Published in final edited form as:

Dev Biol. 2018 February 01; 434(1): 24–35. doi:10.1016/j.ydbio.2017.10.022.

Cell cycle-related kinase regulates mammalian eye development through positive and negative regulation of the Hedgehog pathway

Floria I. Lupu[1], **Jacob B. Burnett**[1], and **Jonathan T. Eggenschwile**[*]

Department of Genetics, University of Georgia, Athens, GA 30602, USA

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.

Keywords

Optic stalk; Retinal pigment epithelium; Neural retina; Ciliogenesis; GLI2; GLI3

1. 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

[*]Correspondence to: Department of Genetics, University of Georgia, Coverdell Bldg., Rm. 210A, Athens, GA 30606, USA. jeggensc@uga.edu (J.T. Eggenschwiler).

[1]These authors contributed equally to the work.

Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ydbio.2017.10.022.

(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; Take-uchi 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.

2. Materials and methods

2.1. 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*^{zfd} and *Smo*^{tm1Amc} alleles were obtained from Alexandra Joyner (Sloan Kettering Institute) and Andrew McMahon (University of Southern California), respectively genotyped as previously described (Matise et al., 1998; Zhang et al., 2001). *Gli3*$^{Xt\text{-}J}$ mice were obtained from the Jackson Laboratories (Bar Harbor, ME) and genotyped as previously described (Hui and Joyner, 1993).

2.2. 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-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). Images were taken with either a Zeiss Axioplan 2 or a Keyence BZ-×710 fluorescence microscope. High-magnification images of cilia are maximum intensity projections from z-stacks taken on a Zeiss LSM510 confocal microscope.

2.3. 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’-GACTCTCGCTCCCAAATGAG-3’, respectively. For sense riboprobes, the Ccrk plasmid

was digested with NcoI 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.

2.4. Optic stalk length measurement

Images of coronal cryosections stained with anti-PAX2 and antiCHX10 (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.

2.5. 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).

3. Results

3.1. CCRK regulates eye morphogenesis and cilia morphology within optic progenitors

Ccrk null mice exhibited microphthalmia by E12.5 (Fig. 1A). Threedimensional 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 the 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 the basal body protein, GAMMMA-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.

3.2. 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.

3.3. 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 elevated 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 repress *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 it also leads to an ectopic increase in Hh activity in the distal optic vesicle.

3.4. 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 and 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.

3.5. 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.

3.6. Ccrk 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 *Ccrk* 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 to act 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).

3.7. 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.

4. Discussion

4.1. 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 execute properly 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; Take-uchi 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 upregulated in cells that would normally exhibit very low, if any, levels of pathway activity. This defect may explain 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 Fig. 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.

4.2. The role of GLI proteins in the Ccrk mutant 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 ligandindependent 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 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 for regulation.

4.3. 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.

4.4. 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 expression and NR specification in *Ccrk* 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 a 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 *Ccrk;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, which 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

The authors wish to thank Julie Gordon and Kristina Buac (UGA) for technical advice. The authors also wish to thank Muthugapatti Kandasamy at the UGA Biomedical Microscopy Core for his assistance with confocal microscopy, as well as the husbandry and veterinary staff of the Coverdell Animal Resources Facility at UGA.

Funding

This work was supported by The National Institute of Child Health and Human Development (grant #R01HD050761, to JTE) and the Office of Vice President for Research at the University of Georgia (grant# 1021RX064569). JBB was supported by a T32 Training Grant (T32GM007103) from the National Institute of General Medical Sciences.

References

Amato MA, Boy S, Perron M, 2004 Hedgehog signaling in vertebrate eye development: a growing puzzle. Cell. Mol. Life Sci 61, 899–910. 10.1007/s00018-003-3370-7. [PubMed: 15095011]

Asleson CM, Lefebvre PA, 1998 Genetic Analysis of Flagellar Length Control inChlamydomonas reinhardtii: A New Long-Flagella Locus and Extragenic Suppressor Mutations.

Bai CB, Joyner a.L., 2001 Gli1 can rescue the in vivo function of Gli2. Development 128, 5161–5172. [PubMed: 11748151]

Bai CB, Auerbach W, Lee JS, Stephen D, Joyner AL, 2002 Gli2, but not Gli1, is required for initial Shh signaling and ectopic activation of the Shh pathway. Development 129, 4753–4761. 10.1242/dev.00115. [PubMed: 12361967]

Bai CB, Stephen D, Joyner AL, 2004 All mouse ventral spinal cord patterning by Hedgehog is Gli dependent and involves an activator function of Gli3. Dev. Cell 6, 103–115. 10.1016/S1534-5807(03)00394-0. [PubMed: 14723851]

Bharti K, Gasper M, Ou J, Brucato M, Clore-Gronenborn K, Pickel J, Arnheiter H, 2012 A regulatory loop involving PAX6, MITF, and WNT signaling controls retinal pigment epithelium development. PLoS Genet. 8, 1–17. 10.1371/journal.pgen.1002757.

Briscoe J, Thérond PP, 2013 The mechanisms of Hedgehog signalling and its roles in development and disease. Nat. Rev. Mol. Cell. Biol 14 10.1038/nrm3598.

Burnett JB, Lupu FI, Eggenschwiler JT, 2017 Proper ciliary assembly is critical for restricting Hedgehog signaling during early eye development in mice. Dev. Biol 430, 32–40. 10.1016/j.ydbio.2017.07.012. [PubMed: 28778798]

Buttitta L, Mo R, Hui C-C, Fan C-M, 2003 Interplays of Gli2 and Gli3 and their requirement in mediating Shh-dependent sclerotome induction. Development 130, 6233–6243. 10.1242/dev.00851. [PubMed: 14602680]

Caspary T, Larkins CE, Anderson KV, 2007 The Graded Response to SonicHedgehog Depends on Cilia Architecture. Dev. Cell 12, 767–778. 10.1016/j.devcel.2007.03.004. [PubMed: 17488627]

Chiang C, Litingtung Y, Lee E, Young KE, Corden JL, Westphal H, Beachy P. a., 1996 Cyclopia and defective axial patterning in mice lacking Sonic hedgehog gene function. Nature. 10.1038/383407a0.

