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Building the Developmental Oculome: Vertebrate Eye Development and Disease

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Abstract

The vertebrate eye is a sophisticated multi-component organ that has been actively studied for over a century, resulting in the identification of the major embryonic and molecular events involved in its complex developmental program. Data gathered so far provides sufficient information to construct a rudimentary network of the various signaling molecules, transcription factors and their targets for several key stages of this process. With the advent of genomic technologies, there has been a rapid expansion in our ability to collect and process biological information, and the use of systems-level approaches to study specific aspects of vertebrate eye development has already commenced. This is beginning to result in the definition of the dynamic developmental networks that operate in ocular tissues, and the interactions of such networks between coordinately developing ocular tissues. Such an integrative understanding of the eye by a comprehensive systems-level analysis can be termed the "oculome", and that of serial developmental stages of the eye as it transits from its initiation to a fully formed functional organ represents the "developmental oculome". Construction of the developmental oculome will allow novel mechanistic insights that are essential for organ regeneration-based therapeutic applications, and the generation of computational models for eye disease states to predict the effects of drugs. This review discusses our present understanding of two of the individual components of the developing vertebrate eye – the lens and retina – at both the molecular and systems levels, and outlines the directions and tools required for construction of the developmental oculome.

Keywords

Eye; Development; Systems biology; *Pax6*; *Six3*; Organogenesis; Oculome; Transcription factor network; Lens development; Retinal development; Microarray analysis

The developmental "oculome"

Significant advances in technologies as distant as robotics, high throughput sequencing and microarrays, together with dramatic increases in computational power and algorithmic innovation, provide the opportunity to conduct high-throughput experiments on multiple "omics" levels [1–6]. Specifically, transcriptome, proteome, glycome and metabolome datasets can now be obtained for any biological specimen. Cells of normal mouse tissue or those from loss-of-function allelic mutants can be subjected to these analyses. Indeed, systems-level studies for organs such as the heart and the kidney have already resulted in

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integrative datasets that constitute the “cardiome” and the “nephrome”, respectively [1, 4]. Similarly, a systems-level, integrative understanding of the eye can be termed the “oculome,” and the “developmental oculome” can thus be defined as a similar comprehensive understanding of serial developmental stages of the eye as it develops from its earliest embryonic origins to a fully functional organ.

Building the developmental oculome is a challenging endeavor since oculogenesis is a complex process involving reciprocal interactions between multiple ocular tissues, which are coordinated in a spatially and temporally precise manner. The ocular network that would emerge from these studies will have a four-dimensional nature as the underlying nodes (genes and their products) involved in this program are under strict spatiotemporal regulation.

Knowledge of the vertebrate oculome will enable the definition of the molecular regulatory circuitry involved in the development as well as the maintenance of ocular tissues. In addition, it should be possible to recognize circuit defects that can lead to disease conditions. Such information may allow the construction of detailed computational models for eye development and for ocular disease states that can be used to predict the effects of drugs. Furthermore, such information may permit the formulation of organ regeneration-based therapies.

Overview of vertebrate oculogenesis

The adult vertebrate eye is a sophisticated organ with multiple cell types and complex tissue components, the latter including the retina, lens, cornea, iris, ciliary body, trabecular meshwork, Schlemm’s canal, the ocular glands, and the optic nerve, each with diverse yet specific functions (Fig. 1). These components form as a result of coordinated, multi-step signaling between the prospective lens ectoderm and neuroretina, the lens and the cornea, iris, ciliary body and the periocular mesenchyme [7]. Gruss and co-workers have proposed that vertebrate eye development can be empirically divided into three phases [8]. In the first phase, the process of induction via localized signaling leads to the regional specification and formation of the major morphological structures of the eye. In the second phase, these structures undergo cellular differentiation. Finally, in the third phase, individual components mature, resulting in the formation of a functional eye.

Eye development has been reviewed previously [7–19], and a detailed description is beyond the scope of this review. However, we do present a brief overview, in order to highlight the critical developmental stages where systems-level approaches can be most readily applied. Overall, oculogenesis is well conserved in vertebrates. However, distinct morphological differences do exist between various model systems and expectedly some of the underlying molecular mechanisms are found to be species-specific [15]. In the event of a species-specific difference in eye development, the process in the mouse is described as a reference.

Specification of the eye field

Vertebrate eye development is initiated early in embryogenesis during late-gastrulation when the ectoderm is divided into four domains: the neural plate, neural crest, pre-placodal

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region (PPR) and epidermis [15, 20–22]. At this stage, the region of the anterior neural plate (ANP) that eventually forms bilateral retinas is a single "presumptive retinal ectoderm" (PRE), which is surrounded by precursor cells of the telencephalon and the hypothalamus (Fig. 1A) [23–26]. The PPR surrounds the ANP and contains multipotent cells that harbor the potential to form all sensory and neurogenic placodes, including the lens placode. The PPR is specified by a combination of FGF-mediated positive regulation and the simultaneous inhibition of the negative regulators of this process, the Bmp and Wnt signaling pathways. The precise positioning of these tissues in developmental space and time is critical for the co-ordinate exchange of signals between them.

While only the genes encoding the transcription factors *Six3* and *Foxg1* are presently known to be expressed in the mouse PPR, many others, namely *Otx2*, *Rx*, *Pax6*, *Sox2*, *Hes1*, *Lhx2*, and *Six3* are expressed in the PRE and are required for eye formation, along with signaling molecules like Sonic hedgehog (Shh) [27, 28]. The fate of the single broad PRE is progressively restricted by Shh secreted by cells of the midline pre-chordal region, which functions to eventually divide the PRE into two distinct retinal fields [29]. Each retinal field region then evaginates to form an optic pit that progressively grows towards the overlying surface ectoderm and subsequently develops into the optic vesicle (Fig. 1B). Meanwhile, as a result of the anterior-most part of the PPR being biased to form the anterior pituitary and olfactory placodes, the lens-forming region of the PPR is also bilaterally divided [20, 22, 30–34].

Coordinated development of the lens vesicle and the optic cup

After neural tube closure, the optic vesicles grow out bilaterally and expand through the surrounding mesenchyme until they physically contact the overlying surface ectoderm, cells of which form the future lens (Fig. 1B). What follows is one of the best-understood examples of reciprocal inductive tissue interactions in development [35, 36]. Each optic vesicle induces the contacted surface ectoderm to form a thickening termed the lens placode (Fig. 1C). Reciprocally, signaling from the pre-lens ectoderm is essential for the optic vesicle neuroepthelium in gaining competence to form the optic cup [37, 38]. In *Pax6* loss-of-function mice - either naturally occurring $Pax6^{Sey1Neu/Sey1Neu}$ (*Small eye*) mutants [39, 40] or $Pax6^{lacZ/Pax6floxLe\text{-}Cre}$ mutants generated by conditional inactivation [37] - the lens placode fails to develop, suggesting a cell autonomous requirement for *Pax6* in the transition of presumptive lens ectoderm (PLE) to future lens. In addition, functional characterization of oculogenesis in mice containing loss-of-function alleles for *Rx* [41], *Lhx2* [42, 43], *Mab21l2* [44] and *Hes1* [45] demonstrates a requirement for these genes and for the optic cup in lens placode development.

