Development/Plasticity/Repair

Suppressor of Fused Is Required to Maintain the Multipotency of Neural Progenitor Cells in the Retina

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The morphogen sonic hedgehog (Shh) plays a crucial role in development of the CNS, including the neural retina. *Suppressor of fused* (*Sufu*) has been recently identified as a critical regulator of Hh signaling in mammals. However, the precise roles that *Sufu* plays in the regulation of proliferation and cell-fate decisions in neural progenitors is unknown. Here, we have addressed these questions by conditionally deleting *Sufu* in mouse multipotent retinal progenitor cells (RPCs). *Sufu* deletion in RPCs results in transient increases in Hh activity and proliferation followed by developmentally premature cell-cycle exit. Importantly, we demonstrate a novel role for *Sufu* in the maintenance of multipotency in RPCs. *Sufu*-null RPCs downregulate transcription factors required to specify or maintain RPC identity (*Rax*, *Vsx2*) and multipotency (*Pax6*) but continue to express the neural progenitor marker *Sox2*. These cells fail to express retinal lineage-specific transcription factors, such as *Math5*, and adopt an amacrine or horizontal cell fate at the expense of all other classes of retinal neurons. Genetic elimination of *Gli2* in *Sufu*-null RPCs attenuates Hh pathway activity and restores multipotency in neural progenitors. These data provide novel evidence that *Sufu*-mediated antagonism of Hh/*Gli2* signaling is required to maintain RPC multipotency and identity.

Introduction

CNS development requires a complex interplay between cell-extrinsic and -intrinsic signals that regulate stem cell/progenitor cell proliferation and diversification. The neural retina is an accessible and relevant model for interrogating the integration of these processes. The seven cell types in the adult retina develop in a temporal sequence from multipotent retinal progenitor cells (RPCs) (Young, 1985). RPC specification, proliferation, and neurogenic competence are regulated by several transcription factors, including *Pax6* (Marquardt et al., 2001), *Rax* (Mathers et al., 1997), *Vsx2* (Burmeister et al., 1996), *Six6* (Li et al., 2002), and *Sox2* (Taranova et al., 2006); retinal lineage commitment is regulated by the coordinated activity of additional transcription factors, including bHLH and homeobox genes (Ohsawa and Kageyama, 2008). *Pax6* is the only gene identified to date that is required for RPC multipotency, as conditional *Pax6* deletion at the optic cup stage causes RPCs to adopt an amacrine interneuron fate exclusively (Marquardt et al., 2001). While a number of cell-extrinsic cues, including Hedgehog (Hh) signaling, also play a role in RPC proliferation and cell-fate specification (Amato et al., 2004), the mechanism(s) that couple determination of retinal fate to extrinsic signaling is not known.

The secreted morphogen Sonic hedgehog (Shh) is a key regulator of CNS development. Binding of Shh to its receptor, Ptch, derepresses Smo and initiates the Hh signaling (Alcedo et al., 1996; Murone et al., 1999) that converges upon the *Gli* transcription factors (*Gli1–3*) and, ultimately, target gene transcription. *Suppressor of fused* (*Sufu*) is a key antagonist of *Hh* signaling in mammals (Cooper et al., 2005; Svard et al., 2006; Varjosalo et al., 2006) and exerts dichotomous effects on Gli function by both cytoplasmic sequestration and increased protein stability (Barnfield et al., 2005; Humke et al., 2010). Embryonic deletion of Sufu results in a strong Hh gain of function phenotype with extensive CNS mispatterning (Svard et al., 2006). However, the precise roles of *Sufu* in the regulation of Hh signaling in developing neuroepithelia are unknown.

In the developing retina, Shh is secreted by retinal ganglion cells (GCs) and signals to RPCs to regulate cell fate, proliferation, and self-renewal (Amato et al., 2004; Wang et al., 2005). Recently, Gli2 has been identified as an important mediator of Hh-induced RPC proliferation and cell fate (Wall et al., 2009). As *Sufu* likely integrates the cell-extrinsic Hh signal and cell-intrinsic *Gli* responses to direct progenitor behavior, we sought to determine the roles of *Sufu* in RPCs. Here, we demonstrate that *Sufu* negatively regulates Hh signaling and proliferation in RPCs. Unexpectedly, *Sufu*-null RPCs also failed to maintain their multipotency and differentiated exclusively as amacrine and horizontal neurons. This

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phenotype was characterized by the downregulation of transcription factors required to maintain RPC identity and multipotency, including *Pax6*, *Rax*, and *Vsx2*, and a failure to initiate expression of transcription factors required for the development of retinal cell lineages. We further demonstrate that these effects were mediated by *Gli2*. These findings identify a novel role for Sufu/Gli2 function upstream of Pax6 in the regulation of RPC multipotency.

Materials and Methods

Mice. All experiments were approved by the University of Ottawa Animal Care Ethics Committee and adhered to the guidelines of the Canadian Council on Animal Care. Both male and female mice were selected randomly for all genotypes. C57BL/6 mice were obtained from the Charles River Laboratory. The *αP0-Cre* transgenic mice, which express *Cre-recombinase* under the control of the αP0 element of the *Pax6* promoter, were obtained from Dr. P Gruss (Max-Planck Institute, Gottingen, Germany) (Marquardt et al., 2001) and were maintained on a C57BL/6 background. $Sufu^{F/F}$ mice harbor loxP sites flanking *Sufu* exons 4–8 (Pospisilik et al., 2010) and were maintained on a C57BL/6 background. Retina-restricted conditional *Sufu* knock-out mice were generated by crossing *αP0-Cre;*$Sufu^{+/Fl}$ mice with $Sufu^{F/F}$ mice to generate *Cre;*$Sufu^{F/F}$ mice. *Gli2* mutant mice were maintained on a CD1 background and genotyped as published previously (Wall et al., 2009). ROSA Cre reporter mice were obtained from Jackson Laboratories. Mice were genotyped by the PCR of tail DNA for wild-type and floxed *Sufu* alleles with the following primers: forward (F)–5′CTGTTTGTACTCATGGTC3′, reverse (R)–5′ CCTACCCTTTCCAGTAAG3′, Del–5′GCTGAATTCTTGACTCACTG3′, Neo–5′GTGTCAGTTTCATCGCCTG3′. Primers directed against the wild-type allele yielded a band size of 300bp, those directed against the floxed allele yielded a band size of 350bp.