Chow RL, Lang RA, 2001 Early Eye Development in Vertebrates. Annu. Rev. Cell Dev. Biol 17, 255–296. [PubMed: 11687490]

Christopher KJ, Wang B, Kong Y, Weatherbee SD, 2012 Forward genetics uncovers Transmembrane protein 107 as a novel factor required for ciliogenesis and Sonic hedgehog signaling. Dev. Biol 368, 382–392. 10.1016/j.ydbio.2012.06.008. [PubMed: 22698544]

Dai P, Akimaru H, Tanaka Y, Maekawa T, Nakafuku M, Ishii S, 1999 Sonic hedgehog-induced activation of the Gli1 promoter is mediated by GLI3. J. Biol. Chem 274, 8143–8152. 10.1074/jbc.274.12.8143. [PubMed: 10075717]

Dale JK, Vesque C, Lints TJ, Sampath TK, Furley A, Dodd J, Placzek M, 1997 Cooperation of BMP7 and SHH in the induction of forebrain ventral midline cells by prechordal mesoderm. Cell 90, 257–269. 10.1016/S00928674(00)80334-7. [PubMed: 9244300]

Deml B, Reis LM, Lemyre E, Clark RD, Kariminejad A, Semina EV, 2015 Novel mutations in PAX6, OTX2 and NDP in anophthalmia, microphthalmia and coloboma. Eur. J. Hum. Genet 24, 535–541. 10.1038/ejhg.2015.155. [PubMed: 26130484]

Echelard Y, Epstein DJ, Mcmahon JA, Mcmahon AP, 1993 Sonic Hedgehog, a Member of a Family of Putative Signaling Molecules, Is Implicated in the Regulation of CNS Polarity. Cell 75, 1417–1430. [PubMed: 7916661]

Ekker SC, Ungar AR, Greenstein P, von Kessler DP, Porter JA, Moon RT,Beachy PA, 1995 Patterning activities of vertebrate hedgehog proteins in the developing eye and brain. Curr. Biol 5, 944–955. 10.1016/S09609822(95)00185-0. [PubMed: 7583153]

Erdmann M, Scholz A, Melzer IM, Schmetz C, Wiese M, 2006 Interacting protein kinases involved in the regulation of flagellar length. Mol. Biol. Cell 17, 2035–2045. 10.1091/mbc.E05. [PubMed: 16467378]

FitzPatrick DR, Van Heyningen V, 2005 Developmental eye disorders. Curr. Opin. Genet. Dev 15, 348–353. 10.1016/j.gde.2005.04.013. [PubMed: 15917212]

Fuhrmann S, 2010 Eye morphogenesis and patterning of the optic vesicle. Curr. Top. Dev. Biol 93, 61–84. 10.1016/B978-0-12-385044-7.00003-5. [PubMed: 20959163]

Furimsky M, Wallace VA, 2006 Complementary Gli activity mediates early patterning of the mouse visual system. Dev. Dyn 235, 594–605. 10.1002/dvdy.20658. [PubMed: 16342201]

Galy A, Néron B, Planque N, Saule S, Eychène A, 2002 Activated MAPK/ERK Kinase (MEK-1) induces transdifferentiation of pigmented epithelium into neural retina. Dev. Biol 248, 251–264. 10.1006/dbio.2002.0736. [PubMed: 12167402]

Goetz SC, Anderson KV, 2010 The primary cilium: a signaling center during vertebrate development. Nat. Rev. Genet 11, 331–344. 10.1038/nrg2774.The. [PubMed: 20395968]

Graw J, 2010 Eye development. Curr. Top. Dev. Biol 90, 343–386. 10.1016/S0070-2153(10)90010-0. [PubMed: 20691855]

Haycraft CJ, Banizs B, Aydin-Son Y, Zhang Q, Michaud EJ, Yoder BK, 2005 Gli2 and Gli3 localize to cilia and require the intraflagellar transport protein polaris for processing and function. PLoS Genet. 1, e53 10.1371/journal.pgen.0010053. [PubMed: 16254602]

Hever AM, Williamson KA, van Heyningen V, 2006 Developmental malformations of the eye: the role of PAX6, SOX2 and OTX2. Clin. Genet 69, 459–470. 10.1111/j.1399-0004.2006.00619.x. [PubMed: 16712695]

Horsford DJ, Nguyen M-TT, Sellar GC, Kothary R, Arnheiter H, McInnes RR,2005 Chx10 repression of Mitf is required for the maintenance of mammalian neuroretinal identity. Development 132, 177–187. 10.1242/dev.01571. [PubMed: 15576400]

Huangfu D, Anderson KV, 2005 Cilia and Hedgehog responsiveness in the mouse.Proc. Natl. Acad. Sci. USA 102, 11325–11330. 10.1073/pnas.0505328102. [PubMed: 16061793]

Huangfu D, Lui A, Rakeman AS, Murcia NS, Niswander L, Anderson KV, 2003 Hedgehog signalling in the mouse requires intraflagellar transport proteins. Nature 426, 83–87. [PubMed: 14603322]

Hui C, Joyner AL, 1993 A mouse model of Greig cephalo-polysyndactyly syndrome: the extra-toesJ mutation contains an intragenic deletion of the Gli3 gene. Nat. Genet 3, 241–246. [PubMed: 8387379]

Hui C-C, Slusarski D, Platt KA, Holmgren R, Joyner AL, 1994 Expression of three mouse homologs of the Drosophila segment polarity gene cubitus interruptus, Gli, Gli-2, and Gli-3, in ectodermand mesoderm-derived tissues suggests multiple roles during postimplantation development. Dev. Biol 162, 402–413. 10.1006/dbio.1994.1097. [PubMed: 8150204]

Kitaura Y, Hojo H, Komiyama Y, Takato T, Chung U Il, Ohba S, 2014 Gli1 haploinsufficiency leads to decreased bone mass with an uncoupling of bone metabolism in adult mice. PLoS One, 9 10.1371/journal.pone.0109597.

