nucleotide motif of 6S01. The final model obtained shows PAX6 bound to DNA that is wrapped around the nucleosome with important residues G18, R26, N50, T63 (PAI domain), R128 (RED domain), and R242 (HD domain) highlighted. The individual histone subunits are shown as H3 (light blue), H4 (dark green), H2A (olive), and H2B (light red). Our data show that there are sequence similarities between Pax6 consensus sequence and other transcription factors raising the possibility that some Pax6 sites evolved from pre-existing cis-sites recognized by stress regulatory transcription factors (Cvekl et al., 2017). C) In vivo DNA-binding motifs of Pax6 proteins based on our ChIP-seq studies (Sun et al., 2015). D) Analysis of Pax6 intrinsically disordered regions (IDRs) (iupred2a.elte.hu)
(Mészáros et al., 2018) showing highly disordered C-terminal transcriptional activation domain, S/T/P-rich intrinsically disordered domain, IDD (Epstein et al., 1994a).
Fig. 7.
Schematic representations of representative model interactions between signaling molecules, DNA-binding transcription factors and patterning processes at the eye field and optic vesicle stages. A) Wnt signaling (canonical and non-canonical) at the anterior neural plate (anterior-posterior, A/P) including the zebrafish eye field (see Esteve and Bovolenta, 2006; Giger and Houart, 2018). B) Complex cross-talks between BMP and Shh signaling in the mouse eye field and its dorso-ventral (D/V) patterning (Zhao et al., 2010). C) and D) Polarization of the distal (proximal) chick optic vesicle by Pax6/Fst/Tgfb2 Turing network (Grocott et al., 2020), respectively.
Six3 and Six6 are jointly required for maintenance of multipotent RPCs through suppressing Wnt/β-catenin signaling and activating retinogenic factors (Diacou et al., 2018). Transcription factors, TFs; signal regulated transcription factors, SRTFs.

Fig. 8.
Model of Six3 and Six6 joint functions in the optic cup formation. Pathways downstream of Six3 and Six6 involved in the mouse optic cup patterning (E14.5-E15.5). Six3 and Six6 are jointly required for maintenance of multipotent RPCs through suppressing Wnt/β-catenin signaling and activating retinogenic factors (Diacou et al., 2018). Transcription factors, TFs; signal regulated transcription factors, SRTFs.
Table 1

A summary of progressive cell fate decision and differentiation steps, assembly of GRNs and cell positions applicable to eye field formation followed by synchronized optic vesicle and lens development.

| Stages of new cell type formation | Transcription factors (TFs) and level of GRN organization | Cell-to-cell positions |
|----------------------------------|----------------------------------------------------------|-----------------------|
| **Competence** (a property of individual cells to interpret inductive signals for new cell fates) | Ability of genes encoding lineage-specific “pioneering” TFs to respond to inductive signaling through appropriate cell surface receptors at single cell level | Early formation of the anterior neural plate and eye field; multipotent cells without definitive positions |
| **Specification** (in this labile phase, cells are specified if they do not alter their fate in neutral environment) | Initiation of novel gene expression including lineage-specific and signal regulated TFs | Eye field formation; migrating cells change their surface receptors and cell adhesion molecules |
| **Determination** (cell type identity is determined by coordinated and stable expression of a combination of lineage-specific DNA-binding TFs and thus these cells differentiate normally even if placed at ectopic positions of the embryo) | Formation of stable GRN in which TFs regulate each other to form committed (lens) and/or highly restricted multipotent progenitors (retinal progenitor cells); autoregulation of key TFs to maintain cell type and formation of epigenetic cell type memory (see 3.1); parallel repression of alternate cell fates; expression of specific cell adhesion molecules, ECM proteins and cell surface receptors | Epithelialization, cell sorting processes and self-organization produce 3D tissue primordia (lens placode and optic vesicle) comprised of proliferating cells, symmetric (lens and retina) and asymmetric cell divisions (retina) |
| **Early differentiation** | Initiation and/or increase of cell-specific gene expression through maintenance of earlier GRNs and formation of novel GRNs that coordinately regulate mature cell phenotype | Cell cycle exit coupled with formation of definitive cell type, possible cell migrations towards their final destinations within the tissue |
| **Terminal differentiation** (stable maintenance of given cell type) | Tissue-specific expression of batteries of genes required for specific phenotype | Generation of final 3D tissue organization, cell-to-cell contacts including neuronal connectivity |
### Table 2

