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Signaling and gene regulatory networks in mammalian lens development

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Abstract

Ocular lens development represents an advantageous system to study regulatory mechanisms governing cell fate decisions, extracellular signaling, cell and tissue organization, and underlying gene regulatory networks. Spatiotemporaly regulated domains of BMP, FGF, and other signaling molecules in the late gastrula-early neurula stage embryos generate the border region between the neural plate and non-neural ectoderm from which multiple cell types, including lens progenitor cells, emerge and undergo initial tissue formation. Extracellular signaling and DNA-binding transcription factors govern lens and optic cup morphogenesis. Pax6, c-Maf, Hsf4, Prox1, Sox1, and a few additional factors regulate expression of the lens structural proteins, the crystallins. A multitude of cross-talks between a diverse array of signaling pathways control the complexity and order of the lens morphogenetic processes and its transparency.

Keywords

Cell determination; crystallins; differentiation; ectoderm; lens; Pax6; pre-placodal region; BMP; FGF; retinoic acid; Wnt signaling

The ocular lens as an experimental model for cell fate decisions and cellular differentiation

Embryonic development originates from a single cell, the fertilized egg, that progresses into an adult organism comprised of as many as 230 distinct cell types. Within these cell types, some of them are further subdivided into numerous regions of morphologically and phenotypically unique subtypes. Recent advances in experimental embryology, powered by genetics, genomics, genome engineering, and live cell imaging have enabled scientists to probe the intriguing mechanisms underlying the birth of novel cell types and how they form

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tissues and organs within a dynamic three-dimensional (3D) cellular space of the early embryo. Ocular lens development has fascinated many generations of biologists due to the relative ease to observe, manipulate, and analyze the entire morphogenetic process [1]. Further, lens morphogenesis [2] is also needed for the formation of other parts of the eye, including the retina and cornea [3–5]. Here we discuss lens development as an advantageous model to elucidate regulatory mechanisms governing cell fate decisions and tissue morphogenesis through the combinatorial action of extracellular signaling and DNA-binding transcription factors.

The mature lens is comprised of an anterior sheet of epithelium and a posterior fiber cell compartment that comprises the bulk of the lens. All lens cells descend from a pool of a few hundred of cells [6] forming the lens placode that was originally derived from the “border” ectoderm surrounding the anterior neural plate [7–10]. The internal lens cellular organization provides a “historic record” of its formation, making the lens one of the best experimental systems to dissect laws governing cellular differentiation. In addition, both the cornea and lens function as unique transparent light-focusing tissues. There are a few additional anatomical features important for lens development and function. While the embryonic lens initially receives direct nutritional support from a special vasculature known as the tunica vasculosa lentis, which is connected to the hyaloid artery of the retina, this vascular system eventually disappears while the space between the lens and retina is filled by the transparent vitreous gel [11]. Consequently, the adult lens is both avascular and not innervated. The outer surface of the lens is covered by a thick extracellular matrix (ECM), the lens capsule, which possesses a range of functions for cell signaling and lens accomodation [12]. Lens transparency and refractive properties depend upon a high accumulation of crystallin proteins in the lens fiber cells that remain water-soluble even when their concentration within the lens “nucleus” reaches 450 mg/ml [13]. Finally, a degradation of mitochondria, endoplasmatic reticula, Golgi apparatuses, and nuclei, within the central fiber cell compartment, the organelle free zone, is needed to minimize light scatterning [14].

Vertebrate lens development appears highly conserved among a variety of species. Notably, in zebrafish, the lens placode does not invaginate to form the lens vesicle but increases its cell number through a delamination process and quickly adopts a multilayered cell mass comprised of cells with three types of morphology: flattened cells at the anterior portion, ovoid cells in the central cell mass, and fiber-like cells at the posterior compartment. Central cells of the placode form the lens fibers while the more peripheral regions reorganize into a single layer of anterior lens epithelium [15]. Here we review vertebrate lens development with a focus on the mouse as a model organism with appropriate references to the chick, frog, and zebrafish as these models provide unique insights into the earliest stages of lens formation due to the size of individual embryos and their experimental accessibility. The architecture of *cis*-acting elements comprising lens-specific transcriptional regulatory regions and wiring of individual genes into the gene regulatory networks (GRNs) that control lens morphogenesis illustrate general mechanisms of tissue-specific gene control. Finally, studies of lens formation are valuable for the general understanding of eye evolution as the lens is the final tissue that emerged during a long evolutionary journey starting from a group of photosensitive cells shielded by pigmented cells into a highly complex organ, the camera-like eye [16].

The “invisible” and “visible” stages of lens development

Lens development can be divided into an “invisible” stage (Figure 1) when early lens progenitor cells are formed followed by a series of “visible” morphogenetic processes first observable when the future bilateral optic vesicles emerge from the anterior neural folds and symmetrically approach the ectoderm to form a pair of lens placodes (Figure 2). At the cellular level, an interplay of extracellular signaling (Table 1), commonly referred to as inductive processes, dictate cell fate decisions of common progenitor cells towards the lens, olfactory epithelium, and part of the adenohypophysis, and possibly to the corneal epithelium and non-neural ectoderm [8, 9, 17]. Throughout lens development, transmembrane receptor serine/threonine and tyrosine kinases bound by bone morphogenetic factors (BMPs) and fibroblast growth factors (FGFs), respectively, are most frequently used to transmit these inductive processes [8, 9, 17]. At the molecular level, cell type identity is determined by a combinatorial expression of lineage-specific (“local activators”, Table 2) and signal-regulated DNA-binding transcription factors (SRTFs, Table 3) [18], including their posttranslational modifications (PTMs) as well as their interactions with chromatin modifiers and remodelers [19–21]. Table 2 provides a summary of transcription factors with well-established roles in lens development and their roles at different stages of lens morphogenesis are discussed below.

The “invisible” beginning of lens development

Lens morphogenesis is traced back to the neural plate stage of early embryogenesis and its three ectoderm-derived domains, including the non-neural ectoderm, the neural plate, and a third transitional region known as the “border” between the neural plate and the non-neural ectoderm (Figures 1A,B) [7, 17, 22]. Formation of both the border region and its antero-posterior patterning into the anterior pre-placodal region (aPPR) and the more posterior neural crest (NC) has been extensively studied in chick, frog, and zebrafish embryos [7, 8, 17, 22]; however, data on mammalian embryos is sparse (see below). Evidence exists to show that there is no demarcated boundary; rather, there exists an overlap between prospective neural, anterior pre-placodal, and epidermal cells [8]. The border formation depends on planar signals from the anterior neural plate and non-neural ectoderm [8] as well as from the dorsolateral and head mesoderm [23] through BMP signaling and secretion of one or more members of the FGF family, e.g. FGF8, from the neural plate. Both BMP4 and BMP7 are involved and BMP signaling is negatively modulated by Noggin to reach the “optimal” levels needed for the border and PPR formation. FGF signaling is required to suppress non-neuronal ectoderm formation [24] and its additional roles in the formation of the PPR remain to be discovered. Wnt and retinoic acid (RA) signaling also control cell fate decisions within the border towards aPPR or NC formation. Blocked Wnt signaling is compatible with pre-placodal (PPR) formation and active Wnt signaling leads to neural crest formation [23, 25–30]. Likewise, RA signaling functions here as a commonly used posteriorizing mechanism [7, 22, 31–34] (Table 1).

Gene expression analysis has identified several markers that are expressed in the early PPR, namely those genes encoding homeodomain (HD) transcription factors such as Six1, Six2, and Six4 [35–37], and their binding partners Eya1, Eya2, Eya3, and Eya4 phosphatase co-

activators [38, 39]. Further, HD factors Dlx2, Dlx5, and Dlx6, forkhead factor Foxg1, Zn-finger factor Gata3, and HMG-box factor Sox2, also appear in the same region; however, their expression domains are more variable compared to the the Six/Eya group. It is noteworthy that these genes are expressed in many other regions of the developing embryo and that loss-of-function studies of these individual genes in mice do not disrupt lens formation at the pre-placodal stage. This observation can most likely be attributed to their functional redundancies [40–46]. The anterior-posterior patterning of the PPR is marked by the directional movements of the cells expressing Gbx2 and Otx2 HD proteins towards either its posterior or anterior regions [47] through their mutual repression [48]. The expression of Pax6 is triggered in an anterior subpopulation of PPR cells and these cells form the placodes. Conversely, Pax6 negative cells contribute to the surrounding epidermis [44, 49, 50]. The formation of the aPPR is induced by BMPs from the lateral-to-ventral non-neuronal ectodermal cells that in parallel inhibit neural formation. FGFs are generated by the telencephalic precursors from the anterior neural plate, and inhibited by Wnt signaling from the posterior neural plate and underlying mesoderm (Figure 1, Table 1) [8]. Evidence that BMP signaling is involved in the earliest stages of lens development is supported by the absence of lens formation in both knockout *Bmp4*$^{-/-}$ mouse embryos, even though olfactory placodes appear normal [51], and in embryos mutated by conditional inactivation of the type I BMP receptor genes *Bmpr1a* and *Acvr1*, encoding serine/threonine kinases, in the prospective lens ectoderm [52]. Similarly, inhibition of FGF signaling in explant experiments diminished the expression of Pax6 visualized through a reporter gene demonstrating that FGF signaling controls lens induction [53]. Recently, conditional inactivation of Bmp4 revealed the necessity of the optic vesicle as a source of this morphogen for lens induction [54].

The cells initially comprising the aPPR give rise to multiple migratory progenitors [47] that ultimatelly diverge into the adenohypophyseal, olfactory, and lens placodes (Figure 1). Interestingly, it has been proposed that lens placode formation is the default state for the cells making up the aPPR, at least in chicken [55]. Data show a common pool of lens/olfactory progenitor cells and that formation of the olfactory cells requires transient inhibition of BMP signaling [24]. More recent studies identified two neuropeptides, nociceptin and somatostatin, as lens and olfactory placode inducing molecules in chicken [56]. Clonal analysis of cells comprising the aPPR identified a down-regulation of Dlx5 in the lens compared to the olfactory progenitor cells [57], Dlx5^{+} cells are ultimatelly excluded from the lens altogether [49]. It has also been proposed that some lens-like Pax6^{+} progenitor cells, namely those in the more peripheral locations, later give rise to the corneal epithelium [58]. In zebrafish, PPR patterning and signaling are similar to that of the chick model [59]. Additionally, both zebrafish and chick studies suggest that the adenohypophysis precursors require high levels of sonic hedgehog (Shh) signaling [59–62]. In contrast, the more distal lens progenitor cells do not require this morphogen (Figure 1) [59, 60, 62]. We conclude that the aPPR is comprised of intermingled cells that through migration and self-sorting mechanisms mediated by cell-adhesion proteins converge at distinct domains within the anterior ectoderm that become discernable as the ectodermal thickenings known as the craniofacial placodes [24, 49, 63, 64].

