INVESTIGATION OF THE MECHANISMS OF VERTEBRATE EYE
DEVELOPMENT THROUGH ANALYSIS OF MOUSE CILIOGENESIS MUTANTS

by

JACOB BRADLEY BURNETT

(Under the Direction of Jonathan T. Eggenschwiler)

ABSTRACT

Development of the vertebrate eye is a multi-step process that requires the integration of morphogen signals, inductive tissue interactions, and intrinsic cell programs. The eye initially contains uncommitted progenitors with the capacity to develop into optic stalk, retinal pigment epithelium, or neural retina, but how these cells commit to a particular fate is not fully understood. Hedgehog (HH) signaling plays a role in this process, in part by contributing to the establishment of the proximo-distal and dorso-ventral axes within the eye. However, it is not clear how establishment of these axes ultimately leads to specification of distinct optic cell fates. This dissertation provides insight into the mechanisms of HH-mediated optic cell fate specification through the functional characterization of several mouse ciliogenesis regulators: IFT122, IFT172, and CCRK. Ift122, Ift172, and Ccrk mouse mutants all exhibited defects in ciliogenesis as well as in optic patterning and morphogenesis. These phenotypes were associated with abnormal HH signaling. Through extensive genetic epistasis experiments with core components of the HH pathway, these HH signaling defects were shown to functionally contribute to the eye phenotypes of these mutants. Interestingly, the levels of HH
pathway activation in these mutants correlated with distinct shifts in optic cell fates, some of which are predicted from the literature and others which are novel. Taken together, this work suggests that optic progenitors sense subtle changes in the levels of HH pathway activity and alter their fate accordingly.

INDEX WORDS: Mouse, Cilia, Hedgehog signaling, Morphogen, Eye development, Optic stalk, Retinal pigment epithelium, Neural retina, Epistasis, Cell fate specification, Morphogenesis, IFT122, IFT172, CCRK, SMO, GLI2, GLI3
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JACOB BRADLEY BURNETT

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by

JACOB BRADLEY BURNETT

Major Professor: Jonathan Eggenschwiler
Committee: Nancy Manley
Douglas Menke
James Lauderdale
Scott Dougan

Electronic Version Approved:

Suzanne Barbour
Dean of the Graduate School
The University of Georgia
December 2017
DEDICATION

This work is dedicated to the mice that were sacrificed during the course of these studies, without whom such research would not be possible.
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Eyes are specialized extensions of brain tissue capable of sensing photons and transmitting this information in the form of neuronal action potentials. Eyes of primitive animals, such as those of the polychaete trochophore, are not much more than a cluster of cells containing one photoreceptor cell type and one pigment cell type (Arendt, 2003). In contrast, the eyes of modern vertebrates are morphologically intricate, consisting of a retinal pigment epithelium that supports homeostasis of the neural retina, the latter of which houses one glial and six neuronal cell types, each with a specialized role to facilitate phototransduction (Graw, 2010). What is astounding is that during embryological development, this complex eye arises from a simple field of seemingly homogenous progenitor cells (Chow and Lang, 2001). How do these progenitors know which specialized cell-type to become? How do they collectively undergo morphogenesis to form an optic cup? This dissertation aims to provide answers to these questions and add to our understanding of the mechanisms of vertebrate eye development.
CHAPTER 1
INTRODUCTION AND LITERATURE REVIEW

EYE DEVELOPMENT

Overview of eye development

Development of the eyes begins with specification of the eye field along the anterior neural plate. The eye field is later subdivided into two separate eye fields, from each of which an optic vesicle arises. As an optic vesicle evaginates, it eventually makes contact with the overlying surface ectoderm (Fig. 1). This contact induces formation of the lens, which forms via invagination of the surface ectoderm. The presumptive lens ectoderm reciprocally induces invagination of the distal optic vesicle to form a two-layered optic cup. The inner layer of the optic cup gives rise to the photoreceptive neural retina (NR), whereas the outer layer gives rise to the retinal pigment epithelium (RPE), which aids in photoreceptor homeostasis. The optic stalk (OS), through which axons from the NR project to regions elsewhere in the brain, forms from the proximal optic vesicle—a region distinct from the presumptive NR and RPE territories (Chow and Lang, 2001; Graw, 2010).

The eye field

Fate mapping experiments indicate that the eyes are derived from a region along the anterior neural plate (Eagleson et al., 1995; Fernández-Garre et al., 2002; Inoue et al., 2000). Early explant experiments in amphibians showed that an eye can form from this
piece of anterior neural plate tissue isolated 6 hours prior to any visible indication of optic
vesicle formation (Lopashov and Stroeva, 1964). Thus, eye identity is imparted to this
region relatively early, before any morphological indication of eye formation. This region,
known as the eye field, is characterized molecularly by the expression of genes in the eye
field transcription factor (EFTF) network, which include ET, Rax, Pax6, Six3, Lhx2, tll,
Six6 (Zuber et al., 2003). The EFTFs are necessary, and in some cases sufficient, for
initiating eye formation (Bernier et al., 2000; Chow et al., 1999; Fish et al., 2014; Hill et
al., 1991; Lagutin et al., 2001; Li, 2002; Mathers et al., 1997; Yu et al., 2000; Yun et al.,
2009; Zuber et al., 2003; Zuber, 2010). Early in development, the EFTFs are expressed in
a single, broad domain along the neural plate (Zuber et al., 2003). As development
proceeds, their expression gradually resolves into two separate domains. Several models
have been proposed for the appearance of two eyes, generally differentiated from each
other by inferring either the existence of two separate eye fields or, conversely, the splitting
of a single eye field into two. The EFTF expression patterns mentioned above have led to
the conclusion that there is one single eye field that is split into two (Li et al., 1997).

Several lines of evidence indicate the prechordal plate is critical for splitting the
eye field. Removal of the prechordal plate in frog or chick embryos prevents the
downregulation of the EFTFs and results in cyclopia (Li et al., 1997; Pera and Kessel,
1997). Additionally, transplantation of prechordal plate adjacent to presumptive retinal
tissue inhibits expression of Pax6, an EFTF (Pera and Kessel, 1997). Thus, it is thought
that the prechordal plate is a source of signals that inhibit retinal fate and ultimately lead
to splitting of the eye field. A leading candidate for one such prechordal plate-derived
factor is the Hedgehog (HH) family member, Sonic hedgehog (SHH). SHH is expressed in
the prechordal plate and the ventral midline. *Shh* mouse mutants are cyclopic (Chiang et al., 1996), and mutation of *Shh* in humans can lead to cyclopia (Dubourg et al., 2007). Such phenotypes highlight the critical role of SHH in splitting the eye field.

**Extrinsic factors patterning the optic vesicle**

Following eye field specification and division, the next major phase of eye development is evagination and patterning of the optic vesicles. An optic vesicle gives rise to OS, RPE, and NR. The OS is derived from the most proximal region of the optic vesicle, whereas the RPE and NR are derived from the intermediate and distal regions, respectively. Many signaling pathways contribute to establishing these cell fates, including the FGF, BMP, WNT, and HH pathways (Fig. 2) (Fuhrmann, 2010; Yang, 2004). This dissertation focuses on the role of the HH signaling in these processes, but others pathways are discussed below.

SHH, in addition to dividing the eye field, plays a crucial role in patterning the optic vesicle into proximo/ventral and dorso/distal regions. Overexpression of *Shh* in several model species results in the expansion of the PAX2+ and VAX1/2+ proximo/ventral region of the optic vesicle at the expense of the PAX6+ and TBX5+ dorso/distal region (Ekker et al., 1995; Macdonald et al., 1995; Sasagawa et al., 2002; Zhang and Yang, 2001). By contrast, inhibition of the HH pathway—either by cyclopamine, SHH antibody, or conditional ablation of the essential HH activator, *Smo*—inhibits development of the proximo/ventral region and allows for the reciprocal expansion of the dorso/distal region (Perron et al., 2003; Take-uchi et al., 2003; Zhang and Yang, 2001; Zhao et al., 2010).
These experiments have helped establish a role for HH activity in promoting proximo/ventral identity and inhibiting dorso/distal identity.

It is not clear how establishment of the proximo/ventral and dorso/distal axes ultimately leads to specification of distinct optic cell fates. One hypothesis is that SHH acts as a morphogen to specify distinct optic cell fates (i.e., OS, RPE, and NR) as it does in other tissues, such as the spinal cord (Dessaud et al., 2008). Under the morphogen model, distinct levels of SHH ligand (or durations of SHH ligand exposure) would translate into distinct levels of HH pathway activity in a proximo/ventral-high, dorso/distal-low gradient (Briscoe and Small, 2015). These distinct levels of HH pathway activity within optic progenitors would lead to different cell fates being adopted along this axis within the optic vesicle. Certain findings are consistent with this model. For instance, loss- and gain-of-function experiments indicate SHH is necessary and sufficient for specification of the OS, which is derived from the region of the optic vesicle in closest proximity to the source of SHH (Ekker et al., 1995; Macdonald et al., 1995; Zhang and Yang, 2001). There is also evidence that SHH promotes RPE specification, as infection of a virus overexpressing SHH leads to distal expansion of the RPE into the NR domain in chick (Zhang and Yang, 2001). Additionally, HH activity is required for RPE specification (Lee et al., 2001; Perron et al., 2003). In this dissertation, I present genetic evidence that distinct levels of HH activity specify OS, RPE, and NR fates, which further supports the hypothesis that SHH acts as a morphogen to impart cell identity in the eye (see Ch. 4 - Conclusions).

In addition to the HH pathway, the BMP pathway is also involved in establishing the proximo/ventral-dorso/distal axis within the eye (French et al., 2009; Gosse and Baier, 2009). BMP4 is expressed in the dorso/distal optic vesicle, and loss- and gain-of-function
experiments indicate it is necessary for dorso/distal retina formation, and that it promotes expression of dorso/distal markers (Pax6 and Tbx2/3/5) at the expense of proximo/ventral markers (Pax2 and Vax1/2) (Sasagawa et al., 2002). Thus, it is possible that BMP4 forms an anti-parallel concentration gradient to that of SHH, and that the integration of these two signaling pathways leads to proper establishment of cell fates (Yang, 2004). Moreover, BMP ligands from the lens can specify NR identity (Pandit et al., 2015). There is also evidence for the involvement of other TGF-β signals in patterning the RPE and NR. Removal of extraocular mesenchyme disrupts RPE specification (i.e. downregulation of MITF expression and loss of pigmentation) and leads to expanded NR specification in the outer optic cup (i.e., upregulation of PAX6 and CHX10 expression). Activin, but neither BMP5 nor BMP7, can suppress these defects in the absence in extraocular mesenchyme, suggesting an Activin-like signal emanating from the extraocular mesenchyme is necessary for RPE specification (Fuhrmann et al., 2000).

WNT signaling plays a critical role in RPE determination. WNT pathway activity is detected in the dorsal RPE and is required for its development, as RPE-specific loss of WNT signaling (via loss of β-catenin) results in the transdifferentiation of RPE to NR (Fujimura et al., 2009; Veien et al., 2008; Westenskow et al., 2009). WNT signaling directly regulates RPE fate, as ChIP and luciferase assays indicate that β-catenin binds to and activates transcription of the RPE determinants Mitf and Otx2 (Westenskow et al., 2009).

FGF signaling is involved in the determination of both NR and RPE fates (Nguyen and Arnheiter, 2000). The surface ectoderm expresses FGFs and the optic vesicle expresses FGF receptors (Hyer et al., 1998). Removal of the surface ectoderm leads to a mixture of
NR- and RPE-fated cells in the inner optic cup, whereas these cells of these two fates are properly segregated following the addition of FGF1 (Hyer et al., 1998). Additional *ex vivo* gain- and loss-of-function experiments indicate that FGFs are necessary and sufficient for promoting NR formation; chick optic vesicles cultured *ex vivo* in the presence of FGFs leads to ectopic NR specification in the outer optic cup, whereas the addition of an FGF2-blocking antibody prevents NR specification (Pittack et al., 1997).

**Cell autonomous control of RPE and NR identity**

Signaling pathways ultimately converge on the transcription factors MITF and CHX10, which are critical for the specification of the RPE and NR, respectively. MITF, a basic-helix-loop-helix transcription factor, is initially expressed throughout the optic vesicle but becomes downregulated in the distal optic vesicle and is eventually confined to the RPE (Bumsted and Barnstable, 2000; Horsford et al., 2005). Loss of MITF leads to defects in RPE development and transdifferentiation of RPE into *Chx10*+ NR (Bumsted and Barnstable, 2000; Horsford et al., 2005). CHX10, a paired-like homeobox transcription factor, is the earliest known marker expressed in the NR (Liu et al., 1994). Loss of CHX10 leads to morphological defects and transdifferentiation of the NR into RPE, which is accompanied by ectopic *Mitf* expression in the inner optic cup (Horsford et al., 2005; Rowan et al., 2004). Additionally, misexpression of *Chx10* in the RPE inhibits *Mitf* expression and RPE development (Rowan et al., 2004). As CHX10 primarily functions as a transcriptional repressor (Dorval et al., 2005), it is tempting to speculate that CHX10 directly inhibits transcription of *Mitf*, though such an interaction has not been reported. Interestingly, disruption of *Mitf* in a *Chx10* mutant background largely rescues NR
specification, suggesting that the primary role for CHX10 in NR specification is to antagonize the expression of Mitf.