Retinal explant culture, electroporation BrdU incorporation, and cell dissociation. Explants were prepared and electroporated as described previously (Matsuda and Cepko, 2004). Briefly, eyes from postnatal day 0 (P0) mice were dissected free of the optic nerve and RPE, and retinas were then electroporated (ECM 830 BTX Harvard apparatus) in a 2 mm gap cuvette (VWR). The DNA plasmids used in this study were *Smo-M2* (CMV promoter) (a gift from G. Fishell, New York University, New York, NY), *Sufu*-Flag (CMV promoter) (Ding et al., 1999), *pUB-GFP*, *Gli-Luciferase* (Sasaki et al., 1997) (a gift from Dr. H Sasaki, Osaka, Japan), *Renilla Luciferase* (a gift from Dr. Alan Mears, Ottawa Hospital Research Institute, Ottawa, Canada), and Cre (β-actin promoter). Plasmids were prepared using a Qiagen Maxiprep kit according to manufacturer's instructions (Qiagen) and were electroporated at a concentration of ~1 μg/μl. After electroporation, the lens was removed and explants were flattened on micropore filters (Millipore) and cultured in serum-free retinal explant medium (Wang et al., 2005). For BrdU incorporation experiments, explants were pulsed with BrdU (Sigma) for 5 h before enzymatic dissociation. To obtain single cell dissociates of retinal explants for IHC and fluorescent activated cell sorting (FACS) analysis, retinal explants were treated with 0.1 mg/ml trypsin (Sigma) in sterile calcium-free PBS (Invitrogen) at 37°C for 15 min. Trypsinization was inhibited with 10% FBS/ DMEM/DNAseI (0.2 mg/ml, Sigma) and tissue was triturated to obtain a single cell suspension. Cells were pelleted at 1000 × *g* for 5 min and resuspended in 350 μl of 10%FBS/DMEM/insulin (0.01 mg/ml, Sigma). Cell suspensions (15 μl) were plated on glass slides, which were then incubated in a humidified chamber for 15 min at room temperature followed by 40 min at 37°C to allow the cells to adhere to the slides. Cells were then fixed with 4% PFA for 5 min, washed three times with sterile PBS and air dried. Cells were either processed immediately for immunocytochemistry or stored at −20°C.

Tissue preparation for in situ *hybridization or immunohistochemistry.* For timed matings, the morning of vaginal plug detection was considered embryonic day 0.5 (E0.5). To label S-phase cells *in vivo*, time-mated females were given two intraperitoneal injections 2 h apart of BrdU at 0.1 mg/g body weight. For processing of embryonic retinae, whole heads were fixed in 4% PFA in PBS overnight at 4°C. For fixation of adult retinae, mice were anesthetized using CO_2, and a cardiac perfusion was performed using ~10 ml of 4% PFA. Eyes were then enucleated and fixed in 4% PFA overnight. Tissue was then washed 3 × 10 min in sterile PBS (0.14 M NaCl, 2.5 mM KCl, 0.2 M Na_2HPO_4, 0.2 M KH_2HPO_4) and immersed in 30% sucrose in PBS at 4°C overnight or up to 3 d. Before embedding, tissue was equilibrated for 1 h in 50:50 30% sucrose in PBS: OCT (Tissue-Tek) and then embedded in the equilibration solution and snap frozen with liquid nitrogen. Adult retinal material was cross-sectioned and embryonic eyes were sectioned in the coronal plane at 10 μm using a Leica 1850 cryostat. Sections were transferred onto Superfrost Plus coated slides (Fischer Scientific), air dried for ~4 h at room temperature, and used immediately for immunohistochemistry or *in situ* hybridization or stored with desiccant at −20°C. Wild-type and mutant tissues were always compared on the same slides. To accurately delineate the mutant region of the retina, images shown for each figure are adjacent sections from a representative animal unless specified otherwise, and the region of *Sufu* deletion in each eye was confirmed by *in situ* hybridization (ISH) on a section from the same series.

Hematoxylin and eosin staining. Slides were rehydrated for 30 min in 1 × PBS and then stained with hematoxylin (Fischer Scientific) for ~7 s. Tissue was then dehydrated through consecutive 2 min incubations in 50% ethanol (EtOH), 70% EtOH, 80% EtOH, 90% EtOH ×2, 100% EtOH ×2. Tissue was then stained with eosin (Fischer Scientific) for 1 min and dehydrated using the same protocol as for hematoxylin. Slides were washed 1× 2 min in xylene (Fischer Scientific) and mounted using PerMount (Fischer Scientific). Slides were visualized using an Axioplan microscope and images were captured using an AxioVision camera 2.05 (Zeiss).

Immunohistochemistry/immunocytochemistry and nuclear labeling and TUNEL staining. Immunohistochemistry (IHC) and immunocytochemistry (ICC) were performed as described previously (Wall et al., 2009; Wang et al., 2005). The primary antibodies used in this study were as follows: αBrn3b (Santa Cruz Biotechnology), αChAT, αGly-T1, αTH2, αProx1 (Millipore Bioscience Research Reagents), αCalretinin (Swant), αVsx2 (a gift from Dr. Rod Bremner, University of Toronto), αCRALBP (a gift from Dr. Jack Saari, University of Washington, Seattle, WA), αGABA, αHPC1 (Sigma), αGFP (Invitrogen), αIsl1 (Abcam), αLim1/2 (4F2) (Developmental Studies Hybridoma Bank), αRhodopsin (B630) (Developmental Studies Hybridoma Bank), αBrdU, αKi67 (BD Bioscience), and αGAD65 (Abcam). Secondary antibodies used were as follows: αRabbit FITC (Invitrogen), αRabbit Cy3, αMouse Cy3 (Jackson ImmunoResearch), biotinylated αGoat, and Cy3-Streptavidin (GE Healthcare). Nuclei were counterstained with bisbenzimide (DAPI) (Invitrogen) and mounted with fluorescent mounting medium (Dako). Terminal uridine deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay was performed using the *In Situ* Death Detection Kit (Roche Diagnostics) according to the manufacturer's instructions. Nuclei were counterstained with DAPI as above and mounted with fluorescent mounting medium (DAKO). Staining was visualized using a Zeiss fluorescent upright microscope and images were captured using an AxioVision 6.05 camera (Zeiss).