Summary of general morphological structures leading to the formation of the optic cups.

| Stage (mouse embryos) | General features                                      | Morphological features, key regulatory genes and processes. |
|-----------------------|-------------------------------------------------------|-------------------------------------------------------------|
| E6.5                  | Pre-streak                                            | Formation of primitive endoderm, Otx2 broadly expressed in epiblast and visceral endoderm, onset of Six3 expression in the anterior end. |
| E7.0                  | Early primitive streak                                 | Anterior definitive endoderm formation, beginning of notochord formation. |
| E7.5                  | Late primitive streak - neural plate Headfold - cephalic neural groove | Head mesoderm formed under the neural plate, restriction of Otx2 expression to the anterior neuroectoderm and mesendoderm, detectable Rax and Pax6 expression. Onset of Shh expression (at ~ E7.75) in prechordal plate, a primitive streak-derived temporal structure. |
| E8.0                  | 1–3 somites – Pre-optic sulcus                        | Notochord generates Shh and initiates the eye field bilateral separation; anterior neural plate is marked by Six3 and Sox2 expression. |
| E8.0                  | 4–7 somites – Cephalic flexure, optic sulci           | Anterior neural ridge generates FGF8; onset of FoxG1 expression; symmetrical optic sulci formed at ~ E8.25. |
| E8.5                  | 8–12 somites – Neural folds begin to close at the level of the cervical/hindbrain boundary, optic vesicles | Floor plate produces Shh; initial formation of prepatterned optic vesicles at ~ E8.5. |
| E9.0                  | 13–20 somites – Anterior neuropore closes, beginning of optic cup and lens placode formation | Optic vesicles reach the surface ectoderm followed by thickening of the ectoderm and formation of lens placodes. |
| E9.5                  | 21–29 somites – Early optic cups                      | Lens placode/optic vesicle invaginations in progress; onset of Vsx2 expression in the distal portion of the optic cups. |
| E10.5                 | Advanced optic cup with invaginating lens placode, completion of the neural tube closure (30 somites) | A bilayered optic cup is formed with the inner and outer layers forming the neuroretina and RPE, respectively. Lens vesicle is inside of the optic cup. The optic cup connection with diencephalon is mediated by the optic stalk. |
| E11                   | Optic cup during the circumferential phase of growth, beginning of optic fissure closure | Proliferation of both RPCs and RPE progenitors and growth of the optic cups. |
| E11.5                 | Maturing optic cup with open ventral optic fissure and lens vesicle inside | Last wave of symmetric cell divisions in the retina. |
| E12.5                 | Fully formed optic cup with onset of optic fissure and primary lens fiber cell formation in the lens vesicle | The CMZ is initially formed at the boundary between the most peripheral retina and RPE (optic cup margin zone). |
| E13.5                 | Full basement cell continuity while completely closed optic fissure is found later at E16.5 | Formation of the earliest retinal ganglion cells underway. |
Table 3

Summary of main activities of canonical signal transduction pathways during optic cup morphogenesis.