**Lens induction**

The lens develops as an invagination of the surface ectoderm, which occurs concomitantly with optic cup formation. In mice, lens-forming competence extends broadly along the periocular surface ectoderm, a region defined as pre-placodal ectoderm (Donner et al., 2006; Smith et al., 2005). The optic vesicle grows toward and eventually forms contact with the surface ectoderm (Hendrix and Zwaan, 1975). Indeed, the optic vesicle is required for induction of the lens placode from the pre-placodal ectoderm, in part through the activity of BMP and FGF ligands (Faber et al., 2001; Furuta and Hogan, 1998; Wawersik et al., 1999). SOX2 and PAX6 are both functional markers of lens placode specification, although PAX6 is also expressed in the pre-placodal ectoderm prior to induction (Kamachi et al., 1998; Lang, 2004; Smith et al., 2009). Mechanisms exist to confine lens induction to a domain adjacent to the optic vesicle, which involve neural crest-derived extraocular mesenchyme (Donner et al., 2006).

**Optic cup morphogenesis**

The distal optic vesicle invaginates to form the two-layered optic cup. There is evidence that the process of optic cup formation requires the pre-lens ectoderm but not lens specification or differentiation (Hyer et al., 2003; Smith et al., 2009). However, the self-directed formation of optic cups *in vitro* from mouse and human embryonic stem (ES) cells suggests that the pre-lens ectoderm isn’t required *per se* for optic cup formation (Eiraku et
al., 2011; Eiraku and Sasai, 2012; Nakano et al., 2012; Sasai et al., 2012). One interpretation of these results is that, in vivo, the pre-lens ectoderm inhibits an inhibitor of optic cup formation, which itself might arise from the extra-ocular mesenchyme or non-lens ectoderm. Such inhibitory factors would not be present in these in vitro optic cups derived from ES cells, thereby forgoing the requirement of the pre-lens ectoderm. Alternatively, the conditions of the ES culture system might include the factor(s) necessary for inducing invagination of the optic vesicle.

Different models have been proposed to account for the mechanism of optic cup morphogenesis. One model invokes principles of a “bimetallic strip”, in which two bonded pieces of metal, with different expansion coefficients, bend upon a temperature increase due to differential expansion rates of each layer (Carpenter et al., 2015). This is analogous to the two-layered optic neuroepithelium, in which the expansion of the RPE layer induces the folding of the neuroepithelium towards the relatively shorter NR (Carpenter et al., 2015). It appears WNT ligands from the surface ectoderm promote distal RPE-specific proliferation, which may in part account for such expansion of the RPE (Carpenter et al., 2015). Another model, termed the “relaxation-expansion” model, suggests the optic vesicle invaginates by following three local rules: (1) relaxation of the NR, (2) apical constriction of the “hinge”, which borders the NR and RPE, and (3) tangential NR expansion (Eiraku et al., 2012). This hypothesis is supported by live-imaging observations, experimental perturbations, and computer simulations incorporating a “relaxation-expansion” mathematical model (Eiraku et al., 2012). These two models are not mutually exclusive, and it is likely that optic cup morphogenesis occurs in vivo using principles from both.
HEDGEHOG SIGNAL TRANSDUCTION

Core components of the Hedgehog pathway

*Hedgehog* (*Hh*) was initially identified in a forward genetic screen in *Drosophila* (Nüsslein-Volhard and Wieschaus, 1980). Since then, three *Hh* homologs have been identified in mice: *Sonic hedgehog* (*Shh*), *Desert hedgehog* (*Dhh*), and *Indian hedgehog* (*Ihh*) (Pereira et al., 2014; Varjosalo and Taipale, 2008). These HH family ligands initiate signal transduction via binding to and inhibiting the cell surface receptor PTCH1 (Stone et al., 1996). Prior to ligand binding, PTCH1 inhibits SMO, an obligate transducer of the pathway (Taipale et al., 2002). Binding of HH ligand to PTCH1 inhibits this repressive activity, allowing SMO to transduce the signal downstream (Corbit et al., 2005). Ultimately, activated SMO leads to the activation of the GLI proteins (GLI1-3), which are the transcriptional effectors of the HH pathway (Ingham and McMahon, 2001). In the absence of HH ligand, GLI2 and GLI3 are processed into transcriptional repressors (Sasaki et al., 1999). HH pathway activation prevents GLI repressor formation and converts them into transcriptional activators, which requires their release from SUFU (Tukachinsky et al., 2010). Although both GLI2 and GLI3 each have transcriptional activator and repressor functions, GLI2 is the primary HH activator, whereas GLI3 is the primary HH repressor (Bai et al., 2004; Buttitta et al., 2003; Lei et al., 2004; McDermott et al., 2005; Pan et al., 2006; Rallu et al., 2002; Yong et al., 2009). GLI1 lacks a repressor domain and only functions as an activator, though its role is relatively minor (Bai et al., 2002; Bai and Joyner, 2001; Dai et al., 1999).
**Hedgehog signaling and primary cilia**

Though not the case in *Drosophila*, transduction of the HH pathway in vertebrates is intricately tied to the primary cilium, a subcellular organelle that projects from cells of most cell types (Fig. 3) (Davey et al., 2006; García-García et al., 2005; Goetz and Anderson, 2010; Huang and Schier, 2009; Huangfu et al., 2003; Huangfu and Anderson, 2005; Yin et al., 2009). For instance, PTCH1 localizes to the cilium in the absence of signaling (Rohatgi et al., 2007). GLI2 and GLI3 are processed into transcriptional repressors at the base of the cilium, in part via phosphorylation by PKA, GSK3β, and CKI family members (Barzi et al., 2010; Briscoe and Thérond, 2013; Fumoto et al., 2006; Mukhopadhyay et al., 2012; Pan et al., 2009; Sillibourne et al., 2002; Tuson et al., 2011). Upon ligand binding, PTCH1 exits the cilium, while SMO, SUFU, GLI2 and GLI3 accumulate within the cilium (Briscoe and Thérond, 2013; Haycraft et al., 2005; Kim et al., 2009; Milenkovic et al., 2009; Wang et al., 2009). Thus, it is not surprising that defects in ciliary assembly interfere with processing of GLI2 and GLI3 into transcriptional activators and repressors (Caspary et al., 2007; Haycraft et al., 2005; Kim et al., 2009; Ko et al., 2010; Liu et al., 2005; Qin et al., 2011).

**Structure of the primary cilium**

The core of the primary cilium is the axoneme, which is composed of nine microtubule doublets and is anchored to the cell by the centriole-derived basal body (Fig. 4). The axoneme is surrounded by the ciliary membrane, which is distinct from the cell membrane with respect to protein and lipid composition. Transition fibers at the base of the cilium regulate the passage of molecules into and out of the cilium (Rohatgi and Snell,
The cilium is built and maintained by a process termed intraflagellar transport (IFT). IFT involves the trafficking of a massive complex of proteins along the axoneme, to and from the tip of the cilium to the base (Taschner et al., 2012). This IFT complex can be subdivided into complex A and B. Anterograde IFT relies primarily on complex B via the molecular motor kinesin-2, while retrograde IFT relies primarily on complex A and is transported by the dynein motor. Mutations in IFT-B subunits usually result in a severely truncated or absent cilium (Gorivodsky et al., 2009; Huangfu et al., 2003). In contrast, mutations in IFT-A subunits lead to an accumulation of cargo at the ciliary tips as a result of malfunctional retrograde IFT (Cortellino et al., 2009; Qin et al., 2011).

THESIS OVERVIEW

In the proceeding chapters, I report the functional characterization of several ciliogenesis regulators (i.e., IFT122, IFT172, and CCRK) with respect to their roles in mammalian eye development. In Chapter 2, I use Ift122 and Ift172 mouse mutants to perturb cilia formation in distinct ways, and in combination with patterning analyses and genetic epistasis experiments with Gli2, I establish a role for primary cilia in restricting HH pathway activity during early eye development. In Chapter 3, I show that CCRK, an evolutionary conserved regulator of cilia length and morphology, controls early eye development by both positively and negatively regulating the HH pathway. From these experiments, I have developed a model for how the levels of HH pathway activity control cell fate specification in the eye, which is detailed in Chapter 4.
FIGURES

Figure 1. Electron microscopic images of the embryonic mouse eye at the optic vesicle (A,D), optic vesicle-optic cup transition (B,E), and optic cup (C,F) stages (adapted from Heavner and Pevny, 2012).
Figure 2. Graphical summary of the signaling pathways that control cell fate specification during eye development (adapted from Heavner and Pevny, 2012). Yellow indicates OS, red indicates RPE, green indicates NR, and blue indicates the lens placode. See text for further details.
Figure 3. Graphical representation of the dynamic localization of HH components within the primary cilium during unstimulated and stimulated conditions (adapted from Eggenschwiler and Anderson, 2007). See text for details.
Figure 4. Graphical representation of the dynamics of IFT within the primary cilium (adapted from Eggenschwiler and Anderson, 2007). See text for details.
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CHAPTER 2

PROPER CILIARY ASSEMBLY IS CRITICAL FOR RESTRICTING HEDGEHOG SIGNALING DURING EARLY EYE DEVELOPMENT IN MICE

Jacob B. Burnett*, Floria I. Lupu* and Jonathan T. Eggenschwiler

*These authors contributed equally to the work.

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ABSTRACT

Patterning of the vertebrate eye into optic stalk, retinal pigment epithelium (RPE) and neural retina (NR) territories relies on a number of signaling pathways, but how these signals are interpreted by optic progenitors is not well understood. The primary cilium is a microtubule-based organelle that is essential for Hedgehog (HH) signaling, but it has also been implicated in the regulation of other signaling pathways. Here, we show that the optic primordium is ciliated during early eye development and that ciliogenesis is essential for proper patterning and morphogenesis of the mouse eye. *Ift172* mutants fail to generate primary cilia and exhibit patterning defects that resemble those of *Gli3* mutants, suggesting that cilia are required to restrict HH activity during eye formation. *Ift122* mutants, which produce cilia with abnormal morphology, generate optic vesicles that fail to invaginate to produce the optic cup. These mutants also lack formation of the lens, RPE and NR. Such phenotypic features are accompanied by strong, ectopic HH pathway activity, evidenced by altered gene expression patterns. Removal of GLI2 from *Ift122* mutants rescued several aspects of optic cup and lens morphogenesis as well as RPE and NR specification. Collectively, our data suggest that proper assembly of primary cilia is critical for restricting the Hedgehog pathway during eye formation in the mouse.
INTRODUCTION

The developing vertebrate eye contains uncommitted progenitors with the capacity to differentiate into optic stalk, retinal pigment epithelium (RPE), or neural retina (NR) (Chow and Lang, 2001). How these cells commit to a particular fate is not fully understood. While much effort has focused on identifying the signaling factors governing eye formation, relatively little is known about how these signals are integrated within optic progenitors to promote distinct cellular behaviors (Adler and Canto-Soler, 2007; Fuhrmann, 2010; Yang, 2004).

One mechanism might involve the primary cilium. This microtubule-based organelle is essential for proper Hedgehog (HH) signaling and has been implicated in the transduction of a number of other signals (e.g., Wnt, PCP, RTK, TGF-β, PDGFα, mTOR and Notch) (Boehlke et al., 2010; Christensen et al., 2016; Ezratty et al., 2011; Goetz and Anderson, 2010; May-Simera and Kelley, 2012; Schneider et al., 2005; Umberger and Caspary, 2015). Indeed, eye defects have been observed in several mouse ciliogenesis mutants, yet there has been no detailed investigation of this phenomenon nor any insight into the underlying mechanism (Gorivodsky et al., 2009; Huangfu and Anderson, 2005; Ko et al., 2010; Qin et al., 2011; Snouffer et al., in press).