In situ *hybridization.* ISH was performed as described previously (Jensen and Wallace, 1997) using digoxigenin (DIG)-labeled antisense RNA riboprobes prepared by *in vitro* transcription from linearized plasmids containing complete or partial cDNA sequences of the following mouse genes: *Sufu*, *Crx*, *FoxN4*, *Gli1*, *Math5*, *Pax6*, *Rax*, and *Vsx2*. Sections were analyzed on an Axioplan microscope and digital images were captured using an AxioVision camera 2.05 (Zeiss).

Fluorescent activated cell sorting. Single cell dissociates of trypsinized retinal explants were resuspended in 2 ml of 10% FBS/DMEM and passed through a 50 μm mesh (Millipore). Cells were then placed on ice and sorted using a Dako Cytomation MOFLO (Dako) by StemCore. Sorting parameters gated out dead cells using forward and side scatter and sorted for GFP+ cells. The apparatus was cleaned beforehand using 10% bleach and DEPC water. Cells were collected on ice in 2 ml of 10% FBS/DMEM.

mRNA isolation and real-time quantitative PCR analysis of gene expression. mRNA from FACS-sorted cells and dissected embryonic retinas was extracted using Tri-Reagent (Invitrogen) according to manufacturer's instructions. mRNA purity and concentration was determined using spectroscopy (Ependorph BioPhotometer). cDNA was then prepared using Moloney murine leukemia virus (Invitrogen) according to manufacturer's instructions.

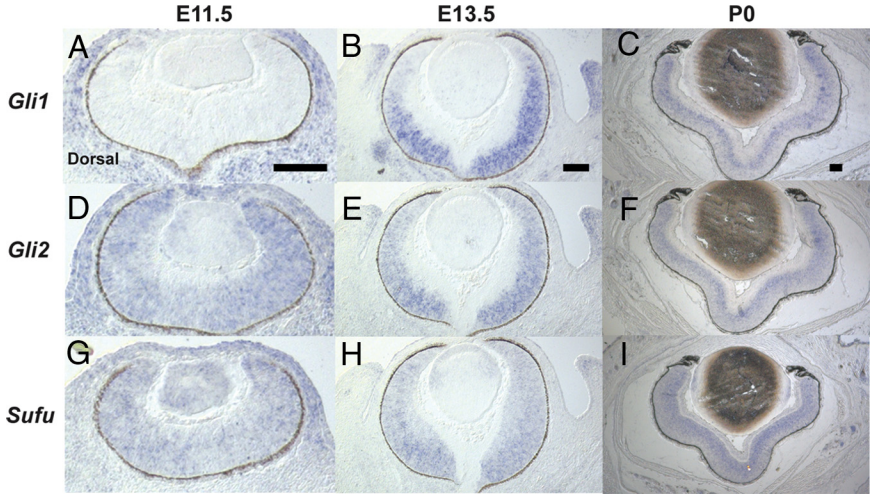

Figure 1. Sufu antagonizes Hh signaling in RPCs. ***A–I***, *In situ* hybridization on serial coronal sections showing *Gli1* (***A–C***), *Gli2* (***D–F***), and *Sufu* (***G–I***) expression in the neuroblast layer at E11.5, E13.5, and P0. Sections are oriented with dorsal on the left. Scale bars, 100 μm.

For quantitative PCR (qPCR), 2 μl of cDNA, 1 μl of 10 μM forward/10 μM reverse primers, and 10 μl of HPLC grade water were added to 15 μl of SYBR-Green qPCR master-mix reagent (Sigma). Reactions were performed using an Mx3000P thermocycler (Stratagene). The primer sequences used were as follows: *CRX*: F–CAGGTCTGGTTCAAGAATCG, R–ACGAGCCTTGGTCTGTGC; *Gli1*: F—CAAAAGGGCAGACCAGAAAG, R—TCAATCCAATGACTCCACCA; *FoxN4*: F—AGCCACACCCCAAACACTAC, R—AAGCTGCCTGTTTTGCTGTT; *GAPDH*: F–5′TGAAGGGGTCGTTGATGG3, R–5′AAAATGGTGAAGGTCGGTGT3′; *Math3*: F—CCATCTCAAGCCTCAACCAT, R—CTAATGCTCAGGGGTGGTGT; *Math5*: F—ATGGCGCTCAGCTACATCAT, R—GGTCTACCTGGAGCCTAGCA; *Pax6*: F—CGGCAGAAGATCGTAGAGC, R –TGGATACGTTTTCATTGTCCAG; *Ptch1*: F—TAGCCCTGTGGTTCTTGTCC, R—TGTGGTCATCCTGATTGCAT; *Rax*: F—TTCATGGACGACACTTCCAG, R—GTTAACCTACCCGAGGTTCG; *Sox2*: F—ACCTCTTCCTCCCACTCCAG, R—CTGGGCCATGTGCAGTCTAC; *Sufu*: F—ATTCAGCCCAACAGTGGAAC, R—CCGTCTGTCTAATGCCTTT. Transcript levels were normalized to GAPDH and fold change was calculated using 2^{-ddCt}. Primer specificity was confirmed by verifying PCR product sizes using electrophoresis and by sequencing PCR products (StemCore) after ligation into pGEM-T plasmid (Promega) as per manufacturer's instructions.

Luciferase activity. Luciferase activity was assayed using the Dual Luciferase Kit (Promega) according to manufacturer's instructions and quantified using a Lumat LB 9507 luminometer (Eg&G Berthhold). Briefly, explants were immersed in 500 μl of 1× passive lysis buffer solution (Promega), homogenized briefly, and rocked at room temperature for 30 min. The lysate was added to 200 μl of luciferin (Promega), luciferase activity was measured, and then 200 μl of Stop and Glo solution (Promega) was added to measure *Renilla* activity.

Image processing, data analysis, and statistics. Images were processed using Adobe Photoshop CS3. Images of control and mutant retinae were taken at the same exposure for each magnification. In the event that contrast/levels were adjusted, they were adjusted for the entire image, and images of both control and mutant retinae were adjusted equally. All scale bars measure 100 μm. For the *in vivo* analysis, at least three mutant mice from each age group were examined and compared with wild-type littermates. When counting dissociated cells, at least 150 transfected, GFP+ cells were scored per retinal explant. Cell quantification in embryonic retinal sections was performed by counting all the Dapi, Ki67, and BrdU-labeled cells in the ventral and dorsal retina in a region measuring 118 μm in length (along the proximal–distal axis of the retina) from three sections/retina at the level of the optic nerve head from a minimum of four eyes/genotype. The position of the *Sufu* knock-out (KO) region for counting was confirmed by ISH for *Sufu* on a serial section from the same retina. Staining was visualized as mentioned above for IHC. All data are presented as mean ± SEM. Statistical significance was evaluated using a two-tailed, unpaired Student's *t* test and $p < 0.05$ was considered statistically significant.

