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Deficient FGF signaling causes optic nerve dysgenesis and ocular coloboma

Zhigang Cai[1], Chenqi Tao[1], Hongge Li[1], Raj Ladher[2], Noriko Gotoh[3], Gen-Sheng Feng[4], Fen Wang[5] and Xin Zhang[1,*]

SUMMARY

FGF signaling plays a pivotal role in eye development. Previous studies using *in vitro* chick models and systemic zebrafish mutants have suggested that FGF signaling is required for the patterning and specification of the optic vesicle, but due to a lack of genetic models, its role in mammalian retinal development remains elusive. In this study, we show that specific deletion of *Fgfr1* and *Fgfr2* in the optic vesicle disrupts ERK signaling, which results in optic disc and nerve dysgenesis and, ultimately, ocular coloboma. Defective FGF signaling does not abrogate Shh or BMP signaling, nor does it affect axial patterning of the optic vesicle. Instead, FGF signaling regulates Mitf and Pax2 in coordinating the closure of the optic fissure and optic disc specification, which is necessary for the outgrowth of the optic nerve. Genetic evidence further supports that the formation of an Frs2α-Shp2 complex and its recruitment to FGF receptors are crucial for downstream ERK signaling in this process, whereas constitutively active Ras signaling can rescue ocular coloboma in the FGF signaling mutants. Our results thus reveal a previously unappreciated role of FGF-Frs2α-Shp2-Ras-ERK signaling axis in preventing ocular coloboma. These findings suggest that components of FGF signaling pathway may be novel targets in the diagnosis of and the therapeutic interventions for congenital ocular anomalies.

KEY WORDS: FGF, Frs2α, Shp2, Ras, Coloboma, Optic fissure, Optic disc, Optic nerve, Pax2, Mitf, Mouse

INTRODUCTION

Eye development follows an elaborate sequence of morphogenetic events, which are highly conserved between humans and mice. At mouse embryonic day 9.5 (E9.5) when the proximal optic vesicle connected to the diencephalon forms the optic stalk (OS), the distal optic vesicle contacting the head surface ectoderm folds into two layers: an outer layer of presumptive retinal pigmented epithelium (RPE) and an inner layer of developing neural retina (NR) (Bharti et al., 2006; Fuhrmann, 2010). This remodeling of optic vesicle into a cup-like structure at E10.5 leaves a transient groove opening at the ventral side of the eye, termed the optic fissure (OF), which will be progressively sealed from the proximal to the distal end through fusion of the lip areas of the ventral optic stalk and neural retina until E12.5 (Silver and Robb, 1979; Chang et al., 2006; Morcillo et al., 2006). The interface between optic stalk and neural retina, however, will be specified as optic disc (OD), a unique ocular structure where blood vessels enter the eye. Moreover, although optic disc is immediately adjacent to neuronal progenitor cells in neural retina, it consists exclusively of astrocyte progenitor cells (APCs), which express multiple axon guidance molecules. These extracellular cues direct the fasciculated axons of retinal ganglion cells to exit the eye at the optic disc as the optic nerve (ON), which connects the eye to the brain (Deiner et al., 1997; Dakubo et al., 2003; Morcillo et al., 2006; Bao, 2008). It has been proposed that aberrant closure of the optic fissure and defective formation of the optic disc in human fetuses can cause ocular coloboma and optic nerve dysgenesis, leading to childhood blindness (Gregory-Evans et al., 2004; Chang et al., 2006).

Multiple signaling pathways have been implicated in ocular coloboma and optic nerve hypoplasia. Early midline-derived Shh governs the proximal-distal patterning of optic vesicle by regulating Pax2 and Pax6 expression, which are necessary for the demarcation of the optic stalk, optic disc and neural retina (Macdonald et al., 1995; Chiang et al., 1996). This is followed after the onset of retinal neurogenesis by retinal ganglion cell-derived Shh, which further promotes proliferation of the optic disc and optic stalk (Dakubo et al., 2003). Studies of knockout mice reveal that *Bmp4* expressed in the dorsal optic vesicle is crucial for dorsal-ventral patterning, whereas *Bmp7* expressed in the ventral optic vesicle and the neighboring periocular mesenchyme is required for initiation of the optic fissure and specification the optic disc (Furuta and Hogan, 1998; Morcillo et al., 2006). Similarly, RA signaling has also been proposed to regulate the closure of the optic fissure by controlling ventral retina morphogenesis and periocular mesenchyme development (Matt et al., 2005; Molotkov et al., 2006; Matt et al., 2008; Lupo et al., 2011). Finally, mutations in Wnt signaling genes *Lrp6*, frizzled 5 (Fzd5) and β-catenin (*Ctnnb1*), which are known to regulate the dorsal-ventral patterning of optic vesicle and the specification of the RPE, all cause ocular coloboma (Liu et al., 2007; Liu and Nathans, 2008; Zhou et al., 2008; Fujimura et al., 2009; Westenskow et al., 2009). Many of these coloboma mutants, as well as human patients, exhibit dysregulation of Pax2, a key homeodomain protein required for the closure but not the initiation of the optic fissure (Torres et al., 1996; Alur et al., 2010; Cross et al., 2011). In addition, mutations in ocular transcription factors, e.g. *Vax2*, *Chx10* and *Mitf*, also result in ocular coloboma (Robb et al., 1978; Silver and Robb, 1979; Scholtz and Chan, 1987; Hero, 1989; Torres et al., 1996; Barbieri et al., 2002; Mui et al., 2005). These animal models provide a framework within which to unravel the etiology of human coloboma and optic nerve defects.

[1]Department of Medical and Molecular Genetics, Stark Neuroscience Institute, Indiana University School of Medicine, Indianapolis, IN 46202, USA. [2]Laboratory for Sensory Development, RIKEN Kobe Institute-Center for Developmental Biology, 2-2-3 Minatojima-Minamimachi, Chuo-ku, Kobe, 650-0047, Japan. [3]Division of Molecular Therapy, Institute of Medical Science, University of Tokyo, 108-8639, Japan. [4]Department of Pathology, School of Medicine, and Section of Molecular Biology, Division of Biological Sciences, University of California San Diego, La Jolla, CA 92093, USA. [5]Center for Cancer Biology and Nutrition, Institute of Biosciences and Technology, Texas A&M, Houston, TX 77030, USA.

*Author for correspondence (xz4@iu.edu)

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Previous studies in chick and zebrafish models suggest that FGF signaling plays essential roles in eye development, but thus far none of the murine *Fgf* mutants have shown significant retinal defects. We recently reported that protein tyrosine phosphatase Shp2 (Ptpn11), a downstream mediator of receptor tyrosine kinase signaling, is required for embryonic neural retina specification and adult retinal survival (Cai et al., 2010; Cai et al., 2011). To further explore the signaling pathway upstream of Shp2, we have now generated a conditional knockout of two FGF receptors, *Fgfr1* and *Fgfr2*, specifically in optic vesicle, which resulted in ocular coloboma and optic nerve dysgenesis. Although BMP and midline Shh signaling appear unaffected, *Fgfr1/2* deletion disrupts Pax2 expression in the developing optic fissure and optic disc, which instead acquire ectopic expression of RPE marker Mitf. This failure of optic fissure and optic disc specification is also phenocopied by the specific deletion of *Frs2a* and *Shp2*, and by mutations that disrupt Fgfr-Frs2α or Frs2α-Shp2 interactions. By contrast, both *Fgfr1/2* and Frs2α/Shp2 mutants can be rescued by constitutively active Ras-ERK signaling. Our results thus demonstrate a novel role for FGF-Frs2α-Shp2-Ras-ERK signaling in the development of the optic fissure, optic disc and optic nerve.

MATERIALS AND METHODS

Mice

Frs2a^{flox}, *Frs2a*^{2F}, *Shp2*^{flox} mice have been previously reported (Zhang et al., 2004; Lin et al., 2007; Gotoh, 2008). *Fgfr1*$^{\Delta Frs}$ mice were derived from the recombinant ES cells kindly provided by Philippe Soriano (Mount Sinai School of Medicine, New York, NY, USA) (Hoch and Soriano, 2006). *Fgfr1*^{flox} mice were obtained from the Jackson Laboratory (Bar Harbor, ME, USA) (Hoch and Soriano, 2006). *Fgfr2*^{flox} mice were kindly provided by Dr David Ornitz (Washington University Medical School, St Louis, MO, USA) (Yu et al., 2003). *Six3-Cre* mice were kindly provided by Dr Yasuhide Furuta (MD Anderson Cancer Center, Houston, TX, USA) (Furuta et al., 2000). *Rx-Cre* mice were kindly provided by Dr Milan Jamrich (Baylor College of Medicine, Houston, TX, USA) (Swindell et al., 2006). *LSL-Kras*^{G12D} mice were obtained from the Mouse Models of Human Cancers Consortium (MMHCC) Repository at National Cancer Institute (Tuveson et al., 2004). All mice were maintained in mixed genetic background. *Six3-Cre* transgene alone or in combination with heterozygous *Fgfr1*^{flox}, *Fgfr2*^{flox}, *Frs2α*^{flox} and *Shp2*^{flox} alleles did not cause any phenotype (data not shown) and they were used as wild-type controls. All experiments were performed in accordance with institutional guidelines.

Histology and immunohistochemistry

Mouse embryos were staged according to the day the vaginal plug was first observed. The samples were fixed in 4% PFA overnight for frozen or paraffin wax-embedded sections before standard immunohistochemistry was performed as previously described (Pan et al., 2006; Pan et al., 2008). Serial sections (frontal or sagittal sections for ~E9.5-E11.5 embryos, transverse or sagittal sections for E13.5 embryos) were always stained with anti-Pax2 to identify optic stalk, optic disc and the lip region of the ventral neural retina (optic fissure). We performed regular double fluorescent staining for Pax2/Mitf or Pax2/NF165 and Tyramide Signal Amplification (TSA)-fluorescent staining for Shp2 and phospho-ERK (TSA Plus Fluorescein System, PerkinElmer, Waltham, MA, USA) (Cai et al., 2010). The antibodies used were anti-Pax2 (1:200, PRB-276P) and anti-Pax6 (1:100, PRB-278P) (both from Covance, Berkeley, CA, USA), anti-Mitf (1:100, #MS-771-P0, Thermo Scientific, Fremont, CA, USA), anti-Sox2 (1:100, #Ab5603, Chemicon, Temecula, CA, USA), anti-Shp2 (1:3000, Sc-280, Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-cleaved-caspase 3 (1:100, #9664), anti-phospho-ERK1/2 (1:1000, #9101) and anti-cyclin D1 (1:100, #2926) (all from Cell Signaling Technology, Beverly, MA, USA), anti-Frs2α (1:1000, ab10425, Abcam, Cambridge, MA, USA), anti-Ki67 (1:10, #550609, BD Pharmingen San Diego, CA, USA) and anti-NF165 (1:100, #2H3, Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA, USA). Anti-phospho-Smad1 (PS1) antibody [1:100, kindly provided by Peter ten Dijke (Leiden University Medical Center, Leiden, The Netherlands) and Carl-Henrik Heldin (Ludwig Institute for Cancer Research, Uppsala, Sweden)]. The secondary antibodies used were goat anti-mouse conjugated with Cy2 and goat anti-rabbit conjugated with Cy3 (both from Jackson Laboratory, West Grove, PA, USA). The fluorescent staining was examined under a Leica DM500 fluorescent microscope equipped with Cy2 and Cy3 filters. The cell proliferation and apoptosis rates were calculated as the ratio of Ki67 and TUNEL-positive cells versus DAPI-positive cells, and analyzed by the Student's *t*-test. For analysis of each marker, at least three embryos of each genotype were fully sectioned and stained.