Ko HW, Norman RX, Tran J, Fuller KP, Fukuda M, Eggenschwiler JT, 2010 Broad-minded links cell cycle-related kinase to cilia assembly and Hedgehog signal transduction. Dev. Cell 18, 237–247. 10.1016/j.devcel.2009.12.014. [PubMed: 20159594]

Lei Q, Zelman AK, Kuang E, Li S, Matise MP, 2004 Transduction of graded Hedgehog signaling by a combination of Gli2 and Gli3 activator functions in the developing spinal cord. Development 131, 3593–3604. 10.1242/dev.01230. [PubMed: 15215207]

Liu A, Wang B, Niswander LA, 2005 Mouse intraflagellar transport proteins regulate both the activator and repressor functions of Gli transcription factors. Development 132, 3103–3111. 10.1242/dev.01894. [PubMed: 15930098]

Long JE, Swan C, Liang WS, Cobos I, Potter GB, Rubenstein JLR, 2009 Dlx1 & 2 and Mash1 transcription factors control striatal patterning and differentiation through parallel and overlapping pathways. J. Comp. Neurol 512, 556–572. 10.1002/cne.21854. [PubMed: 19030180]

Macdonald R, Barth K. a., Xu Q, Holder N, Mikkola I, Wilson SW, 1995 Midline signalling is required for Pax gene regulation and patterning of the eyes. Development 121, 3267–3278. 10.1016/0168-9525(95)90453-0. [PubMed: 7588061]

Martinez-Morales JR, Signore M, Acampora D, Simeone a., Bovolenta P, 2001 Otx genes are required for tissue specification in the developing eye. Development 128, 2019–2030. [PubMed: 11493524]

Martínez-Mo rales JR, Dolez V, Rodrigo I, Zaccarini R, Leconte L, Bovolenta P, Saule S, 2003 OTX2 activates the molecular network underlying retina pigment epithelium differentiation. J. Biol. Chem 278, 21721–21731. 10.1074/jbc.M301708200. [PubMed: 12663655]

Matise MP, Epstein DJ, Park HL, Platt KA, Joyner AL, 1998 Gli2 is required for induction of floor plate and adjacent cells, but not most ventral neurons in the mouse central nervous system. Development 125, 2759–2770, (doi:9655799). [PubMed: 9655799]

McDermott A, Gustafsson M, Elsam T, Hui C-C, Emerson CP, Borycki A-G,2005 Gli2 and Gli3 have redundant and context-dependent function in skeletal muscle formation. Development 132, 345–357. 10.1242/dev.01537. [PubMed: 15604102]

Moon H, Song J, Shin J, Lee H, Kim H, Eggenschwiller JT, Bok J, Ko HW,2014 Intestinal cell kinase, a protein associated with endocrine-cerebroosteodysplasia syndrome, is a key regulator of cilia length and Hedgehog signaling. Proc. Natl. Acad. Sci. USA 111 (23), 8541–8546. 10.1073/pnas.1323161111. [PubMed: 24853502]

Mui SH, Hindges R, O'Leary DDM, Lemke G, Bertuzzi S, 2002 The homeodomain protein Vax2 patterns the dorsoventral and nasotemporal axes of the eye. Development 129, 797–804. [PubMed: 11830578]

Mukhopadhyay S, Rohatgi R, 2014 G-protein-coupled receptors, hedgehog signaling and primary cilia. Semin. Cell Dev. Biol 33, 63–72. 10.1016/j.semcdb.2014.05.002. [PubMed: 24845016]

Muresan V, Joshi HC, Besharse JC, 1993 Gamma-tubulin in differentiated cell types: localization in the vicinity of basal bodies in retinal photoreceptors and ciliated epithelia. J. Cell Sci. 104 (Pt 4), 1229–1237. [PubMed: 7686172]

Nguyen M, Arnheiter H, 2000 Signaling and transcriptional regulation in early mammalian eye development: a link between FGF and MITF. Development 127, 3581–3591. [PubMed: 10903182]

Nishihara D, Yajima I, Tabata H, Nakai M, Tsukiji N, Katahira T, Takeda K, Shibahara S, Nakamura H, Yamamoto H, 2012 Otx2 Is Involved in the Regional Specification of the Developing Retinal Pigment Epithelium by Preventing the Expression of Sox2 and Fgf8, Factors That Induce Neural Retina Differentiation. PLoS One 7, 1–12. 10.1371/journal.pone.0048879.

Ou J, Bharti K, Nodari A, Bertuzzi S, Arnheiter H, 2013 Vax1/2 Genes Counteract Mitf-Induced Respecification of the Retinal Pigment Epithelium. PLoS One, 8 10.1371/journal.pone.0059247.

Palencia-Campos A, Ullah A, Nevado J, Yıldırım R, Unal E, Ciorraga M, Barruz P, Chico L, Piceci-Sparascio F, Guida V, De Luca A, Kayserili H, Ullah I, Burmeister M, Lapunzina P, Ahmad W, Morales AV, Ruiz-Perez VL, 2017 GLI1 inactivation is associated with developmental phenotypes overlapping with Ellis–van Creveld syndrome. Hum. Mol. Genet 0, 1–16. 10.1093/hmg/ddx335.

Pan Y, Bai CB, Joyner AL, Wang B, 2006 Sonic hedgehog Signaling Regulates Gli2Transcriptional Activity by Suppressing Its Processing and Degradation Sonic hedgehog Signaling Regulates Gli2 Transcriptional Activity by Suppressing Its Processing and Degradation. Mol. Cell. Biol 26, 3365–3377. 10.1128/MCB.26.9.3365. [PubMed: 16611981]

Pan Y, Wang C, Wang B, 2009 Phosphorylation of Gli2 by protein kinase A is required for Gli2 processing and degradation and the Sonic Hedgehog-regulated mouse development. Dev. Biol 326, 177–189. 10.1016/j.ydbio.2008.11.009. [PubMed: 19056373]

Park HL, Bai C, Platt KA, Matise MP, Beeghly A, Hui C. c., Nakashima M, Joyner AL, 2000 Mouse Gli1 mutants are viable but have defects in SHH signaling in combination with a Gli2 mutation. Development 127, 1593–1605. 10.1101/gad.10.16.2003. [PubMed: 10725236]

Perron M, Boy S, Amato M. a., Viczian A, Koebernick K, Pieler T, Harris W. a.,2003 A novel function for Hedgehog signalling in retinal pigment epithelium differentiation. Development 130, 1565–1577. 10.1242/dev.00391. [PubMed: 12620982]

Phirke P, Evgeni E, Mohan S, Burghoorn J, Crona F, Bakhoum MW, Trieb M, Schuske K, Jorgensen EM, Piasecki BP, Leroux MR, Swoboda P, 2011 Transcriptional pro fi ling of C . elegans DAF-19 uncovers a ciliary base-associated protein and a CDK / CCRK / LF2p-related kinase required for intra flagellar transport. Dev. Biol 357, 235–247. 10.1016/j.ydbio.2011.06.028. [PubMed: 21740898]

Rallu M, Machold R, Gaiano N, Corbin JG, McMahon AP, Fishell G, 2002 Dorsoventral patterning is established in the telencephalon of mutants lacking both Gli3 and Hedgehog signaling. Development 129, 4963–4974. [PubMed: 12397105]

Ribes V, Balaskas N, Sasai N, Cruz C, Dessaud E, Cayuso J, Tozer S, Yang LL, Novitch B, Marti E, Briscoe J, 2010 Distinct Sonic Hedgehog signaling dynamics specify floor plate and ventral neuronal progenitors in the vertebrate neural tube. Genes Dev, 1186–1200. 10.1101/gad.559910. [PubMed: 20516201]

Rohatgi R, Milenkovic L, Scott MP, 2007 Patched1 regulates hedgehog signaling at the primary cilium. Science 80 (317), 372–376. 10.1126/science.1139740.