These developmental events, which are directed by a series of signaling molecules and transcription factors, lead to the invagination of the lens placode, transforming it into a lens pit. As the lens pit develops, its cells undergo a change in their adhesive characteristics that results in its detachment from the overlying surface ectoderm [46]. At this stage, the lens acquires a globular shape with a hollow cavity, and comprises the lens vesicle (Fig. 1C). Concurrent with invagination of the lens placode, the optic vesicle invaginates to form a bilayered optic cup; the posterior part of the optic cup remains connected to the brain as the

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optic stalk. Cells in the inner layer of the optic cup form the neural retina (NR) while those in the outer layer differentiate into the retinal pigment epithelium (RPE) (Fig. 1C, D) [17]. Cells in the posterior/ventral region of the optic stalk give rise to the optic nerve, while those from the anterior rim region contribute to the epithelial component of the iris and ciliary body (Fig. 1D) [47].

As the lens vesicle develops further, its cells form two morphologically and functionally distinct cell populations. Cells in the anterior part of the lens vesicle form the anterior lens epithelium (ALE) and remain in the cell cycle, while those in the posterior part commence differentiation to form primary fiber cells. Throughout the life of the animal, cells of the ALE exit the cell cycle in the "transition zone" of the lens and undergo terminal differentiation to secondary fiber cells [7, 48]. The fiber cells of the lens are highly differentiated, and lack cellular organelles and contain an extremely high proportion of proteins termed crystallins [49–51]. Crystallins belong to the metabolic enzyme and stress protective protein classes, and serve non-refractive functions in non-ocular tissues [52]. In the lens, however, crystallins have been coapted via the evolution of lens-specific *cis*-regulatory elements (CREs) to exhibit high level expression [53, 54]. This leads to an ordered protein lattice that optimizes the refraction of light and provides the requisite optical transparency.

Key molecular events in lens development

Studies in fish and *Xenopus* indicate the transcription factor genes *Six3* and *Pax6* are at the top of a genetic cascade that drives lens development. Indeed, ectopic expression of *Drosophila* (*eyeless*, *twin of eyeless*) or *Xenopus* orthologs of mammalian *Pax6* in 3- and 16-cell stage *Xenopus* embryos, and ectopic expression of mouse *Six3* in 2–4-cell stage embryos of *Medaka* fish, result in the formation of ectopic lenses [55–58]. However, until recently the hierarchical relationship between these two "master" regulatory genes in lens development has been a source of debate [59]. Early studies that demonstrated the absence of *Six3* expression in presumptive lens ectoderm (PLE) of *Pax6* loss-of-function embryos suggest that *Six3* acts downstream of *Pax6* in stages following lens placode induction [60]. However, recent studies indicate that *Six3* regulates *Pax6* in the PPR region that encompasses the PLE, which at this stage is termed the preplacodal ectoderm (PPE). Six3 is also responsible for regulating the placodal expression of *Pax6* via multiple enhancers [59, 61, 62]. Conversely, the placodal expression of *Pax6* is required for the continued expression of *Six3* at later stages of lens development [37, 61, 62]. Feedback loops of this sort are a common theme in the gene regulatory networks (GRNs) that regulate organogenesis.

Meis proteins, members of the TALE or three amino acid loop extension family of homeodomain transcription factors, have also been implicated in the direct regulation of the lens placode phase of *Pax6* expression. This is thought to occur via their direct binding to a cognate site in the *Pax6* ectodermal enhancer (*Pax6 EE*), a 110 bp element that lies approximately 3.9 kb upstream of the *Pax6* P0 promoter which drives *Pax6* expression in the lens placode [63–66]. Also, recent genetic analysis of mouse *Pou2f1*; *Sox2* compound mutant lenses indicates that the HMG box transcription factor Sox2 interacts with the POU

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family member Oct1 to regulate placode expression of the same *Pax6 EE*, in turn regulating lens placode induction itself [67]. In addition, *Pax6* controls the expression of the Mab gene family member *Mab21l1* that is required for lens placode development, and *Mab21l1* in turn regulates *Foxe3*, a highly lens-enriched transcription factor gene that functions in regulating ALE proliferation, the onset of fiber cell differentiation, and lens vesicle closure [68–72]. In the ALE, *Foxe3* negatively regulates *Prox1*, which is required for lens fiber cell differentiation [69, 73–75]. The proliferating cell nuclear antigen (PCNA) gene is expressed in ALE where its direct interaction with residual levels of Prox1 may prevent *Prox1* from inappropriately activating fiber cell differentiation proteins such as *βB-crystallin*, $p27^{Kip1}$ and $p57^{Kip2}$ [76]. Lastly, the bicoid-like homeodomain transcription factor *Pitx3* is required for ALE maintenance, and functions by regulating *Foxe3*, *Prox1*, $p27^{Kip1}$, $p57^{Kip2}$ and *Pdgfrα* [77].

Recently, *Sox11*, a group C *Sox* family member has been shown to act downstream of *Pax6*. It functions in cell cycle regulation during lens vesicle invagination, and in later stages it regulates *Bmp7* expression [78]. Another candidate regulator that acts downstream of *Pax6* in the lens is the transcription factor *AP-2α*, which is involved in regulating adhesion molecules critical to lens vesicle separation [79]. The bZIP transcription factor Maf controls the lens-specific expression of *crystallin* genes, and is also required for fiber cell differentiation [80, 81]. Finally, analysis of *Sox1* loss-of-function embryonic lenses reveals an inappropriate up-regulation of *Pax6* in fiber cells, suggesting that *Sox1* is responsible for the normal down-regulation of *Pax6* in this tissue [82]. Along with *Prox1*, *Sox1* regulates *γ-Crystallin* expression in fiber cell differentiation [73, 83]. Other transcription factors that function in lens development include AP-1, CREB, Etv1, Etv5, Hsf4 and USF, but the functions of these factors require further analyses [18]. Thus, of the approximately 1800 TFs that are encoded in each mammalian genome, a significant subset - several percent - are implicated in the process of lens development either on the basis of mutational analysis or by a suggestive pattern of gene expression.

In addition to these transcription factors, major signaling pathways play distinct roles in specific stages of lens development [14, 84, 85]. *Bmp7* is expressed in the PLE prior to lens placode induction and analysis of *Bmp7* loss-of-function embryos indicates a requirement for Bmp7 in maintaining *Pax6* placodal expression [86]. In addition, *Bmp4* loss-of-function mice display aphakia, and Bmp4, secreted from the optic vesicle, is critical for induction of the lens placode during the interaction of the optic vesicle with the PLE [87]. Furthermore, this study indicated a requirement for *Bmp4* for *Sox2* expression in the lens placode. The Bmp pathway also plays an important role in the initiation of lens fiber cell differentiation [88, 89]. In early stages of lens induction, signaling via the Fgf pathway is essential for regulating *Pax6* placodal expression [90]. Later in development, Fgf signaling is crucial for cell cycle exit and for the initiation of fiber cell differentiation [14, 48, 85]. The canonical Wnt pathway co-activator Pygopus2 is required for *Pax6* lens placode expression, but in a manner independent of Wnt signaling [91]. However, another canonical Wnt pathway effector, β-catenin, negatively regulates lens fate in periocular ectoderm, but is required for later stages in lens development [92]. In addition, integrin receptors play a critical role in lens development and some of these such as α5 are directly regulated by Pax6 [93]. Finally,

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activation of Notch signaling in *Xenopus* embryos leads to eye duplication and in mice has distinct roles in lens progenitor cell proliferation and fiber cell differentiation [58, 94–96]. Together, this information - along with that described in the following sections - allows construction of a first-order gene regulatory network (GRN) for lens development, as depicted in Fig. 2.