RNA *in situ* hybridization

RNA *in situ* hybridization on cryosections was carried out as previously described (Pan et al., 2006; Cai et al., 2010). After hybridization treatment, the sectioned samples were counterstained with the Pax2 antibody to determine the region of the optic stalk, optic disc and ventral neural retina. Probes for the following genes were used: *Crx*, netrin 1, *Shh* and *Vax2* (from Dr Valerie Wallace, Ottawa Health Research Institute, Ottawa, Ontario, Canada); *BF2* (*Foxd1* – Mouse Genome Informatics), *Bmp7*, *Bmp4* and *Raldh3* (*Aldh1a3* – Mouse Genome Informatics) (from Dr Robert Heuckeroth, Washington University Medical School, St Louis, MO, USA); *Otx1* and *Rx* (*Rax* – Mouse Genome Informatics) (from Dr Naoki Takahashi, Nara Institute of Science and Technology, Nara, Japan); *BF1* (*Foxg1* – Mouse Genome Informatics) (from Marie Fernandes, Albert Einstein College of Medicine, Bronx, NY, USA); *Six3* and *Tbx5* (from Dr Guillermo Oliver, St Jude Children's Research Hospital, Memphis, TN, USA); *Vax1* (from Dr Kapil Bharti, National Institutes of Health, Bethesda, MD, USA); *Nlz2* (*Zfp503* – Mouse Genome Informatics) (from Dr Brian Brooks, National Institutes of Health, Bethesda, MD, USA); *Fgf15* (from Dr Minh-Thanh T. Nguyen, National Institutes of Health, Bethesda, MD, USA); *Math5* (*Atoh7* – Mouse Genome Informatics) (from Dr Tom Glaser, University of Michigan, Ann Arbor, MI, USA); *Brn3b* (*Pou4f2* – Mouse Genome Informatics) (from Dr Lin Gan, University of Rochester, Rochester, NY, USA); and *Chx10* (*Vsx2* – Mouse Genome Informatics) (from Dr Roderick R. McInnes, Hospital for Sick Children, Toronto, Ontario, Canada). A 1.8 kb fragment of *Frs2α* (*Frs2* – Mouse Genome Informatics) exon 5 was used as an in situ probe to confirm the deletion of *Frs2α* gene. At least three independent and fully sectioned eye balls of each genotype were analyzed for each RNA probe.

RESULTS

Conditional knockout of *Fgfr1* and *Fgfr2* by *Six3-Cre* caused ocular coloboma and optic nerve dysgenesis

Previous RNA *in situ* hybridization experiments show that both *Fgfr1* and *Fgfr2* are expressed in embryonic retina, whereas transcripts for *Fgfr3* and *Fgfr4* appear undetectable (Visel et al., 2004). We thus used *Six3-Cre* to ablate *Fgfr1* and *Fgfr2* in optic vesicle to study FGF signaling in eye development (Furuta et al., 2000). We first carefully investigated the timing and specificity of *Six3-Cre* using the *R26R* reporter. As we previously reported, *Six3-Cre;R26R* embryos exhibited only sparse X-gal staining in the optic vesicle at E9.0 (Cai et al., 2010). At E9.5, however, strong X-gal staining was first observed in the ventral optic vesicle, the precursor region of the optic fissure, optic disc and ventral neural retina (supplementary material Fig. S1A-C). By contrast, the dorsal optic vesicle did not stain strongly for X-gal until E10.5 (supplementary material Fig. S1D-F). After that, transverse sections of the *Six3-Cre;R26R* embryos indicated that *Six3-Cre* activity was concentrated in the central retina, optic stalk and optic chiasm (Cai et al., 2010; Cai et al., 2011).

Fgfr1 and *Fgfr2* single mutants were viable and fertile without ocular defects (data not shown). Examination of the adult *Six3-Cre;Fgfr1*$^{flox/flox}$;*Fgfr2*$^{flox/flox}$ double mutants, however, revealed a 'tear-drop'-shaped iris, which mimics uveal coloboma found in humans (Fig. 1A,E) (Chang et al., 2006). More strikingly, the optic nerve was either extremely thin or completely missing from the

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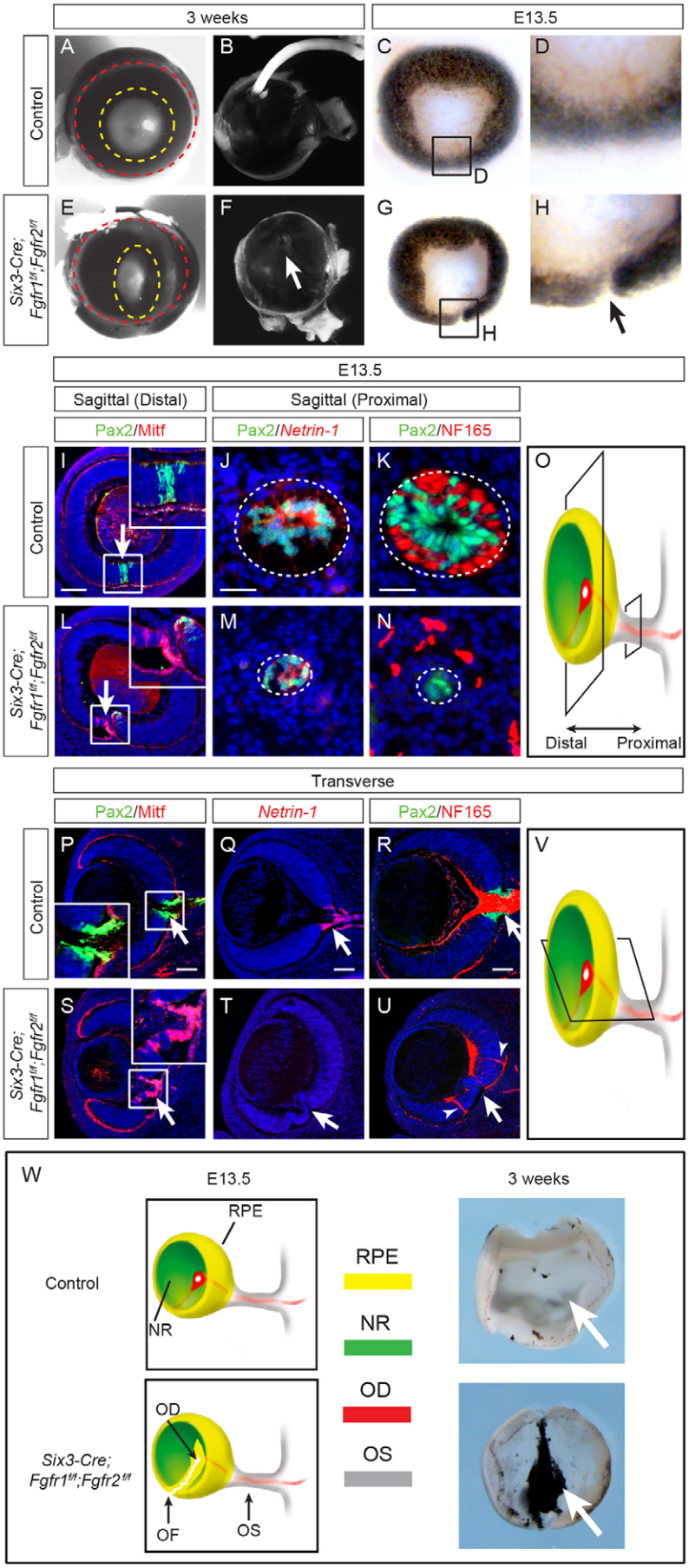

Fig. 1. Ocular coloboma and optic nerve aplasia in the *Six3-Cre;Fgfr1*$^{flox/flox}$*;Fgfr2*$^{flox/flox}$ mice. (**A-H**) The adult *Six3-Cre;Fgfr1*$^{flox/flox}$*;Fgfr2*$^{flox/flox}$ animals displayed a teardrop-shaped iris (circled in yellow) and a loss of optic nerve (ON) (arrow in F). At E13.5, the optic fissure was completely closed in the control eye, but it remained open in the *Six3-Cre;Fgfr1*$^{flox/flox}$*;Fgfr2*$^{flox/flox}$ embryos (arrow in H). (**I-O**) Sagittal sections of the E13.5 mutant eyes revealed ectopic Mitf expression in the open optic fissure (arrow in L), the hypoplastic optic stalk (circled in M) and a loss of optic nerve as indicated by lack of NF165 staining (circled in N). A sagittal section scheme is shown in O. (**P-V**) Transverse sections showed that the presumptive optic disc in *Fgfr1/2* mutant lost Pax2 and netrin 1 expression, but gained Mitf expression (S and T, arrows). As a result, NF165-stained retinal ganglion cell axons were misrouted (U, arrowheads) and optic nerve was not formed (U, arrow). The transverse section through the optic disc region is shown in V. (**W**) (Left) Summary of the ocular phenotypes in the E13.5 *Six3-Cre;Fgfr1*$^{flox/flox}$*;Fgfr2*$^{flox/flox}$ embryos. Adapted, with permission, from Bharti et al. (Bharti et al., 2006). The control eyeball is composed of an outside RPE layer (yellow) and an inside neural retina (green) layer, which fuse at the ventral optic fissure (faint red), but remained open in the posterior optic disc (red). In the *Six3-Cre;Fgfr1*$^{flox/flox}$*;Fgfr2*$^{flox/flox}$ mutants, the ventral neural retina along the optic fissure were transformed to RPE, preventing the closure of the eyeball. A similar transformation of optic disc to RPE prevented the formation of optic nerve. NR, neural retina; OD, optic disc; OF, optic fissure; ON, optic nerve; OS, optic stalk; RPE, retinal pigmented epithelium. (Right) Three-week-old eye balls were dissected to remove RPE covering the back of the eye, revealing abnormal pigmentation in mutant optic disc and optic fissure (arrows). Scale bars: 100 μm in I,L; 25 μm in J,K,M,N; 100 μm in P-U.