Sasagawa S, Takabatake T, Takabatake Y, Muramatsu T, Takeshima K, 2002 Axes establishment during eye morphogenesis in Xenopus by coordinate and antagonistic actions of BMP4, Shh, and RA. Genesis 33, 86–96. 10.1002/gene.10095. [PubMed: 12112877]

Sasaki H, Nishizaki Y, Hui C, Nakafuku M, Kondoh H, 1999 Regulation of Gli2 and Gli3 activities by an amino-terminal repression domain: implication of Gli2 and Gli3 as primary mediators of Shh signaling. Development 126, 3915–3924. 10.1038/386735a0. [PubMed: 10433919]

Snouffer A, Snouffer A, Brown D, Lee H, Walsh J, Lupu F, Norman R, Lechtreck K, Ko HW, Eggenschwiler J, Caspary T, 2017 Cell Cycle-Related Kinase (CCRK) regulates ciliogenesis and Hedgehog signaling in mice. PLoS Genet., 1–30.

Steinfeld I, Bausch A, Coronato N, Hampel M, Depner H, Layer PG, Vogel-ho A, 2017 BMP-induced reprogramming of the neural retina into retinal pigment epithelium requires Wnt signalling. Biol. Open, 979–992. 10.1242/bio.018739. [PubMed: 28546339]

Steinfeld J, Steinfeld I, Coronato N, Hampel M-L, Layer PG, Araki M, VogelHopker A, 2013 RPE specification in the chick is mediated by surface ectodermderived BMP and Wnt signalling. Development 140, 4959–4969. 10.1242/dev.096990. [PubMed: 24227655]

Take-uchi M, Clarke JDW, Wilson SW, 2003 Hedgehog signalling maintains the optic stalk-retinal interface through the regulation of Vax gene activity. Development 130, 955–968. 10.1242/dev.00305. [PubMed: 12538521]

Varjosalo M, Taipale J, 2008 Hedgehog: functions and mechanisms. Genes Dev. 22,2454–2472. 10.1101/gad.1693608. [PubMed: 18794343]

Veien ES, Rosenthal JS, Kruse-Bend RC, Chien C-B, Dorsky RI, 2008 CanonicalWnt signaling is required for the maintenance of dorsal retinal identity. Development 135, 4101–4111. 10.1242/dev.027367. [PubMed: 19004855]

Vokes S. a., Ji H, McCuine S, Tenzen T, Giles S, Zhong S, Longabaugh WJR, Davidson EH, Wong WH, McMahon AP, 2007 Genomic characterization of Gli-activator targets in sonic hedgehog-mediated neural patterning. Development 134, 1977–1989. 10.1242/dev.001966. [PubMed: 17442700]

Wang X, Lupo G, He R, Barsacchi G, Harris WA, Liu Y, 2015 Dorsoventral patterning of the Xenopus eye involves differential temporal changes in the response of optic stalk and retinal progenitors to Hh signalling. Neural Dev. 10, 7 10.1186/s13064-015-0035-9. [PubMed: 25886149]

Wijgerde M, Mcmahon JA, Rule M, Mcmahon AP, 2002 A direct requirement for Hedgehog signaling for normal specification of all ventral progenitor domains in the presumptive mammalian spinal cord. Genes Dev. 1, 2849–2864. 10.1101/gad.1025702.produced.

Yang XJ, 2004 Roles of cell-extrinsic growth factors in vertebrate eye pattern formation and retinogenesis. Semin. Cell Dev. Biol 15, 91–103. 10.1016/j.semcdb.2003.09.004. [PubMed: 15036212]

Yang Y, Roine N, Ma TP, 2013 CCRK depletion inhibits glioblastoma cell proliferation in a cilium-dependent manner. EMBO Reports 14 (8), 741–747. 10.1038/embor.2013.80. [PubMed: 23743448]

Yong P, Chengbing W, Wang B, 2009 Phosphorylation of Gli2 by protein kinase A is required for Gli2 processing and degradation and the Sonic Hedgehog-regulated mouse development. Dev. Biol 326, 177–189. 10.1007/978-3642-30406-4. [PubMed: 19056373]

Zhang XM, Yang XJ, 2001 Temporal and spatial effects of Sonic hedgehog signaling in chick eye morphogenesis. Dev. Biol 233, 271–290. 10.1006/dbio.2000.0195. [PubMed: 11336495]

Zhang XM, Ramalho-Santos M, McMahon AP, 2001 Smoothened mutants reveal redundant roles for Shh and Ihh signaling including regulation of L/R asymmetry by the mouse node. Cell 105, 781–792. 10.1016/S0092-8674(01)00385-3. [PubMed: 11440720]

Zhao L, Saitsu H, Sun X, Shiota K, Ishibashi M, 2010 Sonic hedgehog is involved in formation of the ventral optic cup by limiting Bmp4 expression to the dorsal domain. Mech. Dev 127, 62–72. 10.1016/j.mod.2009.10.006. [PubMed: 19854269]

Zuber ME, Gestri G, Viczian AS, Barsacchi G, Harris WA, 2003 Specification of the vertebrate eye by a network of eye field transcription factors. Development 130, 5155–5167. 10.1242/dev.00723. [PubMed: 12944429]