Retinal differentiation

Retinal differentiation is discussed elsewhere in this issue (see article by Byerly and Blackshaw), and hence we summarize it only briefly. Cells of the inner layer of the bilayered optic cup contain retinal progenitor cells (RPC), which harbor the potential to differentiate into Müller glial cells and into the six types of neurons found in the retina (ganglion, amacrine, horizontal and bipolar cells, and rod and cone photoreceptors). As neurogenesis proceeds, intrinsic changes in the RPC regulate its competence to generate these different cell types [97, 98]. Both proliferation and neuronal differentiation initiate at the center of the inner layer in the prospective retina and proceed outward toward the periphery. The transcription factors Six3, Rx, Chx10, Pax2, Mitf, Otx2 and Pax6 play distinct roles in retinal differentiation [41, 99–104], while Math5 functions in retinal ganglion cell development [105] and Crx, Nrl, Nr2e3 and NeuroD1 are involved in photoreceptor development [106–109]. The Notch, Hedgehog, Egf and Fgf signaling pathways also play a role regulating the differentiation and proliferation of retinal cells [16, 110–114].

Development of the cornea

As the lens vesicle pinches off from the surface ectoderm, the surrounding ectoderm proceeds to close the gap and differentiate into the corneal epithelium, which is accompanied by expression of the marker Keratin12 [115, 116]. Periocular mesenchymal cells and neural crest cells contribute to formation the corneal stroma and endothelium by migrating between the corneal epithelium and the ALE, and differentiating into endothelial cells and keratinocytes [116–118]. This migration is triggered by a signal secreted by the surface ectoderm. In addition, signals from the lens are essential for the mesenchymal-to-epithelial transformation of cells that form the corneal endothelium [119, 120]. Recent lens ablation experiments show that in absence of the lens, the corneal epithelium fails to undergo proper differentiation as indicated by downregulation of Keratin 12 expression, and is replaced by cells that express the epidermal markers Keratin 1 and Keratin 10 [121]. As the cornea develops further, cells of the corneal stroma get arranged in a lamellar manner and undergo compaction, eventually leading to the mature cornea.

Systems-level analyses of eye development

In the following sections, we describe several systems-level approaches, mainly gene expression analysis and network construction, that have been used to study developing ocular tissues. Before going further, we would like to define critical terms and concepts widely used in systems biology. To begin with, systems biology aims to define all the molecular components of biological systems and to integrate this information into a network of interactions that can be used to predict system behavior [2]. An interaction network is

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composed of "nodes", which can represent biomolecules, such as proteins, DNA, RNA, or metabolites, and "edges", which indicate the nature of the relationship between the nodes [6, 122]. A network that operates in an individual cell or tissue at a particular developmental state can be generated in multiple ways, depending on how one defines the nodes and edges. In a transcriptional regulatory network (TRN), nodes represent transcriptional regulators (TRs), which include all the transcription factors, co-factors, and associated chromatin regulators expressed in the cell, as well as their target *cis*-regulatory elements (CREs) [6].

Biological networks appear to be "scale-free" and follow the power law degree distribution wherein most nodes interact with only a few other nodes and a few "critical" nodes interact with numerous nodes. Such critical nodes can be termed "hubs" [122–124]. This nomenclature has been used mainly for describing protein-protein interactions and is not naturally translated to transcription factors, as these proteins tend to have numerous edges due to their intrinsic functional properties. However, certain transcription factors that are positioned at the apex of a developmental program can be viewed as acting as "hubs" in a conceptual sense. For present purposes, we define these critical nodes as "global regulators" or "developmental hubs".

Pax6-transcriptional regulatory network in the postnatal lens: a global perspective

Over the past several years, gene expression profiling by microarrays has been used to acquire a systems-level perspective of the *Pax6*-mediated TRN in lens biology. Early studies used custom-made cDNA microarrays to examine differential gene expression in *Pax6*$^{+/+}$ and *Pax6*$^{+/-}$ lenses from 8-week old mice [125, 126]. More recently these studies have been extended to an earlier time point (P1) and have used commercial microarrays [127]. This approach has resulted in the identification of 559 transcripts that are differentially regulated by *Pax6* in the lens. Furthermore, comparison of this lens dataset with that generated for *Pax6*$^{-/-}$ telecephalonic tissue at E12 and E15 [128] has led to the identification of 178 transcripts that are commonly regulated and 381 transcripts that are differentially regulated by *Pax6* in these diverse tissues. Of nine lens-differentially regulated genes, five (*Cspg2*, *Mab21l2*, *Olfm3*, *Spag5* and *Tgfb2*) were demonstrated to be direct targets of *Pax6* by quantitative chromatin immunoprecipitation (qChIP) assays. This study also found *Pax6* to be involved in the regulation of lesser-characterized components of the Fgf, RA, Tgfβ/Bmp and Wnt signaling pathways in the lens. Furthermore, a comparison of 381 transcripts that are differentially expressed in lens with data from a high-throughput *in situ* hybridization screen that identified 82 putative *Pax6* regulated genes in mid-gestation mouse embryonic tissues [129] revealed eight genes in common (*Cdh8*, *Fgf14*, *Gabrg2*, *Neurod1*, *Neurod6*, *Pdelc*, *Tmem2* and *Wnt2b*) that appear to be *Pax6*-regulated in the lens, in turn validating these candidate genes as downstream targets of *Pax6*.

In addition, a recent study describes microarray analyses on lenses from *AP-2α* conditional loss-of-function mice at (P0) [79]. This study identified 415 transcripts that are differentially regulated in mutant and wild type lenses, which include genes essential for maintenance of the lens epithelium. Since *AP-2α* is a downstream target of *Pax6*, data from this study should allow further understanding of the *Pax6*-gene regulatory network (GRN).

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Gene expression analyses of lens fiber cell differentiation

Gene expression profiling studies on the lens have focused on the entire lens and thus represent data from cells of the ALE and the proliferative zone in addition to differentiating fiber cells. However, a recent study attempts to define and compare the transcriptome of young elongating lens fiber cells with that of mature fiber cells in P5 mouse lenses [130]. This study utilized Laser Capture Microdissection (LCM) and GFP-expressing transgenic reporter mice that express GFP in a variegated mosaic manner in young elongating fiber cells to isolate and characterize young and old lens fiber cells. Gene expression profiling experiments revealed 65 genes that are differentially expressed at 2-fold or greater levels between these cell types, with about 75% of the genes being down-regulated as the fiber cells mature. Interestingly, of the 25% of genes that were up-regulated in maturing fiber cells, several have apoptosis-related functions. These include *Gadd45b*, which encodes an activator of Tgfβ-induced apoptosis [131] and *Dlad*, which encodes a lens-enriched DNase II beta enzyme that is responsible for degrading fiber cell nuclei [132, 133].

The present model of fiber cell differentiation posits that a specific combination of pro- and anti-apoptotic molecules contribute to fiber cells undergoing a regulated form of apoptosis. According to this model, the apoptotic process does not reach completion and eventually results in these cells attaining an unusual differentiation state whereby they have lost all organelles but remain viable [50, 51]. In support of this view, maturing fiber cells exhibit elevated *Bag3* expression, which encodes a chaperone regulator protein that inhibits apoptosis when overexpressed in cell culture [134, 135]. Additionally, this work suggests that the CD9-mediated fusion pathway is active in maturing lens fiber cells, and that this may partly account for how fiber cells fuse to form a syncytium. Further analyses of genes in these datasets should enable a greater understanding of the fiber cell transcriptome and help define the GRNs operative in fiber cell differentiation.

