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Proper ciliary assembly is critical for restricting Hedgehog signaling during early eye development in mice

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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.

1. 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 been 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.

2. Materials and methods

2.1. Mouse lines

All mice were on a C3Heb/FeJ background and were genotyped

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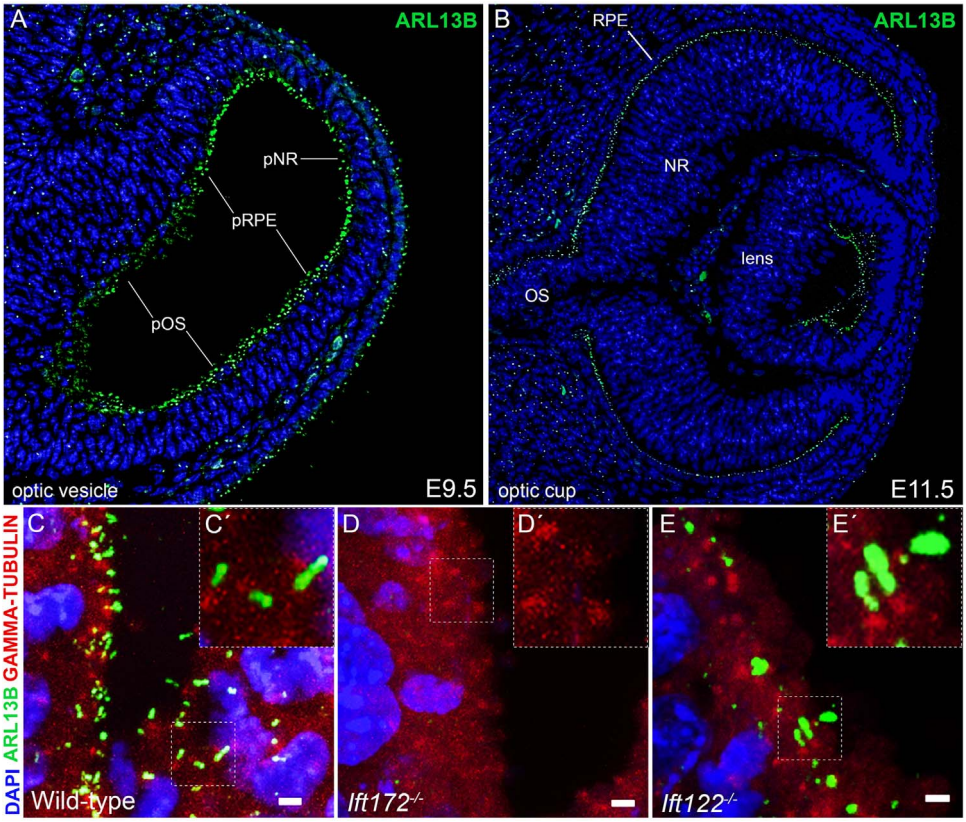

Fig. 1. Optic progenitor cells are ciliated and require IFT122 and IFT172 for proper cilia assembly. (A-B) Sections through a wild-type E9.5 optic vesicle (A) and a wild-type E11.5 optic cup (B). Cilia are visualized with an antibody against ARL13B (green) and DNA counterstained with DAPI (blue). Abbreviations 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).

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*^{sopb} 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'-AACTCATGCGCGGTTCATTG-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*^{wim} embryos were obtained from Tamara Caspary (Emory University) and genotyped by PCR followed by restriction digest with forward and reverse primers 5'-CACTGTGCTGATGA-AAGACTGGAATAGCC-3' and 5'-TCTGCAGGGAGTAACTGGGTGTG-GCGGAAG-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*^{zfd} mice were obtained from Alexandra Joyner (Sloan Kettering Institute) and genotyped as previously described (Matise et al., 1998). *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

Embryos were dissected in PBS, fixed for 2 h with 4% PFA at 4 °C, washed 4 times for 5 min each with PBS, and incubated in 15% sucrose/PBS and 30% sucrose/PBS at 4 °C for 2 h each. Embryos were then embedded in O.C.T. (Tissue-Tek), flash frozen, and sectioned in the coronal plane at 12 μm with a Leica CM1850 cryostat. Slides were washed 3 times for 10 min each in 0.2% Triton X-100/PBS, blocked in 10% serum/0.2% Triton X-100/PBS for 1 h, 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 min each (as above) and incubated for 1 h 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 min 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.