Much less is known about the border and aPPR formation in mammalian organisms [17, 22, 65]. Expression of three DNA-binding transcription factors, including Foxg1 [45], Otx2 [48], and Six3 [44], were shown to occur in E8 mice embryos (the 1–7 somite state) in the presumptive border/PPR and prior to the onset of Pax6 expression [66] (Table 2). Subsequent activation of Pax6 expression in the aPPR and its autoregulation generate a driving force for the entire lens differentiation cascade (see below). Interestingly, human ES cells can be differentiated into cells expressing the pre-placodal markers and their derivatives by the sequential inhibition and activation of BMP signaling [67, 68], which also includes a robust formation of lens progenitor cells driven by BMP4/BMP7/FGF2 [69]. Taken together, the compilation of data support the idea of evolutionarily conserved mechanisms generating the aPPR in vertebrates; nonetheless, future work is needed to isolate all types of cells residing in the pre-placodal ectoderm, establish their transcriptome at single cell and pooled progeny levels, and elucidate their specification towards pituitary, olfactory, and lens placodes, neural, non-neural ectoderm, and neural crest, by activated or repressed extracellular signaling.

The “birth” of “visible” lens embryology

As the formation of neural folds progresses, the prospective neuroectodermal retinal field separates into bilateral regions, morphologically termed the “optic groove” (“optic sulcus”), that deepen to form the optic vesicles. When the optic vesicle reaches the surface ectoderm, mesenchymal cells residing in the space between the ectoderm and neuroectoderm (periocular mesenchyme, POM) are excluded. As the contact between the neuroectoderm and ectoderm is established, a thickening of the ectoderm appears as the lens placode (Figure 2A,B). Reciprocal invagination of the lens placode with the optic vesicle establishes the initial lens and optic cup 3D-structures (Figures 2B,C,D). The lens vesicle is comprised of lens precursor cells under the influence of posterior BMP and FGF signaling and the optic cup is the source of these lens fiber cell differentiation signals (Table 1). The optic cup is patterned along the proximal-distal axis into multiple domains: future neural retina precursor cells, prospective retinal pigmented epithelium, and astrocytes of the future optic nerve (Figure 2C) [70]. The anterior cells of the lens vesicle will give rise to the lens epithelium, while the posterior lens vesicle cells will elongate and produce the primary lens fiber cells (Figure 2E) that form the embryonic lens nucleus (Figure 2F). Mitotic activity within the lens epithelium generates new cells and this process results in posterior cell displacement towards the lens equator. At the equatorial region, a “transitional” zone comprised of 6 to 7 cells is formed in which the posterior cells exit the cell cycle and differentiate into the secondary lens fiber cells (Figure 2G) [71, 72]. These “late” onset lens differentiation processes are discussed in multiple comprehensive reviews [2, 73–75].

In previous sections we discussed how the future lens cells originate from the border/aPPR region and are specified as individual cells together with other progenitors before the optic vesicle physically engages the outer ectoderm/aPPR. This process raises two questions: which cellular and molecular mechanisms control the positioning of the lens placode and optic vesicle and what defect(s) within the optic vesicle impairs lens placode formation? A variety of experimental approaches, ranging from tissue explant cultures to genetic loss-of-function studies in mice, provided key insights into these complex processes. The classical

embryological studies performed using late gastrula, neural plate, and neural fold stage *Xenopus laevis* embryos and microdissected tissues demonstrated that lenses can be formed in the absence of the optic vesicle; however, the lens is ultimatelly formed in the area of direct contact between the neuro-epithelium of the future optic cup and the prospective lens ectoderm [1]. The lens vesicle outgrowth results in exclussion of the POM from the contact area (Figures 2A,B). The best molecular explanation of how the POM inhibits lens formation [76] is that TGF-β and Wnt signaling from the POM limit the expression domain of Pax6 [77].

Loss-of-function studies of multiple mouse genes expressed in the early optic vesicle, including transcription factors Lhx2, Pax6, and Rax, putative RNA-binding protein Mab21l2, and retinaldehyde dehydrogenase Raldh2, revealed critical non-cell autonomous functions of these genes in lens placode formation. The homeobox-encoding Rax gene is expressed in the prospective retina-hypothalamus field of the neural plate [78]. *Rax* null embryos do not form any optic vesicles and consequently do not form optic cups, resulting in a severe anophthalmic phenotype [79]. The *Lhx2* mutants show a derepression of genes specific to the thalamic eminence and anterodorsal hypothalamus coupled with a mislocalization of the optic vesicle [80]. In *Lhx2* null embryos, expression of Pax6 is absent in the prospective lens ectoderm while Pax6 expression in the optic vesicle is unchanged [81]. It is possible that the primary cause of this gross defect could be attributed to the reduced expression of both BMP4 and BMP7 [82]. Spatiotemporally controled depletion of *Pax6* in the optic vesicle also disrupts lens placode formation even though ectodermal Pax6 expression persists in the mutated embryos [83]. Somatic loss of *Mab21l2* also inhibits lens placode formation [84]. Multiple mutations in human *MAB21L2* gene were identified [85, 86] and their mechanisms were tested in zebrafish [86]. The Mab21-like family (Mab21l1 and Mab21l2) includes cyclic GMP-AMP synthase (cGAS) and recent structural analysis of the MAB21L1 protein revealed a potential nucleotidyltransferase domain [87]; nevertheless, apart from the possibility that Mab21l2 binds Smad1 [88], nothing is known about its molecular function. In contrast, although the Mab21l1 is highly expressed in the invaginating lens placode, its formation does not require this gene [89]. Likewise, the *Rax* and *Pax6* loss-of-function models described above need to be extended towards the identification of dysregulated genes both in the optic vesicle and surface ectoderm with a primary focus on those implicated in BMP, FGF, Notch, and Wnt signaling.

Emerging studies also show that RA signaling plays important functions in the formation of the lens placode, beginning with its role in the posteriorization of the border region where it regulates expression of the transcription factor Tbx1, the co-repressor Ripply3, and Fgf8 to set up boundaries within the PPR [33]. In *Xenopus*, both Raldh2 (enzyme catalyzing oxidation of retinaldehyde into retinoic acid) and lipocalin-type prostaglandin D2 synthase (LPDGS, regulator of RA transport) are expressed around the anterior neural plate and their expression is non-cell autonomously regulated by the zinc finger transcription factor Zic1 expressed at the anterior neural plate [90]. Another expression domain of Raldh2 identified in mice includes the POM adjacent to the temporal portion of the optic vesicle, which in this case acts in a paracrine mode towards the optic vesicle [91]. In E8.75 *Raldh2*$^{-/-}$ mouse embryos, the optic vesicles appear normal while at E10.25 no formation of the early optic cup is found with a lack of thickening of the apposed surface ectoderm [92]. Being required

for normal lens morphogenesis, RA signaling evidently regulates multiple processes within the PPR and optic vesicle (Table 1).

Finally, between E8.5 and E8.75, the “stage” represented by a transient population of cells forming the aPPR is ready for Pax6 to assume a number of orchestrating functions that direct lens placode formation and subsequent stages of lens morphogenesis. *Pax6* is currently the only known gene that is essential for the formation of the common adenohypophyseal/olfactory/lens progenitor cells as well as for the formation of the lens placode as evidenced by analysis of $Pax6^{-/-}$ embryos [6] and slightly delayed conditional mutants [3]. Consistent with these loss-of-function data, injection of Pax6 cDNA into 2-cell stage *Xenopus* embryos can produce numerous ectopic lenses without association with neuronal-like tissue [93] as well as ectopic eyes following the injection at the 16-cell stage [94]. In addition, an ectopic lens can be generated by Six3 expression in 2–4-cell stage medaka embryos in the area of otic placode likely by cell non-autonomous process [95].

The role of chromatin and histone PTMs in lens placode formation was probed through the inactivation of two histone acetyltransferases CBP and p300, in the prospective lens ectoderm. The $CBP^{-/-};p300^{-/-}$ embryos display gross reductions of both H3K18ac and H3K27ac histone PTMs in the mutated ectoderm without any evidence of lens placode formation [96] as found earlier in $Pax6^{-/-}$ embryos [3]. Further, both CBP [97] and p300 [97, 98] are established protein-binding partners of Pax6. Given that many genes are commonly downregulated between Pax6, CBP, and p300 mutants, it is reasonable to postulate a mechanistic link between Pax6 and enhancer activation [96]. Importantly, an overlap between $Pax6^{-/-}$ and $CBP^{-/-};p300^{-/-}$ downregulated genes includes well-known regulators of lens morphogenesis (e.g. c-Maf, Meis1, Pitx3, Prox1, and Sox2) and a plethora of crystallin genes [96, 99]. Taken together, studies of Pax6 expression and its directly regulated genes during the transition from the aPPR into the lens placode and alternate states are paramount for deciphering the molecular mechanisms of lens cell formation.

Regulation of Pax6 expression in lens and other cell types, including retinal progenitor cells, pancreas cells, and radial glia cells, is an important subject of current investigations [100, 101]. Earlier studies have shown that the mouse *Pax6* locus resides within a 420 kb region of chromosome 2 [102] and contains a landscape of interdigitated distal enhancers, many of them being highly conserved throughout vertebrate evolution [103]. To elucidate the onset of *Pax6* expression in the aPPR, it is necessary to first identify the earliest active enhancer(s) and corresponding transcription factors that regulate Pax6 expression and link them with the extracellular signaling pathways described above. As transcription of *Pax6* continues throughout all subsequent stages of lens development and other ectodermal derived eye structures (the corneal epithelium and lacrimal gland), it is possible that either additional enhancers may get involved and/or the “triggering” enhancer may be decomissioned. *Pax6* expression in the lens originates from the upstream promoter P0, while the downstream promoter P1 is used by the retina and brain (Figure 3A) [104, 105]. Two distal enhancers of the *Pax6* locus were identified through the evolutionarily conserved non-coding regions [ectodermal enhancer, EE, 106, 107], and through the analysis of DNaseI hypersensitivity and human DNA translocation within the 3’-region of PAX6 locus called SIMO [SIMO, 108, 109]. Genomic deletion of EE partially disrupted *Pax6* expression [110], prompting a

systematic analysis of both 5'-EE and 3'-SIMO (Figure 3A), including their compound effects [100]. The current data support the idea that EE and SIMO function as shadow enhancers and employ several common factors, including Meis1/2 and Pax6 [100, Figure 4B]; nevertheless, whether these enhancers also bind SRTFs downstream of BMP and FGF signaling and trigger *Pax6* expression remain unknown (Figure 3B).