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Table 1. Ocular coloboma and optic nerve (ON) dysgenesis in FGF signaling mutants

	Ocular phenotype			
	Coloboma	ON aplasia	ON hypoplasia	*n*
Wild type	0%	0%	0%	20
Six3-Cre;Fgfr1$^{f/f}$*;Fgfr2*$^{f/f}$	100%	88%	12%	40
Six3-Cre;Fgfr1$^{f/\Delta Frs}$*;Fgfr2*$^{f/f}$	100%	83%	17%	12
Six3-Cre;Frs2a$^{f/f}$*;Shp2*$^{f/f}$	100%	70%	30%	40
Six3-Cre;Frs2a$^{2F/f}$*;Shp2*$^{f/f}$	100%	57%	43%	14
Six3-Cre;Fgfr1$^{f/f}$*;Fgfr2*$^{f/f}$*;LSL-Kras*^{G12D}	31%	0%	63%	16
Six3-Cre;Frs2a$^{f/f}$*;Shp2*$^{f/f}$*;LSL-Kras*^{G12D}	0%	0%	0%	20

back of the eye (Fig. 1B,F, arrow; Table 1). At E13.5, when the control optic fissure is completely fused, there still remained a significant ventral cleft in the mutant optic cup (Fig. 1C,D,G,H, arrow). These results suggest that FGF signaling is required for the closure of the optic fissure and the formation of the optic nerve.

We next examined the optic fissure and optic nerve defects by preparing sagittal sections of the distal and proximal eye, respectively (see Fig. 1O). Mitf is a crucial regulator of RPE, whereas Pax2 is required for ventral neural retina development and optic fissure closure (Torres et al., 1996; Horsford et al., 2005). In the E13.5 control eye, Pax2 expression marked the fusion of optic fissure in the ventral optic cup, whereas Mitf was restricted to the RPE (Fig. 1I, arrow). In the *Fgfr1/2* mutants, however, Mitf was induced in the open optic fissure, where Pax2 expression was significantly downregulated (Fig. 1L, arrow). Further examination of proximal eye sections showed that the mutant optic stalk, marked by Pax2 and netrin 1 expression, was significantly reduced in size (Fig. 1J,M). Although Mitf was no longer ectopically expressed, levels of the axonal marker NF165 were greatly diminished and mislocalized in the proximal eye (Fig. 1K,N; data not shown), consistent with the loss of the optic nerve in the *Fgfr1/2* mutants. As shown in Fig. 1V, we also examined transverse sections of the optic disc, which is necessary for the guidance of retinal ganglion cell axons (also known as optic nerve fibers) to exit the eye as the optic nerve. Instead of expressing Pax2 and the axon guidance gene netrin 1, the presumptive optic disc region in the *Fgfr1/2* mutants was severely deformed with ectopic Mitf expression (Fig. 1P,Q,S,T, arrows). As a result, the NF165-stained optic nerve fibers were misrouted into the sub-retinal space, failing to form a properly bundled optic nerve (Fig. 1R,U, arrows and arrowheads). Additional transverse sections of the ventral eye consistently revealed gaps in ventral retina (data not shown), confirming a complete penetrance of coloboma in these mutants (Table 1). In 3-week-old animals, bright-field microscopy even revealed ectopic pigmentation in the mutant optic fissure and presumptive optic nerve head (Fig. 1W, arrows). Similar coloboma and optic disc defects were also observed in *Rx-Cre;Fgfr1*$^{flox/flox}$*;Fgfr2*$^{flox/flox}$ double mutants, where *Fgfr1* and *Fgfr2* were ablated using another retina-specific Cre driver, *Rx-Cre* (supplementary material Fig. S2) (Swindell et al., 2006). Taken together, our histological and molecular analyses suggest that ectopic pigmentation of optic fissure in *Fgfr* mutants caused ocular coloboma by preventing the fusion of the ventral optic vesicle. Similar conversion of the mutant optic disc region to RPE tissue abolished the proper guidance and assembly of optic nerve, causing optic nerve dysgenesis.

Fgfr1/2 deficiency disrupted the development of the optic fissure and optic disc without perturbing axial patterning of the optic vesicle

To determine the molecular mechanism of *Fgfr1/2* mutant defects, we next investigated the extensive transcriptional network underlying retinal differentiation. *Rx* and *Six3* are transcription factors required for eye field specification and *Otx1* marks RPE, optic disc and ciliary margin. Their expressions in the *Fgfr1/2* mutants expanded into the presumptive optic stalk (Fig. 2A-F′). In E13.5 control embryos, retinal progenitor marker Sox2 labeled the neural retina and optic disc tissue, whereas Pax6 was present only in the neural retina and RPE. In the *Fgfr1/2* mutants, however, the presumptive optic disc region exhibited ectopic Pax6 expression at the expense of Sox2 expression (Fig. 2G-J′, arrows). Finally, *Fgf15* and *Chx10* were specific to retinal progenitor cells, which express *Isl1*, *Math5*, *Brn3b* and *Crx* to promote neuronal differentiation. These retinal-specific markers were excluded from the presumptive optic disc and neural retina adjacent to the optic fissure in the *Fgfr1/2* mutants (Fig. 2K-T′, arrows; data not shown). Taken together, these results further confirmed that the ocular defects in the *Fgfr1/2* mutants were caused by disruption of optic disc and optic fissure specification.

Shh and BMP signaling are known to play important roles in early optic vesicle patterning, which have been implicated in coloboma formation. In the E11.5 *Fgfr1/2* mutants, there was no change in expression of *Shh* and its target gene *Gli1* (Fig. 3A-C′, arrows). Similarly, *Bmp4* and its downstream effector phospho-Smad1 were maintained in dorsal optic vesicle and *Bmp7* was correctly expressed ventrally (Fig. 3D-F′, arrows). Consistent with this, the axial polarity markers *Vax1, Tbx5*, *Vax2*, *Raldh3*, *Nlz2*, *BF1* and *BF2* were all expressed normally (Fig. 3G-L′, arrows; data not shown), suggesting that the proximal-distal, dorsal-ventral and temporal-nasal axes were maintained in the *Fgfr1/2* mutants. The optic vesicle patterning genes *Rx*, *Six3*, *Pax6* and *Otx1* also appeared unaffected at this stage (Fig. 3M-P′, arrows). By contrast, the neural retinal marker *Chx10* was already reduced in the ventral retina adjacent to the optic fissure (Fig. 3Q,Q′, arrows). Importantly, whereas control optic fissure exclusively expresses Pax2 at E11.5, the RPE marker Mitf was ectopically expressed by the Pax2-positive cells in the mutant optic fissure (Fig. 3R,R′, arrows). As shown above in Fig. 1, *Fgfr1/2* mutant optic fissure eventually lost all Pax2 expression and acquired ectopic pigmentation. This apparent transition from Pax2/Mitf co-expressing progenitors to purely Mitf-positive RPE in ventral retina cannot be explained by simple infiltration of RPE in the wake of the failed fusion of optic cup, but instead it points to a genuine fate change of neural retina to RPE tissue. Collectively, these results suggested that the lack of *Fgfr1/2* signaling disrupted the development of the optic fissure without affecting the general patterning of the optic vesicle.

FGF signaling defects were reproduced by *Frs2a* and *Shp2* double, but not single, mutants

To further understand the molecular mechanism of FGF signaling in eye development, we next explored its intracellular signaling cascade. As early as E10.5, before the onset of Mitf

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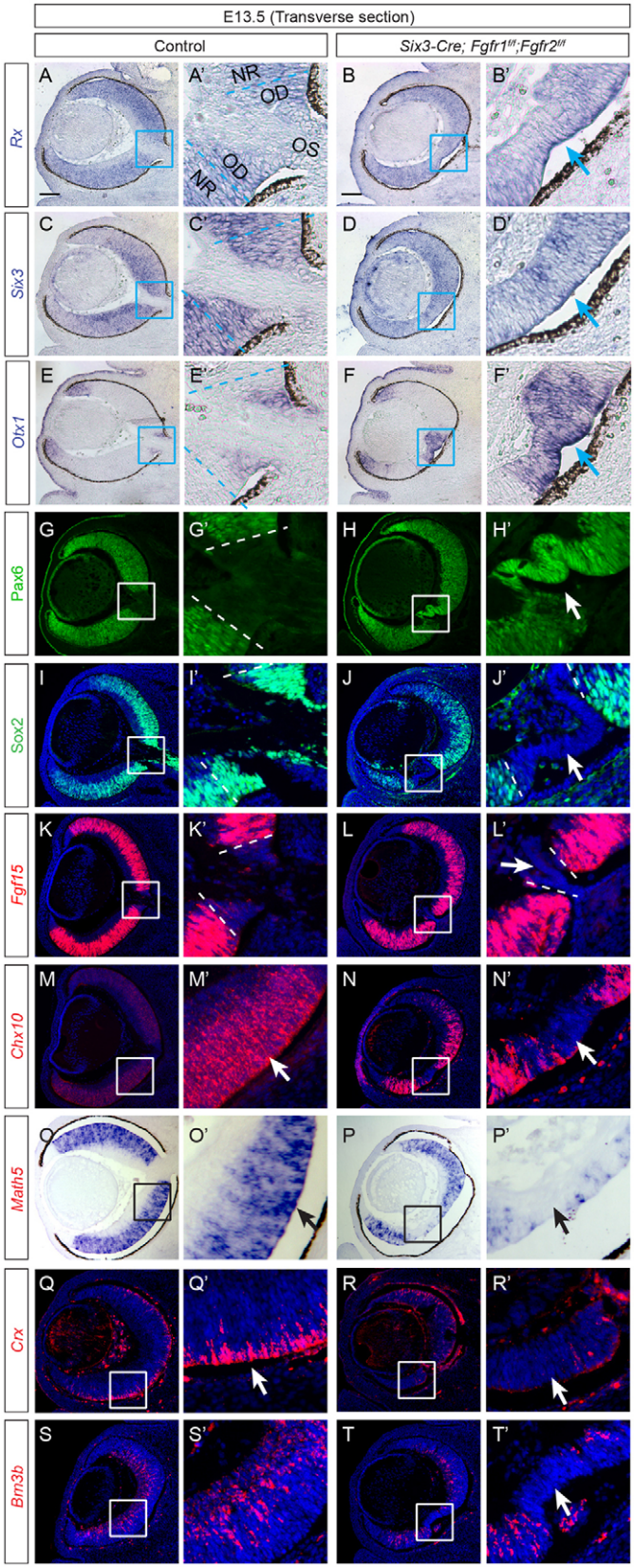

Fig. 2. Defective retina genesis in the *Six3-Cre;Fgfr1*$^{flox/flox}$*;Fgfr2*$^{flox/flox}$ mutants. (**A-F′**) At E13.5, ablation of *Fgfr1* and *Fgfr2* resulted in the expansion of *Rx*, *Six3* and *Otx1* into presumptive optic stalk. (**G-J′**) In *Fgfr1/2* mutants, the expression of Pax6 expanded from the neural retina and RPE into the presumptive optic disc region, where the normal expression of Sox2 was lost. (**K-T′**) The neural retina makers *Fgf15*, *Chx10*, *Math5*, *Crx* and *Brn3b* were excluded from presumptive optic disc and neural retina adjacent to the optic fissure. Dashed lines indicate the boundary of optic disc. Arrows indicate the presumptive optic disc/stalk (A-L′) and neural retina (M-T′) in mutants. NR, neural retina; OD, optic disc; OS, optic stalk. Scale bars: 100 μm.

misexpression, a clear reduction in phospho-ERK staining was observed in the ventral optic vesicle, the presumptive optic disc, the ventral optic stalk and the optic chiasm (Fig. 4A-D,G-J, arrows). At E13.5, when control embryos exhibited strong elevation in ERK phosphorylation in the Pax2-positive optic disc, *Fgfr1/2* ablation abolished both phospho-ERK and Pax2 staining in the presumptive optic disc region (Fig. 4E,F,K,L, arrow). ERK signaling is also known to promote cell proliferation and survival. Consistent with this, the presumptive optic disc region in the *Fgfr1/2* mutants showed a loss of cell cycle regulator cyclin D1 and cell proliferation marker Ki67, but there was a significant increase in apoptotic marker cleaved caspase 3 and TUNEL staining at E11.5 and E13.5 (Fig. 4M-T′, arrows). These results supported the theory that *Fgfr1/2* was required for ERK signaling in the optic fissure and optic disc.