Intraflagellar transport (IFT) is critical for proper cilia formation and function. IFT involves anterograde transport of cargo primarily via IFT-B complex proteins and kinesin-2, as well as retrograde transport primarily by via IFT-A complex proteins and cytoplasmic dynein-2 (Taschner et al., 2012). IFT172 is an IFT-B component that is required for anterograde IFT; Ift172 mouse mutants completely fail to produce cilia in the tissues examined (Gorivodsky et al., 2009; Huangfu et al., 2003). In contrast, IFT122, an IFT-A
component, is required for retrograde IFT; Ift122 mutant cilia are bulbous and accumulate cargo at the tips primarily due to defective retrograde IFT (Cortellino et al., 2009; Qin et al., 2011). In this study, we investigated Ift172 and Ift122 mouse mutants to gain insight into the role of the primary cilium in early eye formation.

MATERIALS AND METHODS

Mouse lines

All mice were on a C3Heb/FeJ background and were genotyped using DNA isolated from tail or yolk sac tissue. For harvesting embryos, noon on the day of finding a vaginal plug was considered embryonic day 0.5 (E0.5). Ift122<sup>sopb</sup> is a recessive null allele (Qin et al., 2011) and mice were genotyped by PCR followed by restriction digest using the forward and reverse primers 5’-AACTCATGCAGCCGTTCCATTG-3’ and 5’-CGCTTTGTCTCTCCACGTCA-3’, respectively. The amplified region of the mutant allele is digested with Hpy99I, producing 125 bp and 35 bp fragments, while the wild-type allele is an uncut 160 bp fragment. Ift172<sup>wim</sup> embryos were obtained from Tamara Caspary (Emory University) and genotyped by PCR followed by restriction digest with forward and reverse primers 5’-CACTGTGCTGATGAAAGACTGGAATAGCC-3’ and 5’-TCTGCAGGGAGTAACTGGGTGTGGCGGAAG-3’, respectively. The amplified region of the WT allele is digested with EarI, producing 163 and 25 bp products, while the amplified mutant allele is an uncut 188 bp product. Gli2<sup>zfd</sup> mice were obtained from Alexandra Joyner (Sloan Kettering Institute) and genotyped as previously described (Matise et al., 1998). Gli3<sup>Xr-J</sup> mice were obtained from the Jackson Laboratories (Bar Harbor, ME) and genotyped as previously described (Hui and Joyner, 1993).
**Immunohistochemistry**

Embryos were dissected in PBS, fixed for 2 hours with 4% PFA at 4°C, washed 4 times for 5 minutes each with PBS, and incubated in 15% sucrose/PBS and 30% sucrose/PBS at 4°C for 2 hours each. Embryos were then embedded in O.C.T. (Tissue-Tek), flash frozen, and sectioned in the coronal plane at 12µm with a Leica CM1850 cryostat. Slides were washed 3 times for 10 minutes each in 0.2% Triton X-100/PBS, blocked in 10% serum/0.2% Triton X-100/PBS for 1 hour, and incubated overnight at 4°C in 1% serum/0.2% Triton X-100/PBS with primary antibodies at the following concentrations: rabbit anti-PAX2 (1:450, Covance Research Products), mouse anti-PAX6 (1:50, Developmental Studies Hybridoma Bank), sheep anti-CHX10 (1:600, Exalpha Biologicals), mouse anti-MITF (1:1500, Abcam), rabbit anti-OTX2 (1:600, Upstate Biotechnology), mouse anti-COUPTFII (1:300, R&D Systems), goat anti-SOX1 (1:100, R&D Systems), rabbit anti-SOX2 (1:300, EMD Millipore), mouse anti-γ-TUBULIN (1:1000, Sigma-Aldrich), rabbit anti-ARL13B (1:3000, T. Caspary, Emory University). Slides were washed 3 times for 20 minutes each (as above) and incubated for 1 hour at room temperature in with Alexa Fluor 488/555–conjugated secondary antibodies (Invitrogen) and Cy2/Cy3/Cy5–conjugated secondary antibodies (Jackson ImmunoResearch) at 1:300. Slides were washed 3 times for 20 minutes each (as above) and mounted with 90% glycerol/PBS. Images were taken with either a Zeiss Axioplan 2 or a Keyence BZ-X710 fluorescence microscope. High-magnification images of cilia are maximum intensity projections from z-stacks taken on a Zeiss LSM510 confocal microscope.
**In situ hybridization**

Embryos were dissected in PBS, fixed overnight with 4% PFA at 4°C, washed 4 times for 5 minutes each with depc-treated PBS, and then dehydrated through a dilution series in methanol. Whole mount in situ hybridization was performed as previously described, with the modification of extended wash times (Lauter et al., 2011). Embryos were sectioned following whole mount staining using methods described above (see Immunohistochemistry). Riboprobes for *Vax2* (Mui et al., 2002), *Shh* (Echelard et al., 1993), *Nkx2.1* (Long et al., 2009) and *Gli1* (Hui et al., 1994) were generated by *in vitro* transcription using a dig-UTP labeling mix (Roche) following manufacturer’s specifications.

**Three-dimensional reconstruction**

Embryos were dissected in PBS, fixed overnight with 4% PFA, washed 4 times for 5 minutes each with PBS, and then dehydrated through a dilution series in ethanol followed by two washes in xylene. Embryos were embedded in paraffin wax and 10µm sagittal sections were cut with a Leica RM2155 microtome. Hematoxylin and eosin (H&E) staining was performed according to standard protocols (Fischer et al., 2008). Three-dimensional reconstruction was performed by stacking tracings of the optic neuroepithelium from serial sagittal sections stained with H&E using Surf Driver™ 3.5.3 software (Surfdriver).
RESULTS

IFT172 and IFT122 regulate ciliogenesis in optic progenitors

To understand how primary cilia contribute to development of the eye, we first asked which cells of the developing eye were ciliated. We used antibodies against ARL13B (Caspary et al., 2007) and γ-TUBULIN (Muresan et al., 1993) to visualize the cilium and basal body, respectively. Primary cilia occupied the surface of cells along the optic neuroepithelium, surface ectoderm and periocular mesenchyme in wild-type embryos during the optic vesicle and optic cup stages (Fig. 1A–C). Ift172−/− embryos lacked ARL13B localization distal to the γ-TUBULIN+ basal body, confirming optic progenitors in these mutants failed to produce cilia (Fig. 1D). By contrast, optic progenitors in Ift122−/− embryos produced cilia that exhibited a swollen, bulbous morphology, resembling cilia previously characterized in Ift122−/− spinal neural progenitors (Qin et al., 2011; Fig. 1E). These data indicate IFT172 and IFT122 are required for proper cilia formation in optic progenitors, consistent with previous reports regarding the roles of these proteins in ciliogenesis in other embryological contexts (Cortellino et al., 2009; Gorivodsky et al., 2009; Huangfu et al., 2003; Qin et al., 2011).

Loss of IFT172 leads to patterning defects consistent with elevated Hedgehog signaling

To determine how the complete loss of primary cilia would impact eye formation, we examined cell fate specification in Ift172 mutants at optic cup stages (E10.5 and E11.5). PAX2 is a marker for proximal (optic stalk) fate, whereas PAX6 is expressed distally in the optic cup and lens (Schwarz et al., 2000). Ift172 mutants expressed PAX2 in a distally-
expanded domain at the expense of the neuroepithelial PAX6 domain at E10.5 (Fig. 2A). This PAX2 and PAX6 expression pattern was exacerbated by E11.5 (Fig. 2B). SOX1 is normally expressed in the optic stalk of wild-type embryos (Wood and Episkopou, 1999). Like PAX2, its expression was expanded distally in Ift172 mutants at both stages. These data suggest that the optic stalk domain is expanded in Ift172 mutants (Fig. 2A,B). COUPTFII is expressed in the dorsal optic stalk, RPE and extraocular mesenchyme (Tang et al., 2010). We detected no change in COUPTFII expression in Ift172 mutants. In wild-type embryos, OTX2 and MITF are expressed in the outer optic cup (RPE), whereas CHX10 is expressed in the inner optic cup (NR) (Horsford et al., 2005; Martínez-Morales et al., 2003; Rowan et al., 2004). In Ift172 mutants, MITF and OTX2 expression extended abnormally into the inner layer of the optic cup at both E10.5 and E11.5, which was accompanied by a reduction in the CHX10 domain (Fig. 2A,B). Additionally, SOX2 was expressed in the NR of controls, but its expression was absent in the Ift172−/− optic cup (Fig. 2A,B). These data suggest the RPE domain is expanded at the expense of the NR in Ift172−/− embryos.

The patterning defects we observed in Ift172 mutants are remarkably similar to those observed when the HH pathway is elevated in other vertebrates (Amato et al., 2004; Ekker et al., 1995; Macdonald et al., 1995; Perron et al., 2003; Sasagawa et al., 2002; Wang et al., 2015; Zhang and Yang, 2001). This prompted us to compare the phenotype of Ift172 mutants to that of Gli3−/− embryos. As GLI3 is the main transcriptional repressor of the HH pathway, its loss results in increased HH pathway activity (Persson et al., 2002; Rallu et al., 2002; Tole et al., 2000). We found optic patterning of Gli3 mutants was strikingly similar to Ift172 mutants at E11.5 (Fig. 2B). The optic stalk markers PAX2 and SOX1
were distally expanded, the RPE markers MITF and OTX2 were expressed in the inner optic cup, and the domains of the NR markers CHX10 and SOX2 were reduced. Together, these data suggest that IFT172 is required for preventing ectopic activation of the HH pathway in the eye.

**Loss of IFT122 leads to patterning defects and prevents optic cup and lens formation**

We next asked whether loss of another IFT protein, IFT122, would lead to a phenotype similar to Ift172 mutants. Through our analyses of Ift122−/− embryos at the optic vesicle–optic cup transition stages, it was evident that these mutants failed to make an optic cup or induce lens formation. We performed three-dimensional (3-D) reconstruction of this eye structure at E10.5, which confirmed that no invagination of the optic vesicle to produce an optic cup occurred in Ift122−/− embryos (Fig. 3A). We focused our subsequent analyses on patterning of the Ift122 mutant optic primordium.

At the optic vesicle stage (E9.5), PAX2 expression was expanded distally throughout the vesicle of Ift122 mutants compared to somite-matched, wild-type controls (Fig. 3B). This PAX2 expansion was maintained at E10.5, whereas PAX6 and COUPTFII expression was only found in the dorsal optic vesicle at both stages (Fig. 3B,C). OTX2 expression, which was expressed normally at E9.5, was confined to a small domain of cells in the distal optic vesicle at E10.5, suggesting RPE specification might be compromised in Ift122 mutants (Fig. 3B,C). Consistent with this, we failed to detect any MITF expression at E9.5 or E10.5 (Fig. 3B,C). Additionally, we did not detect CHX10 expression at either stage, suggesting that Ift122 mutants failed to specify the NR (Fig. 3B,C). To give further insight into the identity of Ift122 mutant optic progenitors, we utilized the expression
patterns of SOX1 and SOX2. Whereas both SOX1 and SOX2 are normally co-expressed throughout the presumptive optic stalk, only SOX2 is additionally expressed in the presumptive NR (Wood and Episkopou, 1999). In *Ift122* mutants, both SOX1 and SOX2 expression was found throughout the entire optic vesicle at E10.5 (Fig. 3B,C), which, in addition to the expansion of PAX2, suggests this structure adopts an optic stalk-like identity.

**Abnormal Hedgehog signaling in the *Ift122* and *Ift172* mutant optic vesicle**

In comparison to the patterning changes in *Ift172* mutants, the *Ift122* phenotype described above was relatively severe. We performed *in situ* hybridization against *Vax2* at E9.5, a gene expressed in the ventral optic vesicle, and found that its expression was expanded dorsally in *Ift122* mutants compared to somite-matched controls (Fig. 4A,B). Dorsal expansion of *Vax* gene expression is a common effect of elevated HH pathway activity (Sasagawa et al., 2002; Take-uchi et al., 2003; Wang et al., 2015; Zhang and Yang, 2001). Thus, we investigated HH pathway activity directly in *Ift122* mutants.

We analyzed the expression of *Shh* ligand and of the direct HH pathway targets *Gli1* and *Nkx2.1* (Vokes et al., 2007) in the optic vesicle of *Ift122* mutants at E9.5 and compared this to somite-matched controls. In this region of wild-type embryos, *Shh* is expressed along the ventral midline (Fig. 4C). SHH ligand is thought to spread laterally where it activates target gene expression in a concentration-dependent manner (Amato et al., 2004). *Nkx2.1* expression is induced by high levels of HH signaling and is expressed in the midline and along the peri-ocular diencephalon, in a domain largely overlapping with that of *Shh* (Fig. 4E). *Gli1* expression is excluded from the midline and is found further
distal along the optic neuroepithelium (Fig. 4G), indicating its expression is induced by moderate levels of HH activity but suppressed by high HH levels, as observed in other studies (Ribes et al., 2010). We found that Ift122 mutants expressed Shh in a distally-expanded domain along the optic neuroepithelium compared to wild-type controls (Fig. 4D). Nkx2.1 was expressed from the midline to the dorso-distal tip of the optic vesicle (Fig. 4F). We also observed ectopic Nkx2.1 expression in the dorsal extraocular mesenchyme, though we cannot rule out this phenomenon is a result of neuroepithelial delamination of Nkx2.1+ cells (Fig. 4F). Ift122 mutants also expressed Gli1 ectopically in the distal optic vesicle (Fig. 4H). We noted the proximo-ventral Gli1 negative domain was expanded distally in Ift122 mutants compared to controls, suggesting that the HH pathway is elevated throughout this entire region. Together, these data provide direct evidence that the HH pathway is strongly elevated in the optic vesicle of Ift122 mutants.