In several vertebrates, a "third" lens within the parietal (pineal) eye and single or multiple ectopic lenses are formed in the head area due to specific mutations and genetic manipulations. The parietal eye is found in most lizards, frogs, lamprey, and some species of fish and is photosensitive via an unorthodox phototransduction mechanism [111], which includes parapinopsin [112], non-visual green-light-sensitive opsin called parietopsin, as well as blue-sensitive pinopsin [113]. In contrast to the ectoderm-derived lateral lenses, the parietal lens is formed from the neuroectoderm, and is comprised of a monolayer of elongated cells; nevertheless, it expresses normal crystallins [114, 115]. The neuroectodermal origin of the parietal lens can be linked to observations that the neuroretina is capable of transdifferentiating into the lens [116]. Recently, it was shown that Notch signaling suppresses this process in the chick retina [117]. As described above, both Shh and Wnt signaling inhibit lens cell formation (Table 1). Thus, it is not surprising that mutants generating one or more ectopic lenses outside of the retina exist. The zebrafish mutant *you-too* (*yot*) disrupts normal function of Gli-2, a negative regulator of Shh signaling, and results in lenses developing at the adenohypophyseal placode area [61, 118]. In chick, canonical Wnt/β-catenin signaling mediates lens repression by NC cells, and TGF-β reduces both function and expression of Pax6 [77]. In contrast, loss of β-catenin in the mouse periocular ectoderm leads to the formation of multiple rudimentary lenses [119, 120]. To counterattack Wnt signaling, Pax6 may form a "defensive block" through direct activation of two negative regulators of Wnt signaling, Sfrp1 and Sfrp2 [120]; however, none of these proteins are involved in modulating canonical Wnt signaling during this stage of lens formation [121]. Taken together, these studies illustrate the involvement of many signaling pathways and their cross-talk to ensure that lens formation is restricted to the area defined by the outgrowing optic vesicle.

Lens morphogenesis and gene regulatory networks

3D-morphogenesis of the lens occurs via multiple interconnected processes, including reciprocal invagination of the lens placode and optic vesicle, cell cycle exit within the posterior compartment of the lens vesicle, extensive cell elongation, abundant crystallin gene expression and protein accumulation, formation of intricate cell-to-cell contacts between individual lens fibers, production of ECM proteins to form the lens capsule, organized degradation of subcellular organelles, and a completion of tissue remodeling. The development of the lens microarchitecture employs a range of highly specific proteins that direct the formation of unique cell membranes, gap junctions, and cytoskeletal structures as summarized in Box 1. In parallel, lens fiber cells degrade their intracellular organelles in order to minimize light scattering (Box 2). Among these processes, both the invagination of the lens placode and the molecular mechanisms underlying crystallin gene transription have been extensively probed and serve as prime models to better understand both synchronized

epithelial cell sheet morphogenetic movements in other organs and transcriptional control of other genes highly expressed in the lens, respectively [122].

A morphogenetic “tango”: Lens placode and optic vesicle invagination to generate the 3D eye primordium

In order to explain placode formation as the ectodermal thickening, it was first shown that the contact area between the ectoderm and neuroepithelium remains constant during the invagination process. This contact is established by the constraining effects of ECM [123] and the formation of F-actin-rich basal temporal filopodia acting as physical tethers [124]. This model was further extended by findings which showed that the ECM causes lens cells to thicken locally in order to facilitate subsequent invagination [99, 125], a process primarily driven by both apical constriction to produce wedge-shaped cells [122, 126] and by BMP signaling [127]. In the invaginating lens placode, the cells near the lens pit circumferentially contract the adherens junctional complexes joining them together, and this actomyosin cytoskeleton remodeling requires RhoA small GTPase, Rho-kinase (Rock), PDZ domain-containing protein Shroom3, and p120-catenin δ1 [122, 126]. The physical forces generated by the epithelial curvature are regulated by balanced Rac1 and RhoA activities [128]. Additionally, planar polarization of the cells near the center of the lens placode is established in order to produce multiple morphogenetic physical forces that orchestrate the invagination process [129]. In medaka, the formation of filopodia that tether the lens to the retina requires YAP via fibronectin 1 (Fn1) and β1-integrin, implicating the Hippo-Yap pathway in actomyosin mediated regulation of tissue tension through the Rho GTPase activating protein ARHGAP18 [130]. Further complexity of the optic cup morphogenesis and regulation of its shape is demonstrated by a conditional inactivation of wntless (Wls) in the presumptive lens ectoderm which elicits major structural changes that yield a soucer-shaped optic cup damaged by a ventral coloboma, perturbation of RA-signaling, and reduction of the number of RPE cells near the optic cup rim [131]. These findings prompted a novel hypothesis that differential cell numbers in a bilayered epithelium, in analogy with a “bimetallic strip”, also regulate the final shape of the optic cup [131].

The role of ECM proteins is illustrated by loss-of-function studies involving Fn1 in mice [99] and laminin α1 (Lama1) in zebrafish lens [132]. Similar constriction mechanisms including the actomyosin dynamics were also established in the neuroepithelium of zebrafish optic cup morphogenesis by live imaging of cell shape changes and movements [129, 133, 134].

BMP signaling continues to play critical functions during the formation of the lens pit and optic cup (Table 1). BMP4 (from E9.0 and attenuated between E9.5/10) and BMP7 (between E9.5–10.5) are expressed in the prospective lens ectoderm-lens placode, BMP7 in the future RPE and BMP4 in the future neuroretina. Active canonical BMP signaling, evaluated by pSmad1/5/8 nuclear stainings, is confined to the dorsal part of the optic cup [54]. In the lens-forming ectoderm, BMP expression is negatively autoregulated [135] and this is independent of Smad1, Smad5, or Smad4 [52]. BMP signals stemming from the lens specify the neuroretina [136], and loss of BMP4 in the optic vesicle is detrimental for lens formation along with the neuroretina being converted into the RPE [54]. It appears that expression of

BMPs in the dorsal optic vesicle is stabilized by Wnt signals from the ectoderm via β-catenin-independent and GSK3β-dependent pathway [137]. The Lhx2-BMP axis described above was further elaborated through a "delayed" inactivation of Lhx2 in the optic cup from E10.5 that disrupted both lens and retinal morphogenesis and reduced the expression of Fgf3, Fgf9, Fgf15, Etv5/ERM, Etv1/ER81, and Bmp4 in the mutated optic cup [138]. Importantly, this study identifies a complement of FGFs acting *in vivo* during lens fiber cell differentiation. Likewise, many critical roles of Pax6 in lens placode formation and invagination are demonstrated by studies showing that Lama1, Fn1, α5- and β1-integrins, and maybe Shroom3, are direct targets of Pax6 [99, 126, 139, 140]. Future studies are necessary to establish the molecular mechanisms of both canonical and non-canonical BMP signaling, including SRTFs and their direct target genes, in lens and retinal compartments during the early stages of eye development.

The invaginating lens placode is prone to severe structural damage as mutations in multiple regulatory genes, including *Mab21l1* [89], *Pitx3* [141], and *Six3* [44], induce lens cell apoptosis and *Pitx3*$^{-/-}$ suffers a "disappearance" of the lens itself (aphakia). Both lens and retinal development are severely impaired in *Hes1*$^{-/-}$ mouse embryos from the E10.5 stage onwards, although the early patterning of the eye appears unaffected as supported by the normal expression of Pax6 and Rax prior to this stage; nonetheless, it is defective progenitor cell proliferation rather than apoptosis that underlies these defects [142, 143]. Hes1, a bHLH transcriptional repressor and downstream target of Notch signaling, is expressed in the lens placode, vesicle and optic vesicle-cup, and both cell and non-cell autonomous Hes1-dependent mechanisms require further clarifications. The survival of cells in the lens placode and lens pit requires FGF and BMP signaling (Table 1) as shown by multiple genetic approaches that inactivate three transmembrane tyrosine kinase FGF receptors, Fgfr1, Fgfr2, and Fgfr3 [144], Bmp receptors Bmpr1a and Acvr1 [52], and a combination of Bmpr1a and Fgfr2 [145]. The pro-survival FGF signaling pathway includes the PI3K-AKT pathway (Table 1) [146]. An important part of these morphogenetic movements is the separation of the lens vesicle from the surface ectoderm that subsequently gives rise to the corneal epithelium. This process is disrupted in AP-2α$^{-/-}$ [147], Cited2$^{-/-}$ [148], Foxe3$^{-/-}$ [149], Pax6$^{+/-}$ [150], Rxra$^{-/-}$;Rarg$^{-/-}$ [151], Rbpj$^{-/-}$ [152], and Sox11$^{-/-}$ [153]. A cooperative action of E- and N-cadherins and spatialy regulated apoptosis are required to initiate and complete these cell separations [154]. Taken together, significant progress has been achieved in the identification of many of the critical components of the "tango", including a comprehensive and challenging genetic analyses of the system.

GRNs that govern crystallin gene expression

Crystallin gene expression ranks at the top of all genes studied in mammalian systems and is directly comparable to the high expression of β-globin genes in erythrocytes and *Cacnad21* gene in neurons [155]. Expression levels of individual crystallins in lens fiber cells are typically one to two orders of magnitude higher compared to their expression in epithelial cells. A notable exception is the αB-crystallin, which is more abundantly expressed in the lens epithelium than the fibers. This protein is also expressed in many other tissues such as the heart, brain, retina, kidney, and lung [156]. The common denominator among the diverse family of crystallin genes is that the lens-specific regulatory elements reside within

remarkably short TATA-box containing promoter fragments, e.g. −88/+46 for αA-crystallin, −115/+45 for αB-crystallin, and −67/+45 for γF-crystallin promoters [157]. Two differentiation-specific enhancers reside within the 16 kb *Cryaa* locus [158, 159] while complete regulatory regions of the *Cryab* locus reside within a 4 kb DNA region upstream from its promoter [160], including a muscle/heart/lung-specific −427/−255 enhancer that also stimulates αB-crystallin expression in the lens [161]. In contrast, no distal enhancers are known in the *β/γ-crystallin* loci though 5'-end truncations modulate their expression domains within the lens fiber cell compartment [157, 162]. Mouse genetic and molecular biology studies have identified Pax6 [163–166], c-Maf [167–169], Hsf4 [170–172], Prox1 [173, 174], and Sox1 [171, 175–177] as lens-specific transcription factors. Further studies support the involvement of CREB [158], RARβ/RXRβ [165, 176–179], and Six3 [180] in at least one or a few crystallin genes (Table 2). Importantly, a number of cataract-causing mutations were identified in MAF [181, 182] and HSF4 [183–185] human patients.