Tyrosine phosphorylation of Frs2 by FGF receptors is known to create docking sites for the adaptor protein Grb2 and protein tyrosine phosphatase Shp2, both of which are important for activating Ras-ERK signaling. Surprisingly, although we confirmed the *Six3-Cre*-mediated ablation of *Frs2a* both by RNA *in situ* hybridization and immunohistochemistry (supplementary material Fig. S3A-D, arrows), ERK phosphorylation was unchanged in the E10.5 *Six3-Cre;Frs2a*$^{flox/flox}$ optic vesicle (supplementary material Fig. S3E,I, arrows). Phosphorylated ERK was eventually downregulated in the central retina at E13.5, but the Pax2-positive optic disc and NF165-positive optic nerve were both formed (supplementary material Fig. S3F-H,J-L, arrows). Consistent with this, no ocular coloboma was observed in *Six3-Cre;Frs2a*$^{flox/flox}$ animals (data not shown). As we have previously reported (Cai et al., 2010), *Six3-Cre;Shp2*$^{flox/flox}$ mutant similarly did not display any defects in embryonic development, including the closure of optic fissure and the formation of optic disc (supplementary material Fig. S3M-P, arrows; data not shown).

To further investigate the functional significance of *Frs2a* and *Shp2* in retinal development, we next generated a combined deletion of these two genes. Interestingly, the *Six3-Cre; Frs2α* $^{flox/flox}$*; Shp2*$^{flox/flox}$ mutants now reproduced the ocular coloboma and optic nerve dysgenesis phenotype observed in the *Six3-Cre; Fgfr1*$^{flox/flox}$*;Fgfr2*$^{flox/flox}$ mutants (Fig. 5A-E′, arrows; Table 1). By immunostaining, we confirmed that both Shp2 and phospho-ERK were lost in the ventral optic vesicle at E10.5 (Fig. 5F-G′, arrows). In the E13.5 frontal sections, the *Frs2a*/*Shp2* mutants appeared to maintain the correct dorsal-ventral polarity as indicated by the ventral expression of *Vax2*, but the Pax2-expressing optic disc structure was absent (Fig. 5H,H′). Similarly, ectopic Mitf expression was again induced in the ventral optic cup and the NF165-labeled optic nerve was severely hypoplastic (Fig. 5I-J′, arrows). Moreover, transverse sections of the *Frs2a*/*Shp2* mutants showed that the presumptive optic fissure and optic disc, and the adjacent ventral retina regions lost the retinal progenitor markers *Sox2*, *Fgf15* and *Chx10*, the optic disc markers Pax2 and netrin 1, and cell cycle markers Ki67 and cyclin D1 (Fig. 5K-O′, arrows). These results demonstrated that *Frs2a* and *Shp2* were essential for retinal development.

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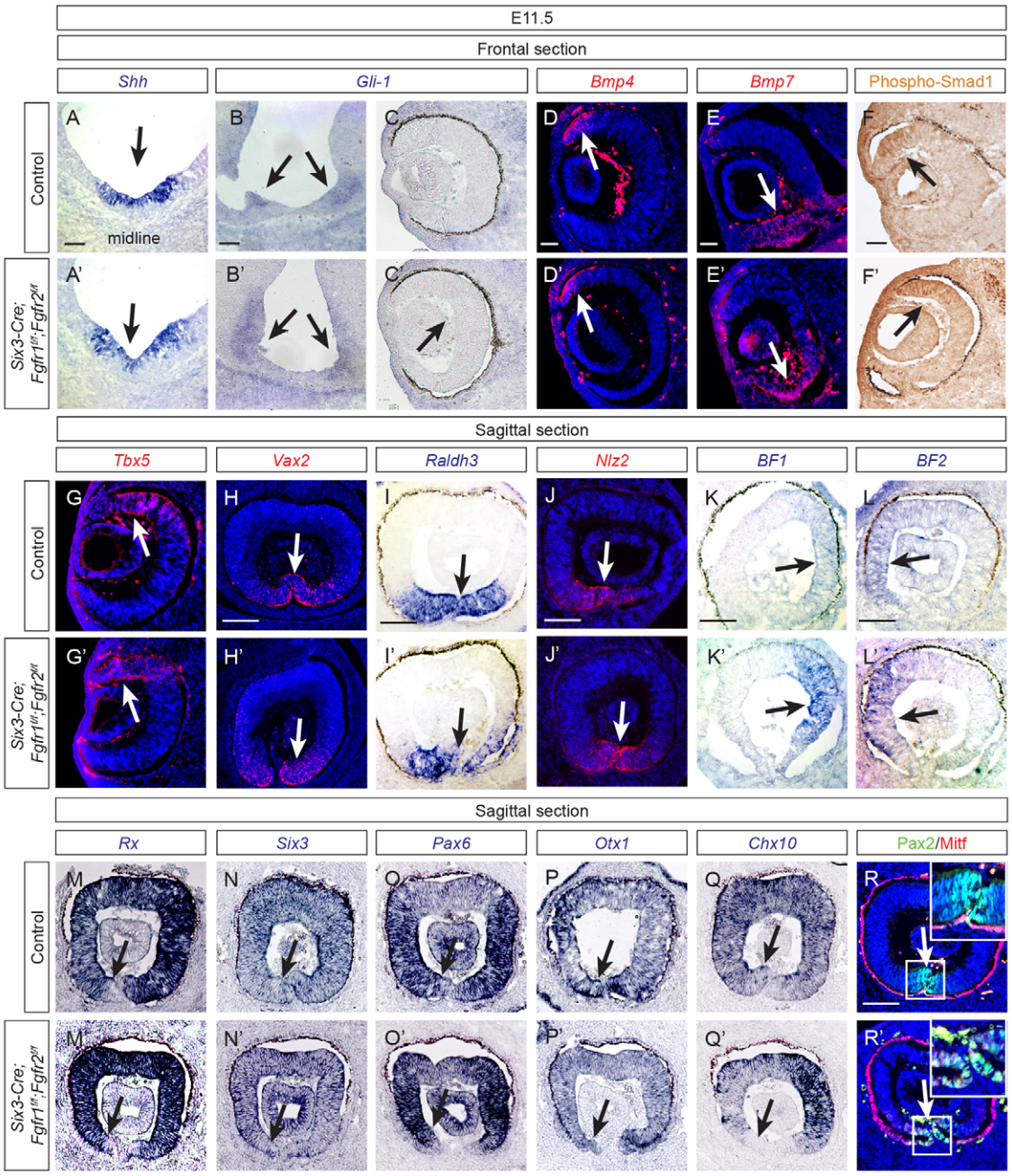

Fig. 3. The *Six3-Cre;Fgfr1*$^{flox/flox}$*;Fgfr2*$^{flox/flox}$ mutant optic vesicle exhibit normal axial patterning but defective optic fissure development. (**A-F′**) At E11.5, the loss of *Fgfr1* and *Fgfr2* signaling did not affect the expression of midline *Shh*, dorsal *Bmp4*, ventral *Bmp7* and their downstream effectors *Gli1* and phospho-Smad1. (**G-L′**) Levels of the dorsal-ventral polarity markers *Tbx5*, *Vax2*, *Radldh3* and *Nlz2*, and the temporal-nasal markers *BF1* and *BF2* were unchanged in *Fgfr1/2* mutants. (**M-R′**) Although levels of the optic vesicle patterning genes *Rx*, *Six3*, *Pax6* and *Otx1* were also unaffected, levels of the neural retinal marker *Chx10* and the optic disc marker Pax2 were downregulated in ventral retina, whereas the RPE maker Mitf was ectopically expressed in the ventral optic fissure in *Fgfr1/2* mutants. Scale bars: 100 μm.

Frs2a and *Shp2* acted epistatically to mediate FGF function in retinal development

The above results show that ocular coloboma appeared only in the *Frs2a*/*Shp2* double mutants but not in the single mutants, which seemed to suggest that these two genes act redundantly in ocular development. That is, *Frs2a* can activate downstream ERK signaling even in the absence of *Shp2*, and *vice versa*. However, an alternative explanation is that *Frs2a* and *Shp2* are indeed both essential for transmitting FGF signaling, but the conditional knockout of either *Frs2a* or *Shp2* resulted in only a gradual loss of either protein, which by itself may not be rapid enough to abolish downstream ERK signaling before the optic disc and optic fissure are developed. In this model, simultaneous depletion of both the Frs2α and Shp2 proteins in the *Frs2a*/*Shp2* double mutants would accelerate the disruption of ERK signaling, resulting in ocular coloboma. This synergistic effect is thus similar to the classic genetic interaction between two hypomorphic systemic alleles on the same pathway, which produces a far more severe phenotype when combined.