Systems-level analyses of cataractous lenses

Alterations in the gene expression of cataractous and normal lenses have been of interest since even before the advent of microarrays. Using reverse transcription-based polymerase chain reaction differential display (RT-PCR-DD), Kantorow and coworkers [136] detected 3 transcripts that were over-expressed and 12 transcripts that were down-regulated in human senile cataractous lenses compared to normal lenses. Of note was the over-expression of the gene encoding a metallothionein IIa protein, known to be involved in detoxification pathways that respond to oxidative stress, while transcripts encoding a protein phosphatase 2A regulatory subunit (P2A-RS), a suppressor of mitosis, were among those that were down-regulated. These results support the observation that both oxidative stress and cell cycle mis-regulation in the lens are associated with the presence of cataract [137–139].

This study has now been extended by the use of gene expression microarrays to assess altered gene expression in human senile cataractous and normal lenses [140]. This approach identified 1031 transcripts that are differentially expressed between cataractous and normal lenses, with the majority of transcripts showing decreased expression in cataractous lenses [141]. A significant number of genes that showed increased expression in cataractous lenses are associated with ionic transport; these may reflect a compensatory mechanism in response

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to decreased calcium channel activity in the lens due to oxidative stress. Proteomics-based approaches also reveal a large number of post-translational modifications on major lens proteins, and some are associated with cataract onset [142]. However, further studies are needed to gain a better understanding of these modifications [143].

A dynamic gene regulatory network (GRN) for lens development

Using the information just summarized, we have begun to assemble a first-order gene regulatory network (GRN) for lens development. This GRN encompasses lens development from the lens bias stage through the lens pit stage, and incorporates temporal aspects of the data into the network, rendering it dynamic in nature (Fig. 2). Our goal is to highlight the relationship between the nodes involved and to demonstrate how this GRN evolves dynamically as the lens develops. Not surprisingly, *Six3* and *Pax6* emerge as critical nodal points that represent "global regulators" or major "developmental hubs" in the lens developmental GRN (Fig. 2) [18, 86, 144]. Both Six3 and Pax6, as well as Sox2 are involved in a transcriptional feed-back regulatory loop that drives and maintains the lens development GRN.

Coincident with the transition of the PLE to lens placode, numerous positive inputs from multiple signaling cascades (Bmp, Fgf, RA, Pygopus) emanate from coordinately developing tissues (optic vesicle and periocular mesenchyme). These signals serve to activate *Pax6* expression in the lens placode by impinging on multiple interacting CREs that reside in the *Pax6* lens enhancers (Fig. 2) [63–65]. The sophisticated regulation of *Pax6* expression, which requires such multiple regulatory inputs, arguably reflects its criticality to the lens developmental cascade. *Pax6* is one of 165 genes whose genomic region is associated with highly conserved non-coding sequences, and it has evolved to acquire multiple lens-specific enhancers supports this concept [63, 64, 145]. This view allows us to suggest that one potential indication of whether a TF constitutes a critical "developmental hub" is not just a large number of downstream regulatory targets, but also a number of distinct upstream inputs that have evolved to regulate its expression.

One conspicuous feature of the lens development GRN is the extensive control of cell cycle molecules, e.g., cyclins. In addition, cell cycle regulators, such as *Foxe3* and *Prox1*, are controlled by multiple regulatory inputs. For example, *Foxe3*, a known positive regulator of the cell cycle, is regulated by multiple positive inputs at this stage when most lens cells are in a proliferative state. In contrast, *Prox1* is critical for cell cycle egress and for subsequent commitment to fiber cell differentiation; therefore, at this early stage of lens development, it is regulated predominantly by negative input signals. Furthermore, Prox1 activity is also negatively regulated by a protein-protein interaction with PCNA, which helps ensure that residual Prox1 levels in ALE cells will not inappropriately initiate fiber cell differentiation. Thus, there are both positive and negative regulatory inputs as well as protein-protein interactions that coordinate cell cycle control.

Several other features of the lens development GRN deserve comment. Regulation of *αB-Crystallin*, an early onset crystallin gene [146] is orchestrated by *Pax6*, *Sox2*, and *RAR/RXR* TFs that are expressed early in the lens. On the other hand, *γ-Crystallin*, a late onset crystallin is predominantly under the control of negative input signals at this stage. As the

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lens placode undergoes invagination at this stage, positive input signals for genes that play a role in this process, e.g., *AP-2α* and *Sox11*, are transduced, and these in turn regulate the expression of cell adhesion molecules such as N-Cadherin and E-Cadherin that are required for morphologic changes in the invaginating lens [46, 78, 79].

Lastly, Sip1 has recently been shown to play a role in lens development [147]. However, the identity of its interacting Smad partner in the lens remains unknown (Fig. 2). Transcript profiling analysis of the developing lens identifies the presence of Smad proteins 1, 2, 3, and 5, indicating them to be potential interacting partners of Sip1 in the lens (Lachke and Maas, unpublished observations). Thus, transcript profiling analysis and network construction can be utilized to make predictions to define specific interactions between molecules.

Systems-level approaches in retinal development and disease

Global gene expression analyses by both serial analysis of gene expression (SAGE) and microarray have identified hundreds of genes that are expressed in retinal development. The availability of these datasets has resulted in more efforts at generating a GRN for retinal development than for any other ocular component [148–154]. The use of motif discovery algorithms has led to the prediction of several rod- and cone-specific CREs and some of these have been experimentally validated [155]. Specifically, promoters of cone-expressed genes are found to contain an enrichment of predicted motifs for Rx and Engrailed family members, while promoters of rod-expressed genes are found to contain predicted IL-6 effector motifs. Using an algorithm for searching binding motifs for the retinal transcriptional regulators Crx, Nrl and Nr2e3 near transcription start sites of retinal-expressed genes, Qian and coworkers [156] have predicted several potential targets and have validated *Rp1*, *Gucy2d* and *Abca4* as novel targets of Crx.

Interestingly, the *Nrl*$^{-/-}$ mouse retina lacks rod photoreceptor cells and displays an extreme "cone photoreceptor cell only" phenotype, thereby presenting a unique opportunity to gain insight into the transcriptomes of these two photoreceptor cell types [109]. Comparative microarray analyses of *Nrl*$^{-/-}$ and wild type retinas indicate alterations in both the Bmp and Wnt signaling pathways, with a downregulation of *Bmp4* and *Smad4* genes in the *Nrl*$^{-/-}$ retina suggests their deployment in rod photoreceptor development [157, 158]. Recently, fluorescence assisted cell sorting (FACS) combined with gene expression profiling of RPCs has led to the identification of over 800 retinal transcripts enriched in these cells [159]. Genes regulating the cell cycle are well represented in RPCs, reflecting the proliferative nature of these cells. Moreover, enrichment of chromatin regulators including ATRX, chromobox homolog 3 (HP1-γ) and histone methyltransferase suggest a role for epigenetic mechanisms in regulating RPC multipotency.