The current data support a general model of lens-specific transcription factors and SRTFs bound to crystallin promoters and enhancers and show that there is a "core" GRN comprised of three DNA-binding transcription factors, including Pax6, c-Maf, and Prox1, and multiple crystallins (Figure 4, Table 2). In the invaginationg lens placode, Pax6 directly regulates the expression of c-Maf [166] through a unique 5'-distal enhancer containing a tandem of Pax6-binding sites. Pax6 also directly regulates Prox1 expression presumably via a pair of 3'-distal enhancers [139]; however, their spatiotemporal activity and their requirement for Pax6 remain to be directly analyzed. The regulatory interaction between Pax6, c-Maf, Prox1, and their crystallin targets is termed as a "feed-forward loop". Evidence also exists that Pax6, Prox1, and Hsf4 directly control the expression of genes encoding Fgfs and their receptors, Fgfrs [139, 170, 186], and that FGF-regulated mitogen-activated protein kinase (MAPK) Erk1/2 phosphorylates Hsf4 to increase its binding to DNA [187]. Likewise, Pax6 can also be phosphorylated by Erk, p38, or by HIPK2 [188]; however, none of these studies were conducted in the context of lens development. Figure 4 shows the wiring of GRNs governing the transcription of mouse αA-, βB1-and γA-crystallin genes. Future studies will be needed to address the following questions: a) Do *β/γ-crystallin* regulatory regions contain AP-1 and Ets binding sites? [189]; b) BMP signaling is known to regulate the expression of *αA-crystallin* gene [190, 191] and maybe other crystallins. Are crystallin genes regulated by Smad proteins? c) Where are the enhancers needed for the onset of *Cryaa* expression at mouse E10.5 located? [159]; d) Does bZIP factor c-Maf form homodimers or heterodimers with c-Jun and/or CREB at different Maf-binding sites? e) What is the role of CREB in lens development? In addition to responding to canonical cAMP signaling, CREB can also be a downstream target of canonical Wnt/β-catenin signaling implicated in advanced stages of lens fiber cell differentiation [192], and f) What PTMs regulate lens transcription factors function? [193–195].

Reiterative use of the same genes and signaling pathways direct lens fiber cell differentiation and build the lens 3D shape and internal structure

The continuous growth of the lens throughout the life of an organism requires the orderly proliferation and differentiation of the lens epithelial cells into secondary the lens fibers.

This process begins at the transitional zone located just anterior to the lens equator, where the lens epithelial cells complete their final mitotic stage before differentiating terminally into fibers (Figure 5). A key regulator of this transition is FGF signaling, which displays biphasic activity on the lens epithelial cells, promoting proliferation at a low dosage and differentiation at a higher one [196]. This is counterbalanced by Notch signaling, which suppresses the differentiation of the lens epithelial cells in order to maintain the progenitor pool. Since the Notch ligand Jag1 is produced by the newly differentiated lens fiber cells, the pathway constitutes an elegant feedback mechanism that properly coordinates the timing and progression of lens differentiation [197, 198]. Interestingly, although the canonical Wnt signaling pathway suppresses the initial acquisition of the lens cell fate as described above, Wnt reporter activity can be detected in the lens epithelium and loss of the Wnt co-receptor Lrp6 causes the eruption of the lens material into the anterior chamber of the eye, demonstrating a positive role of Wnt signaling at this later stage of lens development [199, 200]. As is well known, the key effector of the canonical Wnt signaling pathway is β-catenin, which is also an essential component of the adherens junction anchored by cadherin proteins. Interestingly, ablation of β-catenin does not cause lens herniation, but rather a disruption in polarity and adhesion of the lens epithelial cells, which are similar phenotypes to those observed in cadherin knockouts [119, 154, 201]. Genetic rescue experiments also demonstrated that the cadherin-binding protein NF2 negatively regulates YAP, which is required both to maintain lens epithelial cell polarity and to prevent premature differentiation [202, 203]. YAP has also been shown to be intricately involved in Wnt/β-catenin signaling in other systems. Thus, the lens epithelium is likely regulated by the convergence of Wnt, cadherin and Yap signaling (Table 1).

The newly differentiated lens fiber cells abutting the transitional zone are also the cauldron of multiple signaling pathways, the most extensively studied among them being FGF signaling (Figure 6). Evident by the increasing level of ERK phosphorylation, FGF signaling is significantly elevated in these cells. In contrast to its growth-promoting role in the lens epithelial cells, the high intensity of FGF signaling in the lens fiber cells stimulates cell cycle exit and differentiation. Remarkably, among all the growth factors tested so far, only FGFs are capable of eliciting the expression of fiber specific proteins and a profound change of the cell shape [196, 204, 205]. This raises the interesting question: which intracellular molecules mediate the unique activity of FGF in the lens fiber cells? Recent work suggests that the key transmitters of FGF signaling are Frs2, an adaptor protein specific to FGF tyrosine kinase receptors [206], and Shp2, a protein tyrosine phosphatase [144, 207–209]. Constitutively active Ras signaling can compensate for the loss of Frs2 and Shp2 in cell proliferation and differentiation, whereas PI3K signaling rescues an FGF receptor 2 (Fgfr2) deficiency for cell survival, suggesting that FGF signaling employs a compendium of downstream pathways to regulate lens development [146, 207]. Downstream to Ras, MAP kinases such as Erk phosphorylate and stimulate the activity of AP1 and ETS family transcription factors, which induce the expressions of crystallins and other lens determination genes such as Prox1 and c-Maf [186, 189]. This pathway is also finely tuned by negative regulators including the GTPase activating protein NF1 and the Ras inhibitor Sprouty, as well as by the transcription factor Prox1 that activates the expression of FGF receptors [186, 210–212]. FGF signaling has been shown to induce the expression of Jag1,

the ligand for Notch signaling that suppresses lens epithelial cell differentiation as described above, and Fizzled-6, the receptor for Wnt-planar cell polarity (PCP) signaling that may facilitate the orientation of the lens fibers [213, 214]. FGF signaling also engages in cross-talks with BMP signaling, which promotes cell cycle exit and lens differentiation in an FGF-dependent manner [190, 215, 216]. However, although lens differentiation was arrested in mouse mutants lacking both the BMP receptors Bmpr1a and Acvr1, it was either unaffected or even enhanced by deletion of regulatory Smads (Smad1 and Smad5) and Smad4, which are the mediators of canonical BMP signaling [52, 217, 218]. By contrast, loss of Smad7, the inhibitory Smad, actually disrupted lens differentiation [219]. These disparate phenotypes suggest lens differentiation is likely regulated by Smad-independent non-canonical BMP signaling.

FGF signaling has also been proposed to prime lens cells to differentiate in response to canonical Wnt signaling [192]. In support of this model, deletion of β-catenin disrupted lens fiber differentiation and a combination of Wnt3a and FGF2 were found to be most effective in generating lentoid bodies from human embryonic stem cells [69, 201]. As a caveat, the commonly used TCF/Lef1 reporter for canonical Wnt signaling is only active in the lens epithelium, not in the lens fiber compartment, but this reporter may not fully recapitulate the canonical Wnt acitivity [121, 220]. Finally, FGF signaling in the lens fibers induces expression of Frizzled-6, the Wnt receptor thought to sense a gradient of Wnt to direct migration of the fibers to the anterior pole of the lens, although *in vivo* evidence for such non canonical Wnt/PCP signaling is still lacking [214, 221]. Altogether, these evidence presents FGF signaling as a central director in orchestrating Notch, BMP, and Wnt signaling to regulate lens fiber differentiation and morphogenesis.

Cell adhesion and junction proteins also play important roles in lens differentiation and morphogenesis. E- and N-cadherins are localized at the apical and lateral sides of the lens epithelial cells to maintain their polarity and adhesion to each other, which also requires the RapGAP protein Sipa1l3 and ephrin-A5 signaling [154, 222–225]. In the lens fiber cells, N-cadherin promotes cell elongation and morphogenesis by activating the Src family kinase Fyn, intersecting with EphA2 receptor tyrosine kinases that induce phosphorylation of the Src and F-actin binding protein cortactin to control actin assembly and fiber cell alignment [226, 227]. Also at the lateral side of the lens fiber cells, the gap junction protein Cx50 (Gja8) not only forms channels for ion and metabolite exchange, but also negatively regulates E3 ubiquitin ligase Skp2 to prevent degradation of the cell cycle inhibitor Cdkn1b/p27, promoting cell cycle exit and differentiation [228]. Integrins are the major adhesion receptors at the basal side of the lens cells, connecting the internal cytoskeleton to the extracellular matrix in the lens capsule. In the lens epithelium, the cell differentiation program is suppressed by β1-integrin that antagonizes FGF signaling, but promoted by α6-integrin that activates IGF signaling [229, 230]. Although the exact mechanism for such divergent roles of integrins is not clear, genetic evidence supports the model that integrin signaling is regulated by the transmembrane protein Crim1, mediated by the pseudokinase ILK and ultimately conveyed to small GTPases such as Rac and Rap to control cell adhesion and basement membrane assembly [231–235].

Concluding Remarks

The cell fate decision and differentiation processes described here illustrate both the simplicity and complexity of the ocular lens. At the “simple” level, the current model of lens cell formation is constructed from a tripartite Six3-Pax6-Sox2 network that paves the road for the expression of c-Maf, Prox1, Sox1, and Hsf4 as essential crystallin regulatory genes. Two cell types, the lens epithelium and lens fiber cells, also provide a uniquely “simple” system to understand cellular differentiation at the molecular level. The “complexity” originates from the reiterative use of ubiqitously used signaling pathways (TGF-β/BMP, FGF, Wnt, nuclear receptors, Shh, integrins, Notch, Hippo-Yap, and maybe Jak-Stat) (Table 1); however, it is important to stress that both the sources of these signals and their agonists and antagonists, target receptors, and nuclear SRTFs may differ depending on the precise context. For example, FGF signaling functions first as the early-inductive driver (planar signals from the diencephalon), then as activator of pro-survival mechanisms during lens placode invagination (from the optic vesicle), and finally as the overarching source of differentiation molecules later on in lens development (from the neuroretina). Likewise, we do not fully comprehend the contributions of individual genes for the formation of the aPPR due to the possible effect of “protective” redundancies, species differences, and the wiring of their GRNs within the lens-induction pathway. We also lack a transcriptomic definition of the lens cell fate competence, commitment, and determination. While the majority of extracellular signaling plays positive roles in lens cell formation, the repressive mechanisms seem to be underepresented (Table 1), and various cross-talks between individual pathways remain poorly understood [131, 190, 236]. Other levels of unresolved complexities relate to the contradictory findings that optic cup formation requires ectoderm-derived signals [137], anopthalmia is caused by various mutations of genes either in the ectoderm or optic vesicle [3, 51, 54, 79, 82, 83, 237], and that optic cup formation proceeds without any rudimentary lens in retinal organoid cultures [125, 238]. These and other outstanding questions previously discussed will be resolved in the upcoming years. Nonetheless, experimental challenges remain high due to both the scant number of cells comprising the early embryonic structures and requirements for their purity.