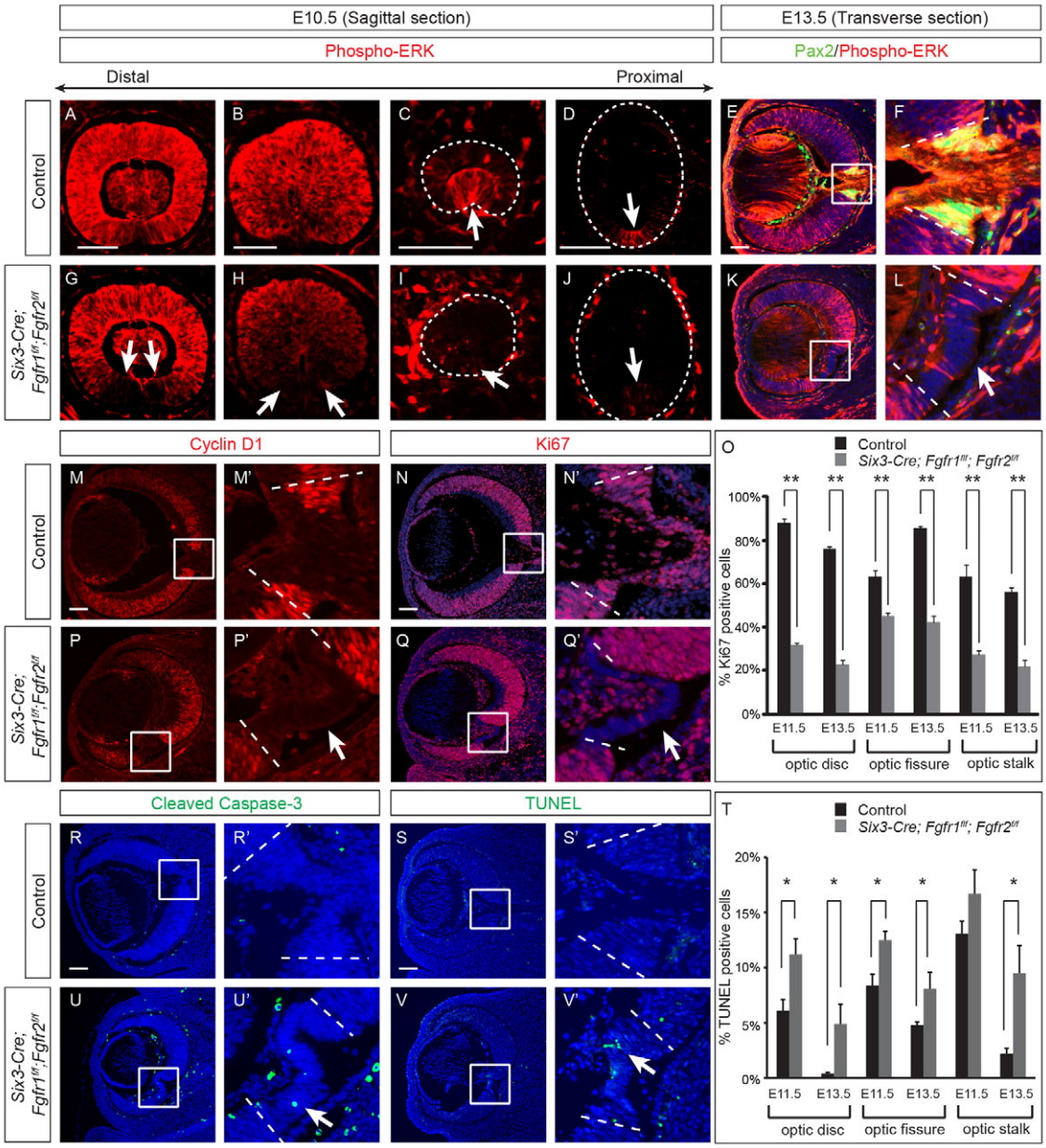

Fig. 4. *Fgfr1/2* ablation disrupts ERK signaling. (**A-D**,**G-J**) In the E10.5 *Six3-Cre;Fgfr1*$^{flox/flox}$*;Fgfr2*$^{flox/flox}$ embryos, phospho-ERK staining was lost in the ventral optic vesicle (G, arrows), the developing optic disc (H, arrows), the ventral optic stalk (I, arrow) and optic chiasm (J, arrow). The optic stalk and optic chiasm were outlined in white. (**E**,**F**,**K**,**L**) The control optic disc marked by Pax2 at E13.5 displayed strong phospho-ERK staining, which was abolished in the *Fgfr1/2* mutants. (**M-Q′**) Consistent with the deficient ERK signaling, the cell cycle regulator cyclin D1 and proliferation marker Ki67 were lost in the mutant optic disc region. (**R-S′**,**U-V′**) Increased cell death in the mutant optic disc region was shown by cleaved caspase 3 and TUNEL staining. Broken lines mark the boundaries of the optic disc. (**O**,**T**) The ratio of Ki-67 and TUNEL-positive cells versus DAPI-positive cells were measured in the indicated region and analyzed using Student's *t* test. *n*=8 for each genotype, **P*<0.05. ***P*<0.01. Scale bars: 100 μm.

To test this hypothesis, we used the well-characterized *Frs2a*^{2F} allele, which contained two point mutations in *Frs2a* that disrupted the Shp2 binding (Fig. 6A) (Gotoh et al., 2004). We reasoned that if Frs2α could act independently of Shp2, disabling the Frs2α and Shp2 interaction in the *Six3-Cre;Frs2a*$^{2F/flox}$*;Shp2*$^{flox/flox}$ mutants would not produce a more severe phenotype than the *Six3-Cre; Shp2*$^{flox/flox}$ single mutants. However, if Frs2α must recruit Shp2 to activate downstream signaling, the *Six3-Cre;Frs2α*$^{2F/flox}$*;Shp2*$^{flox/flox}$ mutants would phenocopy the same ocular coloboma defects in the *Six3-Cre;Frs2α*$^{flox/flox}$*;Shp2*$^{flox/flox}$ mutants. As shown in Fig. 6B-P, the *Six3-Cre;Frs2α*$^{2F/flox}$*;Shp2*$^{flox/flox}$ mutants indeed lost both Shp2 and phospho-ERK staining in the central retina. As a result, Mitf was ectopically induced in the presumptive optic disc region, whereas Pax2, netrin 1 and NF165 were downregulated. Although the adult *Six3-Cre;Frs2α*$^{2F/flox}$ animals did not display any eye phenotype (data not shown), the *Six3-Cre;Frs2α*$^{2F/flox}$*;Shp2*$^{flox/flox}$ mutants presented with ocular coloboma and optic nerve dysgenesis (Table 1). The phenotypic similarities between the *Six3-Cre;Frs2α*$^{2F/flox}$*;Shp2*$^{flox/flox}$ and the *Six3-Cre;Frs2α*$^{flox/flox}$*; Shp2*$^{flox/flox}$ mutants support the hypothesis that the recruitment of

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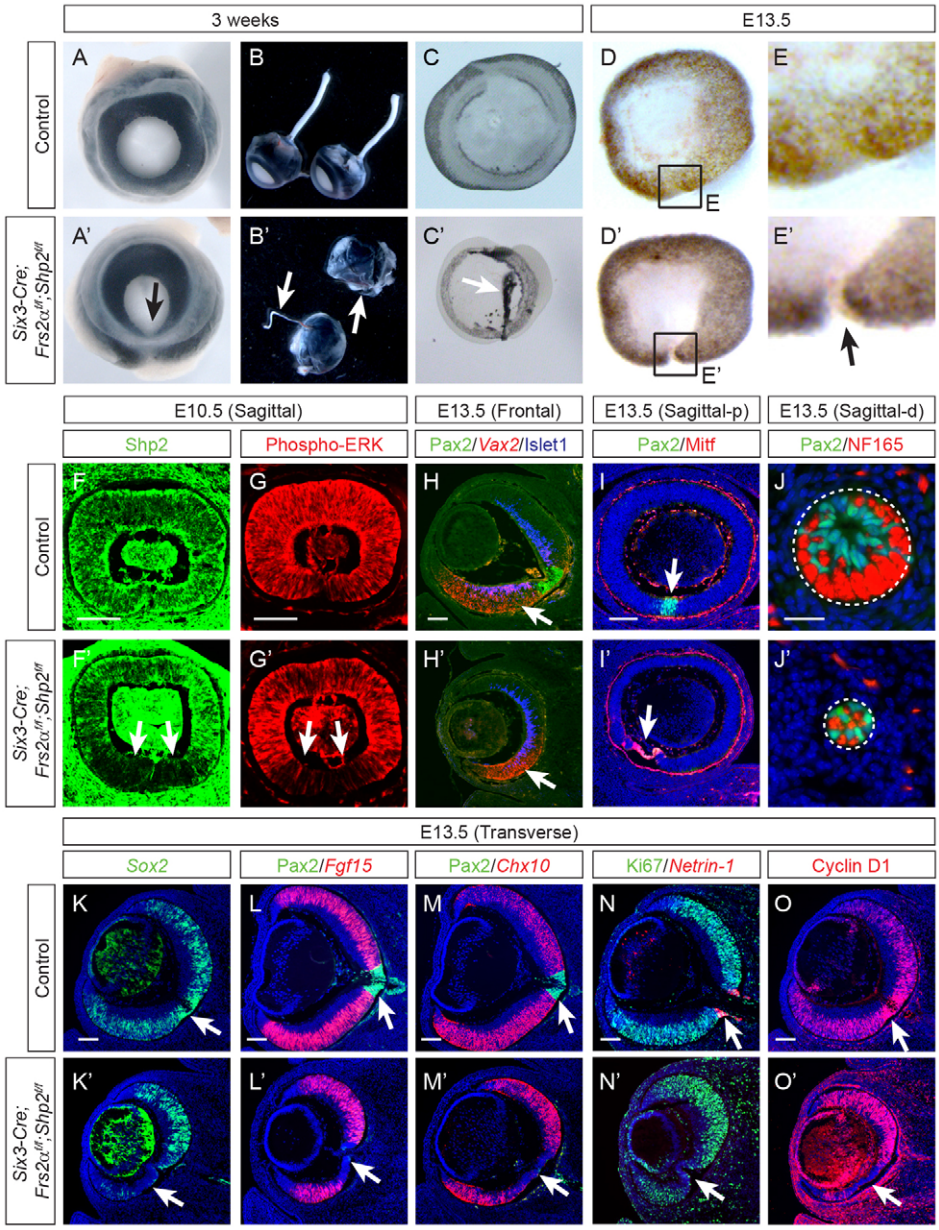

Fig. 5. Concomitant ablation of *Frs2a* and *Shp2* caused coloboma defects. (**A-E′**) The *Six3-Cre;Frs2a*$^{flox/flox}$*;Shp2*$^{flox/flox}$ mutants exhibited ocular coloboma (A′, arrow), optic nerve hypoplasia/aplasia (B′, arrows) and ectopic pigmentation (C′, arrow) at 3 weeks, and an open optic fissure at E13.5 (E′, arrow). (**F-J′**) Shp2 and phospho-ERK staining was downregulated along the ventral optic fissure in the E10.5 *Frs2a/Shp2* mutants. At E13.5, *Vax2* expression was unchanged in the ventral retina (H′, arrow), but the optic fissure was transformed to Mitf-positive RPE (I′, arrow) and the neurofilament marker NF165 was diminished in the optic stalk (circled in J′). (**K-O′**) The retinal progenitor markers *Sox2*, *Fgf15* and *Chx10* were lost along optic fissure in the ventral neural retina (K′,L′,M′, arrows). The optic disc region lost the cell proliferation marker Ki67, cyclin D1 and the optic disc marker netrin 1 (N′,O′, arrows). Scale bars: 100 μm in F-J′; 25 μm in K-O′.

Shp2 by Frs2α is essential in mediating FGF signaling during retinal development.