| Signaling pathway | Neural plate including eye field | Separation of the eye field | Optic vesicle | Optic cup |
|-------------------|---------------------------------|-----------------------------|---------------|----------|
| **BMP** | Inhibition of neural plate formation by noggin and chordin; repressor of eye field formation via Rax gene Rx3; BMP-regulated chemokine receptor Cxcr4 is involved in eye field formation | **Neural plate including eye field** | **Separation of the eye field** | Coordination of BMP signals from the invaginating lens placode; BMP7 involved in optic fissure closure |
| **FGF/MAPK** | Dose-specific FGF8 forebrain organizing center, repression of BMP4, and Wnt8b via FoxG1, activation of Shh via Nkx 2.1, formation of eye field via FGFR2 and ephrinB1 | **FGF8 is regulated by Nodal and Smad2 (TGF-β signaling) and Shh** | **FGF1 is generated by surface ectoderm and demarcates neural retina from RPE; FGF9 in distal region of optic vesicle forming the neural retina; FGF8/FGF3/FGF24 regulate proximal-distal patterning; Shp2, a protein tyrosine phosphatase, mediates FGF/MAPK signaling in the optic vesicle** | Lhx2-dependent FGFRs in the neural retina regulate lens cell proliferation, survival and differentiation; optic cup coloboma due to defective FGF signaling |
| **TGF-β** | Gradient of Activin/Nodal ligands is used for eye field formation: [High] → Rax, Vsx2 (eye field), [Low] → FoxG1, Emx2 (forebrain) | **Nodal signaling and TF FoxG1 involved; Nodal-related Ndr2 regulates Smad2/3 and eye-field separation; Tgf1 and Tgf2 HD TFs required for Shh signaling** | **TGF-βs produced by the pericellular mesenchymal cells pattern optic vesicle into neural retina and RPE; proximal-distal polarization of optic vesicle by Pax6/Fzd/Tgfβ2 Tuning network** | TGF-β2 protects cells in the optic cup from apoptosis; TGF-β signaling positive cells move from the optic stalk into both optic cup fissure margins |
| **Wnt/β-catenin (canonical)** | Anterior neuroectoderm: Wnt signaling promotes posterior fates; inhibitors Dkk1 and Tlc generated at the most anterior portion; repression required for eye field formation via Six3-mediated inhibition of Wnt1 and Wnt6α and broadly expressed Sfrp1 (Wnt antagonist) | Not applicable (N/A) | Activity in dorsal portion of the optic vesicle to promote RPE cell fate via Mif and Otx2; loss of Wnt2b disrupts RPCs and promotes CMZ and iris formation; disruption of co-receptor Lrp6 impairs dorso-ventral patterning of the optic vesicle; inactivation of both Sfrp1 and Sfrp2 genes prevents Wnt canonical signaling in the optic | Active Wnt/β-catenin signaling in CMZ (marked by Wnt2b, Fzd4, Lrf1); mutation of Lrp6 causes optic cup coloboma |
| **Wnt (noncanonical) via Rho family of GTPases and JNK pathway** | Wnt4 and Wnt11 expression domains are posterior to the eye field (zebrafish) and act though Fzd3 and Fzd5 receptors to activate non-canonical Wnt signaling within the eye field; Wnt4 regulates Eaf2 (an RNA polymerase II elongation factor) | N/A | Fzd3/Pax2/Jnk directly regulate cell adhesion molecule Alcam; loss-of-function of Fzd3 and Fzd5 causes optic cup coloboma; Nlk1 and Nlk2 act upstream of Pax2 and positively regulate Wnt11 |
| **Retinoic acid (RA)** | Regulates anterior-posterior patterning via repression of Otx2 expression in the posterior neuroectoderm | N/A | Raklh2 generates RA in the optic vesicle | RA generated by pericellular mesenchymal cells is needed for optic cup invagination; dorsal conversion of RPE to neural retina in double Raklh2/Raklh3 mutants, regulates optic fissure closure |
| **Sonic hedgehog (Shh)** | Six3 → Shh in anterior diencephalon ventral midline, secreted from the prechordal plate | Central role of axial produced Shh in the separation process via Gli1, Gli2 and Gli3 | Proximal-distal patterning of the optic stalk by Cdon, a cell surface protein and Hh coreceptor; crosstalk with Shh | JNK → BMP4 and Shh → Pax2; Cdon and Pax2 mutations cause optic cup colobomas |
### Signaling Pathways and Their Roles in Eye Development