In recent years, lens studies have greatly benefited from the implementation of a full repertoire of genome-wide and proteomic studies, including RNA-seq, ChIP-seq, and mass spectrometry, and a growing number of genes available for mouse genetic studies that include double and triple knockouts [52, 91, 96, 100, 144, 145, 239, 240]. Use of the CRISPR-Cas9 system allows an effective dissection of regulatory mechanisms at the DNA level including complex enhancers [100]. A major gap exists in our understanding of the posttranscriptional, translational, and posttranslational regulatory mechanisms functioning in the lens [241–243], as well as numerous cross-talks occuring between individual signal transduction pathways [221, 244], nuclear organization [245, 246], and how noncoding RNAs regulate nuclear organization and gene expression [247]. Ongoing studies are focused on lens chromatin dynamics by ATAC-seq, identification of lncRNAs and microRNAs and their lens functions. Transcriptional processes and their modulation are intimatelly connected to PTMs, interactions with chromatin modifying and remodeling enzymes, and half-lifes of individual transcription factors. Pioneering studies on protein-protein

interactions of Pax6 [97] and its proteosomal degradation in neuronal cells [248, 249] should be followed by similar studies of other transcription factors in lens cells. Genetic studies of lens abnormalities [250], candidate gene approach facilitated by the iSyTE database [251], and studies of the microphthalmia-anopthalmia-coloboma [237] will also assist in the expansion of lens-specific GRNs.

Lastly, the process of lens fiber cell elongation encounters a series of impediments, such as the production of a large amount of protein and lipid building materials per each individual cell in a short time-window that is restricted by organelle degradation. Requirements for their intracellular transport, quality control, and assembly provide numerous excellent opportunities to study fundamentals of cytoplasmic membrane biogenesis, organelle degradation and turnover [252–254], recycling of materials via autophagy [252, 255], developmental endoplasmatic reticulum stress and unfolded protein response [256], function of the ubiquitin proteasome systems [228, 257, 258], protein sorting into raft and nonraft bilayers [259, 260], remodeling of membrane cytoskeleton [261, 262], and formation of mechanical stiffness and elasticity of the lens [263–265] (Box 1, 2) and to expand studies of GRNs and extracellular signaling to include genes that either control (e.g. Gata3, Hsf4, Prox1, Sox1, and etc) or function in these processes (e.g. aquaporin 0/MIP), Bfsp1, Bfsp2/filensin, Dnase2b, Gja8/Cx50, Lim2, and etc). Paramount to these studies is the implementation of assays to visualize and track single molecules within the lens cells including individual organelles using fluorescent techniques, visualization of nascent transcription and RNA transport, and development of antibodies that recognize specific PTMs of lens proteins, including crystallins. We conclude that the next decade of lens research is poised to provide novel insights into the general cellular and molecular mechanisms of tissue morphogenesis and will explain mechanisms of congenital eye defects as well as “late” aging defects originating from disrupted lens homeostasis.

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ABBREVIATIONSGlossary

aPPR	Anterior pre-placodal region
bZIP	basic domain-leucine zipper
BMP	bone morphogenetic protein
ECM	extracellular matrix
ES	embryonic stem
GRN	gene regulatory network
FGF	fibroblast growth factor

HD	homeodomain
MAPK	mitogen-activated protein kinase
NC	neural crest
OFZ	organelle free zone
POM	periocular mesenchyme
PPR	pre-placodal region
PCP	planar cell polarity
PTM	postranslational modification
RPE	retinal pigmented epithelium
RA	retinoic acid
SRTF	signal regulated transcription factor
Shh	sonic hedgehog
TGF-β	tranforming growth factor-β
3D	three-dimensional, 3D

Anterior pre-placodal region (aPPR)
a horseshoe shape-like transitional region of cells around the anterior neural plate from which progenitors of anterior pituitary, olfactory placode, and lens placode emerge.

Cell fate competence
competence is a property of individual cells to interprete inductive signals to which cell fates of the progenitor cells were programmed.

Cell fate commitment
cells are committed if they do not alter their fate regardless of their environment.

Cell fate determination
cell identity is determined by a coordinated expression of multiple lineage- and tissue-specific DNA-binding transcription factors (“local activators”) and universal signal-regulated transcription factors.

Core (minimal) promoter
a short sequence around the transcriptional start site comprised of sequences recognized by transcription factors that are sufficient for recruitment of RNA polymerase and an initiation of a low basal transcription. Additional sequences flanking the core promoter increase the promoter’s strength and may provide a tissue-specificity, such as in the crystallin promoters.

Crystallins

crystallins are the major lens structural and regulatory proteins of the ocular lens. Vertebrate crystallins are divided into the α-, β- and γ-crystallin families. The α-crystallins arose from gene duplication events involving small heat shock proteins; the β/γ-crystallin subfamilies trace back their history to microbial calcium-binding proteins.

Embryonic induction
inductive processes (competence → specification → determination) represent an interplay of extracellular signalins that collectivelly elicit transcriptional responses of genes encoding lineage- and tissue-specific transcription factors required to produce a novel cell type.

Enhancer
a sequence of DNA composed of concentrated clusters of transcription factor recognition sites enhance the level of transcription from a target promoter by increasing the transcription burst frequency. Enhancers elicit tissue-specificity of minimal promoters or respond to specific differentiation signals if the promoter is tissue-specific such as in the case of many crystallin promoters.

Gene regulatory network (GRN)
a regulatory relationship established between at least three different genes to form a simple GRN module. Multiple modules are combined together and explain how individual regulatory genes and their targets work together to form a regulatory hierarchy.

Histone posttranslational modifications
a set of covalent modifications, including acetylation, methylation, phosphorylation, ubiquitination, and others, of core histone proteins, most often including their N-terminal tails.

Lens placode
an ectodermal thickening formed from palisades of lens progenitor cells formed in the head area of early embryos as the optic vesicle approaches the surface ectoderm.

Lens vesicle
a transitional polarized hollow structure comprised of lens precursor cells. Its anterior cells give rise to the lens epithelium, while cells forming its posterior domain differentiate into the primary lens fiber cells.

Optic cup
a transitional bilayered structure comprised from the neuroretina and retinal pigmented epithelium located in the inner and outer cell layers of the cup, respectively. The neuroretinal cells are the source of growth factors that control lens differentiation.

Organelle free zone
lens fiber cells' terminal differentiations are marked by the highly organized degradation of their endoplasmatic reticulum, Golgi apparatus, mitochondria, and nuclei, in the central part of the lens, the organelle free zone.

Primary and secondary lens fibers

following the formation of primary lens fiber cells that occupy the lumen of the lens vesicle, the lens continues its life-long growth by adding novel outer layers of secondary lens fibers. In this process, the primary lens fiber cells become compacted and form the lens nucleus where the organelle free one is initially formed.

Tissue specification
a stable cell fate in a neutral medium.

Transitional zone
lens epithelial cells are subdivided into four regions, which include the central zones (cells arrested at G0) and symmetric pregerminative, germinative, and transitional zones. The cells comprising the transitional zone consist of a narrow latitudinal band that is comprised of cells just exiting the cell cycle and initiating their elongation.

Transcription factors
proteins that bind promoters and enhancers at 6–20 bp DNA sites of sequence-specificity using one or more DNA-binding subdomains and either activate or repress transcription. Many lens regulatory factors contain the homeodomain (HD) or basic domain-leucine zipper (bZIP) as their DNA-binding subdomains.

Transmembrane receptor kinases
upon ligand binding, transmembrane receptor serine/threonine and tyrosine kinases form catalytically active homo- or hetero-dimers that trigger a cascade of cytoplasmatic signaling processes which ultimately converge into the specific transcriptional responses that involve gene activation and repression within the nucleus.

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Box 1

Lens “in vivo engineering”: Materials needed to build the lens and organelles to be destroyed

Ocular lens functions through its higher refractive index compared to the surrounding medium [14]. This is accomblished by accumulation of crystallin proteins even though many other proteins can serve similar purpose [115]. Transparency requires maintenance of short-range spatial order of crystallin proteins, as in dense liquids or glasses [309]. The hexanonally-shaped lens precursor cells give rise to beams of flattened concentric lens fiber cells that fill more than 95% of the lens volume. Lens differentiation produces 10^5–10^6 lens cells (mouse-human) with lens fibers extending their length by a factor of 1,000 or even more [310]. Correspondingly, increase in length is paralleled by increase of the surface area, further magnified by lateral interdigitations, and increase of the cell volume [310, 311]. The optical properties and lens size change throughout life [311, 312]. Human lens exhibit a biphasic growth rate, the first phase is completed by the first year as lens reaches a mean weigth of 149 mg, and passes value of 200 mg at age 38 of linear growth [313]. The entire morphogenetic process also encounters several important limitations regarding the resources and time needed to produce a perfect tissue for the rest of lifespan. In contrast to material lens, biological lens actively maintains its transparency through a microcirculation system of ions and water [311]. To transport water, aquaporin 0/major lens intrinsic protein (MIP) is the most abundant lens membrane protein, followed by Lim2, and the lens-specific connexins Gja3, Gja8, and Gje1 [314]. Lens membrane are rich in cholesterol and sphingolipids, resulting in the formation of lipid rafts [259, 260]. Lens cytoskeleton is comprised of spectrin-actin system anchored by membrane protein Band 3 similar to other cell types [315]. The intermediate filament proteins Bfsp1 (filensin) and Bfsp2 (phakinin) are highly lens-specific while vimentin is a ubiqitously expressed protein [316–318]. The main cytoplasm building materials are the lens-preferred α- and β/γ-crystallin proteins [319]. Lens also contains high concentrations of reduced glutathione (GST) to provide protection against membrane and protein oxidation [320].

Box 2

Lens “in vivo engineering”: Structures and organelles to be destroyed

During lens embryogenesis, lens is supported from outside through a transient microvasculature system; however, following its regression, nutrients and oxygen can only enter lens epithelial cell layer from the anterior and lens fibers from the posterior side through the aqueous humor and vitreous gel, respectively [11]. To prevent light scattering, lens fiber cells degrade their intracellular organelles, including mitochondria, Golgi apparatus, endoplasmatic reticulum, and nuclei, to generate the “organelle free zone” (OFZ) within the inner most fiber cell compartment, the lens nucleus and this process continues throughout the secondary lens fiber cell differentiation [321]. Consequently, both transcription and translation are terminated as the nuclei dissintegrate (around E18.5 of mouse embryonic development). Production of lens structural materials is thus limited when transcriptional-translational systems are operational, and lens had to evolve an intricate system of quantitity and quality control for its special structural materials, and, ultimatelly “clean” the house from organelles. Even though formation of the OFZ eliminates nearly all vital functions of living cells, the mature lens fiber cells undergo a series of remodeling steps and are ultimatelly transformed into a functional “biological glass” [14]. For example, fiber cell compaction concentrates proteins to levels above 450 mg/ml in the central lens fibers and protein gradient across the lens is needed for correction of spherical aberration [13]. In summary, lens as a tissue employs a range of biophysical solutions to meet multiple demands on its mechanical and optical functions for which evolution found numerous elegant solutions [14].