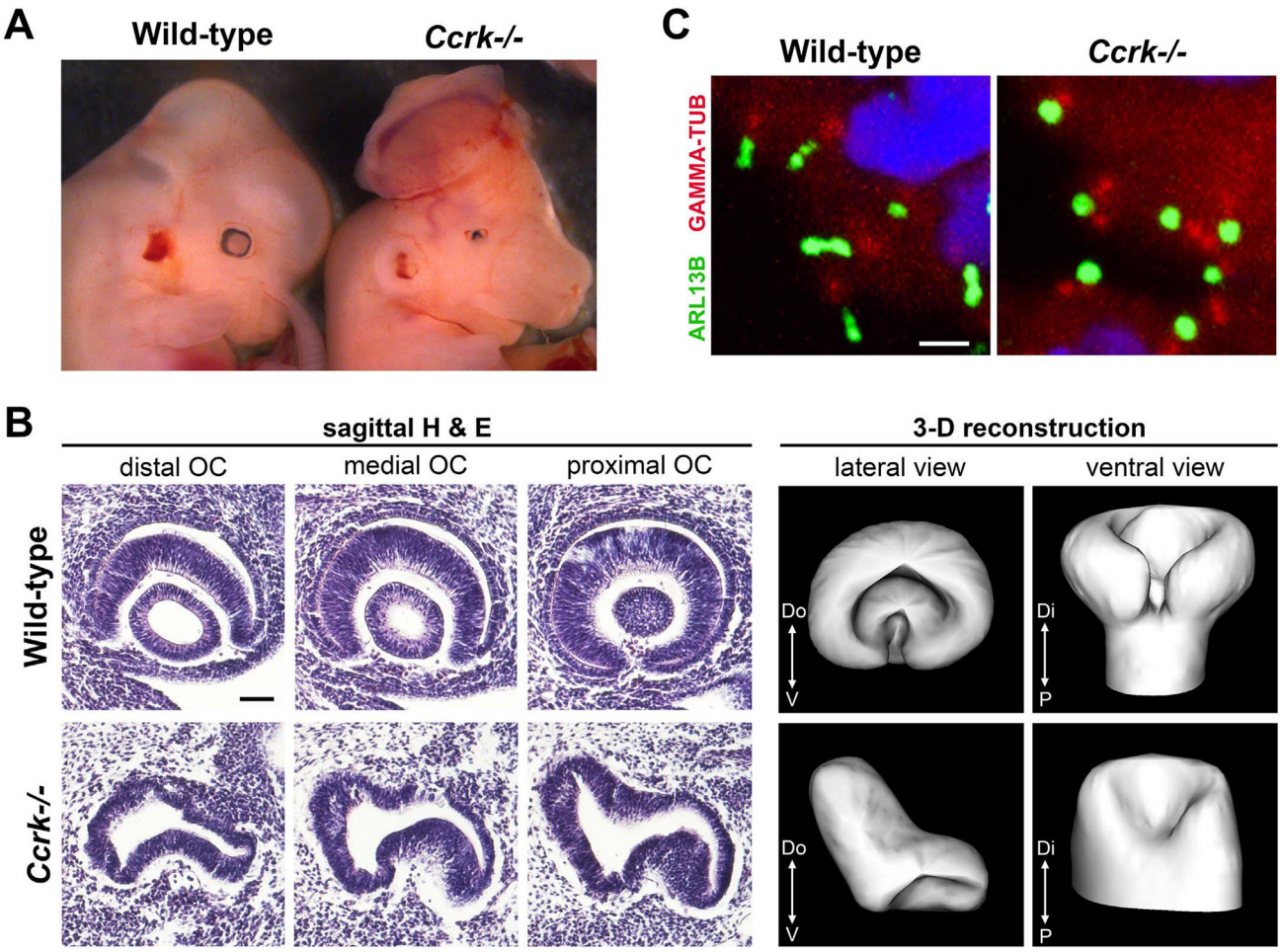

Fig. 1.

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 50 μm. (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 2 μm.