Results

Sufu antagonizes Hh signaling downstream of *Smo* in RPCs

Previously, we showed that the induction of *Shh* expression and *Hh* pathway activation in the developing mouse retina follows the central to peripheral wave of GC differentiation (Wang et al., 2005). Therefore, at early stages in retinal development, Hh activation is suppressed in RPCs that are located peripheral to the wave of GC differentiation. Although the expression patterns of *Gli1* and *Gli2* in the developing retina have been characterized (Wang et al., 2005; Furimsky and Wallace, 2006), we sought to compare the expression domains of these Hh pathway components with the pattern of *Sufu* expression. At E11.5, a stage before the development of Shh-expressing ganglion cells (Wang et al., 2005), the expression of *Gli1*, which is a *Hh* target gene (Lee et al., 1997), is undetectable in the retina (Fig. 1*A*). In contrast, both *Gli2* and *Sufu* are expressed throughout the neuroblast layer at this stage (Fig. 1*D*,*G*). At E13.5, the region of *Gli1* expression does not extend to the peripheral-most part of the retina (Fig. 1*B*), while both *Gli2* and *Sufu* continue to be expressed throughout the neuroblast layer (Fig. 1*E*,*H*). In the P0 retina, *Gli1*, *Gli2*, and *Sufu* are all coexpressed in the neuroblast layer throughout the retina (Fig. 1*C*,*F*,*I*). Thus, the expression of *Sufu* and *Gli2* in RPCs precedes Hh pathway activation in the retina and expression is maintained in the region of active Hh signaling.

To determine whether Sufu can functionally antagonize Smo signaling in RPCs, we investigated the effect of increased Sufu expression on Hh pathway activation in retinal explants. Coelectroporating explants with an expression vector for Smo-M2, a constitutively active form of *Smo* (Xie et al., 1998) that cell-autonomously activates the Hh pathway in RPCs (Yu et al., 2006; Wall et al., 2009) and a *Gli*-Luciferase reporter (*Gli*-Luc) (Sasaki et al., 1997) resulted in a significant increase in *Gli*-Luc activity compared with controls, and this effect was attenuated approximately threefold when explants were coelectroporated with a *Sufu* expression vector (Ding et al., 1999). (supplemental Fig. 1*A*, available at www.jneurosci.org as supplemental material). We next asked whether conditional inactivation of *Sufu* in RPCs was sufficient to activate the Hh pathway. Electroporating explants from $Sufu^{F/F}$ mice with an expression vector for Cre recombinase reduced wild-type levels of *Sufu* after 2.5 days *in vitro* (DIV) and 4 DIV in the transfected population (supplemental Fig. 1*B*, available at www.jneurosci.org as supplemental material) and increased *Gli*-luciferase activity by fivefold and 16-fold after 2.5 and 5 DIV, respectively (supplemental Fig. 1*C*, available at www.jneurosci.org as supplemental material). Thus, *Sufu* inhibits Hh signaling in RPCs, which is consistent with its previously reported antagonistic function on the Hh pathway (Ruel and Thérond, 2009).

Sufu is required *in vivo* to antagonize Hh signaling in RPCs

To study the functions of *Sufu in vivo*, we generated a retina-specific conditional knock-out (CKO) of *Sufu* by crossing $Sufu^{F/F}$ mice with a transgenic line that expresses *Cre recombinase* under