The fact that Ift172 mutants phenocopy Gli3 mutants with respect to eye development suggested Ift172 mutants exhibit elevated HH levels in the optic vesicle. To test this directly, we analyzed Gli1 and Nkx2.1 expression in Ift172 mutants. Ectopic Gli1 expression was found distally in the Ift172/- optic vesicle (Fig. 4I,J,I’,J’). We noted that Gli1 was expressed in the proximal optic vesicle of Ift172 mutants, whereas Gli1 expression was not observed in similar proximal regions of Ift122 mutants. This suggests that the ectopic HH pathway activity in Ift172 mutants is not as high as in Ift122 mutants. In support of this, the Nkx2.1 expression domain was ventrally restricted in Ift172 mutants, rather than distally expanded as seen in Ift122 mutants (Fig. 4K-M). Collectively, these data indicate that the HH pathway is indeed ectopically active in the distal optic
primordium of both *Ift172* and *Ift122* mutants, but the degree of activity is much higher in the latter. This likely contributes to the difference in eye phenotypes of the two mutants.

**Loss of GLI2 in *Ift122* mutants rescues optic cup patterning and morphogenesis**

In mice, the HH pathway is mediated by the GLI family of transcription factors (GLI1-3). GLI1 has only activator function, which is redundant with GLI2 (Bai et al., 2002; Bai and Joyner, 2001). While GLI2 and GLI3 both have activator and repressor functions, GLI2 primarily acts as a HH target transcriptional activator, whereas GLI3 acts primarily as a repressor (Bai et al., 2004; Buttitta et al., 2003; Dai et al., 1999; Lei et al., 2004; McDermott et al., 2005; Persson et al., 2002; Rallu et al., 2002; Sasaki et al., 1999; Yong et al., 2009). Thus, if the eye defects we observed in *Ift122* mutants are indeed due to elevated HH pathway activity, we hypothesized that simultaneous genetic ablation of *Gli2* in *Ift122* mutants should mitigate these defects and rescue aspects of eye formation. It has been previously reported that optic cup patterning and morphogenesis occur normally in *Gli2* mutants, with the only defect being a slightly shortened optic stalk (Furimsky and Wallace, 2006). We first analyzed *Gli2* mutants at E11.5 and confirmed that optic cup patterning and morphogenesis occurs similarly to wild-type controls (Fig. 5).

We found optic patterning of *Ift122* mutants at E11.5 resembled that at E10.5, with a few exceptions. At E11.5, PAX2 expression did not extend to the distal edge of the optic vesicle, but rather was found proximo-ventrally, while PAX6 expression was completely absent from the optic primordium (Fig. 5). COUPTFII expression was found throughout the optic vesicle, whereas OTX2 was expressed in a few cells along the distal optic region. Similar to E10.5, CHX10 and MITF expression remained absent at E11.5, whereas the
entire optic primordium co-expressed SOX1 and SOX2. These data indicate the RPE and NR are not specified in Ift122 mutants even at E11.5, arguing against the possibility of a developmental delay.

We then generated Ift122;Gli2 double mutants at this same stage. Remarkably, these embryos formed optic cup-like structures that expressed PAX6, MITF and OTX2 in the distal region of the optic cup (Fig. 5). CHX10 and SOX2 were expressed in the inner layer of the optic cup, while PAX2, COUPTFII and SOX1 were expressed in the proximal optic stalk region (Fig. 5). While all double mutants analyzed expressed these markers (N=5/5), we noted variability in the extent lens morphogenesis, with some having an obvious lens vesicle (N=2/5; Fig. 5) and others having no apparent lens (N=3/5; data not shown). Though we cannot rule out the possibility that a small lens was formed in these mutants, we propose this variability stems from differences in the ability of the optic vesicle to induce lens formation, or from differences in the competence of the surface ectoderm to respond to the inductive signals (Chow and Lang, 2001). Regardless, reducing HH levels by removal of GLI2 was an effective strategy for rescuing specification of the NR and RPE as well as for the initiation of the optic cup development in Ift122 mutants, arguing that the eye defects due to loss of IFT122 largely, if not entirely, stem from elevated HH pathway activity.

DISCUSSION

We find two IFT proteins, IFT172 and IFT122, are required for proper eye development in mice. Ift172 mutants lack optic primary cilia and show evidence of modest ectopic HH activity in the distal optic vesicle, as revealed by Gli1 expression. Such mutants
adopt a proximalized optic cup with an expanded RPE at the expense of the NR, defects that are consistent with elevated HH signaling (this study; Amato et al., 2004; Ekker et al., 1995; Macdonald et al., 1995; Sasagawa et al., 2002; Zhang and Yang, 2001; Perron et al., 2003). *Ift122* mutants, which have malformed optic primary cilia, do not specify RPE or NR cell types, nor do they form an optic cup or lens. Our *in situ* hybridization data indicate that both *Ift172* and *Ift122* mutant optic vesicles show ectopic HH pathway activity. However, the dramatic expansion of the *Nkx2.1* expression domain in *Ift122* mutants suggests that they experience much higher HH activity than *Ift172* mutants, and therefore *Ift122* mutants exhibit a much more severe optic phenotype. Consistent with the causative role of excessive HH pathway activity in the eye phenotype of *Ift122* mutants, we observed substantial rescue of eye formation in *Ift122* mutants upon reducing HH pathway activity through simultaneous genetic ablation of *Gli2*. While it is possible that both IFT172 and IFT122 have roles outside of the cilium, the most parsimonious interpretation of our data is that the eye defects in these mutants result from their inability to generate normally functioning cilia.

Based on these findings, we propose that the modest ectopic activation of the HH pathway in *Ift172* mutants leads to expansion of optic stalk and RPE fates at the expense of NR fates. In contrast, strong ectopic HH pathway activity in *Ift122* mutants completely blocks both formation of the optic cup and specification of the NR and RPE identities. The entire optic vesicle adopts an optic stalk-like identity. This model is summarized in Figure 6. Of interest, our data provide some insight into the mechanism of RPE specification. In chick, HH ligand overexpression leads to the expansion of RPE into the NR domain (Zhang and Yang, 2001), and our analysis of *Gli3* and *Ift172* mutants supports a role for HH
signaling in the promotion of RPE fate. In contrast, our data on Ift122 mutants indicate further elevation of HH pathway activity completely suppresses RPE fate in the optic vesicle. This suggests HH signaling can both promote and inhibit RPE specification depending on the level of activity.

In other contexts, primary cilia control HH pathway activity by regulating the balance of GLI activator and repressor formation (Caspary et al., 2007; Chang et al., 2016; Huangfu et al., 2003; Huangfu and Anderson, 2005; Liu et al., 2005; Millington et al., 2017). Thus, loss of IFT172 or IFT122 could cause increased HH activity within the eye through several possible mechanisms, which are not mutually exclusive. First, diminished GLI repressor (GLIRep) formation could lead to a de-repression of the HH pathway. Indeed, both Ift122 and Ift122 mutants generate reduced levels of GLI3Rep (Huangfu and Anderson, 2005; Qin et al., 2011). Alternatively, constitutive activity of GLI activator (GLIAct) may lead to hyperactivation of the HH pathway. Previous work suggests that GLIAct cannot be generated in the absence of cilia (i.e. in Ift172 or Ift88 mutants; Huangfu and Anderson, 2005; Ocbina and Anderson, 2008). However, genetic and molecular analysis of Ift122 mutants, which generate (abnormal) cilia, suggests that such mutants exhibit ligand-independent activation of GLI2 (Qin et al., 2011). Indeed, the observed rescue of eye development by removal of GLI2 in Ift122 mutants supports this argument with respect to eye development. Other findings indicate that Ift122 mutant cilia fail to localize the HH pathway antagonist GPR161, which indirectly activates Protein Kinase A (Mukhopadhyay et al., 2013). Thus, Ift122 mutant cilia retain the ability to produce GLIAct, and the loss of ciliary GPR161 in such mutants allows this to occur independently of ligand-mediated pathway activation. As a result, we suggest that the high levels of
ectopic HH pathway activity in *Ift122* mutants are caused by the combination of failure to inhibit GLIAct function together with failure to produce sufficient levels of GLI3Rep activity.

We favor the hypothesis that *Ift122* mutants generate significantly reduced levels of GLI3Rep as an explanation for the incomplete rescue of eye development in *Ift122;Gli2* double mutants. We have no reason to believe that this aspect of the *Ift122* mutant phenotype would be affected by loss of GLI2. Thus, although *Ift122;Gli2* mutants may no longer be able to generate ectopic GLI2Act, the defect in GLI3Rep formation should still cause low levels of ectopic HH activity in these double mutants. Indeed, the eye phenotype of *Ift122;Gli2* double mutants (Fig. 5) is remarkably similar to that of *Gli3* mutants (Fig. 2B), which lack GLI3Rep function. Nevertheless, we acknowledge there are other possible explanations for the lack of a complete rescue in the double mutants. Other signaling pathways associated with cilia (e.g. Wnt, Notch; Ezratty et al., 2011; Goetz and Anderson, 2010) could be directly affected by loss of IFT122, and such a defect would not be rescued by disruption of GLI2.

Our findings indicate that primary cilia play an important role in early mammalian eye development and that different aspects of ciliary assembly make unique contributions to the process. Such contributions are largely, if not entirely, a result of restricting HH pathway activity to different extents and by different means. Appropriate control of HH pathway activity, in turn, directs both morphogenesis and patterning of the eye primordium through mechanisms that are both known and yet to be understood.
Figure 1. **Optic progenitor cells are ciliated and require IFT122 and IFT172 for proper cilia assembly.** (A-B) Sections through a wild-type E9.5 optic vesicle (A) and a wild-type E11.5 optic cup (B). Cilia are visualized with an antibody against ARL13B (green) and DNA counterstained with DAPI (blue). Abbreviations for optic progenitor territories: pOS, presumptive optic stalk; pRPE, presumptive retinal pigment epithelium; pNR, presumptive neural retina; OS, optic stalk; RPE, retinal pigment epithelium; NR, neural retina. (C-E) Maximum intensity projections of confocal z-stacks of wild-type (C), Ift172 mutant (D) and Ift122 mutant (E) eyes in the presumptive optic stalk at E10.5 stained with antibodies against ARL13B (green) and γ-TUBULIN (red); DNA counterstained with
DAPI (blue). Scale bars are 2 µm. Insets (C’-E’) are magnifications of the area marked by the dotted box in the corresponding image (C-E).
Figure 2. IFT172 is required for patterning and morphogenesis of the optic cup. (A) Sections through E10.5 wild-type and Ifi172 mutant eyes stained with markers for cell fates. Note the dorsal expansion of PAX2, dorsal restriction of PAX6, expansion of MITF and OTX2 into the inner optic cup, reduction in CHX10+ cells and loss of SOX2 expression in the Ifi172 mutant. Abbreviations: OS, optic stalk; RPE, retinal pigment epithelium; NR, neural retina; P, proximal; Di, distal; Do, dorsal; V, ventral. (B) Sections through E11.5 wild-type, Ifi172 mutant and Gli3 mutant eyes stained with markers for cell fates. Note the dorsal expansion of PAX2, dorsal restriction of PAX6, expansion of MITF and OTX2 into the inner optic cup, reduction of CHX10+ cells, distal expansion of SOX1, and loss of SOX2 expression in both Ifi172 and Gli3 mutants.
Figure 3. IFT122 is required for optic cup and lens formation and for specification of the RPE and NR. (A) 3-D reconstruction of wild-type and Ift122 mutant eyes at E10.5. Compass abbreviations: A, anterior; Po, posterior; Di, distal; Pr, proximal; V, ventral; D, dorsal. (B) Sections through E9.5 (20-22 somite stage) wild-type and Ift122 mutant optic vesicles stained with markers for cell fates. Note the dorsal expansion of PAX2 and the loss of MITF and CHX10 expression in the Ift122 mutant. (C) Sections through E10.5 (36-40 somite stage) wild-type and Ift122 mutant eyes stained with markers for cell fates. Note the loss of MITF and CHX10 expression, reduction of OTX2+ cells, and the distal expansion of SOX1 expression in the Ift122 mutant.
Figure 4. Elevated Hedgehog signaling in the Ift122 and Ift172 mutant optic vesicle. 