2.3. In situ hybridization

Embryos were dissected in PBS, fixed overnight with 4% PFA at 4 °C, washed 4 times for 5 min 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*

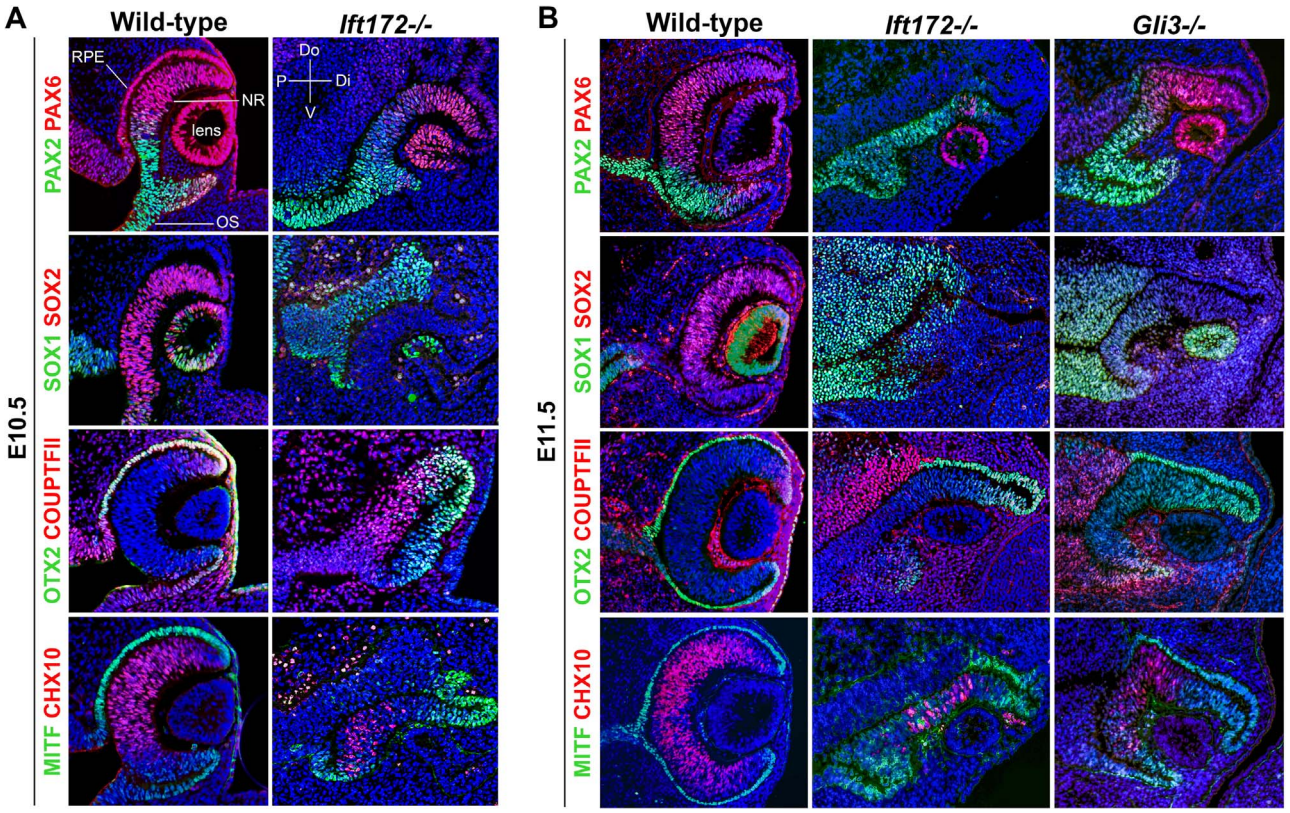

Fig. 2. IFT172 is required for patterning and morphogenesis of the optic cup. (A) Sections through E10.5 wild-type and *Ift172* mutant eyes stained with markers for cell fates. Note the dorsal expansion of PAX2, dorsal restriction of PAX6, expansion of MITF and OTX2 into the inner optic cup, reduction in CHX10+ cells and loss of SOX2 expression in the *Ift172* 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, *Ift172* 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 *Ift172* and *Gli3* mutants.

(Hui et al., 1994) were generated by *in vitro* transcription using a dig-UTP labeling mix (Roche) following manufacturer's specifications.

2.4. Three-dimensional reconstruction

Embryos were dissected in PBS, fixed overnight with 4% PFA, washed 4 times for 5 min 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 10 μm 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).

3. Results

3.1. 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).