Trends

Recent progress based on RNA-seq and ChIP-seq has provided novel insights into the chromatin and RNA expression landscape dynamics and can be scaled-down towards studies of including the pre-placodal cells and their populations.

Use of innovative technology to visualize movements and cell fate decisions at the single cell and single molecule level.

Genome engineering using CRISPR-Cas9 system accelerates studies of gene function, function of lncRNAs, and distal enhancers.

The nuclear organization changes drammatically during lens fiber cell differentiation as a result of the preparation of nuclei for their organized degradation.

Development-controling and congenital cataract-causing genes can be reliably predicted using the iSyTE database.

Outstanding questions

The cells comprising the lens placode are thought to be the first “stable” lens cells of the lens differentiation cascade. What are the minimal requirements to achieve this initial state of lens differentiation? How is this cell type defined at the level of transcriptome and proteome?

The extracellular signals, functioning as lens inducers, and their target DNA-binding transcription factors still remain poorly understood. Lens as a default state for aPPR predicts multiple “inhibitory” mechanisms. Which genes are primary downstream targets for this competition process and where are the target enhancers located?

The pre-placodal cells specified towards different cell fates are intermingled and through cell migration they reach their anatomical location. At the single cell level, are these cells fully committed or there is a range of plasticity?

What is the molecular mechanism underlying the unique dosage-dependent function of FGF signaling in lens cell proliferation and differentiation?

What are the biological functions and molecular mechanisms downstream of ncRNAs, including lncRNAs and miRNAs, and their RNA-RNA and RNA-protein interactions, in lens morphogenesis?

What is the cellular basis for the dramatic one thousand fold elongation of the lens fiber cells? How do the signaling and transcription factors impinge on the cytoskeletal machinery in this process?

Lens regeneration and transdifferentiation from cornea, iris, neuoretina, and RPE can occur in a variety of vertebrates including adults. Are the similar or unique molecular pathways employed like in embryogenesis?

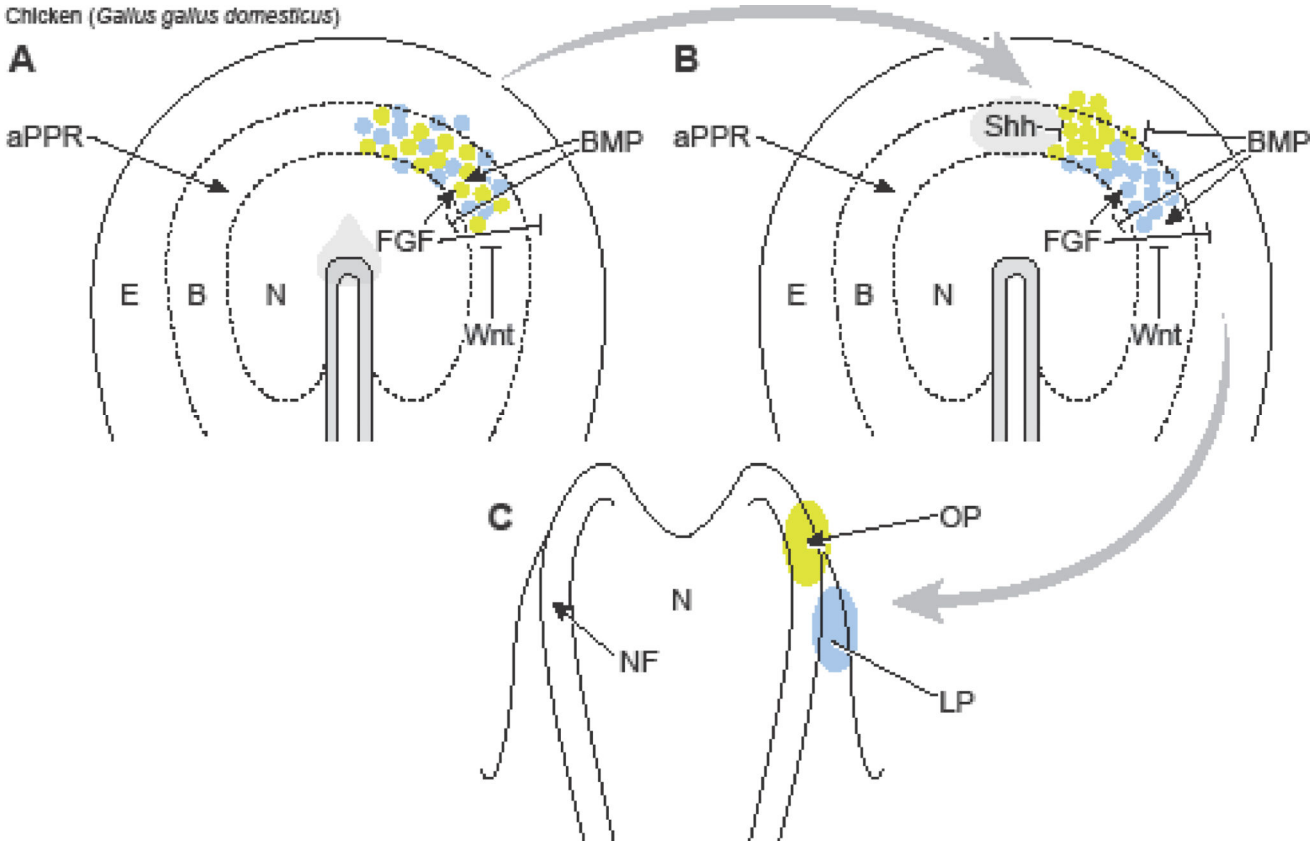

Figure 1. The earliest stages of vertebrate lens formation from the pre-placodal ectoderm

(A) Model of chicken lens cell specification based on the inductive signaling of BMP, the necessity of FGF signaling, and the inhibitory role of Wnt signaling. The precursors for lens and olfactory placodes are intermingled and surround the anterior neural plate domain. Additional cell types, including cells moving into the neural plate/tube and outside towards the ectoderm, are not shown for simplicity. (B) The choice between lens/olfactory and adenohypophyseal cell fates is regulated by Shh signaling promoting adenohypophyseal placode formation, is temporaly regulated the subsequent activation and inhibition of BMP signaling needed for olfactory placodal progenitors. Sustained BMP signaling is required for lens cell formation. (C) Schematic of chicken embryo at the neural fold stage (4–5 somites), Figures A–C adapted and modified from [8].

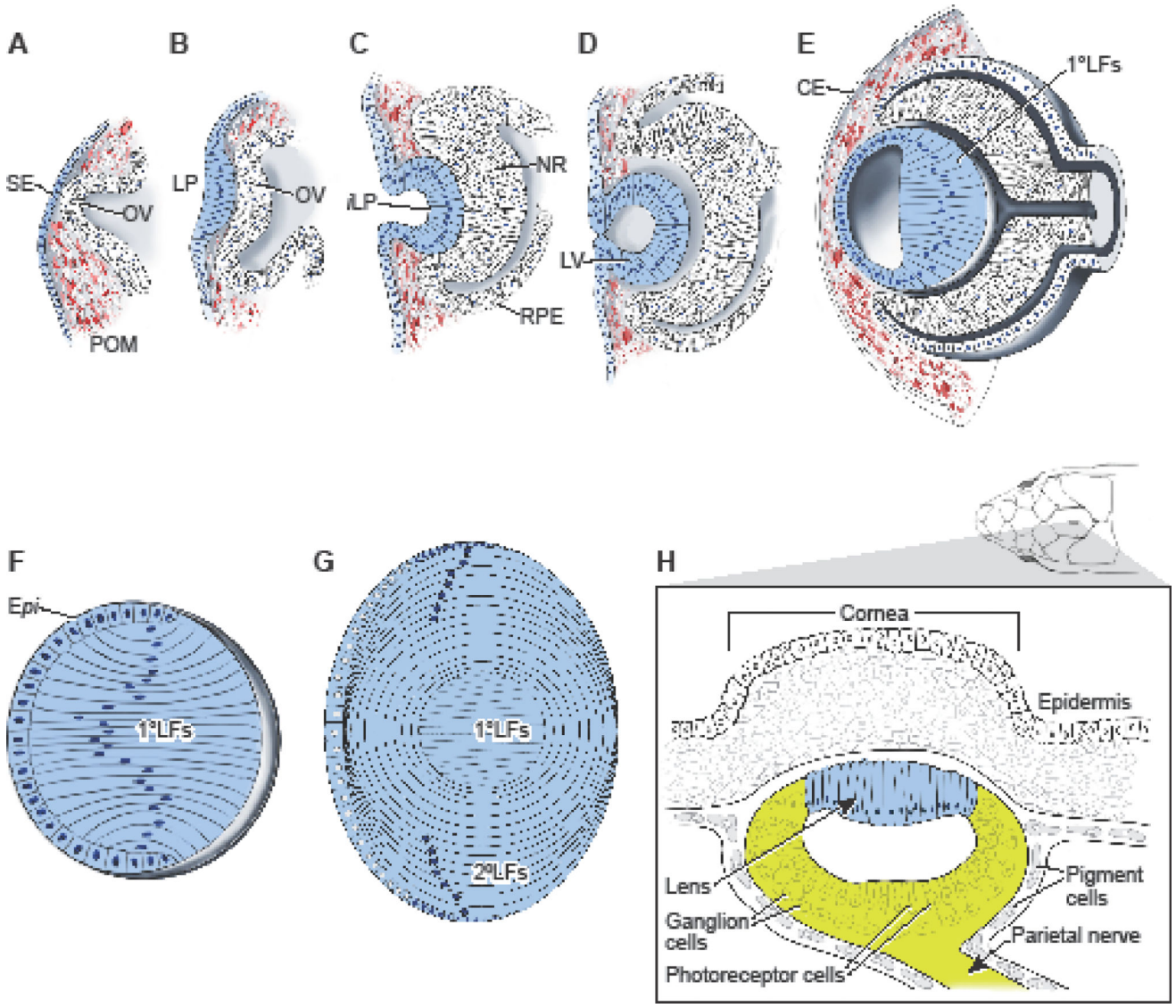

Figure 2. Stages of lens formation in mouse embryos from the prospective lens ectoderm, and eye/lens structure in the parietal "third" eye in lizard

(A) Mouse E8.5 (surface ectoderm). (B) Mouse E9.0 (prospective lens ectoderm). (C) Mouse E10.5 (invaginating lens placode - lens pit). (D) Mouse E11 (open lens vesicle). (E) Mouse E12.5 (primary lens fiber cell differentiation). (F) Mouse E13.5–E14.5 lens (completion of primary lens fiber cell elongation – initiation of secondary lens fiber cell formation). (G) Mature mouse lens. Note that the initial compartment formed by the primary lens fibers (E12.5–E14.5, panel F) now forms the central lens nucleus devoid of intracellular organelles. H) The parietal "third" eye of lizards. The lens is of neuroectodermal origin and is comprised from elongated nucleated cells immediatelly bellow a transparent layer resembling the cornea. The retina is comprised of photoreceptors and ganglion cells. Anterior lens epithelium, ALE; corneal epithelium, CE; invaginating lens placode, iLP; lens capsule, LC; lens epithelium, Epi; lens placode, LP; neuroretina, NR; optic vesicle, OV; periocular mesenchyme, POM; primary lens fibers, 1^0 LFs; prospective lens ectoderm, PLE;

retinal pigmented epithelium, RPE; secondary lens fibers, 2^0 LFs; surface ectoderm, SE. Panels (A–E) are from [75], (F–G) [72, 322] and (H) is based on a scheme [323].