(A-H) Sections through the eyes of wild-type (A,C,E,G,) and Ift122 mutants (B,D,F,H) at the indicated somite stages (ss) following whole-mount in situ hybridization against Vax2 (A,B), Shh (C,D), Nkx2.1 (E,F) and Gli1 (G,H). Note the dorsal expansion of Vax2 and the distal expansion of Shh, Nkx2.1 and Gli1 expression in the Ift122 mutant optic neuroepithelium. (I-J) Whole-mount in situ hybridization against Gli1 on wild-type (I) and Ift172 mutants (J) at the indicated somite stages; (I’-J’) coronal sections of these embryos following whole-mount in situ hybridization. Note the wild-type distal optic vesicle is Gli1 negative (arrows), whereas the entire Ift172 optic vesicle expresses Gli1 (arrowheads). (K-M) Whole-mount in situ hybridization against Nkx2.1 on wild-type (K), Ift172 (L), Ift122
(M) mutants at the indicated somite stages. Note that Nkx2.1 expression is proximally restricted in Ift172 mutants, rather than distally expanded as in Ift122 mutants.
Figure 5. Loss of GLI2 in *Ift122* mutants rescues optic cup patterning and morphogenesis. Sections through wild-type, *Ift122* mutant, *Gli2* mutant and *Ift122;Gli2* double mutant eyes at E11.5. Note the rescue of optic cup and lens formation, the rescue of PAX6, MITF and OTX2 expression in the distal optic cup as well as the rescue of CHX10 and SOX2 expression in the inner optic cup of the *Ift122;Gli2* double mutant.
Figure 6. Levels of Hedgehog activity bias optic progenitor cell fates. In wild-type, HH activity forms a proximal-high, distal-low gradient within the optic vesicle. This leads to normal specification of the OS, RPE, and NR. In Ift172 mutants, HH activity is moderately elevated within the optic vesicle, which results in the expansion of the OS into the proximal optic cup as well as the expansion of RPE into the inner optic cup. In Ift122 mutants, HH activity is strongly elevated throughout the entire optic vesicle, which results in optic progenitors adopting an optic stalk-like identity.
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CHAPTER 3

CELL CYCLE-RELATED KINASE REGULATES MAMMALIAN EYE DEVELOPMENT THROUGH POSITIVE AND NEGATIVE REGULATION OF THE HEDGEHOG PATHWAY

Floria I. Lupu*, Jacob B. Burnett*, and Jonathan T. Eggenschwiler

*These authors contributed equally to the work.

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ABSTRACT

Cell cycle-related kinase (CCRK) is a conserved regulator of ciliogenesis whose loss in mice leads to a wide range of developmental defects, including exencephaly, preaxial polydactyly, skeletal abnormalities, and microphthalmia. Here, we investigate the role of CCRK in mouse eye development. Ccrk mutants show dramatic patterning defects, with an expansion of the optic stalk domain into the optic cup, as well as an expansion of the retinal pigment epithelium (RPE) into neural retina (NR) territory. In addition, Ccrk mutants display a shortened optic stalk. These defects are associated with bimodal changes in Hedgehog (Hh) pathway activity within the eye, including the loss of proximal, high level responses but a gain in distal, low level responses. We simultaneously removed the Hh activator GLI2 in Ccrk mutants (Ccrk-/-;Gli2-/-), which resulted in rescue of optic cup patterning and exacerbation of optic stalk length defects. Next, we disrupted the Hh pathway antagonist GLI3 in mutants lacking CCRK (Ccrk-/-;Gli3-/-) which lead to even greater expansion of the RPE markers into the NR domain and a complete loss of NR specification within the optic cup. These results indicate that CCRK functions in eye development by both positively and negatively regulating the Hh pathway, and they reveal distinct requirements for Hh signaling in patterning and morphogenesis of the eyes.
INTRODUCTION

Eye development begins with specification of the eye field along the anterior neural plate. The single eye field is divided into two, and each side evaginates to form optic vesicles. Each optic vesicle grows outward and makes contact with the overlying surface ectoderm, where it induces lens formation. The lens placode induces invagination of the optic vesicle to form a two-layered optic cup. The inner layer of the optic cup forms the neural retina (NR), while the outer layer forms the retinal pigment epithelium (RPE). During optic cup formation, as the optic vesicle and lens are undergoing coordinated invagination, the proximal region of the optic neuroepithelium forms the optic stalk, which will later contribute to the optic nerve (Chow and Lang, 2001; Graw, 2010).

Such morphogenetic events are governed in part by a group of transcription factors termed the eye field transcription factor network, which include RAX, LHX2, PAX6 and OTX2 (Chow and Lang, 2001; Zuber et al., 2003). This network coordinates with signaling centers secreting FGFs, Wnts, BMPs, RA and Hedgehog (Hh) family members to control both cell fate specification and morphogenesis of the developing eye (Fuhrmann, 2010; Yang, 2004). Mutations in these factors result in major morphological abnormalities including anophthalmia, microphthalmia, and coloboma (Deml et al., 2015; FitzPatrick and Van Heyningen, 2005; Hever et al., 2006).

Sonic Hedgehog (SHH) is expressed in the ventral midline of the eye region and plays a critical role in patterning and morphogenesis of the eye. Ablation of Shh in mice results in cyclopia, likely due to a failure to split the eye field (Chiang et al., 1996). Milder perturbations of the Hh pathway reveal that Hh activity contributes to the establishment of the proximo-distal and dorso-ventral axes within the eye (Amato et al., 2004; Ekker et al.,
1995; Macdonald et al., 1995; Perron et al., 2003; Sasagawa et al., 2002; Takeuchi et al., 2003; Wang et al., 2015; Zhang and Yang, 2001; Zhao et al., 2010). However, the manner in which different levels of Hh pathway activity contribute to proper ocular morphogenesis or specification of distinct cell fates within the eye is not well understood.

In mice, the Hh pathway is mediated by three GLI transcription factors (GLI1-3). GLI1 is required for aspects of bone/skeletal development (Kitaura et al., 2014; Palencia-Campos et al., 2017), but in the early mouse nervous system (including the eye), this requirement is not observed, apparently due to redundancy with GLI2 (Park et al., 2000). However, the Gli1 gene is a direct transcriptional target of the Hh pathway (Bai et al., 2002; Bai and Joyner, 2001; Dai et al., 1999) making it a useful indicator of pathway activity. In the mouse embryo, GLI2 and GLI3 appear to be the primary effectors of the pathway (Bai et al., 2004; Sasaki et al., 1999). Both GLI2 and GLI3 have activator and repressor activity; however, GLI2 acts predominantly as a transcriptional activator, while GLI3 acts mainly as a repressor (Bai et al., 2004; Buttitta et al., 2003; Lei et al., 2004; McDermott et al., 2005; Rallu et al., 2002; Yong et al., 2009).

Vertebrate Hh signal transduction is regulated within the primary cilium (Briscoe and Thérond, 2013; Goetz and Anderson, 2010). In the absence of Hh ligands, the receptor Patched (PTCH1) localizes to cilia and prevents activation of the obligate transducer Smoothened (SMO). Upon ligand binding, PTCH1 is inactivated and exits the cilium, allowing for the localization of activated SMO within the cilium. This leads to an accumulation of GLI2 and GLI3 within the cilium, and ultimately to their modification into transcriptional activators. In the absence of Hh signaling, GLI2 and GLI3 are proteolytically processed into transcriptional repressors at the base of the cilium. Indeed,
failure of optic progenitors to undergo proper ciliogenesis results in severe ocular defects resulting from ectopic Hh activity within the eye (Burnett et al., 2017).

Cell cycle-related kinase (CCRK) and its orthologs regulate the assembly of the primary cilium in organisms ranging from green algae to mammals (Asleson and Lefebvre, 1998; Erdmann et al., 2006; Ko et al., 2010; Phirke et al., 2011). In mice, loss of CCRK results in abnormal cilia structure and length regulation, which are associated with widespread developmental defects including exencephaly, preaxial polydactyly, skeletal abnormalities, and microphthalmia (Moon et al., 2014; Snouffer et al., 2017; Yang et al., 2013).

Here we find that CCRK is expressed in the eye and is essential for proper ciliogenesis in optic progenitors. We present evidence that CCRK controls patterning and morphogenesis of the eye by regulating Hh pathway activity both positively and negatively. We demonstrate that this regulation occurs downstream of SMO and upstream of the GLI transcription factors. Furthermore, our data suggest that different cell fates within the eye are specified by distinct levels of Hh activity.

MATERIALS AND METHODS

Mouse lines

All mice were on a C3Heb/FeJ background. Genotyping was performed from DNA isolated from yolk-sac or tail tissue. When harvesting embryos, noon on the day of finding a vaginal plug was determined to be embryonic day 0.5 (E0.5). Ccrk is a null allele generated from Cre-mediated recombination of lox-P sites flanking the first two exons and were genotyped as previously described (Snouffer et al., 2017). Gli2^{zf} and Smo^t1Amc
alleles were obtained from Alexandra Joyner (Sloan Kettering Institute) and Andrew McMahon (University of Southern California) and were genotyped as previously described (Matise et al., 1998; Zhang et al., 2001). Gli3\(^{Xx-j}\) mice were obtained from the Jackson Laboratories (Bar Harbor, ME) and genotyped as previously described (Hui and Joyner, 1993).

**Immunohistochemistry**

Immunohistochemistry of coronal cryosections was performed as previously described (Burnett et al., 2017). Primary antibodies were used at the following concentrations: rabbit anti-PAX2 (1:450, Covance Research Products), mouse anti-PAX6 (1:50, Developmental Studies Hybridoma Bank), sheep anti-CHX10 (1:600, Exalpha Biologicals), mouse anti-MITF (1:1500, Abcam), rabbit anti-OTX2 (1:600, Upstate Biotechnology), mouse anti-COUP TFII (1:300, R & D Systems), goat anti-SOX1 (1:100, R & D Systems), rabbit anti-SOX2 (1:300, EMD Millipore), mouse anti-\(\gamma\)-TUBULIN (1:1000, Sigma-Aldrich), rabbit anti-ARL13B (1:3000, T. Caspary, Emory University). Images were taken with either a Zeiss Axioplan 2 or a Keyence BZ-X710 fluorescence microscope. High-magnification images of cilia are maximum intensity projections from z-stacks taken on a Zeiss LSM510 confocal microscope.

**In situ hybridization**

*In situ* hybridization of whole embryos was performed as previously described (Burnett et al., 2017). A 509 bp region of the 3’ UTR of Ccrk was cloned into pGEM-T Easy (Promega) using the forward and reverse primers 5’-
ACACCTGGTCCTGCTCCTTA-3’ and 5’-GACTCTCGCTCCCCAAATGAG-3’, respectively. For sense riboprobes, the Ccrk plasmid was digested with NeoI and transcribed using the Sp6 promoter. For antisense riboprobes, the Ccrk plasmid was digested with SacI and transcribed using the T7 promoter. Antisense riboprobes for Vax2 (Mui et al., 2002), Shh (Echelard et al., 1993), Nkx2.1 (Long et al., 2009) and Gli1 (Hui et al., 1994) were generated by in vitro transcription using a dig-UTP labeling mix (Roche) following manufacturer's specifications.

**Optic stalk length measurement**

Images of coronal cryosections stained with anti-PAX2 and anti-CHX10 (as above) from E11.5 embryos were used to determine the length of PAX2+ tissue (i.e., optic stalk) measured with FIJI software. N=5 individuals were analyzed for each genotype. Statistical analysis was performed using Student’s t-test.

**Three-dimensional reconstruction**

Three-dimensional reconstruction of the optic cup was performed as previously described (Burnett et al., 2017). Briefly, serial sagittal sections of the eyes were stained with H&E, and traces of the optic neuroepithelium were rendered into three dimensions using Surf Driver™ 3.5.3 software (Surfdriver).
RESULTS

CCRK regulates eye morphogenesis and cilia morphology within optic progenitors

*Ccrk* null mice exhibited microphthalmia by E12.5 (Fig. 1A). Three-dimensional reconstruction of *Ccrk* mutant eyes at E10.5, at which point there is no noticeable size discrepancy compared to wild-type, revealed that *Ccrk* mutants initiate optic cup formation by invagination of the distal optic vesicle (Fig. 1B). However, the resulting structure is morphologically abnormal and exhibits coloboma (Fig. 1B). We observed variability in lens formation and optic cup morphology in *Ccrk* mutants. In some embryos, we did not detect a lens structure (Fig. 1B), whereas a lens was present in others (as shown in Fig. 2B). Interestingly, *Ccrk* mutants exhibited this variability in optic cup and lens formation even between the eyes of the same embryo (as shown in Fig. 6A), indicating that such phenotypic variability can be independent of embryonic stage and genetic background.