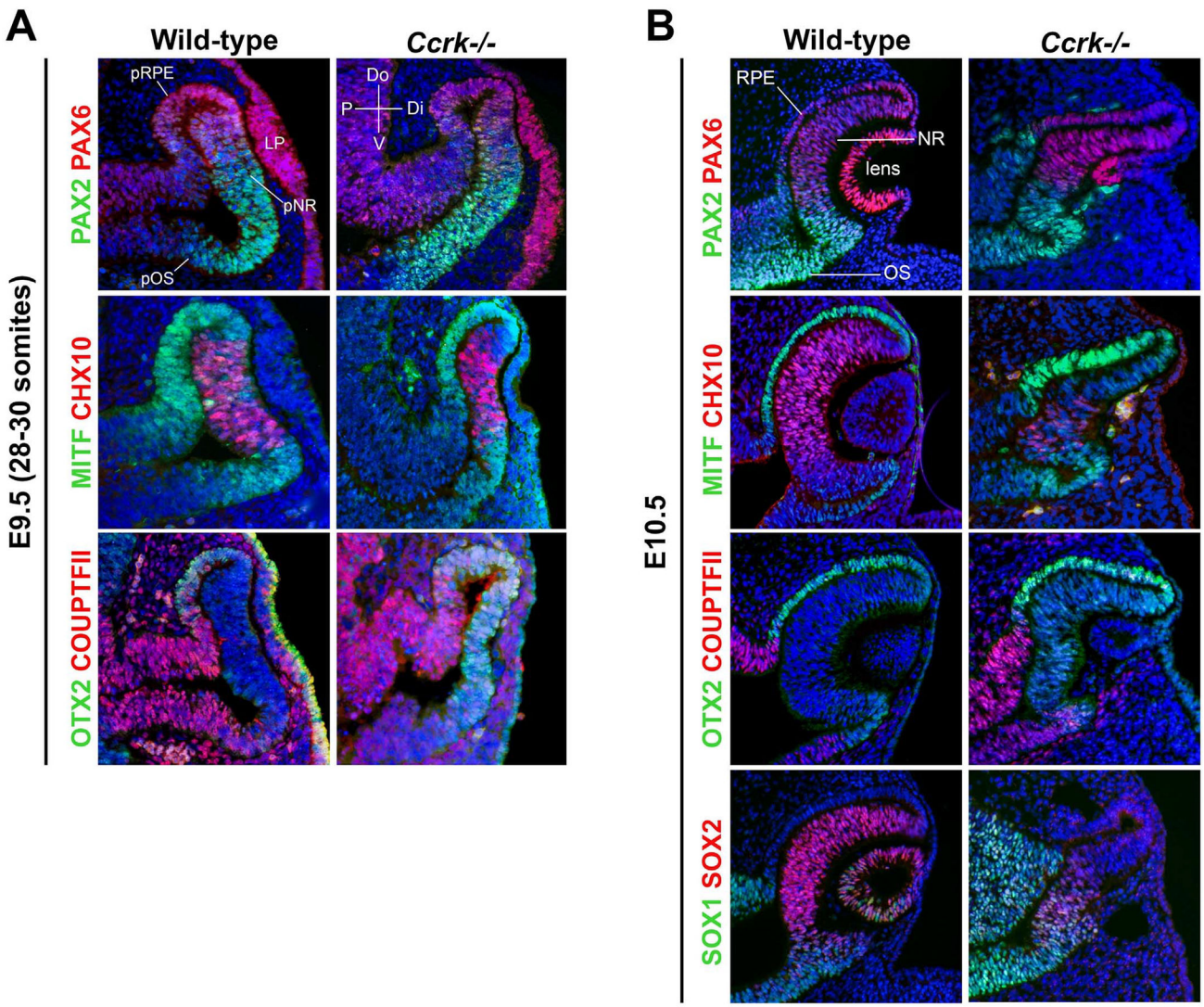

Fig. 2.

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.

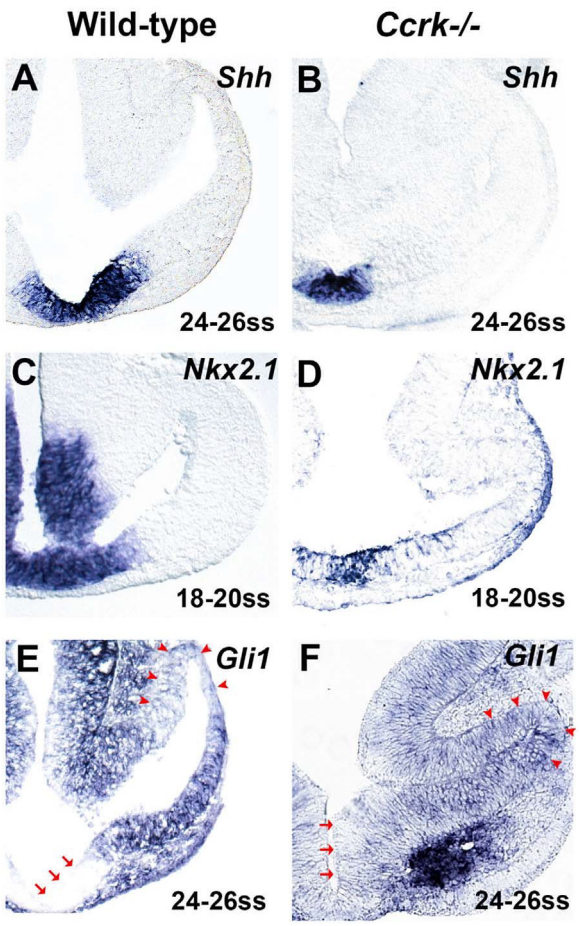

Fig. 3.

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 somitestages (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.

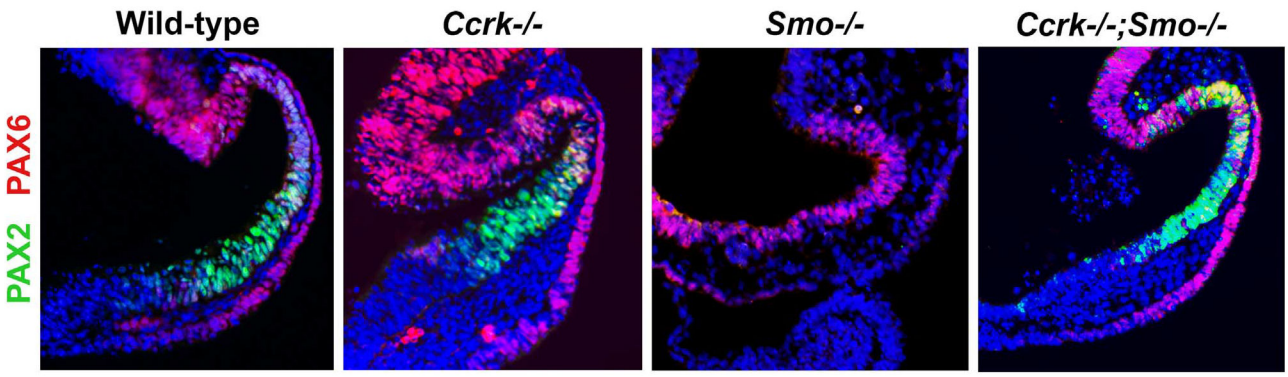

Fig. 4.

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.