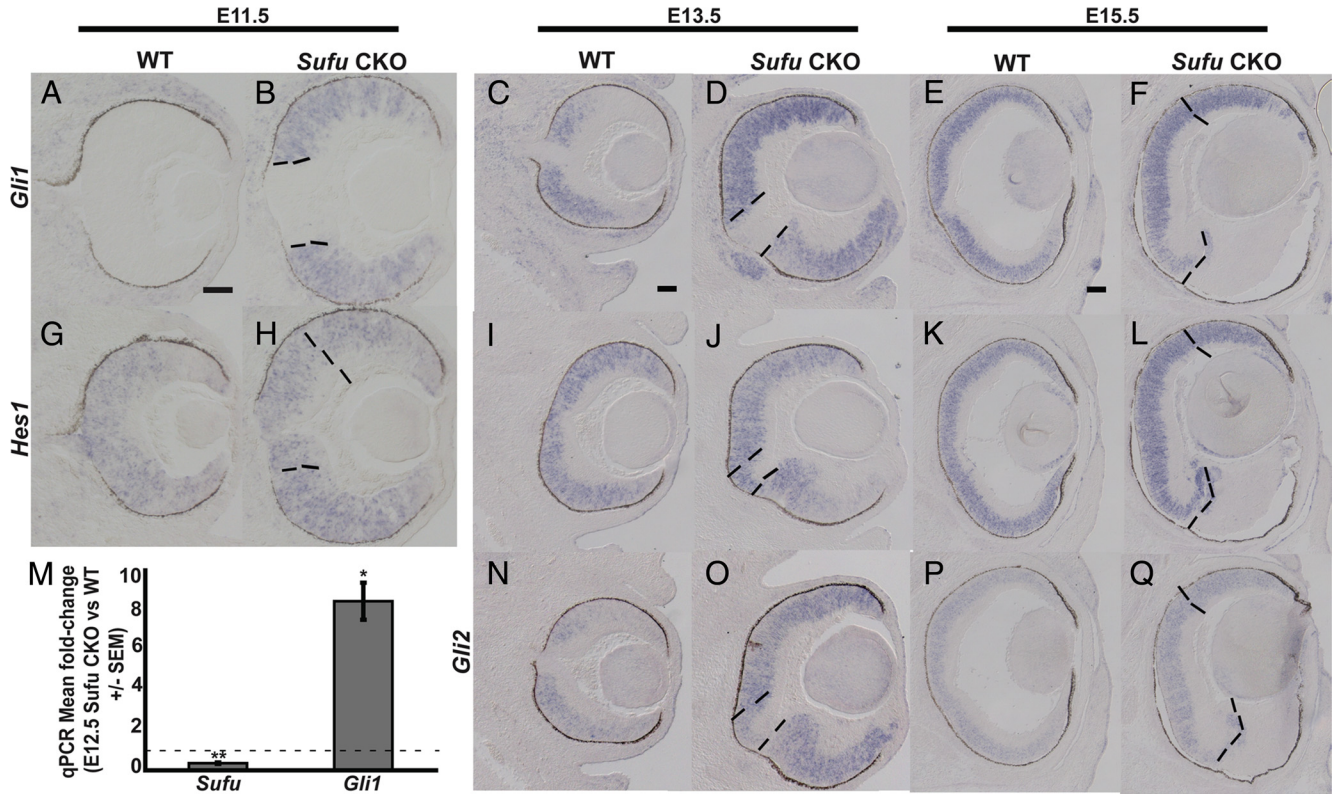

Figure 2. Increased levels of Hh signaling in *Sufu* CKO retinae. ***A–Q***, *In situ* hybridization for *Gli1* (***A–F***), *Hes1* (***G–L***), and *Gli2* (***N–Q***) mRNA in control and mutant retinae at E11.5, E13.5, and E15.5. Dorsal is up. Note the expanded domain of *Gli1* expression in the *Sufu* CKO retina. The dashed lines indicate the border of the mutant region in the peripheral ventral retina, which was determined by ISH for *Sufu* on a serial section from the same eye. Because of the sectorial gaps in Cre activity in this line, not all retinal sections will have *Sufu* deletion in the dorsal retina. Scale bars, 100 μm. ***M***, Mean fold-changes of *Sufu* and *Gli1* cDNA levels in whole *Sufu* CKO retinae compared with whole WT retinae at E12.5 normalized to GAPDH. Error bars represent SEM. $*p < 0.05$, $**p < 0.005$.

the control of the αP0 element of the *Pax6* promoter (*α-Cre*), which induces *Cre* expression in RPCs of the peripheral retina beginning at ~E10.5 (Marquardt et al., 2001) (supplemental Fig. 2*A*, available at www.jneurosci.org as supplemental material). For simplicity, Cre^{+}; $Sufu^{F/F}$ mice will be referred to as *Sufu* CKO mice, and control Cre^{-}; $Sufu^{F/F}$ mice will be referred to as wild type (WT). As we were unable to detect any overt differences in morphology or gene expression when comparing Cre^{+}; $Sufu^{+/F}$ and WT mice (data not shown), we restricted our analysis to WT and *Sufu* CKO mice. The *α-Cre* line exhibits a gap in cre-recombinase activity in the dorsal–temporal quadrant of the retina (Xu et al., 2007), such that Gfp expression (from the α-Cre transgene) (supplemental Fig. 2*B*, available at www.jneurosci.org as supplemental material) and *Sufu* downregulation are more prominent in the ventral compared with the dorsal retina in coronal sections from the temporal side of the eye (supplemental Fig. 2*B*, compare *E*, *F*, available at www.jneurosci.org as supplemental material). Therefore, because of the consistency of Cre activity across the ventral region of the retina, we have focused primarily on the phenotype in the ventral retina, and the region of *Sufu* deletion in the embryonic *Sufu* CKO retinas was confirmed by ISH for *Sufu* on serial sections.

To determine whether *Sufu* deletion in the embryonic retina resulted in Hh pathway activation, we examined the expression of *Gli1*. At E11.5 and E13.5, the domain of *Gli1* expression was expanded in the ventral and dorsal regions of the *Sufu* CKO retinae compared with WT (Fig. 2*B*,*D*). The increase in *Gli1* expression was confirmed by reverse transcription (RT)-qPCR analysis of whole mutant retinae at E12.5 (Fig. 2*M*). In contrast, the expression of *Hes1*, which is regulated by Hh during later stages of development (Wall et al., 2009), was expressed in the mutant retinae at E11.5 (Fig. 2*H*) but appeared to be reduced in the ventral region of the mutant retina at E13.5 (Fig. 2*J*). *Gli2* was expressed throughout the mutant regions of the retina at E13.5 (Fig. 2*O*). However, by E15.5, the expression of *Gli1*, *Hes1*, and *Gli2* was undetectable in the ventral region of the mutant retina (Fig. 2*F*,*L*,*Q*) and the expression of *Gli1* was patchy in the dorsal retina (Fig. 2*F*). Therefore, consistent with the effect observed in explant culture, genetic elimination of *Sufu* in RPCs *in vivo* results in ectopic Hh pathway activation, although this activation in the ventral retina appears to be transient *in vivo* as Hh signaling is not sustained in the *Sufu*-null region of the retina at later gestational and postnatal stages (data not shown).

Sufu deletion resulted in the formation of a prominent cellular mass in the peripheral ventral retina that was visible by E12.5 and that persisted in the adult eye (Fig. 3*A–H*) (data not shown). By contrast, the dorsal adult retina did not exhibit hyperplasia and appeared thinner and disorganized (Fig. 3*F*). Whole eyes of mutant mice were frequently larger than those of WT littermates (Fig. 3*I*). The *α-Cre* transgene is also expressed in amacrine cells in the central retina (Marquardt et al., 2001). The central retina of P0 *Sufu* CKO mice appeared relatively normal, with well demarcated ganglion and neuroblast layers, and the expression of the *α-Cre* transgene, marked by Gfp, in amacrine cells was readily apparent (supplemental Fig. 3*A–K*, available at www.jneurosci.org as supplemental material). By P21, the retina located outside the peripheral mass in the mutants exhibited extensive abnormalities that included rosettes, folds, and disrupted lamination