To determine the tissues in which CCRK might function, we performed *in situ* hybridization for *Ccrk* mRNA at E9.5 (24-26 somites) and found *Ccrk* transcripts throughout the embryo, including the optic vesicle (Fig. S1). As CCRK and its homologs have been shown to control ciliogenesis in other contexts (Snouffer et al., 2017), we analyzed cilia within the eye by immunohistochemistry using antibodies against the axonemal marker, ARL13B, and basal body protein, γ-TUBULIN (Caspary et al., 2007; Muresan et al., 1993). We found that the *Ccrk* mutant optic neuroepithelium had abnormally short, spherical cilia (Fig. 1C). We observed no change in cilia morphology as a function of position within this tissue. These data are consistent with the hypothesis that CCRK function within the eye is linked to its role in cilia formation.
**CCRK regulates specification of the optic stalk, RPE, and NR**

To understand better the developmental basis for these eye defects, we examined cell fate specification within the early optic vesicle of *Ccrk* mutants. *Ccrk* mutants expressed the core eye field transcription factors *Rax* and *Lhx2* at E9.5 (Fig. S2A). We focused our analyses on specification of the optic stalk, RPE, and NR. Patterning of the *Ccrk* mutant optic vesicle was largely normal at E9.5, at which point there was no notable morphological divergence from wild-type (Fig. 2A). Specifically, we detected no clear change in expression of PAX2 or PAX6 (Fig. 2A), which mark the proximal and distal optic territories, respectively. Similarly, there was no change in expression of the RPE marker, MITF, nor of the NR marker, CHX10 (also known as VSX2) (Fig. 2A). We also found no apparent change in COUP-TFII expression, which is found in the dorsal optic stalk and RPE (Fig. 2A). However, we did observe ectopic OTX2 expression in the distal optic vesicle of *Ccrk* mutants (Fig. 2A). Normally, OTX2 is initially expressed throughout the entire optic vesicle, but it is then down-regulated distally and is eventually confined to the RPE (Martinez-Morales et al., 2001). Thus, while most of the early *Ccrk* mutant optic vesicle is correctly patterned, these data raise the possibility of ectopic RPE specification in the distal optic vesicle.

We then analyzed patterning of *Ccrk* mutants during optic cup formation at E10.5. We found ectopic MITF and OTX2 expression in the inner layer of the optic cup, providing further evidence that the RPE domain is expanded into the NR territory in *Ccrk* mutants (Fig. 2B). Interestingly, CHX10 and SOX2 were expressed in a smaller domain within the inner layer of the optic cup, suggesting that NR specification is compromised in *Ccrk* mutants (Fig. 2B). At this stage, the PAX2 or PAX6 expression domains continued to
appear normal, despite the abnormal tissue morphology (Fig. 2B). However, SOX1 expression appeared to be expanded distally, raising the possibility that the optic stalk domain is expanded distally in Ccrk mutants. These data indicate that loss of CCRK leads to misspecification of the optic stalk, RPE, and NR upon the transition into the optic cup.

**CCRK regulates the Hh pathway within the eye**

The distal expansion of optic stalk markers and expansion of RPE markers into the inner optic cup in Ccrk mutants is consistent with elevated levels of Hh pathway activity within the eye (Amato et al., 2004; Ekker et al., 1995; Macdonald et al., 1995; Perron et al., 2003; Sasagawa et al., 2002; Zhang and Yang, 2001). In addition, we found that expression of the ventral marker, Vax2, was expanded dorsally in Ccrk mutants (Fig. S2B). Dorsal expansion of Vax2 expression is another hallmark of hyperactive Hh activity in the eye (Sasagawa et al., 2002; Take-uchi et al., 2003; Wang et al., 2015; Zhang and Yang, 2001). These results led us to investigate Hh pathway activity directly in Ccrk mutants.

In the optic region of wild-type embryos, SHH is expressed in the ventral midline and is thought to promote proximo-ventral fates and inhibit dorso-distal fates in a concentration dependent manner (Amato et al., 2004). High levels of SHH induce expression of the direct target Nkx2.1 while inhibiting Gli1 (Ribes et al., 2010; Vokes et al., 2007). Lower levels of SHH induce expression of the direct target Gli1.

In wild-type embryos at E9.5, Shh expression, which is induced by Shh signaling from the prechordal plate (Dale et al., 1997), was found in the ventral midline (Fig. 3A). Nkx2.1 expression was found in the ventral midline and periocular neuroepithelium (Fig. 3C). In contrast, Gli1 expression was absent in the midline and was found distal to the
Nkx2.1 domain in the optic vesicle (Fig. 3E). Importantly, Gli1 expression was very low in the dorso-distal tip of the optic vesicle, indicating this region normally experiences little, if any, Hh pathway activity (Fig. 3E).

We found that Ccrk mutants expressed Shh in a proximally restricted domain compared to somite-matched, wild-type controls (Fig. 3B). We observed a similar proximal restriction of Nkx2.1 expression in Ccrk mutants (Fig. 3D). These data suggest that high levels of Hh activity are compromised in Ccrk mutants. We observed Gli1 expression in the midline of Ccrk mutants, whereas no Gli1 expression was observed in the wild-type midline. Very high levels of Shh signaling in the ventral midline normally represses Gli1 expression (Ribes et al., 2010), yet intermediate-to-low levels of Hh signaling further from the ligand source normally induce Gli1 expression. The finding that Gli1 expression is retained in the mutant midline suggests that signaling in this domain is not high enough to repress Gli1 expression in Ccrk mutants (Fig. 3F). In conjunction, we observed elevated Gli1 expression in the dorso-distal optic vesicle of Ccrk mutants compared to somite-matched controls (Fig. 3F). Taken together, these results suggest that loss of CCRK not only prevents full activation of the Hh pathway, but that it also leads to an ectopic increase in Hh activity in the distal optic vesicle.

**CCRK restricts the Hh pathway downstream of SMO within the optic vesicle**

The ectopic Gli1 expression in the distal optic vesicle of Ccrk mutants suggested that CCRK is required to fully restrict Hh activity. To test this hypothesis, we performed an epistasis experiment with Smo. SMO is cell-autonomously required for cells to respond to Hh ligands (Wijgerde et al., 2002). As a consequence, Smo mutants fail to specify the
floor plate or other ventral cell types. We found that Smo mutants failed to induce PAX2 expression, while PAX6 was expressed ectopically throughout the entire optic vesicle (Fig. 4). We generated Ccrk;Smo double mutants to test whether loss of CCRK could rescue the Smo phenotype. Such double mutants showed restoration of proximal PAX2 expression and dorso-distal restriction of PAX6 expression, similar to wild-type and Ccrk mutants (Fig. 4). Thus, CCRK indeed restricts the Hh pathway within the eye, and it does so at a step downstream of SMO.

**Rescue of eye patterning in Ccrk mutants by loss of GLI2**

To determine whether the ectopic Hh activity in the Ccrk mutant optic vesicle underlies the patterning or morphological defects, we performed another epistasis experiment. As GLI2 is the primary transcriptional activator of the Hh pathway, we hypothesized that ablation of Gli2 in a Ccrk mutant background would reduce Hh levels to allow for normal eye development. Our analysis of ocular patterning of Gli2 mutants at E11.5 was consistent with previous analyses indicating Gli2 mutants undergo normal cell fate specification and optic cup development (Fig. 5; Furimsky and Wallace, 2006; Burnett et al., 2017). Patterning and morphology of the Ccrk mutant optic cup at E11.5 was similar to that at E10.5: optic stalk markers were expanded distally, RPE markers were ectopically expressed in the inner optic cup, and NR markers were present in a reduced domain (Fig. 5). Although PAX2 and PAX6 expression was normal at E10.5, by E11.5, PAX2 expression was expanded distally into the optic cup, while PAX6 expression was distally restricted, indicating the proximo-distal axis of the optic cup is not properly established at this stage in Ccrk mutants (Fig. 5). We generated Ccrk;Gli2 double mutants, which showed
rescue of both optic cup morphogenesis and optic stalk, RPE and NR patterning (Fig. 5). These data suggest that elevated levels of Hh activity are responsible for the optic cup patterning and morphogenesis defects resulting from the loss of CCRK.

**Cerk mutants exhibit shorter optic stalks, a phenotype exacerbated by loss of GLI2**

The angle of the optic cup in *Ccrk;Gli2* double mutants appeared to be shifted ventrally, suggesting a possible defect in optic stalk outgrowth (Fig. 5; Furimsky and Wallace, 2006). This prompted us to compare the lengths of the ventral optic stalk of wild-type, *Ccrk, Gli2*, and *Ccrk;Gli2* mutants at E11.5. As PAX2 marks the optic stalk territory, we measured PAX2+ tissue in coronal sections that encompassed both eyes (see Materials and Methods). We found that *Cerk* mutants exhibited a significantly shorter optic stalk than wild-type embryos (p<0.01; Fig. 6A,B). Interestingly, *Ccrk;Gli2* double mutant optic stalks were not only shorter than those of wild-type (p<0.0001), but also shorter than those of *Ccrk* single mutants (p<0.01). *Ccrk;Gli2* double mutants also lacked a PAX2-negative ventral midline, whereas this domain was present in both *Ccrk* and *Gli2* single mutants (Fig. 6A). *Ccrk* mutants also exhibited hypotelorism, which was further exacerbated by loss of GLI2 (Fig. 6A). Thus CCRK appears to be required for optic stalk outgrowth and acts synergistically with GLI2 in this process (Fig. 6A,B).

Collectively, these experiments suggest that, in addition to restricting Hh activity, CCRK promotes proper responses to high levels of Hh signals. In *Ccrk* mutants, the gain of low-level, ectopic Hh activity contributes to the distal patterning defects, whereas the diminished response to high levels of Hh signals proximally leads to incomplete outgrowth of the optic stalk (see Discussion).
**Exacerbation of eye defects in Ccrk mutants by simultaneous loss of GLI3**

Our patterning analysis of Ccrk and Ccrk;Gli2 double mutants suggested that Hh activity influences RPE and NR cell fates. To test the hypothesis that these cell fates are indeed sensitive to elevated levels of Hh activity, we compared patterning of Ccrk mutants to that of Gli3 mutants. GLI3 is the primary Hh transcriptional repressor, whose loss leads to an elevation of the Hh pathway. At E11.5, Gli3 mutants phenocopied Ccrk mutants, including the variability of the phenotype. In both mutants, the optic stalk was expanded into the optic cup, the RPE was expanded into the NR, and the size of the NR domain was reduced (Fig. 7; Burnett et al., 2017). Further elevating the Hh pathway in Ccrk mutants by simultaneously removing GLI3 (Ccrk-/-;Gli3-/-) led to a greater expansion of the RPE marker MITF into the NR domain and a complete loss of CHX10+ cells (Fig. 7). Furthermore, whereas most Ccrk and Gli3 single mutants generated a lens by this stage (n=8/10 and 7/8, respectively), we did not observe a lens in any of the Ccrk;Gli3 double mutants analyzed (n=0/6). Thus, disruption of Ccrk and Gli3 together resulted in a phenotype exacerbated beyond that seen in either single mutant.

Collectively, the data suggest that optic progenitors are sensitive to subtle changes in the levels of Hh activity, and that progressively lower doses of Hh activity are required for proper specification of the optic stalk, RPE, and NR, respectively.

**DISCUSSION**

**CCRK controls early eye development and Hedgehog signaling**

Our results indicate that CCRK regulates morphogenesis and patterning of the late optic vesicle/early optic cup in the mouse embryo. Ccrk null mutants properly execute the
earliest stages of eye development: by E9.5, Ccrk mutants have specified and split the eye field and have generated optic vesicles whose patterning is largely normal. However, by E10.5, invagination of the optic vesicles and the lens placodes to form the optic cup and lens, respectively, is variably disrupted. By E11.5, specification of the neural retina (NR), characterized by CHX10 and SOX2 expression and the absence of SOX1, is significantly reduced. Concomitantly, the mutants show ectopic expression of the retinal pigment epithelium (RPE) markers MITF and OTX2 within the domain that would normally adopt the NR fate. Moreover, Ccrk mutants show distal expansion of PAX2+ optic stalk (OS) identity and failure to close the optic fissure (coloboma). As cells adopting the NR, RPE, and OS fates arise from the distal, intermediate, and proximal portions of the optic vesicle, respectively, these results suggest that cell fates in the Ccrk mutant eye are shifted towards a more proximal identity at the expense of distal identity.