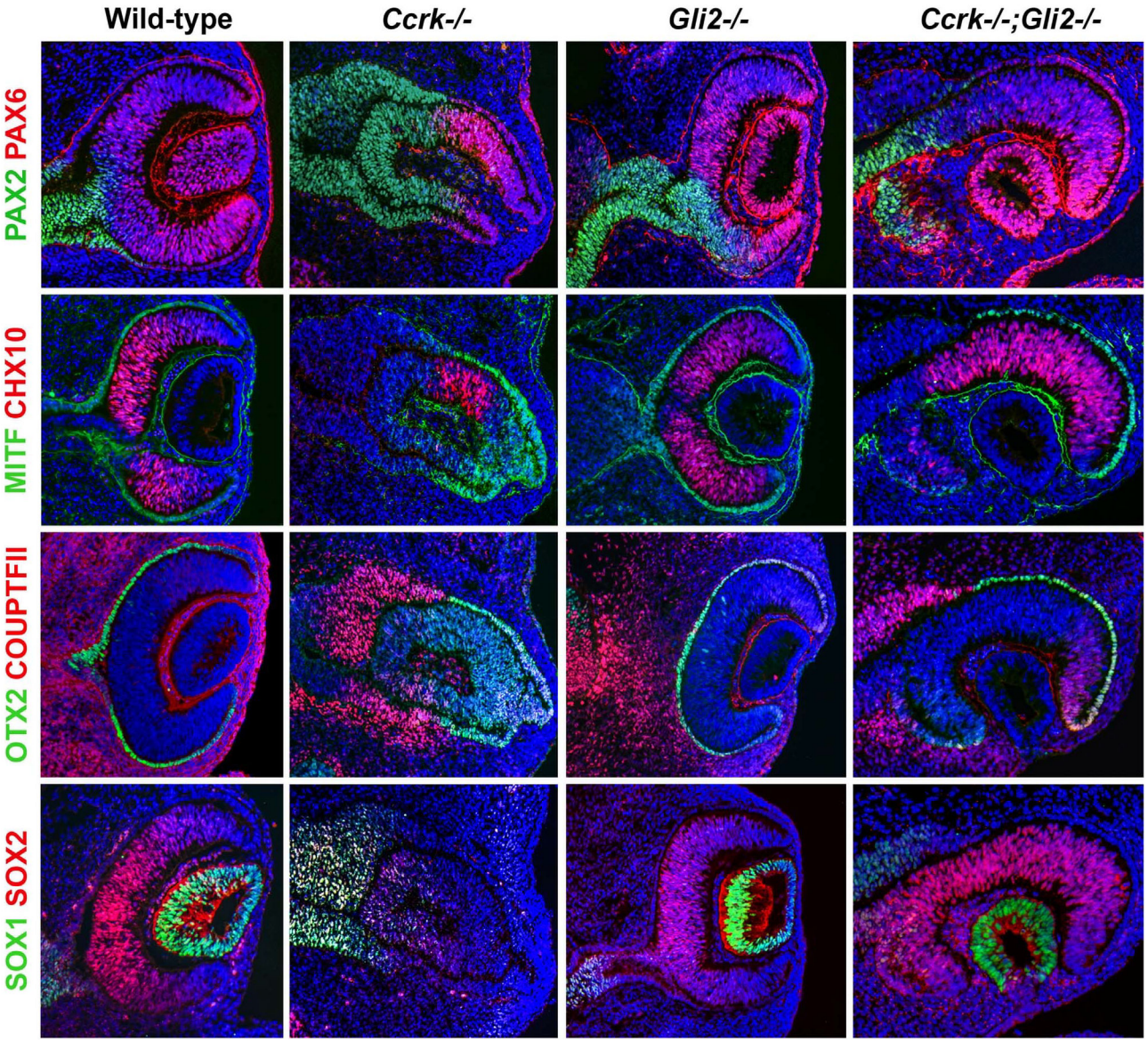

Fig. 5.

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 abnormal patterning in the *Ccrk;Gli2* double mutant. 4–6 embryos per genotype were analyzed.

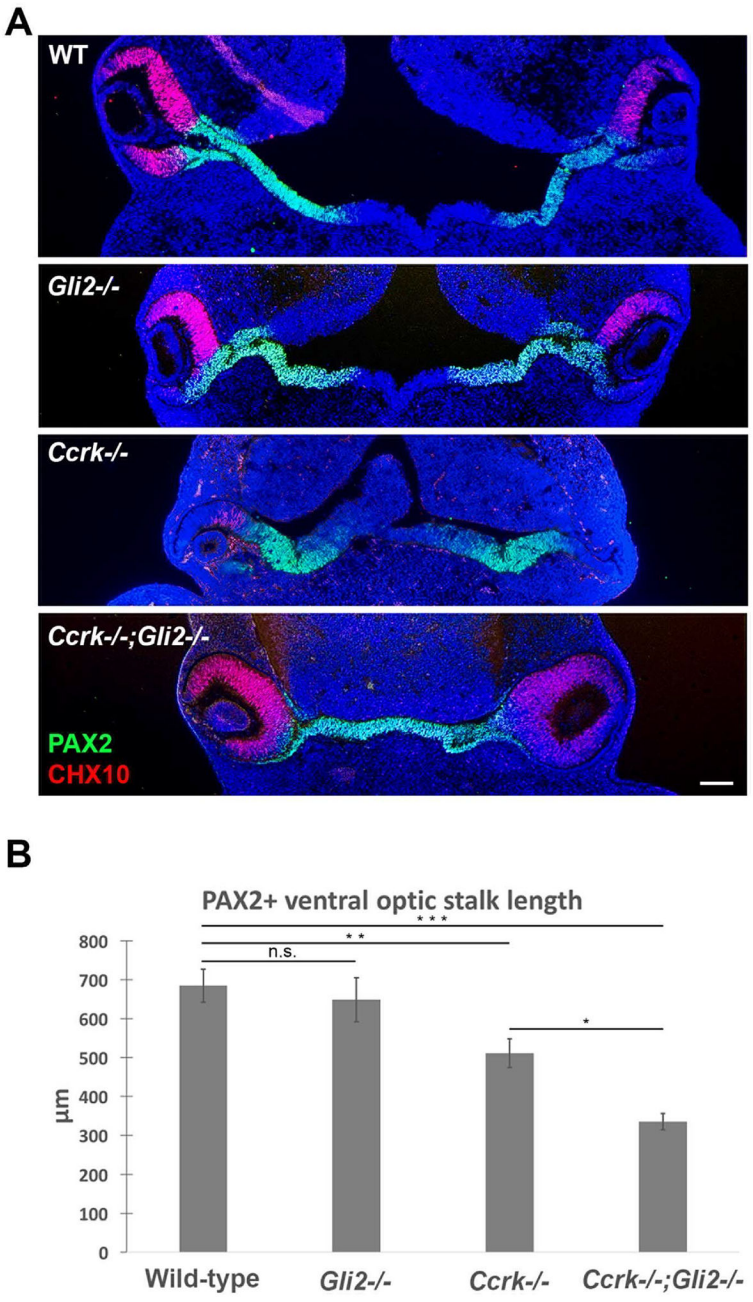

Fig. 6.

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 100 μm. (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.); $p < 0.05$ (*); $p < 0.01$ (**); $p < 0.001$ (***).