3.2. 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,

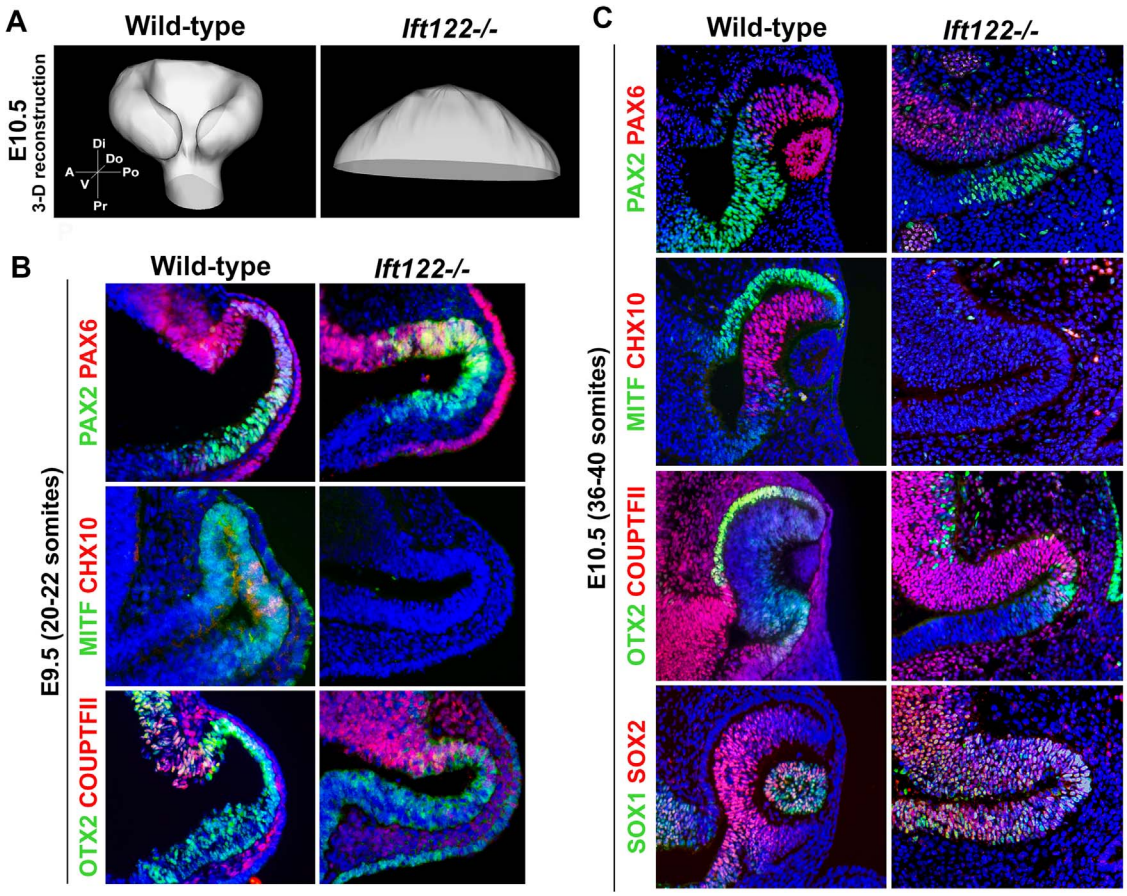

Fig. 3. IFT122 is required for optic cup and lens formation and for specification of the RPE and NR. (A) 3-D reconstruction of wild-type and *Ift122* mutant eyes at E10.5. Compass abbreviations: A, anterior; Po, posterior; Di, distal; Pr, proximal; V, ventral; D, dorsal. (B) Sections through E9.5 (20–22 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.

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.

3.3. 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.