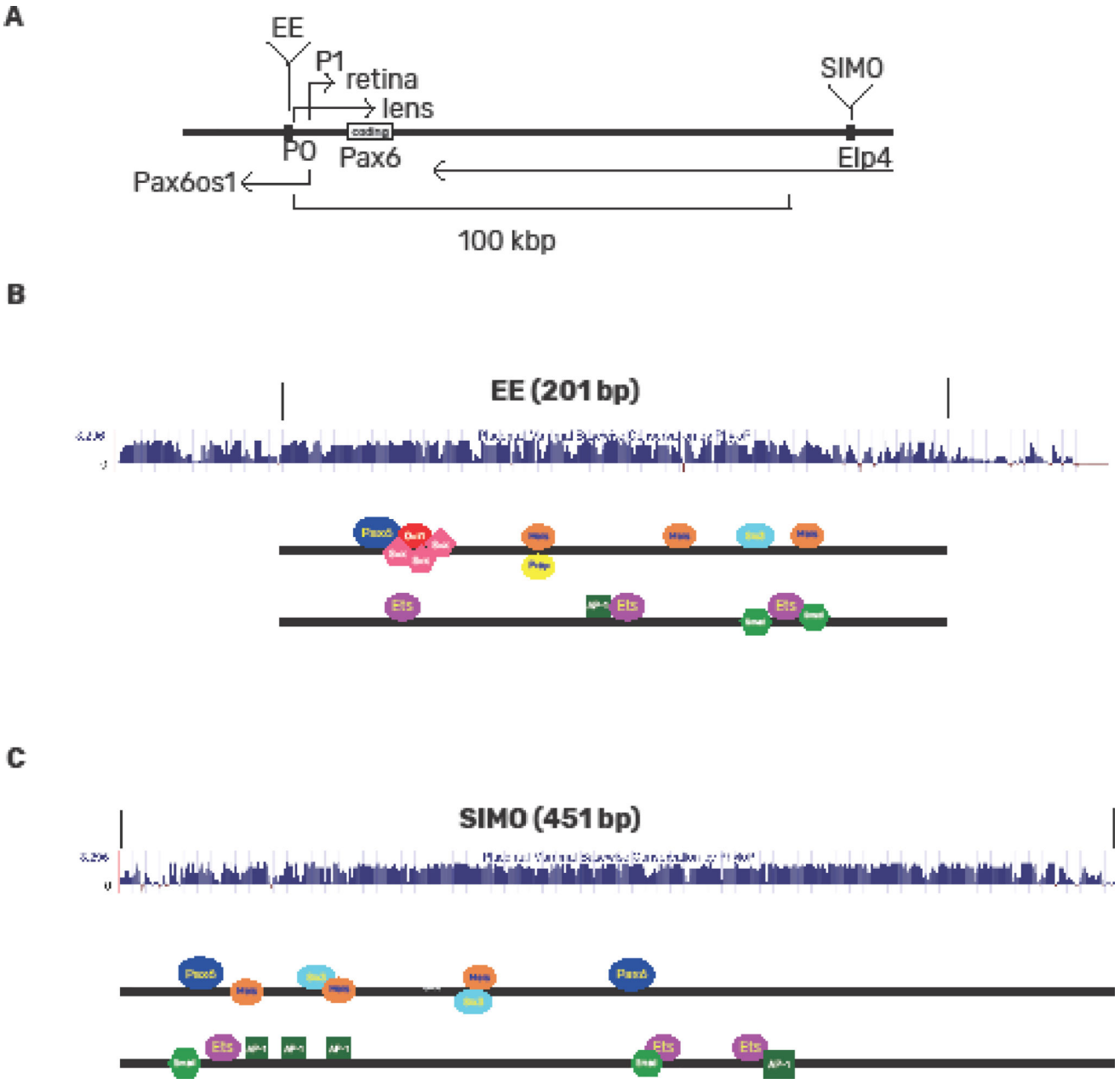

Figure 3. Transcriptional regulation of Pax6 in the lens

(A) Regulatory regions of the *Pax6* locus. The mouse *Pax6* locus resides within a 420 kb region of chromosome 2. The promoters P0 and P1, and EE and SIMO enhancers are indicated. (B) Transcription factors bound to EE include Meis1/2 [100, 284], Pou2f1(Oct1), Sox2 [287], Pax6 [324], Pknox1 [286], and Six3 [44]. Candidate binding sites for AP-1, Ets, and Smads are indicated. (C) Transcription factors bound to SIMO include Meis1/2 [100, 325], Pax6, and Six3 [44]. Candidate binding sites for AP-1, Ets, and Smads are indicated (see also Tables 2 and 3).

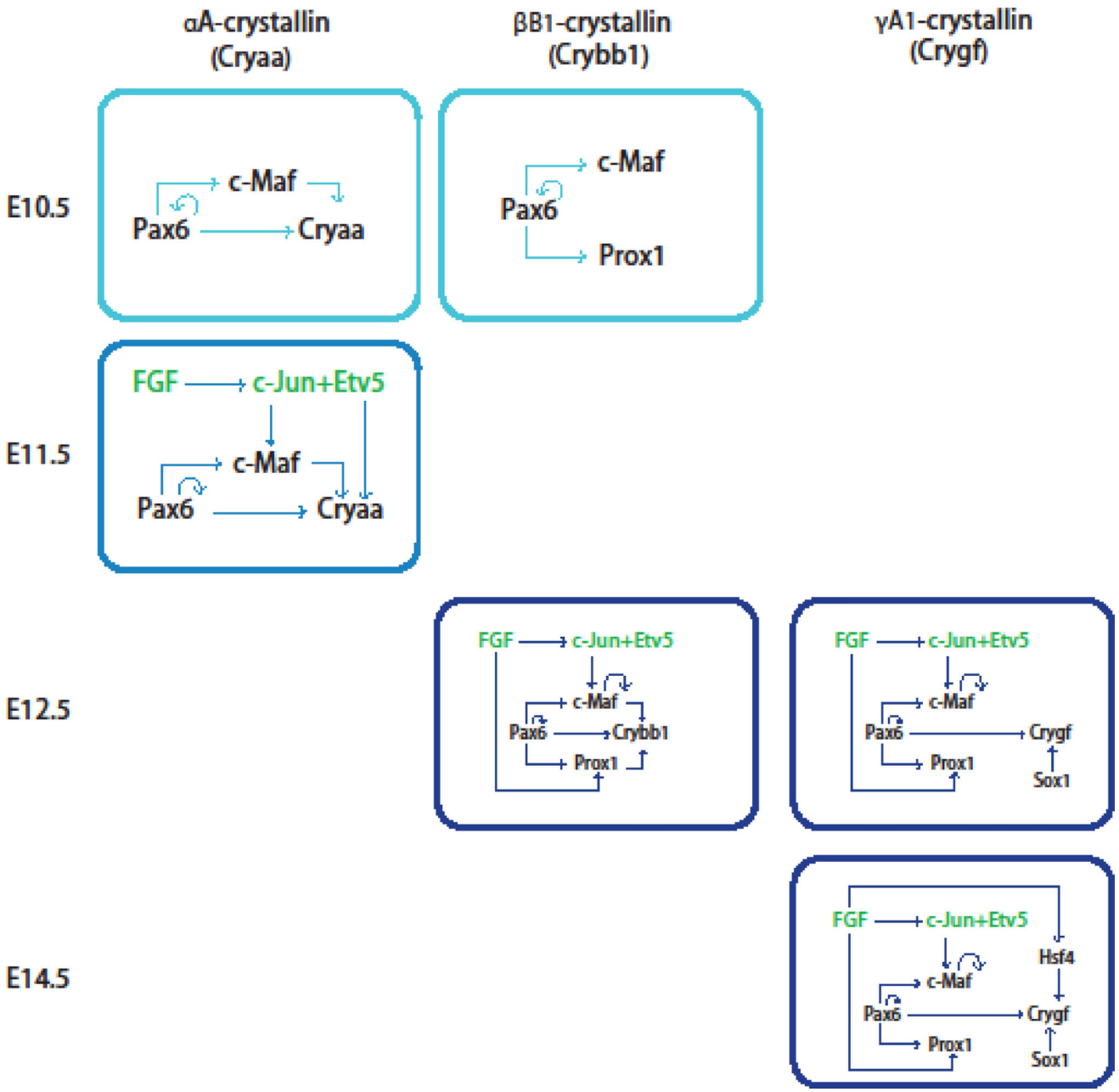

Figure 4. GRNs of αA-, βB1-, and γA-crystallin gene expression

Lens cell differentiation (light-moderate-dark blue contours) is illustrated at mouse embryonic stages E10.5, E11.5, E12.5, and E14.5. The individual GRNs are formed from the "core" GRN comprised of a feed-forward loop consisting of Pax6, c-Maf, and αA-crystallin [189] from E10.5. From E11.5 in posterior cells of the lens vesicle, FGF signaling activates the αA-crystallin indirectly via a regulatory region in the c-Maf promoter and through both the αA-crystallin upstream enhancer DCR1 [158] and promoter [189]. Transcriptional regulation of βB1-crystallin is comprised from the common "core" module, expanded by a Pax6 → Prox1 module [139] active from E12.5. Expression of γA-crystallin requires Sox1 [171, 175] and from E14.5 is further augmented by Hsf4 [170] and the FGF/

MAPK pathway [187]. Other regulatory layers are involved as Hsf4 inhibits the expression of Fgf1, Fgf4, Fgf7, and Fgfr1 [170], and Prox1 regulates the expression of Fgfr3, Fgfrl1, and Lctl and its expression is downstream of FGF/MAPK cascade [186].