| Signaling Pathway | Neural plate including eye field | Separation of the eye field | Optic vesicle | Optic cup |
|-------------------|---------------------------------|----------------------------|--------------|-----------|
| **Insulin-like growth factor (IGF)** | Regulatory subunit of phosphatase PP2A complex, Ppp2r5e, is required for eye field formation via IGF/PI3K/Akt pathway | Ppp2r5e regulates Shh and is required for eye field separation | Early activity continues to maintain cell segregation and disrupted Eph/ephrin signaling delays optic vesicle expansion | Ephrin A5-EphB2-JNK signaling to close the optic fissure (see Shh above) |
| **Eph/ephrin** | Eph/ephrin signaling maintains eye field segregation; EphrinB1 cooperates with FGF signaling; multiple receptors and ligands (ephrinB1) are regulated by Rx3 | N/A | Expression of Yap1 in the optic vesicle, nuclear expression in the future optic stalk | RPE specification (loss of Yap induces expression of Vsx2); controls normal 3D eye shape; mutations in YAP1 cause optic cup coloboma |
| **Hippo/Yap** | N/A | | | |
| **Notch** | Notch-mediated lateral inhibition is involved in repression of neurogenesis | Dll1 → Notch1 → Rbpj → Hes1 promotes RPC proliferation and inhibits neurogenesis | | Hes1 inhibits proneural gene Atoh7/Math5 |

#### References (see text and below):

**BMP signaling:** Mouse: Luukko et al., (2001), Behesti et al., (2006), Morcillo et al., (2006); mouse/chicken: Huang et al., (2015); chicken: Crossley et al., (2001); Adler and Belecky-Adams, 2002, Pandit et al., (2015); zebrafish: Dick et al., (2000), Bielen and Houart (2012), Heermann et al., (2015), Motahari et al., (2016); Eckert et al., (2019).

**FGF/MAPK signaling:** Mouse: Zhao et al., (2001), Storm et al., (2006), Chen et al., (2012); Thein et al., (2016), Cai et al., (2010); chicken: Herzer et al., (1998), Vogel-Höpker et al., (2000), Crossley et al., (2001), Kobayashi et al., (2002), Ohkubo et al., (2002); frog: Willardson et al., (2014); zebrafish: Picker and Brand (2005); Picker et al., (2009); reviews: Turner and Grose (2010), Makrides et al., (2022).

**TGF-β signaling:** Mouse: Taniguchi et al., (2012), Braunger et al., (2013); chicken: Fuhrmann et al., (2000), Grocott et al., (2020); zebrafish: Dick et al., (2000), Eckert et al., (2019).

**Wnt/β-catenin and Wnt/non-canonical signaling:** mouse: Zhou et al., (2008); Esteve et al., (2011), Carpenter et al., (2015), Hägglund et al., (2013); Fujimura et al., (2009); Fuhrmann et al., (2009); Westenskow et al., (2009); chicken: Cho and Cepko (2006); frog: Maurus et al., (2005); Seigfried et al., (2017); zebrafish: Dutta et al., (2015); review: Gordon and Nusse (2006).

**RA signaling:** Mouse: Simeone et al., (1995), Mic et al., (2004), Molotkov et al., (2006), Matt et al., (2008), Halilagic et al., (2007); zebrafish: Lupo et al., (2011), Valdivia et al., (2016).

**Shh signaling:** Mouse: Chiang et al., (1996), Weston et al., (2003), Furinsky and Wallace (2006); Geng et al., (2008), Zhao et al., (2010), Fiore et al., (2020); chicken: Kobayashi et al., (2002); chicken and zebrafish: Cardozo et al., (2014).

**IGF signaling:** Frog: Rorick et al., (2007), Luehders et al., (2015).

**Eph/ephrin signaling:** Mouse: Noh et al., (2016); frog: Moore et al., (2004); zebrafish: Cavodeassi et al., (2013); review: Fagotto et al., (2014).

**Hippo/Yap signaling:** Mouse: Kim et al., (2016); medaka: Porazinski et al., (2015); zebrafish: Miesfeld et al., (2015).

**Notch signaling:** Mouse: Lee et al., (2005), Riesenberg et al., (2009), Dixit et al., (2013); Maurer et al., (2014); review: Favarolo and López, (2018).