3.4. Abnormal Hedgehog signaling in the Ift122 and Ift172 mutant optic vesicle

In comparison to the patterning changes in *Ift172* mutants, the

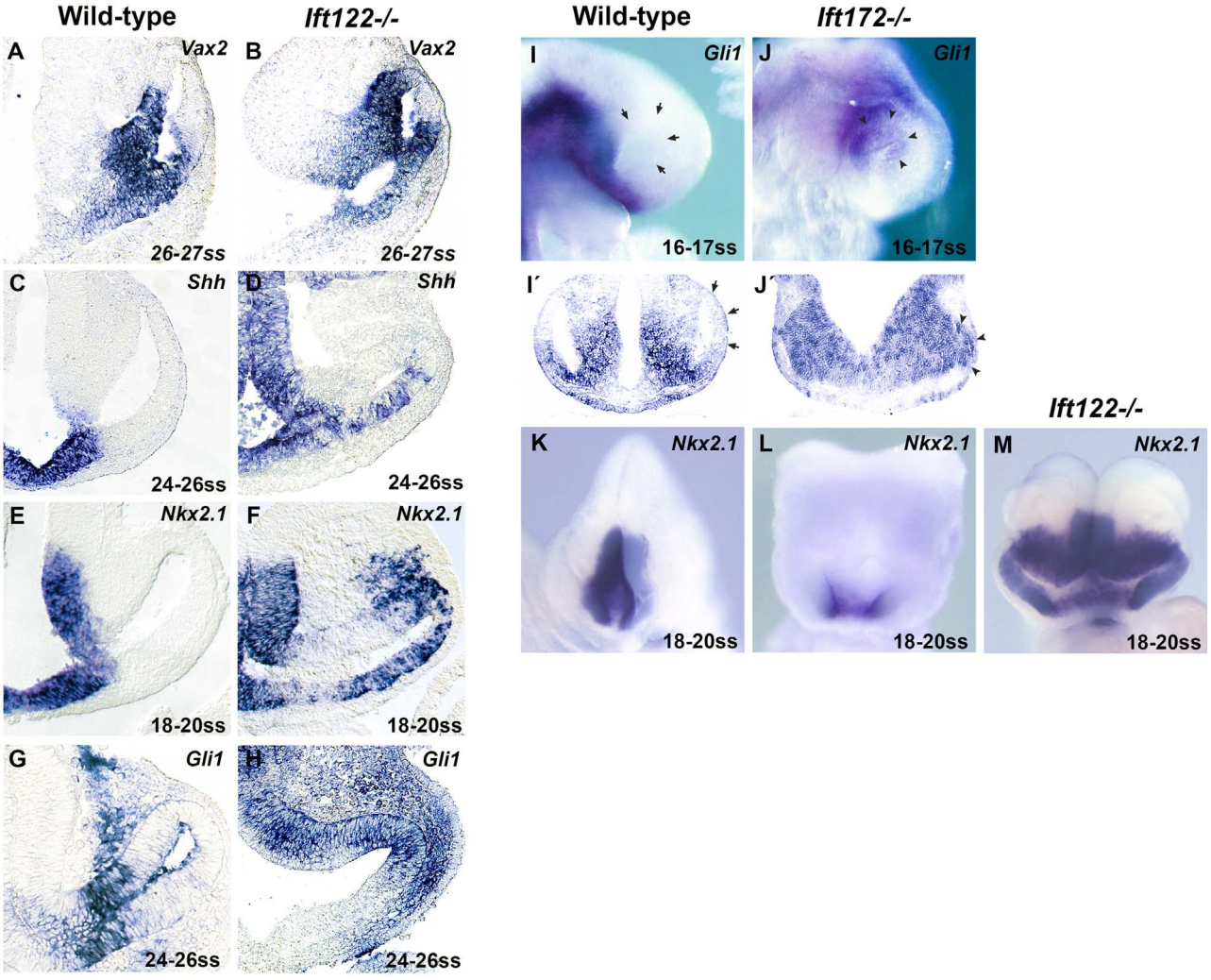

Fig. 4. Elevated Hedgehog signaling in the *Ift122* and *Ift172* mutant optic vesicle. (A-H) Sections through the eyes of wild-type (A,C,E,G,) and *Ift122* mutants (B,D,F,H) at the indicated somite stages (ss) following whole-mount *in situ* hybridization against *Vax2* (A,B), *Shh* (C,D), *Nkx2.1* (E,F) 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.

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 phenocopied *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*