Crx null mice are blind at birth and have no detectable photoreceptor function. Photoreceptors do not form in the outer segment that is necessary for phototransduction, and 46% of photoreceptor-enriched genes are Crx-dependent, particularly opsin genes (Blackshaw 2001). Peng and Chen [160] used ChIP assays to demonstrate the inability of Nr2e3, a rod-photoreceptor determining "orphan nuclear receptor" to bind its target CREs in *Crx*$^{-/-}$ mice, revealing how complex interactions among key TFs can contribute to a retinal phenotype. Recently, Hennig and coworkers [161] used ChIP and quantitative RT-PCR

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combined with existing knowledge of retinal photoreceptor development to analyze the TRN of five TFs that are key to this process. They studied the ability of Crx (necessary for photoreceptor cell lineage determination), Nrl and Nr2e3 (necessary for rod cell lineage determination), and the HLH factor NeuroD1 (necessary for survival of photoreceptor cells) to regulate each other via direct binding to regulatory elements in retinal tissues from wild type, *Crx*$^{-/-}$, *Nrl*$^{-/-}$ and *Nr2e3*$^{-/-}$ mice. Their model transcriptional regulatory network (TRN) of these photoreceptor transcription factors is summarized in Fig. 3.

An alternative approach towards defining the Crx, Nrl, and Nr2e3 TRN in photoreceptor cell development employs gene expression and *in situ* hybridization datasets, combined with novel computational tools [162]. This approach has led to the construction of a network that comprises 600 components and includes novel transcription factors that were previously uncharacterized in photoreceptors cells. An algorithm that identifies photoreceptor-specific CREs of these nodes (photoreceptor transcription factors) was used to define the edges of this network. This allowed for the derivation of a *cis*-regulatory grammar rule, which renders high-level photoreceptor-specific gene expression. Moreover, Hsiau and colleagues used this information to deduce photoreceptor CREs by purely computational means, and have demonstrated the capacity of such modules to drive photoreceptor-specific expression *in vivo* [162]. This study illustrates how convergent datasets can be used to construct a highly informative developmental TRN.

Finally, systems-level analysis has also provided insight into retinal disease. For example, age related macular degeneration (AMD) is the most common form of macular disease and leads to the degeneration of central retina and loss of vision [163]. To achieve comprehensive gene expression profiling of the macula, SAGE and microarray analyses have been carried out and have identified candidate genes for ocular genetic disorders [164]. Although further studies are needed to validate the function of these candidate genes, comparison of these datasets with those for surrounding extra-macular tissue have identified 21 differentially expressed genes that may play a role in AMD [165].

In addition, systems level approach has been initiated to the study of glaucoma. Physiological changes in retinal ganglion cells and glial cells contribute to the pathology of glaucoma. Transcript profiling analyses of these cell types have provided insights into the pathology of glaucoma [166–168]. Moreover, several candidate genes have been identified that are present within loci linked to this complex multifactorial disease [167].

Future directions: construction of a developmental oculome

Although systems-level analytical tools are gaining increasing power, the datasets available for ocular tissues in developmental stages are presently incomplete. Furthermore, there have been only a handful of attempts to build TRNs and GRNs using such systems-level data. A complete understanding of oculogenesis will require a complete definition of all the nodes and edges for each distinct tissue and cell type of the eye as a function of development. In the sections that follow, we examine experiments that need to be performed to provide such a systems-level understanding of ocular development.

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Constructing systems-level datasets for eye components

As summarized above, gene expression datasets exist for specific tissue components of the eye, especially the retina [148–154], and in certain cases, the expression profiling of single cell-types has been successfully attempted [152–154]. However, there is nonetheless a significant lack of cell type specific datasets for serial stages of ocular tissues at the transcript and protein levels. To help remedy this situation, it is now possible to employ LCM or FACS separation, coupled with cell type-specific enhancer driven GFP reporters, to isolate individual tissue components or specific cell-types of the mouse embryonic eye for further analyses. This is especially critical for the analysis of highly heterogeneous retinal cell populations [98]. Recently, a similar approach has been successfully utilized in a systems-level analysis of the developing kidney [169]. Brown and coworkers [170] have conceived of an analogous approach utilizing LCM coupled with expression profiling to identify two novel genes that serve an essential function in optic fissure closure.

Thus, individual ocular components from mouse embryos separated by 0.5-day intervals can be isolated by LCM or FACS sorting, and subjected to global gene expression and proteomic analyses to obtain serial datasets at both the mRNA and protein levels. Using any of a number of relatively new computational algorithms (a few of which are described below), the data gathered can then be processed to generate a network for each stage and tissue of ocular development. Such analyses may demonstrate how individual ocular tissue specific GRNs regulate the development of specific ocular components, and also enable predictions about how the systems level behavior of ocular development depends the underlying network architecture. Furthermore, systems-level analyses of mouse models with ocular defects should provide a more detailed understanding of the structure of individual tissue component and cellular GRNs. For example, with LCM it is now possible to isolate and analyze the surface ectodermal region prior to induction of the lens placode from *Pax6*$^{-/-}$, *Pax6*$^{+/-}$ and *Pax6*$^{+/+}$ embryos. This should allow the identification of critical early events in the *Pax6*-GRN. Similar experiments could be performed for *Six3* conditional loss-of-function embryonic PLE.

Networks of RNA-binding proteins, microRNA and their targets

In recent years, RNA-binding proteins (RBPs) and small non-coding RNA molecules and their associated protein machinery have emerged as mediators of gene regulation [171]. The microRNA (miRNA) mediated gene-silencing pathway that is conserved across many species has been discovered to play a central role in RNA interference (RNAi). Recently, the miRNA pathway has been shown to operate in cytoplasmic RNA granules that are classified as P-bodies; these serve as sites of mRNA decay and degradation [172]. This observation raises the question of whether specific RNA granule components or miRNAs that function in oculogenesis show tissue-specific expression.

Recent data addresses this question. For example, a specific class of RNA granules are expressed in the developing lens (Lachke et al., 2009 submitted), and deficiency of a lens-enriched RNA granule component of this class of RNA granules, Tdrd7, leads to mis-regulation of key transcription factor genes and produces cataract formation. This establishes that RNA binding proteins that constitute tissue-specific components of RNA

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granules play a critical role in eye morphogenesis. Similarly, a role for miRNA-mediated gene regulation in oculogenesis has been indicated in *Xenopus laevis*, where overexpression of a specific miRNA (miR-196a) negatively affects eye development by downregulating the expression of *ET*, *Rx1*, *Six3*, *Pax6*, *Lhx2*, *Optx2*, and *Ath5* in the PRE [173]. In addition, miRNAs and natural antisense transcripts for several regulators of eye development have recently been described and exhibit tissue-specific expression [174–176]. In view of these findings, the identification of RBPs and their mRNA targets, and the generation of an miRNA expression profile for miRNAs and the identication of their targets, will be required to gain a complete understanding of the GRNs that control ocular development.

Novel tools and approaches in systems-level analysis

As outlined above, large-scale gene expression datasets for several ocular tissues exist. However, attempts to process this information, either for GRN construction or disease gene identification, are more limited [177]. Understanding the entire protein-protein interaction network during oculogenesis is essential in building the oculome. However, we will focus on approaches and technologies that would enable building of transcriptional regulatory networks via study of *cis*-regulatory modules, since this represents the logical first step in building a network [178]. Below, we outline several systems-level strategies and computational tools that can be used to efficiently identify ocular disease genes and to construct TRNs for specific ocular tissues.

Systems approaches to ocular disease gene discovery

We have formulated a method for processing mouse lens developmental microarray datasets that enables efficient gene discovery in lens development and disease. The underlying idea is that selectively enriched gene expression in a particular organ tissue above background levels increases the probability that the gene of interest plays a role in the development or function of that organ or tissue component [148, 177]. To pursue this goal, we first generated a developmental profile of the mouse lens transcriptome as the lens transitions from the stage of lens placode invagination to that of lens vesicle formation, when fiber cell differentiation has commenced (Lachke et al. 2009, submitted). Then, to identify differentially regulated genes, we established a normalization protocol by which lens microarray datasets are subjected to *in silico* subtraction with a developmentally matched microarray dataset representing the whole embryo minus ocular tissue. This *in silico* normalization protocol ranks probe datasets based on their lens enrichment *p*-values.