To further confirm the functional significance of Frs2 in FGF signaling, we next took advantage of a previously characterized *Fgfr1*$^{\Delta Frs}$ allele (Hoch and Soriano, 2006), in which the Frs2 binding site was deleted in the Fgfr1 cytoplasmic domain (Fig. 7A). By generating the *Six3-Cre;Fgfr1*$^{\Delta Frs2/flox}$*;Fgfr2*$^{flox/flox}$ mutant, we essentially created a retinal specific *Fgfr1*$^{\Delta Frs}$ mutant in a *Fgfr2*-null background. This strategy allows us to probe Fgfr-Frs2 interaction in a tissue-specific manner. The *Six3-Cre;Fgfr1*$^{\Delta Frs2/flox}$*;Fgfr2*$^{flox/flox}$ mutants completely phenocopied the *Six3-Cre;Fgfr1*$^{flox/flox}$*;Fgfr2*$^{flox/flox}$-null mutants (Table 1). As shown in Fig. 7B-E, phospho-ERK staining was abolished in the presumptive optic disc region in the E13.5 *Six3-Cre;Fgfr1*$^{\Delta Frs2/flox}$*;Fgfr2*$^{flox/flox}$ mutants. Mitf was ectopically induced at the expense of Pax2 expression, which led to misrouting of NF165-positive retinal ganglion axons and an absence of the optic nerve (Fig. 7F,G,J,K, arrows). In sagittal sections, the mutant optic fissure remained open with clear expression of Mitf but not Pax2, whereas the residual optic stalk appeared hypoplastic without an NF165-positive optic nerve (Fig. 7H,I,L,M, arrows). Therefore, the recruitment of Frs2 to FGF receptor was essential for optic disc and optic fissure development.

Constitutive Ras signaling rescues the ocular anomalies in the *Fgfr1/2* and the *Frs2a/Shp2* mutants

Although FGF signaling is capable of activating multiple downstream signals *in vitro*, our study above showed that both the *Fgfr1/2* and the *Frs2a/Shp2* coloboma mutants converged upon a Ras-ERK signaling deficiency. To test whether the activation of Ras-ERK signaling alone can account for the role of FGF signaling *in vivo*, we next took a gain-of-function approach by employing an oncogenic *LSL-Kras*^{G12D} allele (Tuveson et al., 2004), which can be induced by Cre recombinase to express a constitutively activated *Kras*^{G12D} isoform (Fig. 8A). Induction of *Kras*^{G12D} alone in the

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Six3-Cre; LSL-Kras^{G12D} animals did not cause any overt phenotype (Cai et al., 2010; Cai et al., 2011). However, in the *Six3-Cre;Frs2a*$^{flox/flox}$*;Shp2*$^{flox/flox}$*;LSL-Kras*^{G12D} and the *Six3-Cre; Fgfr1*$^{flox/flox}$*;Fgfr2*$^{flox/flox}$*;LSL-Kras*^{G12D} mutants, Kras activation not only led to a recovery of phospho-ERK staining in the central retina, but also restored the formation of the optic disc and optic nerve, as indicated by Pax2, netrin 1 and NF165 staining (Fig. 8B-S, arrows). In adult animals, the occurrence of optic nerve dysgenesis or optic coloboma was significantly reduced in the *Six3-Cre;Frs2a*$^{flox/flox}$*;Shp2*$^{flox/flox}$*;LSL-Kras*^{G12D} mutants and completely eliminated in the *Six3-Cre;Fgfr1*$^{flox/flox}$*;Fgfr2*$^{flox/flox}$*;LSL-Kras*^{G12D} mutants (Table 1). These genetic rescues thus supported an Fgfr-Frs2α-Shp2-Ras-ERK signaling cascade in ocular development.

DISCUSSION

In this study, we provided mouse genetic evidence that ocular ablation of *Fgfr1* and *Fgfr2* caused coloboma and optic nerve dysgenesis, which has not been observed when FGF signaling is disrupted in any other vertebrate organisms. This result is particularly noteworthy, because unlike previous studies of FGF signaling in *Xenopus* and zebrafish (Take-uchi et al., 2003; Lupo et al., 2005; Picker and Brand, 2005; Picker et al., 2009), our *Fgfr1/2* mutants did not display any axial polarity defects, suggesting that the observed ocular coloboma and optic nerve dysgenesis was not secondary to abnormal patterning of early optic cup. As indicated by the misexpression of the ocular astrocyte progenitor cell (APC) marker *Pax2* and the RPE gene *Mitf*, loss of *Fgfr1/2* signaling transformed the presumptive optic disc and optic fissure into RPE, suggesting a crucial role for FGF signaling in promoting the astrocyte progenitor cell fate versus the RPE fate. This function of FGF signaling is probably mediated by the reciprocal expression of *Pax2* and *Mitf*, mutation of either one is known to cause ocular coloboma (Scholtz and Chan, 1987; Torres et al., 1996). Interestingly, the potentially antagonistic interaction between Pax2 and Mitf in optic disc and optic fissure formation contrasts with the early role of Pax2 in stimulating Mitf expression in optic vesicle development (Bäumer et al., 2003). Such function reversals are not without precedence, as Pax2 and Pax6 also transition from redundant to antagonistic interactions from early to late retinal development (Schwarz et al., 2000; Bäumer et al., 2003). Similarly, Chx10 and Mitf are co-expressed in early optic vesicle, but later become antagonistic in specifying the neural retina versus the RPE under the influence of FGF signaling, loss of which results in conversion of neural retina into RPE (Nguyen and Arnheiter, 2000; Horsford et al., 2005; Cai et al., 2010). The role of FGF signaling in preventing ectopic RPE formation appears to be unique to mouse and chick, as inhibiting FGF signaling has never produced ectopic pigmentation or coloboma in amphibian or fish. Conversely, none of the axial polarity phenotype observed when FGF signaling is inhibited in amphibian and fish is detected in our FGF signaling mouse mutants. Taken together, our study reveals a distinctive role of FGF signaling in higher organisms in promoting neuronal against glial fate during eye development.

It is notable that our *Fgfr1/2* mutants displayed a restricted transformation into the RPE only at the optic disc/fissure and a limited neurogenesis defects only in the lip region of the ventral neural retina adjacent to the optic fissure. One possible explanation is the compensatory signaling by *Fgfr3* and *Fgfr4*, which may be expressed at low levels in retina to mediate FGF signaling. However, we suggest that the developmental timing may also restrict the retinal defects in *Fgfr1/2* mutants. From our Cre reporter assay, it was clear that the *Six3-Cre* deletor in the E9.5 optic vesicle was activated in a ventral-to-dorsal order. Consistent with this, the loss of phospho-ERK staining in the *Fgfr1/2* mutants at E10.5 was also limited to the ventral optic vesicle that formed the future optic fissure and optic disc. We

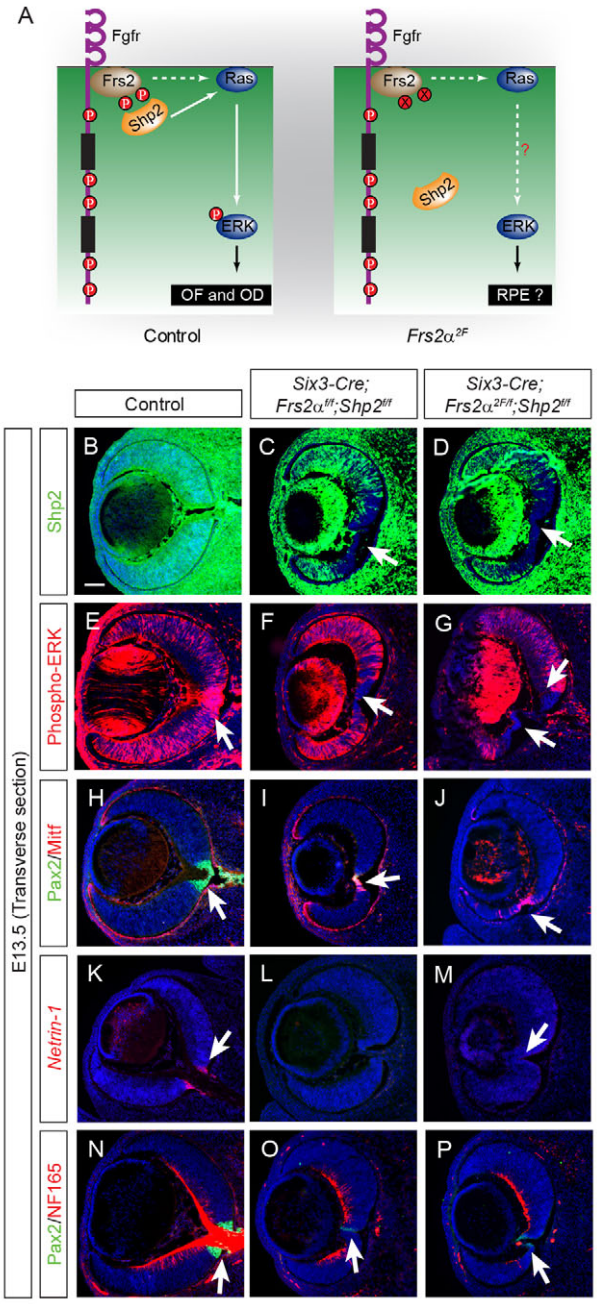

Fig. 6. Deletion of the Shp2-docking site on Frs2α disrupted ERK signaling in retinal development. (**A**) Frs2α-Shp2 interaction. In control retina, Frs2α recruits Shp2 to activate downstream Ras-ERK signaling for optic fissure and optic disc development. Mutating the two phospho-tyrosine residues in Frs2α abrogates the Shp2 docking site in the *Frs2α*^{2F} allele, but leaves the Shp2-independent function of Frs2α intact. This allows a direct test of Frs2α-Shp2 interaction in regulating retinal development. (**B-P**) The *Six3-Cre;Frs2α*$^{2F/flox}$*;Shp2*$^{flox/flox}$ mutants phenocopied the *Six3-Cre;Frs2α*$^{flox/flox}$*;Shp2*$^{flox/flox}$ mutants in the loss of Shp2 and phospho-ERK in central retina around the optic disc region (C,D,F,G, arrows). Both mutants gained ectopic Mitf and lost the optic disc marker Pax2 and netrin 1 (I,J,M, arrows). As a result, the optic nerve was not formed in either mutant (O,P, arrows). Scale bar: 100 μm.