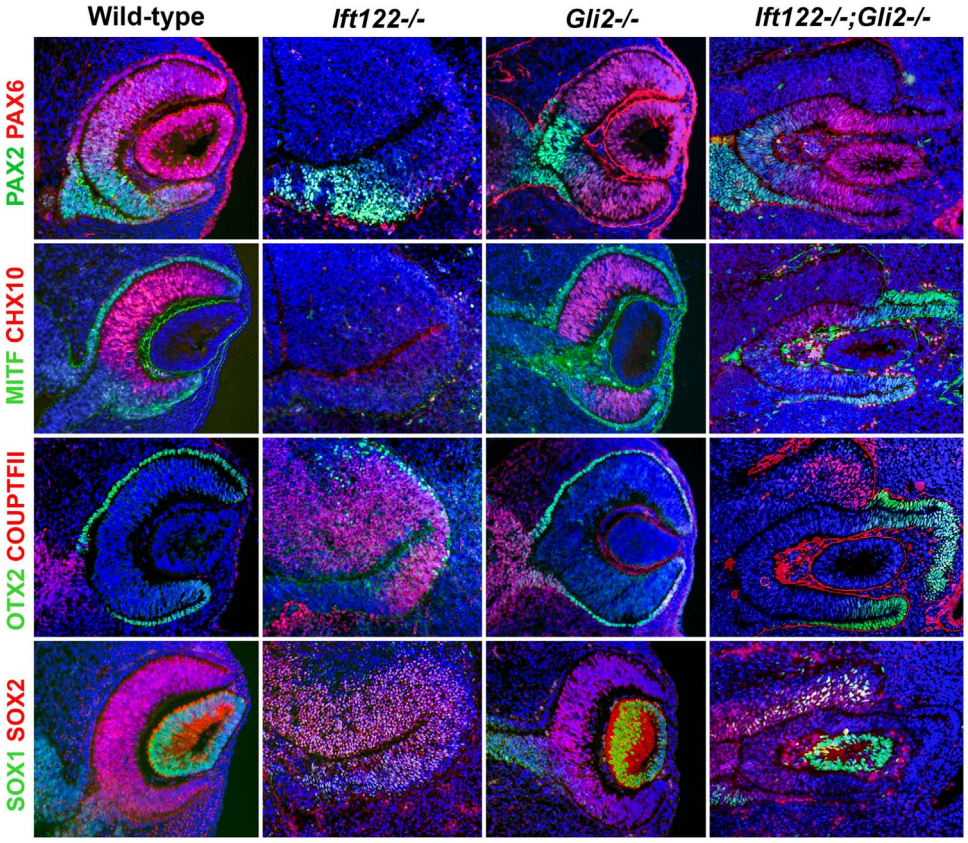

Fig. 5. Loss of GLI2 in *Ift122* mutants rescues optic cup patterning and morphogenesis. Sections through wild-type, *Ift122* mutant, *Gli2* mutant and *Ift122;Gli2* double mutant eyes at E11.5 stained with markers for cell fates. Note the rescue of optic cup and lens formation, the rescue of PAX6, MITF 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.

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.

3.5. 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, while 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 of 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.

4. Discussion