The resulting normalized mouse lens database can be efficiently used to identify lens-enriched genes. The implicit hypothesis is that such genes are likely to play important roles in lens biology. For example, genes that survive this filter for lens enrichment can be used to identify and prioritize potential candidate genes that fall in the vicinity of mapped human cataract loci. This is achieved by ranking all orthologous human genes within a mapped interval for a particular human cataract locus based on their murine homolog developing lens gene expression “enrichment score” as assigned by the normalized database. When this approach is used, all the previously known human congenital cataract genes are readily identified. Furthermore, we have applied this protocol to identify a novel human cataract gene, *TDRD7* (Lachke et al 2009, submitted). Thus, by constructing developmental

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microarray datasets and subsequently normalized databases for various tissue compartments of the developing eye, one can identify novel ocular disease genes.

Tools for building transcriptional regulatory networks (TRNs)

Two major limiting factors in building TRNs for transcription factors (TFs) have been: (1) identification TF DNA-binding motifs, and (2) determination of the biological significance of such motifs throughout the genome. The development of protein binding microarrays (PBMs) has led to the identification of motifs for several hundred DNA binding proteins [179–185]. This technology provides an opportunity to identify *cis*-regulatory "codes" or "modules" for co-regulated genes. The premise of this approach is that the most parsimonious way for nature to accomplish the coordinate expression of genes in overlapping spatiotemporal domains is to co-evolve binding motifs for the same or similar ensembles of transcription factors. Thus, the flanking and intronic genomic regions of co-regulated genes are expected to display an enrichment of DNA binding motifs for particular groups or ensembles of TFs [5, 186, 187]. Recently, two algorithms, *PhylCRM* and *Lever*, have been developed that work together to identify *cis*-regulatory modules (CRMs) for specified combinations of transcription factors in developmental gene expression datasets [188]. *PhylCRM* interrogates genomic sequences for combinations of evolutionarily conserved transcription factor motifs, while *Lever* uses this information to map overrepresented regulatory modules in gene expression datasets for a cell or tissue type at a specific developmental state.

Application of this type of strategy should allow for the efficient building of TRNs for major lens transcription factors as the lens transits from the placode stage to the primary fiber cell differentiation stage. By mining the database of genes that are differentially expressed in the lens, it should be possible to identify previously characterized as well as uncharacterized TFs for which binding motifs can be determined using PBMs. Then, by using *Lever* and *PhylCRM*, one can perform a motif-search for various combinations of the above transcription factors in genomic regions near differentially regulated genes in the lens. A biologically tested model for some of these combinations already exists (e.g., for highly lens-specific crystallin gene promoters or enhancers), and these CRMs can act as "positive controls." Theoretically, this approach can predict novel lens enhancers, and it can also help to define the nodes and edges in a TRN for lens TFs and their targets. Of course, predictions based on such a model must be tested experimentally. However, this strategy can be repeated for other components of the eye to build up an increasingly comprehensive and well-integrated TRN of ocular transcription factors and their targets (Fig. 4).

Clinical applications of the oculome

The approaches outlined above would lead to the identification of a majority – if not all – of the genes that are involved in ocular disease conditions. Eventually, this information can be used to design a signature "ocular disease gene set", which can be used for diagnostic purposes. With the advent of newer and cheaper technologies for sequencing, the entire ocular disease gene set can be sequenced from individual patients to determine the identity of the mutant gene(s). Furthermore, non-coding regulatory regions of ocular disease genes, which will be identified by the TRN analysis, can also be incorporated in such a gene set.

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Thus, information derived from the oculome has the potential to not only advance our fundamental understanding of oculogenesis, but to also contribute to the diagnosis and treatment of ocular diseases.

Conclusion

The past few years have witnessed a dramatic increase in the application of systems analysis to the study of vertebrate eye development. However, our present understanding of oculogenesis still derives largely from the application of molecular genetic tools. Yet even with such approaches, it is already possible to establish simple networks for some of the major molecular components involved in lens and retinal development. Certain essential nodes, termed "global regulators" or "developmental hubs" in the lens GRN such as *Pax6* and *Six3* are known, and analyses of mouse loss-of-function models for key genes in the ocular GRN should provide further insight into the identities and toplogy of the nodes and edges in this network. Finally, application of this approach to serial developmental stages of each tissue compartment of the developing eye should provide insight into how the individual GRNs for each ocular tissue mature during development. This will represent a first step towards understanding how such networks interact with each other in the complex context of vertebrate oculogenesis, and help to elucidate the vertebrate oculome.

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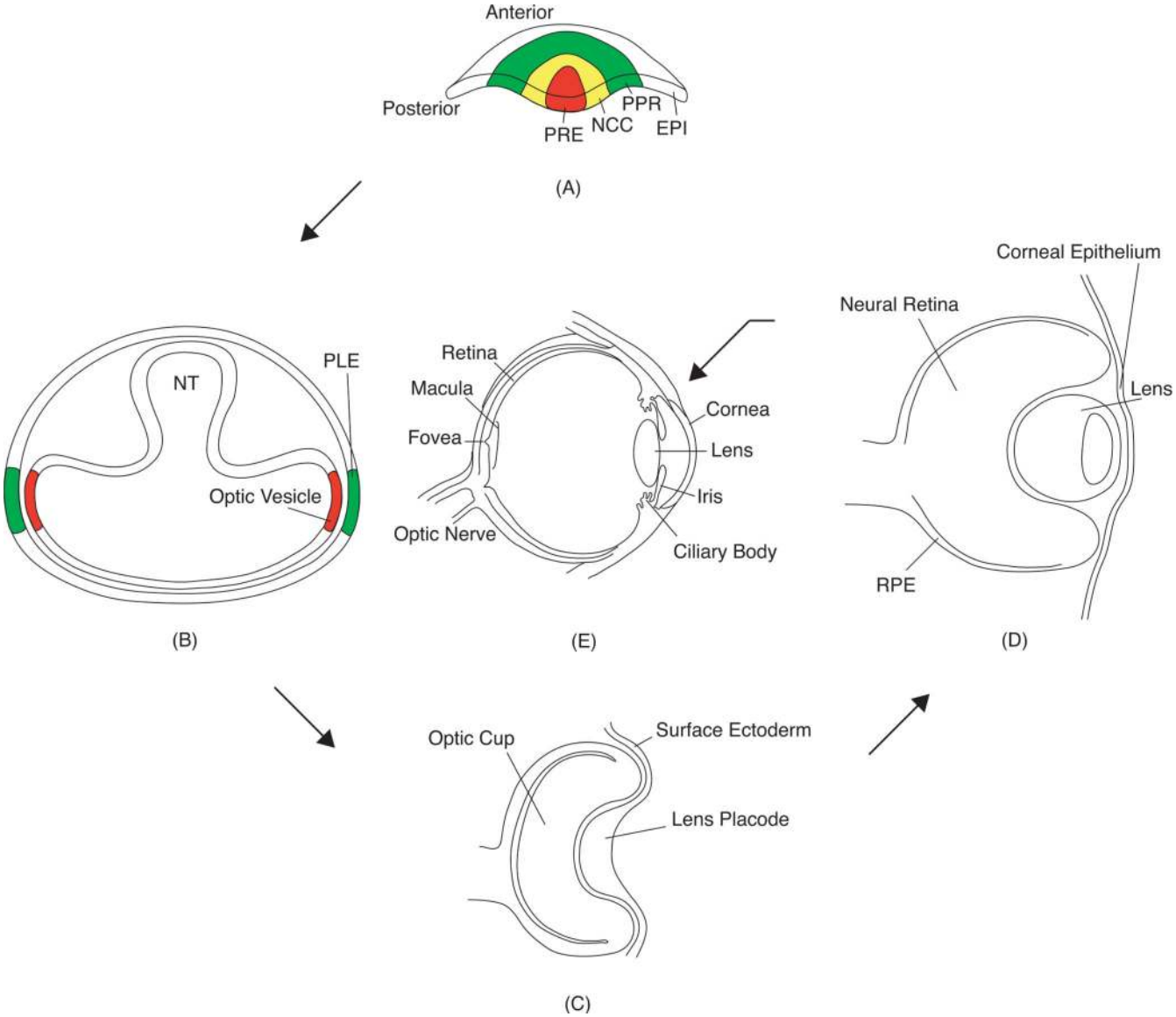

Fig. 1.