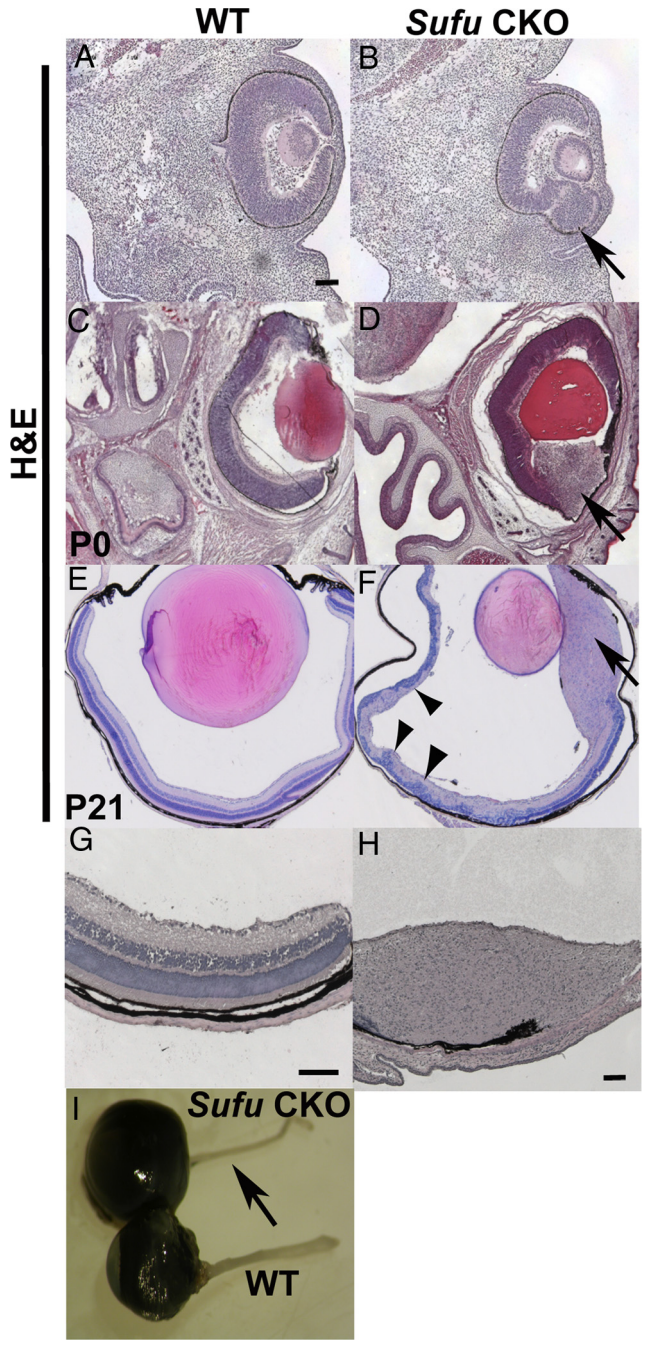

Figure 3. The retinae of *Sufu* CKO mice display severe morphological defects. ***A–H***, Hematoxylin and eosin (H&E) staining of wild-type and mutant retinas at E12.5 (***A***, ***B***), P0 (***C***, ***D***), P21 (***E***, ***F***), and P18 (***G***, ***H***). Note the formation of a cellular mass in the ventral peripheral retina in ***B***, ***D***, ***F*** (arrow). At P21, the remaining retina appears thinner with areas of abnormal lamination (***F***, arrowheads) (***H***). High-magnification view of the ventral mass of the mutant eye at P18 reveals absence of lamination in this region (***I***). Scale bars, 100 μm. ***I***, Dissected eyes and optic nerves from a WT and *Sufu* CKO mouse at P18. Note that the mutant eye is larger than the WT eye, and the optic nerve of the mutant eye is notably thinner (arrow).

(Fig. 3*F*; supplemental Fig. 3*L*, available at www.jneurosci.org as supplemental material). Whether these central retinal abnormalities are a direct consequence of *Sufu* deletion in amacrine cells or are secondary to the hyperplasia in the peripheral retina is difficult to determine, and for this reason we restricted our analysis to the phenotype associated with *Sufu* deletion in the peripheral retina.

Sufu-null progenitors differentiate exclusively into horizontal and restricted classes of amacrine interneurons

To identify the types of differentiated cells in the *Sufu* CKO ventral retina, we performed IHC for lineage-specific markers. At P0, the *Sufu*-null region of the retina can be identified based on the distinct morphological characteristics as well as by the expression of the ROSA-β-Gal cre reporter, which overlaps completely with the hyperplastic region in the ventral retina (supplemental Fig. 4*A*,*B*, available at www.jneurosci.org as supplemental material). At P0, we found that expression of syntaxin, which marks amacrine and horizontal cells, was expanded in the ventral retina (supplemental Fig. 4*C*,*D*, available at www.jneurosci.org as supplemental material), suggesting that the cells differentiated as interneurons. Cells within the *Sufu*-null region of the retina also expressed the amacrine/GC marker Isl1 (supplemental Fig. 4*E*–*F*′, available at www.jneurosci.org as supplemental material) and the horizontal interneuron marker Lim1/2 (supplemental Fig. 4*G*–*H*′, available at www.jneurosci.org as supplemental material). Because several of the cell-type specific markers are not expressed until late in development, we conducted further analysis of P18–P21 retinae, a stage when retinal histogenesis is complete. The mutant region of the adult *Sufu* CKO retina was identified morphologically, based on the increase in thickness and abnormal spacing of the cells that was a consistent feature of the peripheral mutant retina at this stage. Consistent with our analysis at P0, the majority of the cells in the *Sufu*-null region of the retina at P18 expressed syntaxin (Fig. 4*B*,*C*). Significant numbers of cells stained for the amacrine marker calretinin (Fig. 4*E*,*F*), although at lower intensity compared with the WT retina (Fig. 4*D*). A subset of cells also expressed the amacrine/horizontal/bipolar marker Prox1 (Fig. 4*H*,*I*) and the amacrine/horizontal marker calbindin (data not shown). In contrast, very few, if any, of the cells in the mutant region expressed markers exclusive to other differentiated cell types, including GCs (data not shown), bipolar cells, photoreceptors or Müller glia (supplemental Fig. 5*A*–*H*, available at www.jneurosci.org as supplemental material). Amacrine cells are extremely diverse, and can be subclassed on the basis of their expression of neurotransmitters or proteins involved in neurotransmitter metabolism (Nguyen-Legros et al., 1997; Demb, 2007). Some interneurons in the *Sufu*-null region were dopaminergic, based on the expression of tyrosine hydroxylase (TH2) (Fig. 4*K*,*L*). In contrast, very few, if any, of the cells within this region were glycinergic or cholinergic, based on the absence of glycine transporter 1 (Gly-T1) (Fig. 4*N*,*O*) or choline acetyltransferase (ChAT) (Fig. 4*S*,*T*), respectively. Although we detected expression of GAD65, which converts glutamate to GABA, within the mutant region (Fig. 4*W*,*X*), the expression of GABA (Fig. 4*Q*,*R*) was weak to undetectable, suggesting that GABAergic differentiation was aberrant. Our results suggest that *Sufu* deletion leads to a loss of cell diversity and nearly all cells in the *Sufu*-null region of the retina exhibit an interneuron phenotype. Importantly, although we observed a consistent phenotype in the mutant region of the adult *Sufu* CKO retinae, we cannot rule out the possibility that the effects observed at or near the WT-mutant border occur due to non-cell autonomous processes, such as cell mixing or disruption of the physical architecture of the retina.