We find two IFT proteins, IFT172 and IFT122, are required for proper eye development in mice. *Ift172* mutants lack optic primary

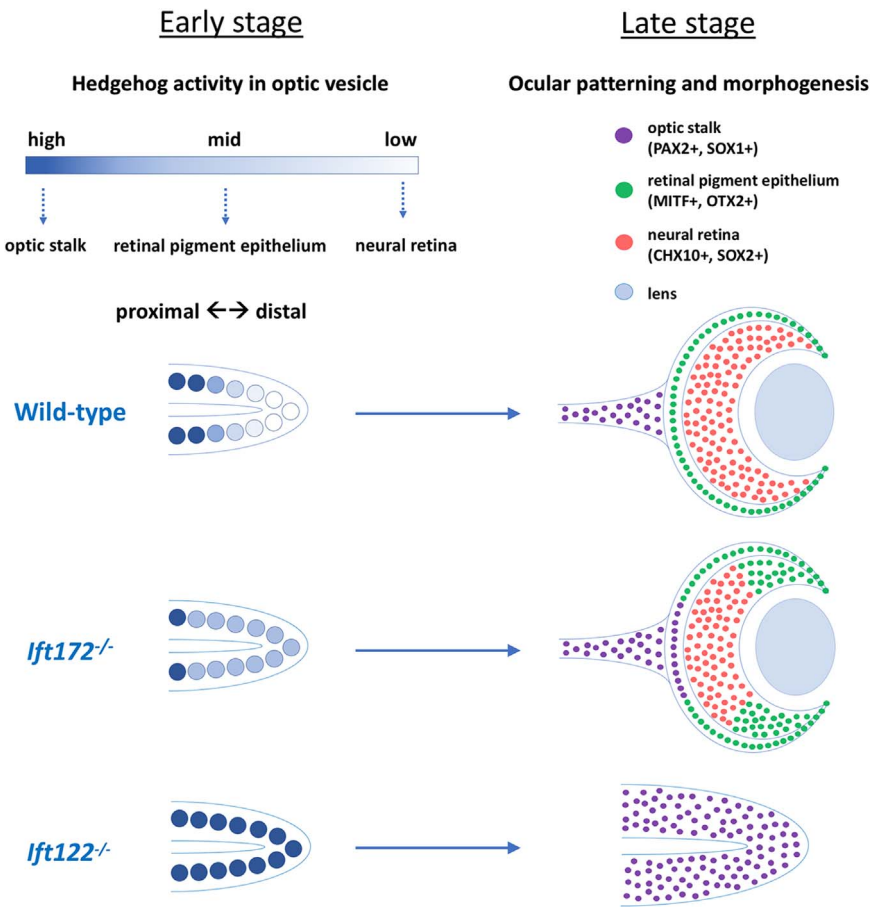

Fig. 6. Levels of Hedgehog activity bias optic progenitor cell fates. In wild-type, Hh activity forms a proximal-high, distal-low gradient within the optic vesicle. This leads to normal specification of the OS, RPE, and NR. In *Ift172* mutants, Hh activity is moderately elevated within the optic vesicle, 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.

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 Fig. 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 *Ift172* 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.

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