Vertebrate ocular development. (A) In late gastrulation, the anterior neural plate contains the presumptive retinal ectoderm (PRE) surrounded by neural crest cells (NCC), pre-placodal region (PRR), and the epidermis (EPI). (B) As the neural tube (NT) closes, the diencephalon bilaterally develops into the optic vesicles, which contact the presumptive lens ectoderm (PLE) on either side. (C) Co-ordinate signaling leads to induction of the PLE to form the lens placode, while the optic vesicle invaginates to form the optic cup. (D) The lens placode invaginates and forms the lens pit that detaches from the surface ectoderm to form the lens vesicle. At this stage the optic cup starts to differentiate into the neural retina and the retinal pigment epithelium (RPE). (E) The adult vertebrate eye thus contains multiple tissue compartments. It is important to note that in fish and frogs, the lens placode does not form a hollow lens vesicle, but instead develops into a solid aggregate of cells.

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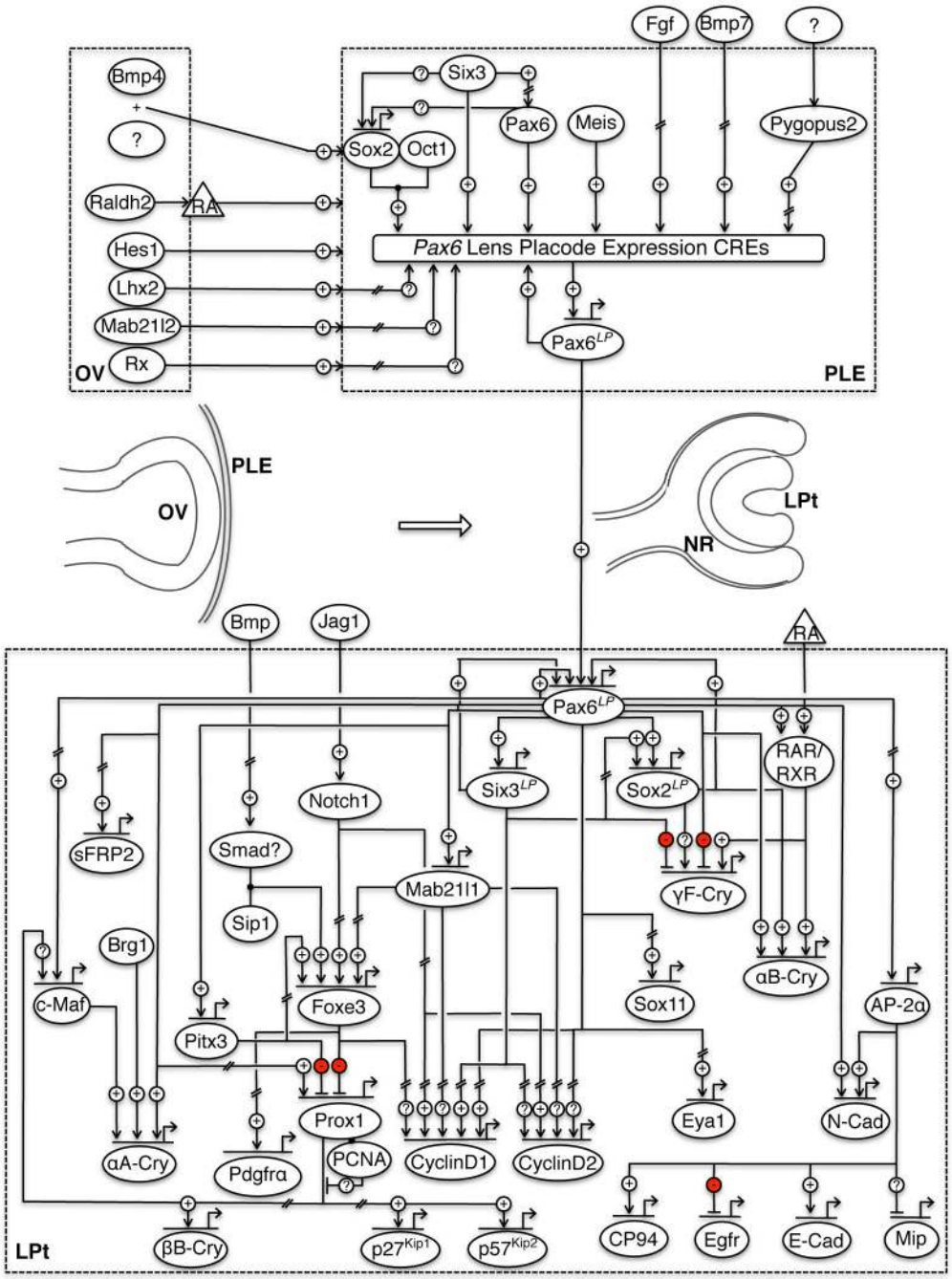

Fig. 2.

The molecular circuitry of early mammalian lens development. Numerous signals from the optic vesicle (OV) along with other molecules converge on multiple *cis*-regulatory elements (CREs) to induce the placodal expression of *Pax6* (*Pax6*^{LP}) in the presumptive lens ectoderm (PLE). As the PLE develops into the lens pit (LPt), *Pax6*^{LP} turns on the circuitry necessary for lens formation. *Pax6*^{LP}, *Six3*^{LP} and *Sox2*^{LP} indicate expression of these genes in stages following lens placode induction. Hatches in lines indicate that the interaction may involve intermediate steps.