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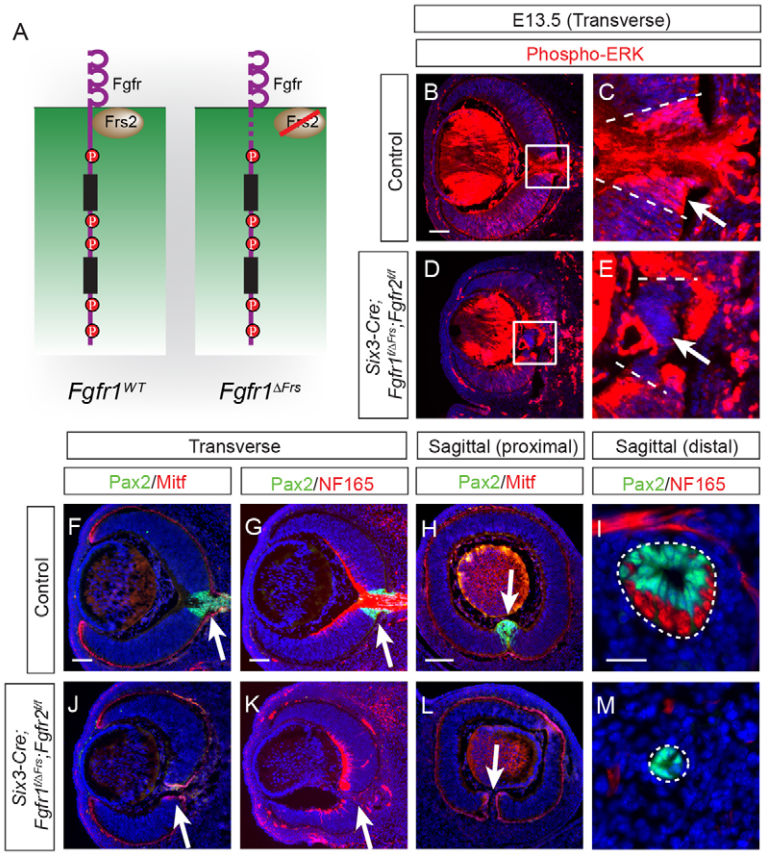

Fig. 7. The Frs2α-binding site on Fgfr1 is required for ERK signaling and the development of the optic disc and optic fissure. (**A**) The *Fgfr1*$^{\Delta Frs}$ allele retains all phosphotyrosine residues, but lost the domain for interacting with Frs2. (**B-E**) ERK phosphorylation was lost in the deformed optic disc region in the *Six3-Cre;Fgfr1*$^{\Delta Frs2/flox}$*;Fgfr2*$^{flox/flox}$ retina (E, arrow). (**F-M**) The *Six3-Cre;Fgfr1*$^{\Delta Frs2/flox}$*;Fgfr2*$^{flox/flox}$ mutant exhibited ectopic Mitf along the optic fissure (J and L, arrows) and lost Pax2 at optic disc (K, arrow). The Pax2-labeled mutant optic stalk is hypoplastic without NF165-stained optic nerve (circled in M). Scale bars: 100 μm in B-E; 25 μm in F-M.

propose that the mutant optic fissure and optic disc regions underwent Mitf-mediated transformation into the RPE because they lost FGF signaling by E10.5, whereas the rest of retina that lost FGF signaling after E10.5 was unable to alter their retinal fate. In this model, FGF signaling is required for the initial demarcation of the optic vesicle, but it becomes dispensable for the maintenance of retinal progenitor cell fate after a limited time window. Our results therefore challenge the view that FGF signaling is required for retinal neurogenesis after E12, but instead support our previous study that FGF signaling is necessary only for the establishment of neuronal fate in optic vesicle at E10.5 (Cai et al., 2010).

The transient requirement of FGF signaling in early optic vesicle development is also supported by the lack of an ocular phenotype in the *Frs2a* and *Shp2* single mutants. As we have previously described and have also shown in this study, *Six3-Cre;Shp2*$^{flox/flox}$ single mutants did not display any embryonic retinal defects (Cai et al., 2010). By contrast, we have previously demonstrated that the retinal specific ablation of *Shp2* by *Rx-Cre*, a Cre deletor that acts earlier than *Six3-Cre*, resulted in the transformation of the neural retina into the RPE. Comparing the *Six3-Cre;Shp2*$^{flox/flox}$ and the *Rx-Cre;Shp2*$^{flox/flox}$ mutants showed that there was a significant perdurance of Shp2 protein after the onset of Cre activities, which resulted in a considerable delay in ERK signaling disruption. As a result, only *Rx-Cre* was able to abrogate Shp2-ERK signaling early enough to interfere with the determination of the neural retina fate (Cai et al., 2010). In this study, we similarly showed that the *Six3-Cre;Frs2a*$^{flox/flox}$ mutants did not significantly downregulate ERK phosphorylation until E13.5. In fact, only by depleting both Frs2α and Shp2 simultaneously in the *Six3-Cre;Frs2a*$^{flox/flox}$*;Shp2*$^{flox/flox}$ mutants were we able to disrupt ERK signaling in the ventral retina at E10.5. This established a genetic interaction between *Frs2α* and *Shp2*, which form a binding complex in mediating FGF signaling in eye development. Such a binary complex is expected to be relatively resistant to a slow depletion in one of its binding partners, but the complex will disintegrate when the concentrations of both proteins are reduced below a certain threshold. Therefore, only *Frs2a*/*Shp2* double mutants displayed ocular coloboma, albeit still at a slightly lower penetrance than that of *Fgfr1/2* mutants (Table 1), which is likely because of the slower recycling rates of intracellular proteins when compared with membrane receptors. Our study thus presents a caution for future investigation of FGF signaling in embryonic development that protein perdurance may be an important confounding factor in interpreting conditional knockout results.

FGF receptors are known to recruit multiple adaptor proteins, including Frs2, Crk and PLCγ, to activate downstream signaling. In particular, recent studies suggest Frs2α, a lipid-anchored protein that is constitutively bound to FGF receptors, mediates some but not all FGF downstream pathways (Eswarakumar et al., 2006; Hoch and Soriano, 2006; Sims-Lucas et al., 2011). It has been previously reported that the *Frs2α*$^{2F/2/F}$ mutant, which disrupts the Frs2α-Shp2 interaction, displayed microphthalmia or even anophthalmia (Gotoh et al., 2004). However, as *Frs2a*$^{2F/2/F}$ is a systemic mutant, it is not clear whether these severe ocular phenotypes are due to cell autonomous defects or to abnormal tissue-tissue interaction. In this study, we have combined systemic deletion mutants with conditional alleles to disrupt protein-protein interactions in a tissue-specific manner. In the *Six3-Cre;Fgfr1*$^{\Delta Frs2/flox}$*;Fgfr2*$^{flox/flox}$ mutants, for example, the *Fgfr1*$^{\Delta Frs}$ mutation was compensated for by the *Fgfr1*^{flox} and *Fgfr2*^{flox} alleles in every tissue other than the retina, where these conditional alleles were ablated by *Six3-Cre*. This allowed us to demonstrate that the Fgfr1-Frs2 interaction within retinal progenitor cells was crucial in coloboma formation.

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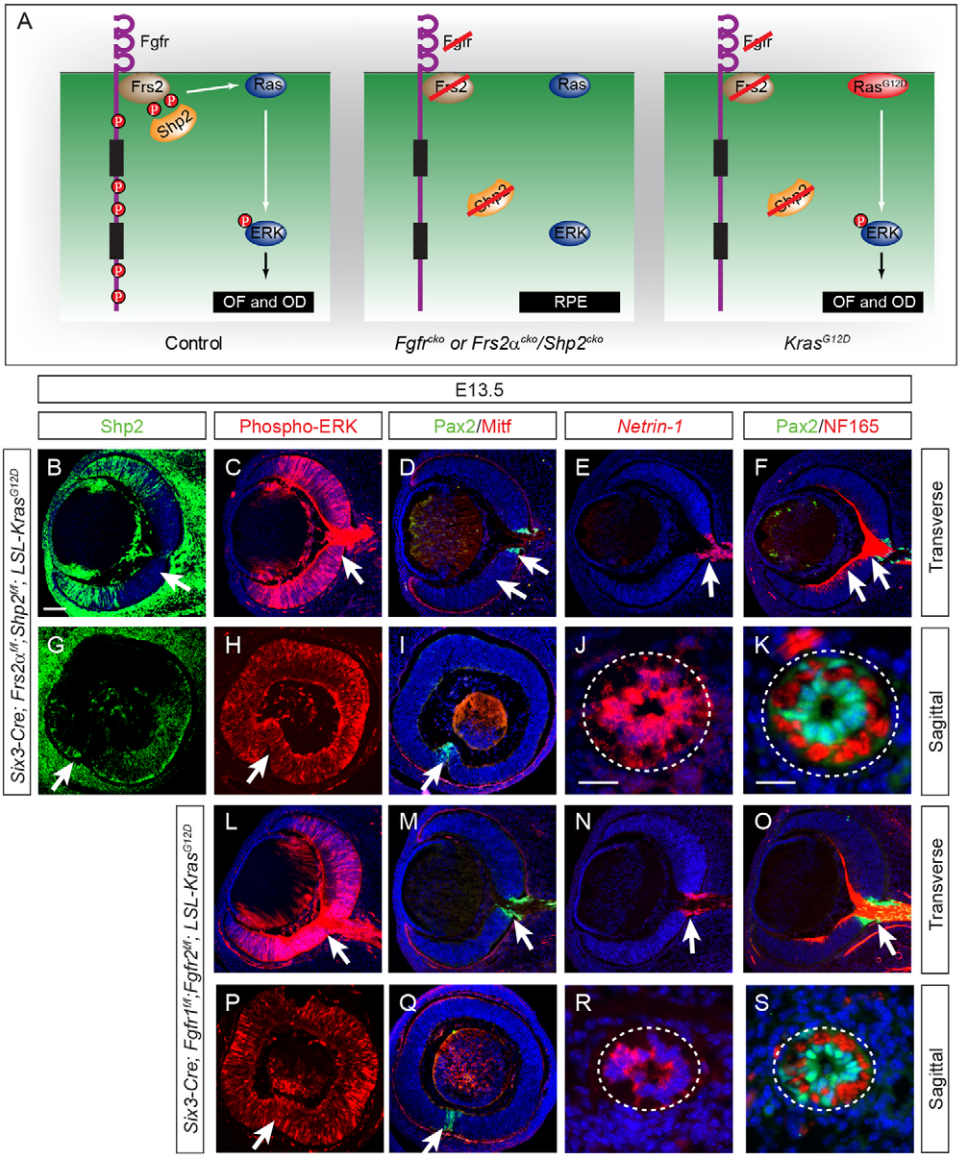

Fig. 8. Retinal FGF-Frs2α-Shp2 FGF signaling can be substituted by constitutive Kras signaling. (**A**) Kras rescue experiments. In the absence of upstream Fgfr-Frs2-Shp2 signaling, the constitutively activated *Kras*^{G12D} mutant can still activate ERK signaling to promote optic fissure and optic disc development. (**B-K**) Despite of loss of Shp2 staining (B,G, arrows), phospho-ERK staining was recovered in the *Six3-Cre;Frs2α*$^{flox/flox}$*;Shp2*$^{flox/flox}$*;LSL-Kras*^{G12D} mutant retina (C,H, arrows). This led to the formation of Pax2- and netrin 1-expressing optic disc and NF165-expressing optic nerve (D-F,I-K, arrows). (**L-S**) Similarly, the *Six3-Cre;Fgfr1*$^{flox/flox}$*;Fgfr2*$^{flox/flox}$*;LSL-Kras*^{G12D} mutant eye also displayed increased ERK phosphorylation (L,P, arrows), formation of optic disc (M-O, arrows), fusion of optic fissure (Q, arrow) and establishment of optic nerve. Scale bars: 100 μm in B-I,L-Q; 25 μm in J,K,R,S.

Similarly, the coloboma phenotype in the *Six3-Cre;Frs2a*$^{2F/flox}$*;Shp2*$^{flox/flox}$ mutants revealed the importance of Frs2α-Shp2 interaction in the retinal-specific FGF signaling. Finally, we showed that constitutive Kras signaling was sufficient to prevent ocular coloboma in these FGF signaling mutants, demonstrating a remarkably specific pathway from Fgfr to Frs2-Shp2 to Ras-ERK signaling in retinal development.