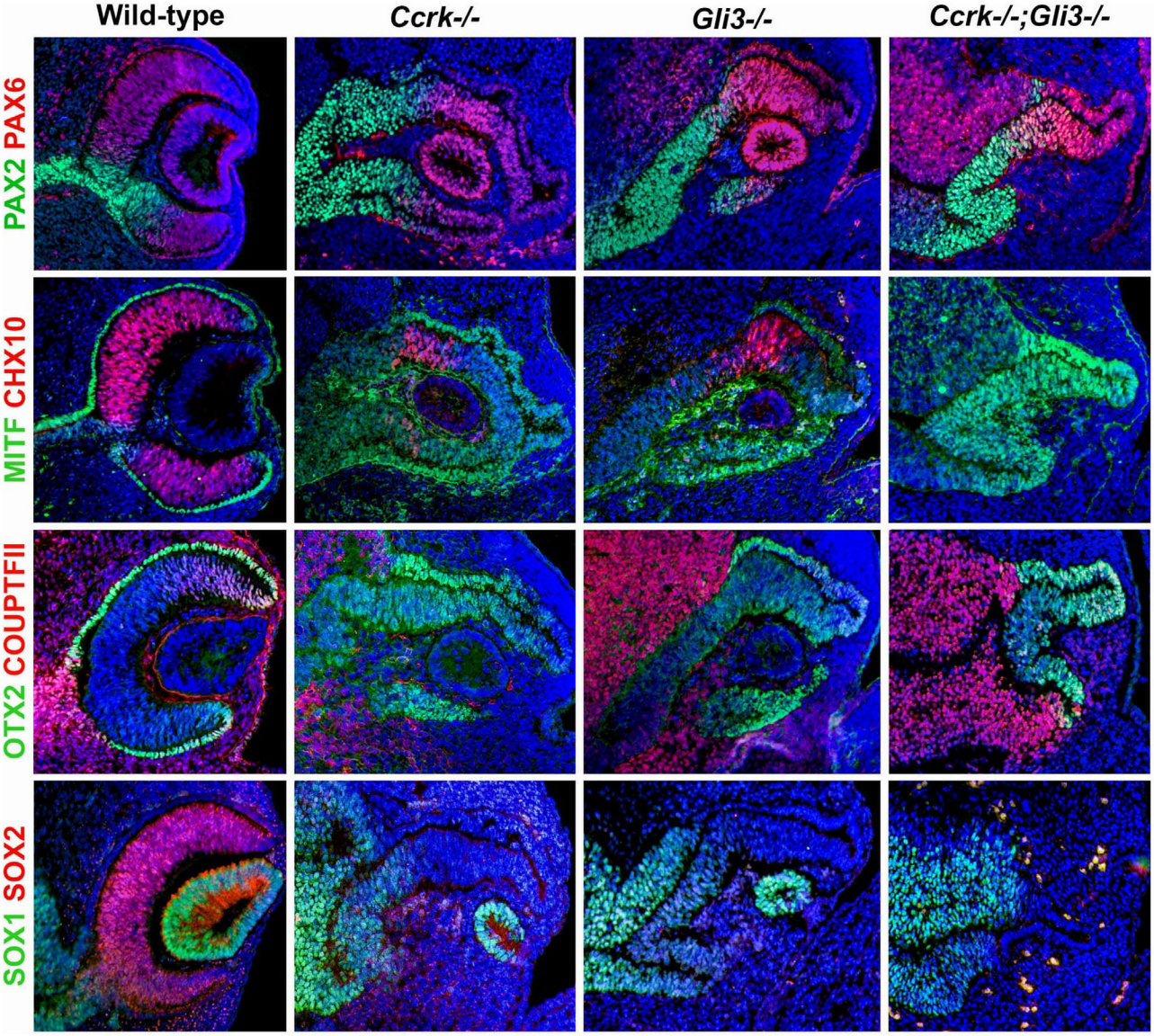

Fig. 7.

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.

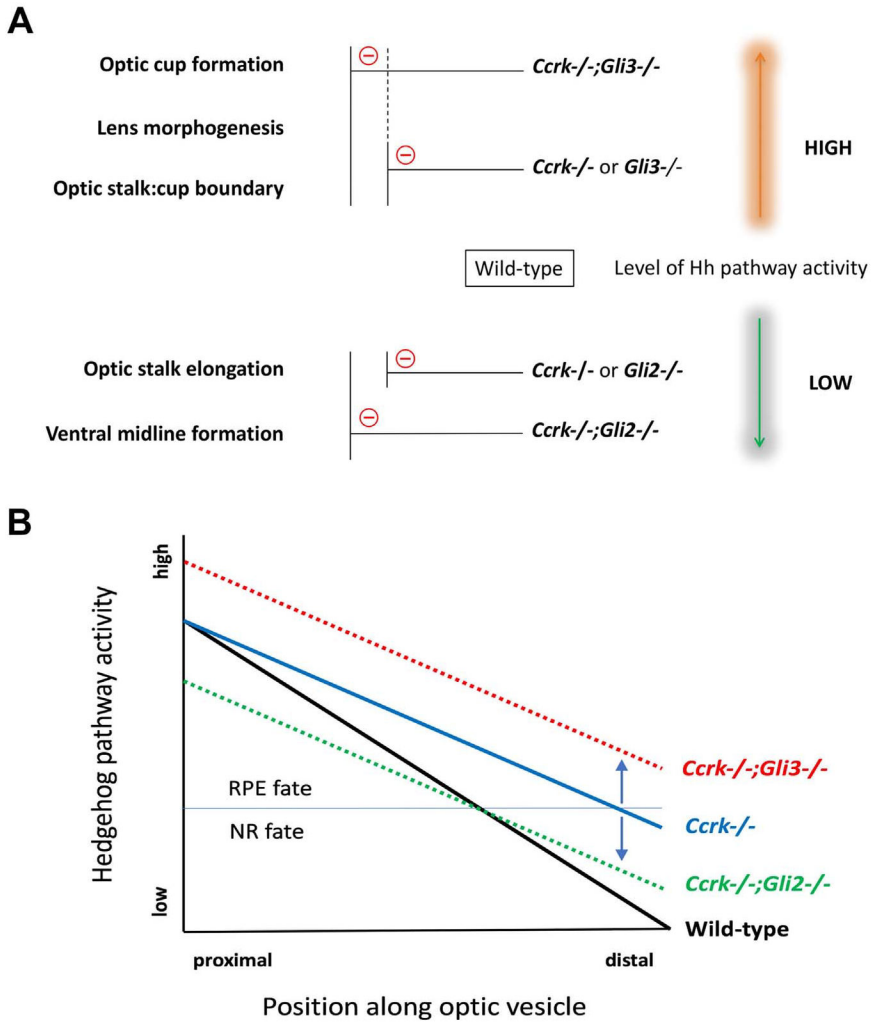

Fig. 8.

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 Hh pathway 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. This also compromises lens formation. Loss of GLI3 in a *Ccrk* mutant background, which further elevates the 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.