Sufu is required to maintain RPC multipotency

To gain insight into the developmental origin of the postnatal *Sufu* CKO phenotype, we addressed whether the effect of *Sufu* deletion in the ventral retina was associated with perturbed expression of transcription factors that regulate RPC proliferation,

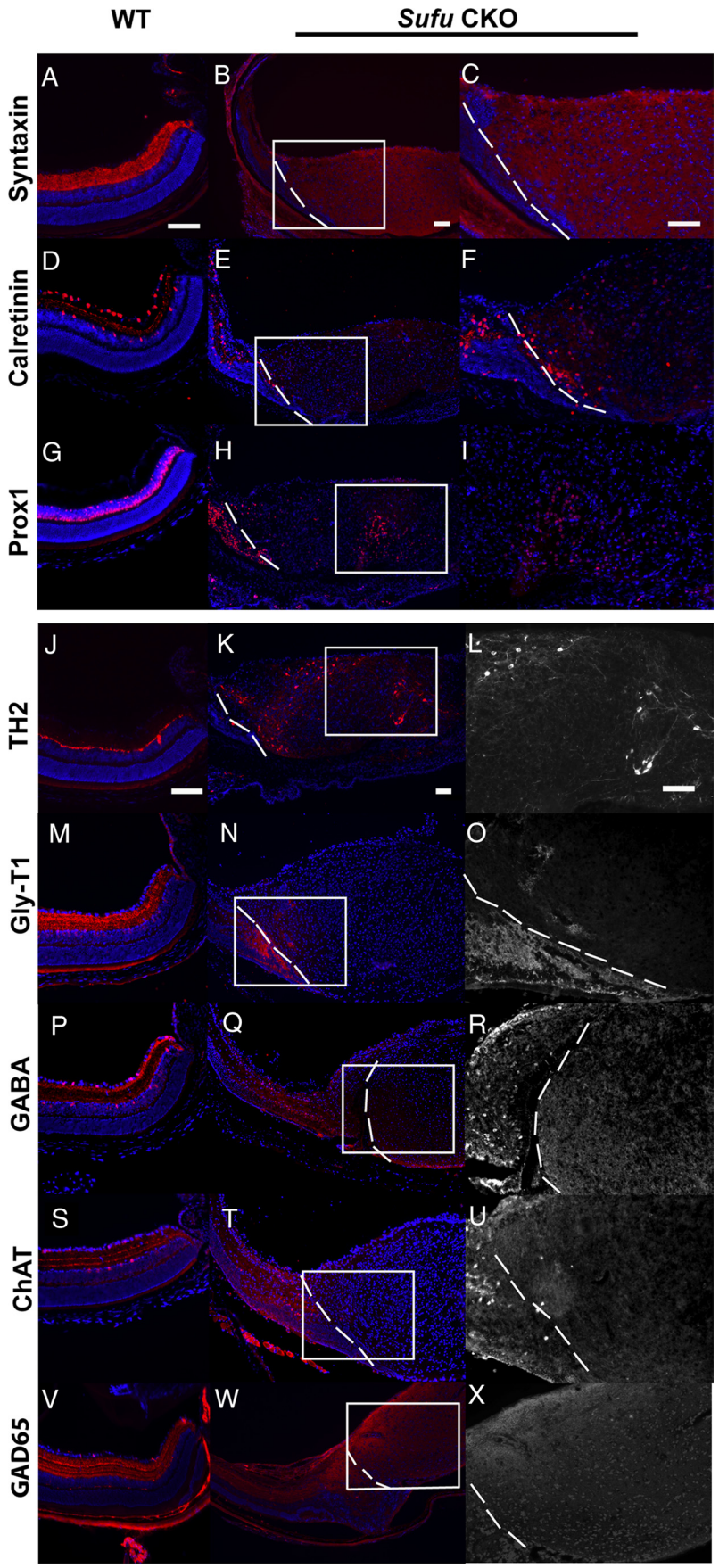

Figure 4. Cells in the peripheral retina of *Sufu* CKO mice differentiate into a restricted subset of interneurons. ***A–X***, Fluorescent immunohistochemistry for interneuron markers syntaxin (***A–C***), calretinin (***D–F***), Prox1 (***G–I***), TH2 (***J–L***), Gly-T1 (***M–O***), GABA (***P–R***), ChAT (***S–U***), and GAD65 (***V–X***) in the retinas of 3-week-old wild-type and Sufu CKO mice. Nuclei are counterstained with DAPI. ***C***, ***F***, ***I***, ***L***, ***O***, ***R***, and ***U*** are high-magnification views of the boxed region in the images to the left of those panels. Dashed lines in ***C***, ***F***, ***O***, ***R***, ***U***, and ***X*** delineate the border between WT (left) and mutant tissue (right) in the *Sufu* CKO retina to provide comparison of the immunofluorescent signal in the WT and mutant regions of the *Sufu* CKO retinae. Scale bars, 100 μm.

neurogenesis, and multipotency at earlier stages in development. Cells in the embryonic *Sufu* CKO ventral retina expressed Sox2, consistent with their neural precursor status (Fig. 5*B*,*D*). Unexpectedly, however, *Sufu* deletion was associated with the dysregulation of transcription factors that are required to maintain RPC function. The expression of *Pax6*, *Rax*, and *Vsx2* was downregulated in the *Sufu* CKO peripheral retina at E12.5 and E14.5 (Fig. 5*E–P*). Quantification of transcript levels in whole mutant eyes confirmed the reduction in *Pax6* and *Rax* expression at E12.5 and E14.5 and an upregulation of *Sox2* expression (Fig. 5*Q*). Based on the requirement for *Pax6* in the maintenance of RPC multipotentiality (Marquardt et al., 2001; Oron-Karni et al., 2008), the downregulation of *Pax6* in the *Sufu*-null region of the retina suggested that RPC multipotency is compromised.