In summary, our present study identifies deficient FGF signaling as a novel cause of ocular coloboma and optic nerve dysgenesis. We show that FGF-activated Ras-ERK signaling is essential for the development of the optic nerve, optic fissure and optic disc, the abnormalities of which have been implicated in not only ocular birth defects but also in adult glaucoma. It is notable that iris and optic disc colobomas have also been observed in some individuals with Noonan syndrome, who have gain-of-functions mutations in Ras-MAPK signaling pathway (Kleanthous et al., 1987; Ascaso et al., 1993; Rudolph et al., 2001; Legius et al., 2002; Carvalho et al., 2003; Tartaglia and Gelb, 2005). Interestingly, we have recently shown that deletion of the genes associated with Noonan syndrome, *Shp2* and *Nf1*, caused lens development defects (Pan et al., 2010; Carbe and Zhang, 2011). These findings suggest that both the gain- and loss-of-function mutations in FGF-Ras-MAPK signaling may have deleterious consequences in human ocular development. Therefore, FGF signaling components should be considered in the diagnosis and therapeutic interventions for ocular anomalies.

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Competing interests statement
The authors declare no competing financial interests.

Author contributions
Z.C. and X.Z. conceived the project. Z.C., C.T. and H.L. performed the experiments. R.L., N.G., G.-S.F. and F.W. provided mouse mutants. Z.C. and X.Z. wrote the manuscript.

Supplementary material

Supplementary material available online at
http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.089987/-/DC1

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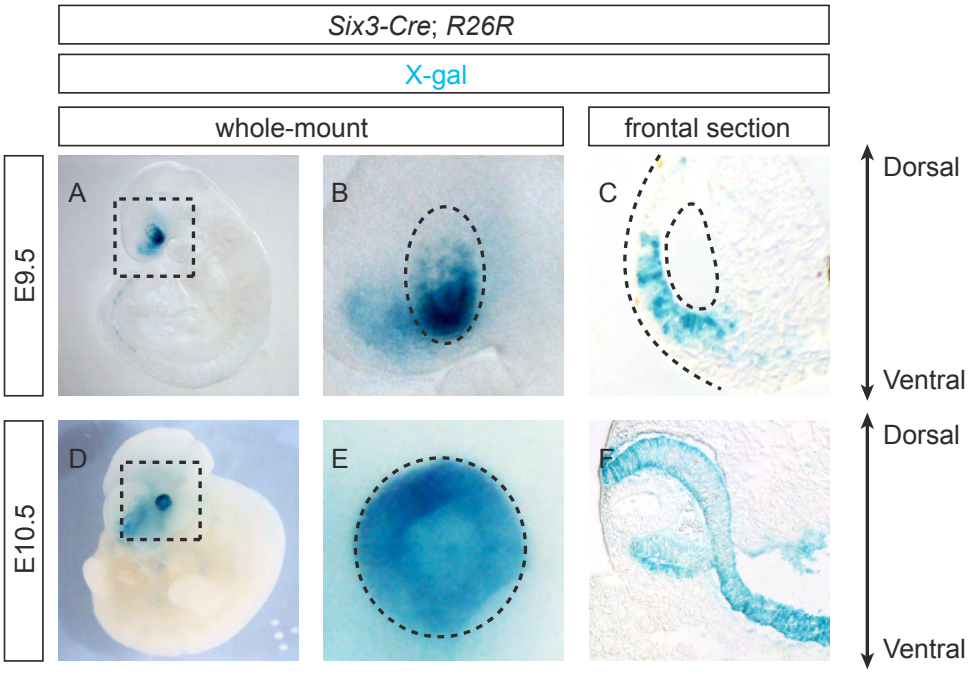

Fig. S1. Spatial and temporal mapping of *Six3-Cre* activity by the *R26R* reporter. (**A-C**) At E9.5, strong X-gal staining was first observed on the ventral side, but not on the dorsal side of the optic vesicle in *Six3-Cre;R26R* embryos. The boxed area in A is magnified in B. Frontal sections of the X-gal stained optic vesicle further demonstrate that *Six3-Cre* is initially active in the ventral side of the optic vesicle (C). (**D-F**) At E10.5, X-gal staining expands to the entire optic cup. The boxed area in D is enlarged in E. The X-gal stained eye in the D was further analyzed with frontal section (F).

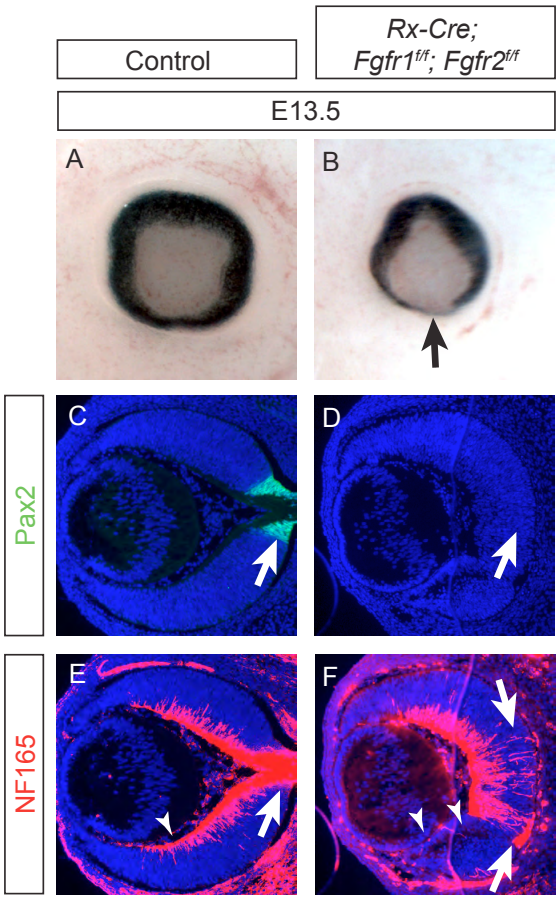

Fig. S2. Coloboma and optic disc dysgenesis in the *Rx-Cre;Fgfr1$^{flox/flox}$;Fgfr2$^{flox/flox}$* mutants. (**A**,**B**) *Rx-Cre*-mediated deletion of *Fgfr1* and *Fgfr2* resulted in coloboma at E13.5 (arrow in B). (**C-F**) The *Rx-Cre;Fgfr1$^{flox/flox}$;Fgfr2$^{flox/flox}$* mutants lose the Pax2-positive optic disc (arrow in D) and display misrouting of NF165-staining retinal ganglion cell axons (arrows in F). Neurogenesis defects are indicated by the loss of NF165 expression (arrowhead in F).

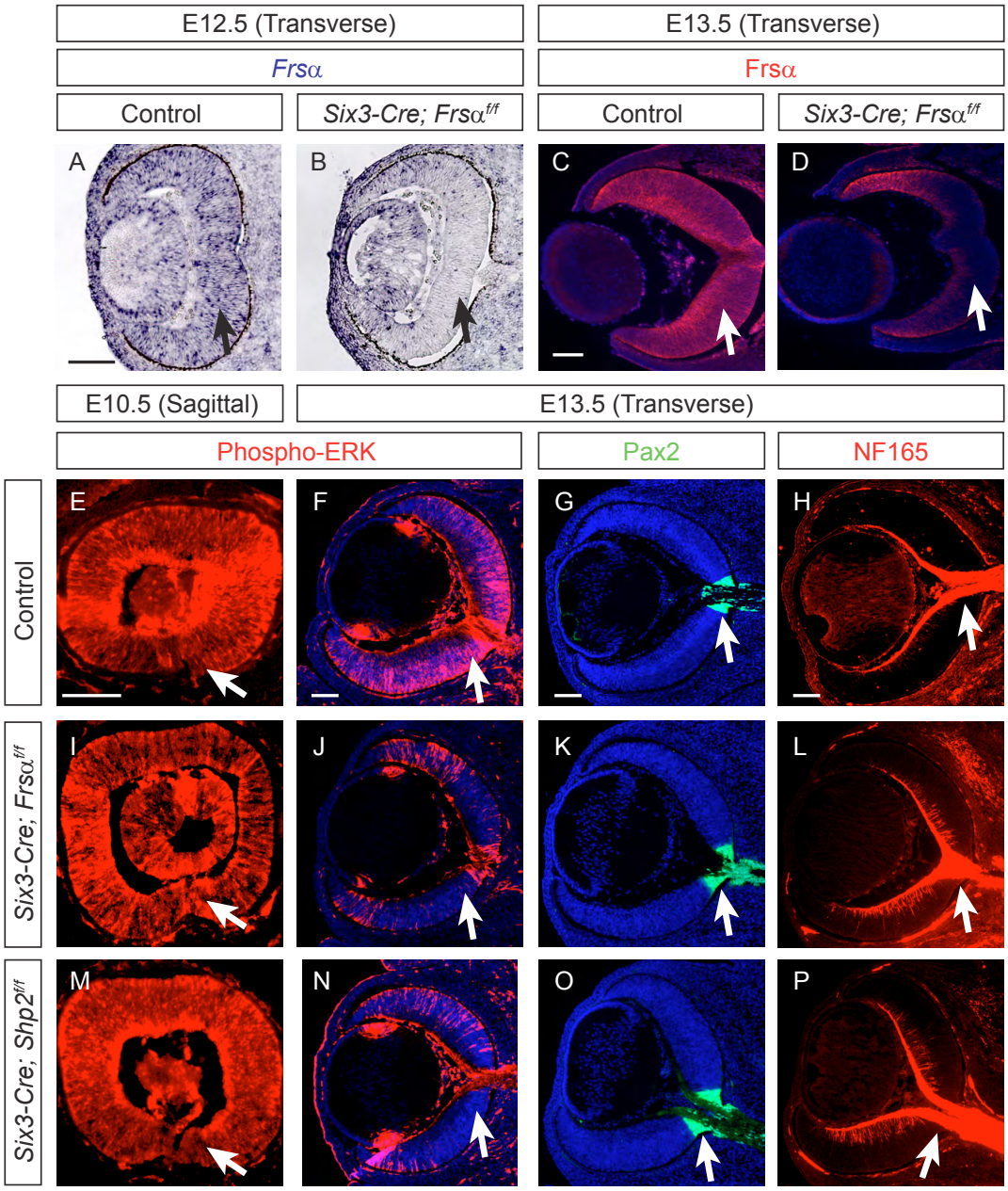

Fig. S3. Lack of ocular phenotypes in the *Six3-Cre; Frs2α*$^{flox/flox}$ and in the *Six3-Cre; Shp2*$^{flox/flox}$ mutants. (**A-D**) *Six3-Cre*-mediated ablation of *Frs2a* was confirmed in central retina by RNA *in situ* hybridization at E12.5 (A and B, arrows) and by immunostaining at E13.5 (C and D, arrows). (**E-P**) Deletion of *Frs2a* or *Shp2* alone reduced ERK phosphorylation in central retina at E13.5, but not at E10.5 (E,F,I,M,N, arrows). Normal optic disc formation as indicated by Pax2 staining and optic nerve genesis, as shown by NF165 labeling, was observed in both mutants (G,H,K,L,O,P, arrows). Scale bars: 100 µm.