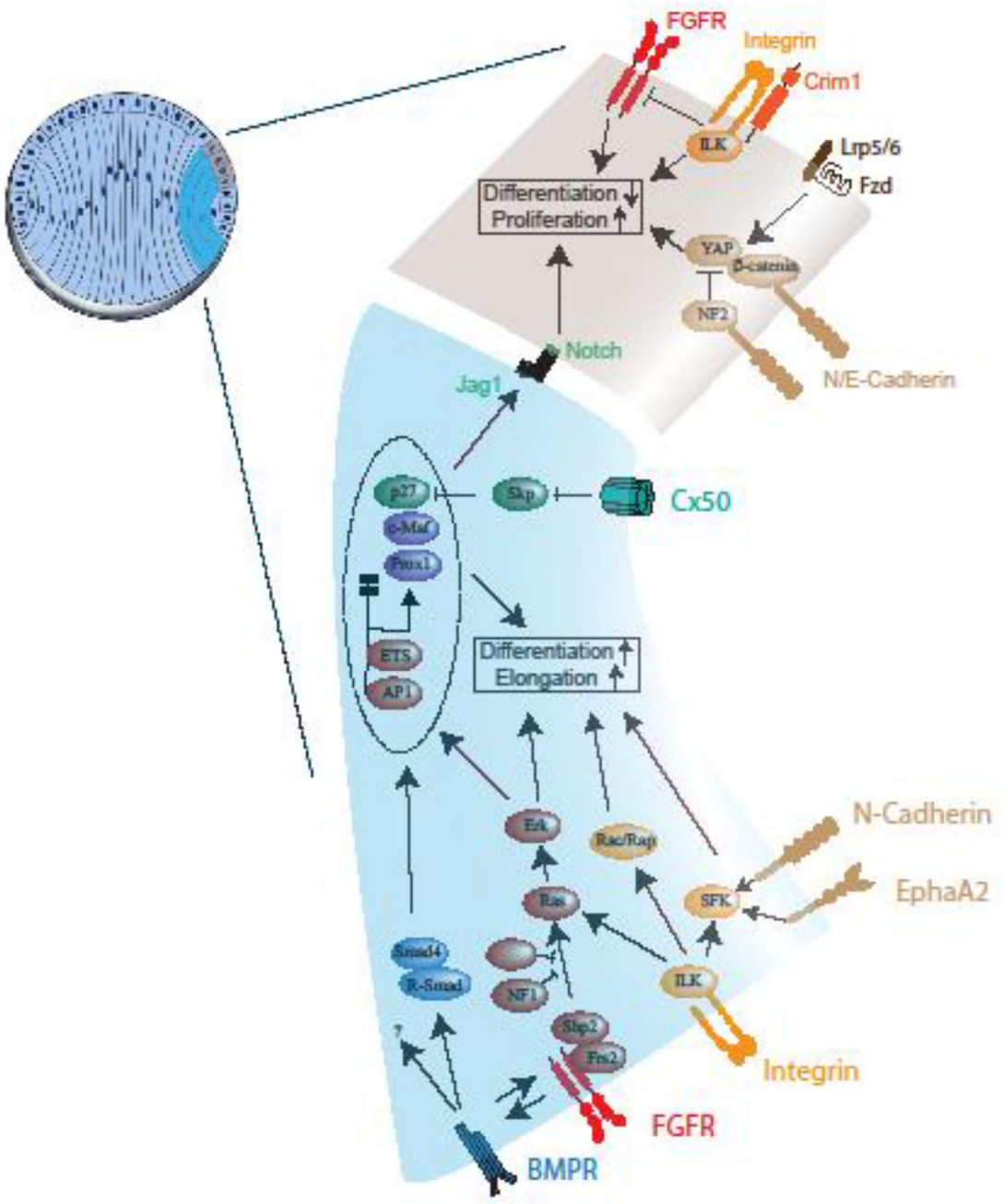

Figure 5. Lens development and extracellular signaling

(A) Schematic diagram of signaling pathways in the epithelial cells at the transitional zone of the lens. FGF and integrin signaling promote cell proliferation and survival, whereas Notch signaling induced by adjacent lens fiber cells suppresses cell differentiation. In addition, Wnt signaling transmitted by Lrp5/6 and Fzd receptors converges with cadherin and Yap to regulate cell differentiation, polarity and adhesion. (B) In differentiating fiber cells, FGF signaling through the Ras-MAPK pathway activates ETS and AP1 family transcription factors to induce expression of lens differentiation factors such as Prox1 and c-Maf. FGF also synergizes with Smad-mediated BMP signaling in cell differentiation and

cooperate with Cx50 in p27-mediated cell cycle exit. Cell adhesion molecules integrin and N-cadherin act with Eph/Ephrin receptors to induce small GTPases and Src family kinases to promote actin assembly and cell elongation.

Table 1

Signal Transduction Pathways in Lens Development.

Signaling pathway	Pre-placodal region	Lens placode Invagination	Lens epithelial cells	Lens fiber cells
BMP [8, 23, 51, 52, 127, 217, 218, 266, 267]	Inducer (↑)	Pro-survival and apical constriction (↑)	Cell proliferation and survival tiation (↑)	Cell cycle exit (↑)
BMP-noncanonical [52, 217, 218]		Smad-independent mechanism (↑)		Differentiation (↑)
FGF-MAPK [8, 9, 24, 53, 196, 204, 205, 207, 208, 268, 269]	Inducer (↑)		Proliferation (↑)	Differentiation (↑)
FGF-PI3K [145, 146]		Pro-survival (↑)	Pro-survival (↑)	
Wnt/β-catenin [8, 9, 23, 77, 192, 199, 201, 270, 271]	Inhibitory signal from periocular ectoderm (↓), posteriorization towards NC cells (↓)		Epithelial cell adhesion, integrity, and polarity (↑)	Differentiation (↑)
Wnt/PCP [214, 272, 273]			Lens GPS *) (↑)	Lens GPS (↑)
RA [7, 22, 33, 34, 92, 151, 274, 275]	Lens placode inducer (↑), posteriorization towards NC cells (↓)	Separation of lens vesicle from the prospective corneal epithelium (↑)	Attenuated between E12.5–E14.5	
Shh [61, 118, 276]	Inhibitory signal (↓)			Promotes towards epithelial at the expense of fiber differentiation (↓)
Notch [197, 198, 213, 277]			Control of lens growth and differentiation (↑)	Differentiation (↑)
Integrins [229, 230, 234, 278, 279]			Early differentiation (↓), cell adhesion and lens capsule assembly (↑)	Differentiation, cell adhesion, and lens capsule assembly (↑)
Cadherins [154, 227]		Separation of lens vesicle from the prospective corneal epithelium (↑)	Polarity, adhesion, and cell survival (↑)	Elongation (↑)
Eph/Ephrin [222, 225, 226]			Adhesion and polarity (↑)	Fiber alignment (↑)
TGF-β [77, 280]	Inhibitory signal from POM (↓)			
Hippo-Yap [202, 203]			Control of lens growth and size (↑)	
JAK-STAT [56]	Nociceptin receptor Oprl1 activates Stat3 and may operate in this process (↑)			

*) Global positioning system.

Table 2

A summary of selected DNA-binding factors and their roles in lens development *)

Pax6 Networks and EE-regulatory factors		**Cell Survival of Lens**	
Pax6	From E8.5 in the aPPR [66], HD	**ATF4**	Alternate name: CREB2 [281], bZIP
Six3	From E8.0 in the aPPR [44], HD	**C/EBPγ**	bZIP heterodimer ATF4-C/EBPγ [282]
Sox2	From E9.5 in the lens placode [50]	**Hif1α**	[283]
Meis1	Regulator of both EE and SIMO [100, 284], HD	**Pitx3**	Aphakia (ak) mouse [141], HD
Meis2	Regulator of both EE and SIMO [100], HD	**Psip1**	Alternate name: LEDGF [285]
Pknox1	Regulator of EE [286], HD	**Cell Cycle Exit and Denucleation**	
Pou2f1	Alternate name: Oct1, Regulator of EE [287], HD	**Gata3**	[288]
Regulators of Pax6 in the aPPR?		**Trp53**	[289]
C/EBPβ	Highly expressed in the aPPR [35], bZIP	**Crystallin GRNs**	
Dlx2	Marker of the aPPR [35, 67, 290], HD	**c-Maf**	[166–169, 171, 177, 189], bZIP
Dlx5	Marker of the aPPR [35, 67, 290], HD	**Hsf4**	[170] [172], also involved in denucleation
Foxg1	[67, 291], regulates Pax6 in brain [292]	**Prox1**	[173, 174, 186], HD
Myb	Regulates Pax6 in neuroretina [293]	**Sox1**	[171, 175]
Otx2	Expressed in the aPPR [294], HD	**Proliferation and Differentiation**	
		MafG	[295], bZIP
		MafK	[295], bZIP
		Myc	[296]
		N-Myc	[297], also involved in denucleation
		Zeb2	Alternate name: Sip1 [298]
		Lens Vesicle Separation	
		AP-2α	[147]
		FoxE3	Dysgenetic lens (dyl) mouse [149], denucleation defects [299]
		Sox11	[153]

*) Genes shown in blue belong to the microphthalmia-anophthalmia-coloboma group [237].

Table 3

A sumarry of selected SRTFs and their roles in lens development *)

FGF-MAPK		RA	
c-Jun	[300, 301], regulator of αA-crystallin [189]	**RARα**	[302]
FosB	[303]	**RARβ**	[237, 304], regulator of αB-crystallin [178]
Etv1	[145]	**RARγ**	[151, 304]
Etv4	[305]	**RXRα**	[274]
Etv5	[145, 306], regulator of αA-crystallin [189]	**RXRβ**	Regulator of αB-crystallin, [178]
BMP/TGF-β		**Notch**	
Smad1	[52, 54, 307]	**Hey1**	Alternate name: Herp2 [308]
Smad3	[77, 280]	**Rbpj**	[152, 198]
Smad4	[52, 218, 267]	**Hippo-YAP**	
Smad5	[52, 217, 307]	**Yap1**	[130, 202, 203]
Smad7	[219]		
SHH			
Gli2	[276] [61, 118]		

*) Genes shown in blue belong to the microphthalmia-anophthalmia-coloboma group [237].