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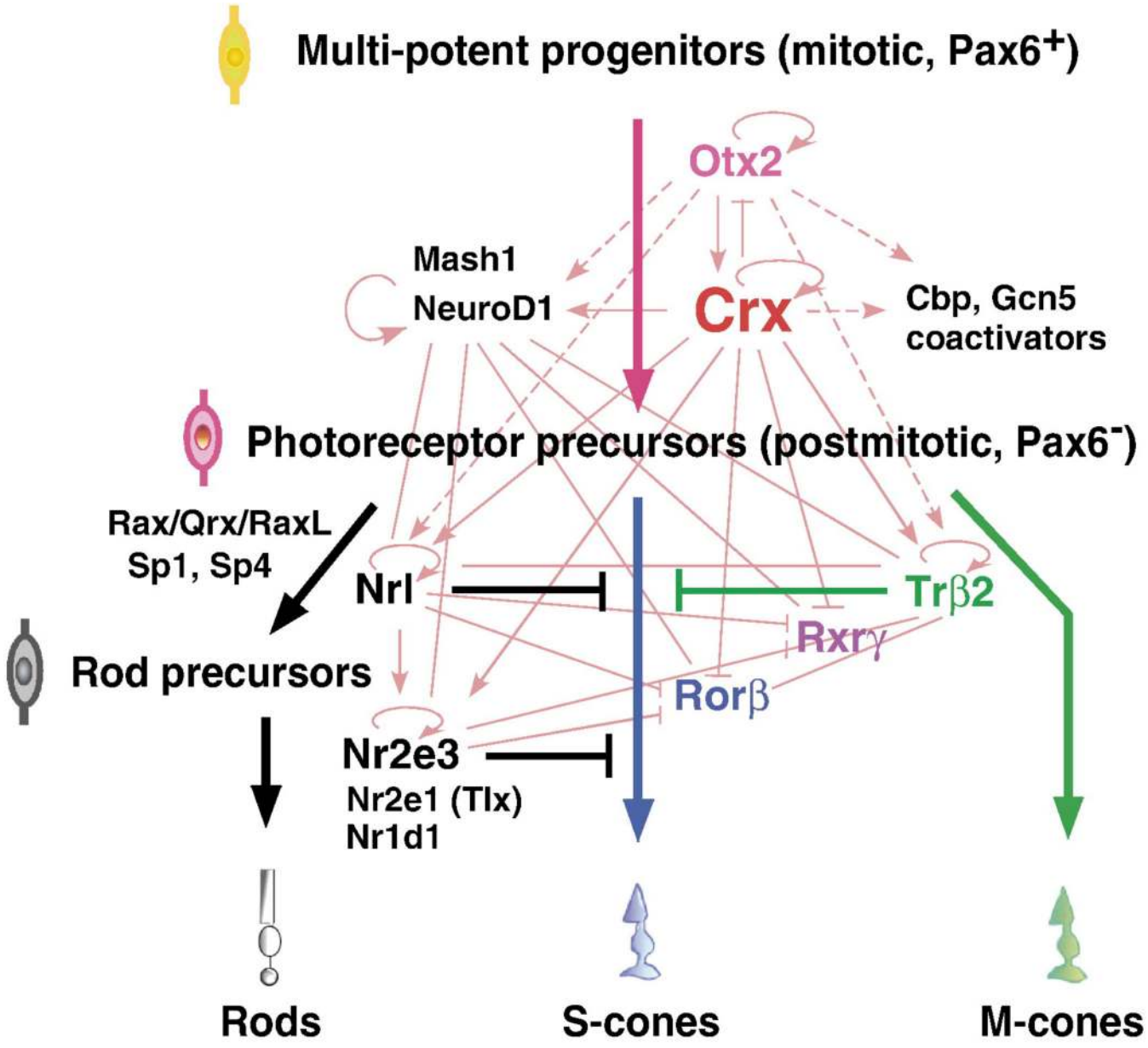

Fig. 3.

Transcription regulatory network (TRN) for retinal photoreceptor cell development. Multipotent, photoreceptor precursor cells differentiate into individual photoreceptor cell types via three separate pathways. Thin lines indicate protein-promoter interactions; solid lines indicate published data and dotted lines are from unpublished data of Hennig et al 2008 [145]. Reprinted from Brain Research 1192, Hennig, A.K., Peng, G-H., Chen, S., Regulation of photoreceptor gene expression by Crx-associated transcription factor network, 114–133, (2008) with permission from Elsevier.

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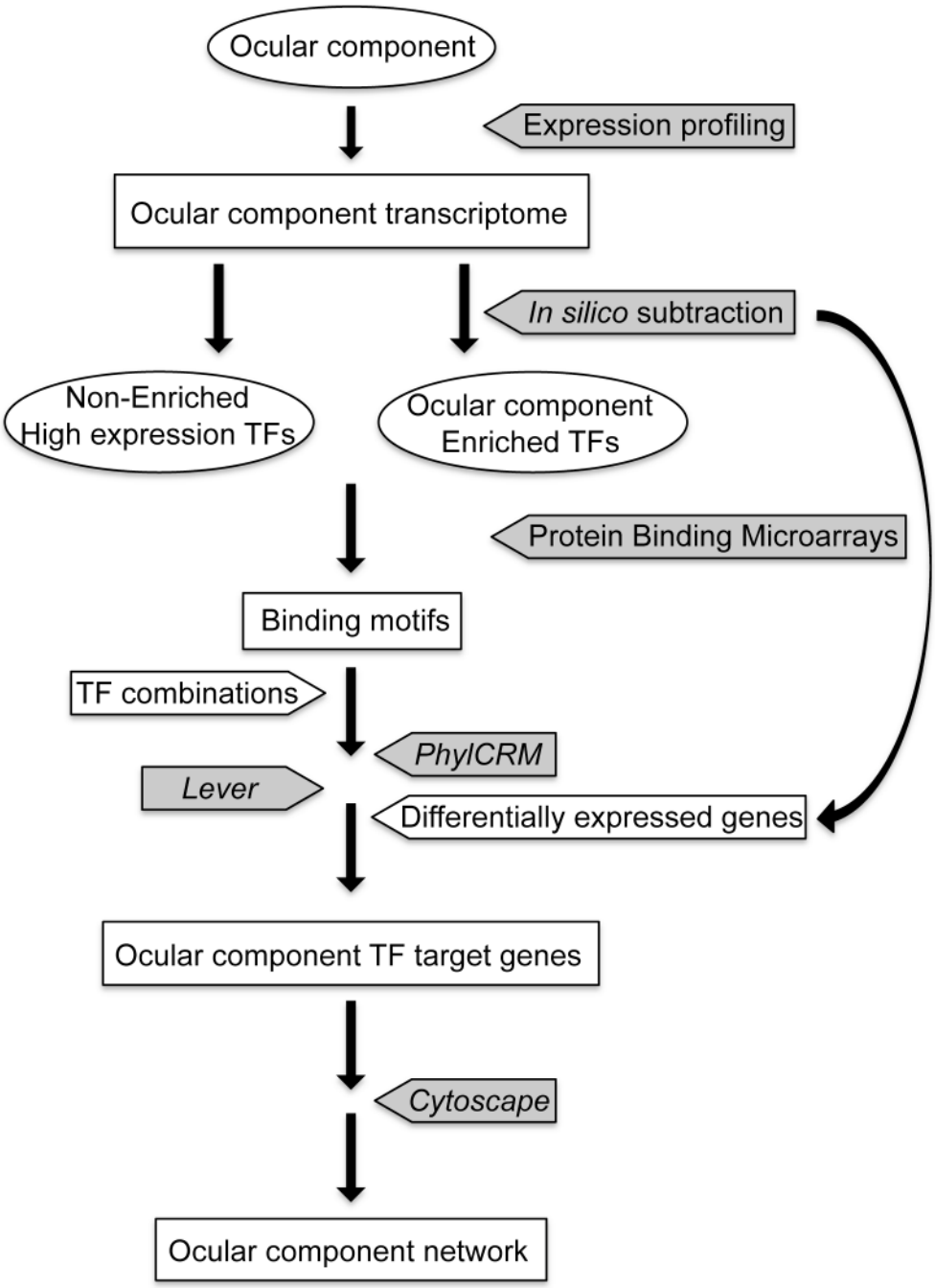

Fig. 4.

Strategy outlining the construction of networks for specific ocular compartments using gene expression profiling and computational tools. Experimental steps are indicated in gray boxes and names of computational software are italicized.