Such defects in patterning are consistent with changes in Hh pathway activity, which has been shown to regulate proximo-distal identity in the mouse eye (Amato et al., 2004; Ekker et al., 1995; Macdonald et al., 1995; Perron et al., 2003; Sasagawa et al., 2002; Takeuchi et al., 2003; Wang et al., 2015; Zhang and Yang, 2001; Zhao et al., 2010). Indeed, the expression of Shh and downstream targets, Nkx2.1 and Gli1, in Ccrk mutants indicates that the Hh pathway is dysregulated in the absence of CCRK. These findings suggest there are two aspects to the effect of the Ccrk mutation on Hh pathway activity. First, the expansion of Gli1 expression throughout the distal optic vesicle suggests the pathway is inappropriately up-regulated in cells that would normally exhibit very low, if any, levels of pathway activity. This defect may explain the distal shift in RPE specification and the reduction of NR specification and disruption of optic cup formation. In addition,
the expression domains of Shh and Nkx2.1 in the ventral midline of Ccrk mutants are reduced. As both genes are normally activated by the highest levels of Hedgehog signaling, these data suggest that CCRK is also required for strong Hh responses. This aspect of CCRK’s function may explain the finding that CCRK and GLI2 act together to promote the length of the PAX2+ ventral optic stalk. These results are consistent with recent work from our laboratory (Snouffer et al., 2017) showing that, in the developing spinal neural tube and in embryonic fibroblasts, CCRK has two functions in Hh signaling: it acts in Hh signal-receiving cells to promote responses to the highest level of signals, while it also inhibits pathway activity in cells that are distant from the source of SHH and are not exposed to such signals. Our hypothesis, summarized in Figure 8, helps to explain how CCRK, through its control of Hh pathway activity, allows for proper patterning and morphogenesis in the distal eye while also promoting ventral midline specification in conjunction with GLI2.

The role of GLI proteins in the Ccrk phenotype

We suggest that the major, if not sole, mechanism by which CCRK controls early eye development is through regulation of the activity of the Hh pathway. The phenotype in the distal portion of the eye is consistent with ectopic activation of the pathway, which typically promotes proximal fate specification and the expense of distal fate specification. As the expression domain of the Shh ligand is restricted in Ccrk mutants, we hypothesized that loss of CCRK causes ligand-independent activation of the pathway. In support of this hypothesis, we found that Hh-dependent repression of PAX6 expression in the ventral midline and activation of PAX2 expression were rescued by disruption of Ccrk in a
background that lacks SMO (which otherwise shows complete lack of Hh pathway activity).

Hh signaling is transduced by the GLI transcription factors, with GLI2 acting primarily as an activator and GLI3, after being proteolytically processed, functioning primarily to repress Hh target gene expression. *Gli2* null mutants show a rather subtle phenotype in the eye (reported as slight shortening of the optic stalk) with distal development remaining largely normal (Furimsky and Wallace, 2006; Fig. 5). This is not surprising as distal optic tissues show little, if any, Hh pathway activity at the early optic vesicle stage, so a reduction of such activity would have little consequence. *Gli3* null mutants, however, show disruption of distal eye development and exhibit a phenotype remarkably similar to that of *Ccrk* mutants (Furimsky and Wallace, 2006; Fig. 7). This is likely due to ectopic Hh activity in the distal eye, which would inhibit NR specification and promote RPE and OS identity.

Consistent with our hypothesis, removing GLI2 function from *Ccrk* mutants rescues morphogenesis and patterning of the distal eye. Our interpretation is that removing GLI2 reduces the level of ectopic Hh activity in *Ccrk* mutants to near normal levels, thus allowing distal development to occur normally. Thus, CCRK may prevent GLI2 from ectopically activating the Hh pathway. However, in the ventral midline and OS, where *Ccrk* mutants exhibit lower than normal levels of activity, further reduction of activity by removing GLI2 results in shortening of the PAX2+ OS and hypotelorism.

Further support for the hypothesis comes from the fact that removal of GLI3 from *Ccrk* mutants exacerbates the distal developmental defects, resulting in robust, ectopic RPE specification in the distal eye and a complete failure of NR specification. Thus, the
simultaneous removal of two repression mechanisms, executed by CCRK and GLI3, would lead to even higher levels of ectopic pathway activity and further proximalization of distal eye development.

In principle, activation of the pathway in CCRK mutants may be caused by loss of GLI repressor functions. The importance of GLI3 repressor (GLI3Rep) in restricting Hh signaling has been well established (Rallu et al., 2002; Wijgerde et al., 2002). However, the fact that the Ccrk/Gli3 double mutant eye is much more severely affected than that of Gli3 single mutants suggests that ectopic activity caused by the Ccrk mutant is not due simply to loss of GLI3Rep. Indeed, the levels of processed GLI3Rep forms are reduced only to a minor extent in Ccrk mutants (Snouffer et al., 2017). Processed GLI2 has also been reported to have a repressor function (GLI2Rep), but this remains controversial and its importance appears to be minor (McDermott et al., 2005; Pan et al., 2009, 2006). The fact that loss of GLI2 rescues, rather than exacerbates, the Ccrk distal eye phenotype does not support a role for CCRK in promoting GLI2Rep function. Furthermore, if the Ccrk distal eye phenotype were to be caused by the loss of both GLI3Rep and GLI2Rep function, one would expect that the distal eye phenotypes of Ccrk/Gli2, Ccrk/Gli3, and Gli2/Gli3 double mutants to be similar to one another, which is not the case. Gli2/Gli3 double mutants (Furimsky and Wallace, 2006) show subtle disruption of distal eye development—a phenotype that is less severe than that seen in Ccrk/Gli3 double mutants and is more severe than that seen in Ccrk/Gli2 double mutants. Taken together, the data suggest that CCRK restricts Hh pathway activity in the distal eye primarily by inhibiting constitutive activity of GLI activators (mainly GLI2 activator). This does not rule out an additional role for CCRK in promoting GLIRep function, but this cannot be the sole mechanism.
The cell biological function of CCRK in Hh pathway regulation

CCRK likely controls the Hh pathway in the eye via its function in ciliogenesis. A large body of evidence indicates that many steps in Hh signaling occur within, and depend upon, the primary cilium (Caspary et al., 2007; Christopher et al., 2012; Goetz and Anderson, 2010; Haycraft et al., 2005; Huangfu et al., 2003; Huangfu and Anderson, 2005; Liu et al., 2005; Mukhopadhyay and Rohatgi, 2014; Rohatgi et al., 2007). Loss of CCRK disrupts the overall morphology and length of primary cilia in the optic primordium (Fig. 1), as well as in the spinal neuroepithelium and embryonic fibroblasts (Snouffer et al., 2017). This function of CCRK is consistent with those of its homologs in other systems, such as *Chlamydomonas* (LF2), *C. elegans* (DYF18), and *Danio rerio* (CCRK). Moreover, we recently showed that loss of primary cilia or defective ciliary transport in cells of the optic primordium leads to ectopic activity of Hh pathway activity and disruption of distal eye development (Burnett et al., 2017). Collectively, the data suggest that CCRK regulates GLI function and Hh pathway activity by virtue of its role in assembling normal cilia.

Although the precise mechanism by which CCRK contributes to ciliary regulation of Hh signaling remains unclear, our recent findings suggest that CCRK promotes the efficient import of key Hh pathway regulators, such as GLI2 and SMO, into primary cilia (Snouffer et al., 2017). The mechanisms by which CCRK controls ciliary transport and the reason why GLI proteins achieve this intermediate state when transport is perturbed are still to be elucidated.
Control of early eye development by Hh signaling

The specification of RPE and NR identity is under the control of a variety of signaling pathways, such as the Wnt, BMP, Notch and FGF pathways, as well as a gene regulatory network involving a number of transcription factors, such as CHX10, MITF, OTX2, VAX2, and PAX6 (Bharti et al., 2012; Galy et al., 2002; Martínez-Morales et al., 2003; Nishihara et al., 2012; Ou et al., 2013; Steinfeld et al., 2017, 2013; Veien et al., 2008). Our results indicate that the Hh pathway also regulates RPE and NR identity. These findings are consistent with previous work showing that overexpression of Hh ligands promotes RPE at the expense of NR specification in chick embryos (Zhang and Yang, 2001), and work on Xenopus showing that at least some Hh signaling is normally required for RPE development (Perron et al., 2003). The phenotype of Ccrk mutants, as well as those of Ccrk;Gli2 and Ccrk;Gli3 double mutants, collectively support the view that, whereas high levels of Hh activity promote midline and OS fate specification, low-to-intermediate levels of Hh activity promote RPE specification and inhibit specification of the NR fate (Fig. 8).

Because our understanding of the rather complex regulatory networks controlling RPE and NR fate remains incomplete, there are many possibilities as to the role of the Hh pathway in this decision. We think it likely that Hh signaling directly controls few aspects of the network while influencing other aspects indirectly. For example, although Hh signaling could act through parallel mechanisms to promote RPE fate and inhibit NR fate, it is perhaps more likely that Hh signaling directly controls one of these aspects, with effects on the other being secondary. This suggestion is supported by the data indicating that MITF, a transcription factor expressed by RPE cells, and CHX10, a NR
transcription factor, inhibit each other’s expression (Horsford et al., 2005; Nguyen and Arnheiter, 2000). Thus, it is possible that down-regulation of Chx10 and NR specification in Cerk mutants is secondary to ectopic Mitf expression induced by low-level Hedgehog signaling. Alternatively, ectopic Mitf expression may be secondary to a more direct inhibitory effect of the Hh pathway on Chx10 expression. Still, other scenarios are also possible whereby Hh activity controls the expression or activity of factors upstream of MITF and CHX10 expression. The direct and indirect roles of Hh signaling in this specification process should be clarified by future studies of the candidate regulatory factors and pathways.

In the absence of Hh signaling (e.g., in Shh-/- or Smo-/- mutants), no discernable pattern within the optic system is apparent (Chiang et al., 1996; Fig. 4). This indicates that there is, at minimum, a permissive role for Hh signaling in establishing proximo-distal pattern. Additionally, our data strongly support the view that different levels of Hh signaling contribute instructively to the specification of distinct cell fates in the developing eye, as both increasing and decreasing Hh pathway activity clearly alter cell fate choice (summarized in Fig. 8). Nevertheless, our data also indicate that differences in the levels of signaling by the graded distribution of Hh ligands cannot be the sole source of positional information that distinguishes between cell fate choices. For instance, increasing Hh pathway activity by removal of CCRK in a background lacking SMO still allows for patterning along the proximal-distal axis (Fig. 4). According to existing data, cells should not be able to sense Hh ligands (via the canonical pathway) in the absence of SMO (Varjosalo and Taipale, 2008; Zhang et al., 2001), and yet cells in the ventral midline of Cerk;Smo double mutants express neither PAX6 nor PAX2; cells towards the distal region
of the optic vesicle express both PAX6 and PAX2; and cells in the dorsal optic vesicle express PAX6 alone. This hypothesis is also supported by the phenotype of Ccrk mutants, who appear to express uniform low levels of Gli1 (Fig. 3F); despite this apparent lack of graded Hh activity, cells in this region are still able to make distinct regionally-defined choices. How such positional information is imparted to cells in the absence of a gradient of Hh activity is unclear, but it could occur through graded BMP, Wnt, or FGF signals from sources external to the optic vesicle, such as the surface ectoderm or extraocular mesenchyme.
Figure 1. *Ccrk* mutants show defects in eye morphology and ciliogenesis. (A) Whole-mount images of wild-type and *Ccrk/-* embryos at E12.5. Note the microphthalmia in the *Ccrk* mutant. (B) Representative images of sagittal serial sections of wild-type and *Ccrk* mutant eyes at E10.5 stained with H & E from a set used to generate the 3-D models shown. Note the coloboma and abnormal optic cup shape in the *Ccrk* mutant. Abbreviations: OC, optic cup; Do, dorsal; V, ventral; Di, distal; P, proximal. Scale bar indicates 50µm. (C) Confocal maximum intensity projections of cilia in both wild-type and *Ccrk* mutant optic stalk at E10.5, visualized with antibodies against ARL13B (green) and GAMMA-TUBULIN (red); DNA is counterstained with DAPI (blue). Note that cilia in *Ccrk* mutants appear abnormally short and round. Scale bar indicates 2µm.
Figure 2. Abnormal cell fate specification in the Ccrk mutant optic vesicle and optic cup. (A) Sections through somite-matched E9.5 wild-type and Ccrk mutant optic vesicles stained with markers for cell fates. Note that Ccrk mutant patterning is largely normal except for expansion of OTX2 into the pNR territory. Abbreviations: pOS, presumptive optic stalk; pRPE, presumptive retinal pigment epithelium; pNR, presumptive neural retina; Do, dorsal; V, ventral; P, proximal; Di, distal. (B) Sections through E10.5 wild-type and Ccrk mutant eyes stained with markers for cell fates. Note the reduced size of the CHX10 and SOX2 domain within the NR and the expansion of MITF and OTX2 into the inner optic cup. 3-6 embryos per genotype per stage were analyzed. Abbreviations: OS, optic stalk; RPE, retinal pigment epithelium; NR, neural retina.
Figure 3. Loss of CCRK results in results in bimodal changes in Hh pathway activity.

(A-F) Sections of wild-type (A,C,E) and Ccrk-/- embryos (B,D,F) at the indicated somite-stages (ss) following whole-mount in situ hybridization against Shh (A,B), Nkx2.1 (C,D), and Gli1 (E,F). Note the proximal restriction of Shh (B) and Nkx2.1 (D) expression, but the proximal and distal expansion of Gli1 expression (F), in the Ccrk mutant. Arrows and arrowheads in (E) mark the Gli1-negative midline and Gli1-low distal optic vesicle, respectively, in wild-type embryos. Arrows and arrowheads in (F) mark ectopic Gli1 expression in the midline and distal optic vesicle, respectively, in Ccrk mutants. 3 embryos per genotype per assay were analyzed.
Figure 4. Loss of CCRK activates the Hh pathway independently of SMO. Sections through the optic vesicle of wild-type, Ccrk, Smo, and Ccrk;Smo double mutants stained with antibodies against PAX2 and PAX6. Note that the loss of PAX2 expression and the proximal expansion of PAX6 expression in the Smo mutant is suppressed in the Ccrk;Smo double mutant. 3-4 embryos per genotype were analyzed.
Figure 5. Eye patterning defects in Ccrk mutants are suppressed by loss of GLI2.
Sections through E11.5 wild-type, Ccrk, Gli2, and Ccrk;Gli2 double mutant eyes stained with markers for cell fates. Note the dorsal expansion of PAX2, dorsal restriction of PAX6, expansion of MITF and OTX2 into the inner optic cup, reduction of CHX10+ cells, distal expansion of SOX1, and loss of SOX2 expression in the Ccrk mutant, yet the rescue of normal patterning in the Ccrk;Gli2 double mutant. 4-6 embryos per genotype were analyzed.
Figure 6. *Ccrk* mutants show shortened optic stalks, and loss of GLI2 exacerbates this defect. (A) Low-magnification images of coronal sections through the eyes of E11.5 wild-type, *Gli2*, *Ccrk*, and *Ccrk;Gli2* double mutants stained with antibodies against PAX2 and CHX10 to mark the optic stalk and neural retina, respectively. Scale bar indicates 100µm. (B) Lengths of the ventral optic stalks of wild-type, *Gli2*, *Ccrk*, and *Ccrk;Gli2* double mutants at E11.5 as indicated by PAX2 expression. 5 embryos analyzed per genotype. Error bars indicate standard error. Data were analyzed using Student’s t-test: not significant (n.s.); p<0.05 (*); p<0.01 (**); p<0.001 (***).
Figure 7. Disruption of *Gli3* exacerbates the eye patterning phenotype of *Ccrk* mutants. Sections through E11.5 wild-type, *Ccrk*, *Gli3*, and *Ccrk;Gli3* double mutant eyes stained with markers for cell fates. Note the loss of CHX10 and SOX2 expression in the inner optic cup and the extensive expansion of MITF into the inner optic cup in the double mutant. 5-7 embryos per genotype were analyzed.
Figure 8. CCRK controls eye development by both promoting proximal, high-level activation of the Hh pathway and by restricting distal, low-level Hh activity. (A) Model for the role of CCRK-dependent Hh activity during eye morphogenesis. In the absence of CCRK, the proximal, high levels of Hh activity are not achieved (green arrow).
This leads to a shorter optic stalk compared to wild-type. Loss of GLI2 in a Ccrk mutant background, which further reduces high-level Hh activity, shortens the optic stalk even further while also preventing formation of a ventral midline. In the absence of CCRK, there is also ectopic, low-level Hh activity in the distal eye, as in Gli3 mutants (red arrow). This shifts the boundary between the optic stalk and optic cup distally, as evidenced by distal PAX2 expansion and distal PAX6 restriction. This also compromises lens formation. Loss of GLI3 in a Ccrk mutant background, which further elevates Hh pathway activity, completely abolishes lens formation and severely impairs optic cup morphogenesis. (B) Model for the role of CCRK-dependent Hh activity in specification of the RPE and NR. The elevation of Hh activity in the distal eye of Ccrk mutants results in an expansion of RPE fate and a corresponding restriction of NR fate. Loss of GLI2 in Ccrk mutants reduces Hh activity and restores normal patterning of the RPE and NR. In contrast, loss of GLI3 in Ccrk mutants further elevates the Hh pathway, which leads to a complete expansion of the RPE and abolishes NR specification.
Figure S1. Expression of *Ccrk* at E9.5. *In situ* hybridization using *Ccrk* sense and antisense probes of E9.5 (24-26 somites) wild-type embryos. Sections are through the optic vesicle of embryos following whole-mount *in situ* hybridization.
Figure S2. Additional patterning analysis of Cerk mutants. (A) Whole-mount in situ hybridization of the eye field transcription factors Rax and Lhx2 in wild-type and Cerk mutant embryos at E9.5. (B) Whole-mount in situ hybridization of Vax2 at the indicated somite stages (ss). Note the lack of dorsal Vax2 expression in the wild-type (arrows) and the dorsal expansion of Vax2 expression in the Cerk mutant (arrowheads).
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CHAPTER 4
CONCLUSIONS AND FUTURE DIRECTIONS

EVIDENCE FOR THE REGULATION OF THE HH PATHWAY BY IFT122, IFT172, AND CCRK WITHIN OPTIC PROGENITORS

In this dissertation, I have shown that IFT122, IFT172, and CCRK are critical for patterning and morphogenesis of the early mammalian eye (Fig. 1). Loss of IFT122 prevented formation of the optic cup and lens, and prevented specification of the RPE and NR; the developing eye arrested at the optic vesicle stage and adopted an optic stalk-like fate. These defects were associated with high levels of HH pathway activity throughout the entire optic vesicle, as evidenced by the ectopic expression of Shh ligand and targets Gli1 and Nkx2.1 in the distal optic vesicle. Disruption of Gli2, the primary HH activator, in Ift122 mutants allowed for the initiation of optic cup and lens formation as well as the specification of RPE and NR fate.

Likewise, loss of IFT172 or CCRK also lead to defects in optic cup patterning and morphogenesis, albeit less severely than loss of IFT122. Ift172 and Ccrk mutants formed a lens and optic cup, though the optic cup adopted an abnormal morphology. Whereas the entire eye of Ift122 mutants expressed markers for optic stalk fate (i.e., extreme proximalization of the eye), Ift172 and Ccrk mutants showed a distal expansion of RPE markers into the NR domain and a distal expansion of OS markers into the optic cup (i.e., less extreme proximalization). Like Ift122 mutants, both Ift172 and Ccrk mutants
ectopically expressed the low-threshold HH target, *Gli1*, in the distal optic vesicle. However, there was no such distal expansion of the high-threshold HH target, *Nkx2.1*, in *Ift172* or *Ccrk* mutants. Thus, such differential activity of the HH pathway might explain the differences in the severity of ocular defects between these two phenotypic classes. *Ift172* and *Ccrk* mutants also phenocopied *Gli3* mutants, which lack the primary HH repressor. Disruption of *Gli2* in *Ccrk* mutants fully rescued patterning of the OS, RPE, and NR. Collectively, these data support the argument that IFT122, IFT172, and CCRK control eye development through regulation of the HH pathway.

**THE CELL BIOLOGICAL ROLES OF IFT122, IFT172, AND CCRK IN THE EYE:**

**EVIDENCE FOR CILIA-DEPENDENT FUNCTION**

Several lines of evidence suggest that the function of IFT122, IFT172, and CCRK in eye development is at the level of the primary cilium. Previous data indicate IFT122 and IFT172 are members of the IFT-A and IFT-B complexes, respectively (Pedersen et al., 2005; Tsao and Gorovsky, 2008). These IFT complexes are trafficked throughout the cilium and carry cargo needed to build and maintain the cilium. Though not ciliary-localized, the role of CCRK in controlling cilia length and morphology has been well established (Asleson and Lefebvre, 1998; Erdmann et al., 2006; Ko et al., 2010; Phirke et al., 2011). Consistent with these previous studies, in this dissertation I have showed that optic progenitors are ciliated and that loss of IFT122, IFT172, and CCRK lead to defects in optic progenitor ciliogenesis: *Ift122* mutants displayed swollen, bulbous optic cilia; *Ift172* failed to generate any optic cilia; *Ccrk* mutants displayed short, round optic cilia. While these factors could have roles outside of the cilium, the most parsimonious
interpretation of these data are that they control eye development via their roles in ciliary assembly, especially given the relationship between cilia and HH signaling (Goetz and Anderson, 2010).

**A MODEL FOR HEDGEHOG-MEDIATED OPTIC PATTERNING: EVIDENCE FOR SHH MORPHOGEN ACTIVITY IN THE EYE**

The early eye is composed of three fates OS, RPE, NR. Using data presented in this dissertation (Fig. 1), I argue that OS fate requires high levels of HH activity, RPE fate requires moderate levels of HH activity, and NR fate requires low levels of HH activity. The evidence for this is as follows. Of the ciliogenesis mutants examined, *Ift122* mutants have the highest levels of HH activity and the entire eye is OS-fated. Reducing HH activity by simultaneously disrupting *Gli2* (*Ift122<sup>-/-</sup>;*Gli2<sup>-/-</sup>*) results in an eye that has all three fates (OS, RPE, NR), though the OS and RPE are distally expanded and the NR domain is reduced. *Ccrk* and *Ift172* mutants have lower levels of HH activity than *Ift122* mutants and phenotypically resemble *Ift122;Gli2* and *Gli3* mutants: the eyes of these mutants also show a distal shift in OS and RPE fate and a reduction in the NR domain. Thus, RPE and NR fates require lower levels of HH activity than OS fate. Further elevating HH activity in *Ccrk* mutants by simultaneously disrupting *Gli3* (*Ccrk<sup>-/-</sup>;Gli3<sup>-/-</sup>*) results in a complete distal expansion of the RPE and a complete absence of the NR. Conversely, reducing HH activity in *Ccrk* mutants by simultaneously disrupting *Gli2* (*Ccrk<sup>-/-</sup>;Gli2<sup>-/-</sup>*) results in a virtually complete rescue of normal patterning: the OS and RPE are no longer distally expanded and the NR domain is of normal size. Thus, NR fate requires lower levels of HH activity than RPE fate. While a number of other signals can also influence RPE and NR identity (see
Ch. 1), the data presented here suggest that distinct levels of HH activity, likely via SHH ligand, play an instructive role in ocular patterning and likely integrate with these other signals to canalize cell fate decisions.

If SHH indeed functions as a morphogen in ocular patterning, then one would expect that distinct levels of HH pathway activity can cell-autonomously control expression of OS-, RPE-, and NR-fate determinants. One way to test this is through chimera analysis with ES cells of different genotypes. If HH activity functions cell autonomously, then mutant cells of a genotype that have increased HH activity (e.g., $Ccrk^{-/-}$, $Gli3^{-/-}$, or $Ccrk^{-/-};Gli3^{-/-}$) should adopt a more proximal fate (i.e., OS or RPE) even though they might be located more distally (i.e., in the presumptive NR) within a chimeric optic vesicle. Conversely, cells of a genotype that have reduced HH activity (e.g., $Gli2^{-/-}$, $Ccrk^{-/-}$; $Gli2^{-/-}$; or $Smo^{-/-}$) would likely show the opposite effect, with mutant cells residing in proximal regions adopting a more distal fate. Another way to address this question is through the use of an in vitro ES-derived optic cup system (Eiraku and Sasai, 2012), in which the system is dosed with different levels of SMO-agonist (SAG) or recombinant SHH. Such a system will lack the influence of tissues external to the optic vesicle. The expectation is that increasing doses of SAG or SHH will lead to a dose-dependent proximalization of distal fates. For example, at lower concentrations of SAG or SHH, one might observe RPE-fated cells in the presumptive NR domain, whereas at higher concentrations, one might observe OS-fated cells in the presumptive RPE and/or NR domain. The converse loss-of-function experiment can be performed using cyclopamine, with the expectation of the reciprocal dose-dependent distalization of eye fates.
Experiments such as these may further support the morphogen model of SHH activity in patterning of the eye.
Figure 1. Summary of the level of HH pathway activity and how this impacts key aspects of eye development for all single and double mutants analyzed in this dissertation. See text for details.
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