To investigate the consequences of *Sufu* deletion on cell-fate diversification, we monitored the expression of transcription factors that are involved in lineage determination or that are expressed in postmitotic lineage-committed precursor cells. *Math5*, which is required to specify GC fate (Brown et al., 2001; Wang et al., 2001), was downregulated in *Sufu*-null region of the ventral retina at E12.5 and E14.5 (Fig. 6*A–D*). Consistent with the loss of *Math5* expression, we did not detect GC differentiation in the *Sufu*-null region of the ventral retina during embryogenesis (Fig. 6*E–H*) or at P0 (supplemental Fig. 4*I–J′*, available at www.jneurosci.org as supplemental material), indicating that GC differentiation in this region was abrogated, rather than delayed. Similarly, the expression of *Foxn4* and *Crx*, which are required for amacrine/horizontal cell (Li et al., 2004) and rod/cone photoreceptor development (Furukawa et al., 1999), respectively, was also undetectable in the *Sufu*-null region of the ventral retina at E12.5 and E14.5 (Fig. 6*I–O*) (data not shown). Consistent with the loss of *Foxn4* expression, the expression of its downstream targets, *Math3* and *NeuroD1* (Li et al., 2004), was also undetectable in the *Sufu*-null region (Fig. 6*O*) (data not

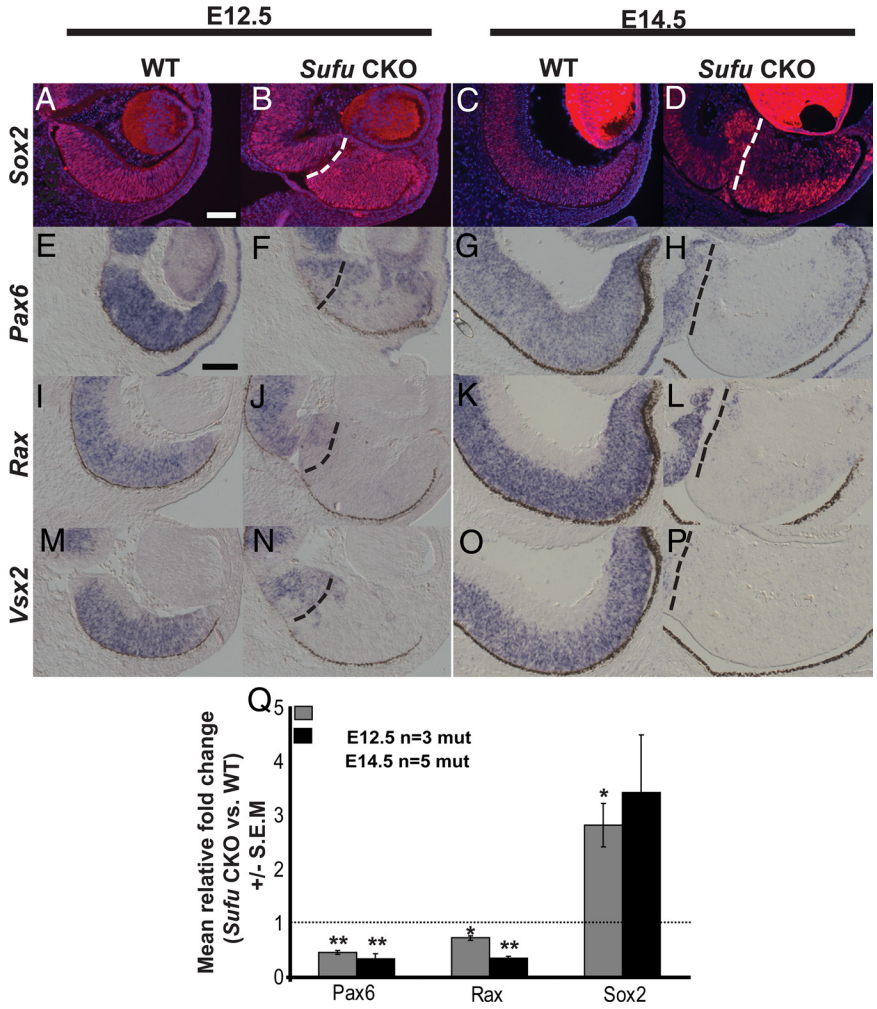

Figure 5. Cells in the peripheral ventral retina of *Sufu* CKO mice exhibit aberrant expression of transcription factors required to maintain the multipotent and proliferative capacity of RPCs. ***A–P***, Fluorescent immunohistochemistry for *Sox2* (***A–D***) and *in situ* hybridization for *Pax 6* (***E–H***), *Rax* (***I–L***), and *Vsx2* (***M–P***) in the retinae of wild-type and mutant (mut) mice at E12.5 and E14.5. Dashed lines delineate the border of the mutant region in the peripheral ventral retina. Note the downregulation or absence of *Pax6*, *Rax*, and *Vsx2* in the peripheral retinae of *Sufu* CKO mice. Scale bars, 100 μm. ***Q***, RT-qPCR was used to determine mean fold-changes of *Pax6*, *Rax*, and *Sox2* cDNA levels in whole *Sufu* CKO retinae compared with whole WT retinae at E12.5 and E14.5 normalized to GAPDH. Error bars represent SEM. $^*p < 0.05$, $^{**}p < 0.005$.

shown). The absence of *Rax* and *Vsx2* expression, which are required for Müller glia (Furukawa et al., 2000) and bipolar cell (Burmeister et al., 1996; Livne-Bar et al., 2006) development, respectively, was also consistent with the absence of these differentiated cell types at later stages in the *Sufu* KO retina. The downregulation of these transcription factors in E14.5 *Sufu* CKO retinae was confirmed with RT-qPCR (Fig. 6*O*). In contrast to the ventral retina, in retinal sections where there was clear deletion of Sufu in the dorsal retina, we observed a consistently weak reduction of *Math5* (supplemental Fig. 2, compare *H*, *J*, available at www.jneurosci.org as supplemental material) (data not shown) *FoxN4*, *NeuroD*, and *Crx* (data not shown) expression.

The dramatic changes in gene expression in *Sufu*-null cells were not associated with altered cell survival, as we did not detect an increase in TUNEL-positive cells in the ventral portion of the retina at E12.5, E14.5, or E15.5 (data not shown). We detected normal expression patterns of *Pax2* and *Otx1*, which are markers of optic stalk and ciliary margin, respectively (supplemental Fig. 6, available at www.jneurosci.org as supplemental material), indicating that cells in the mutant region had not transdifferentiated into other ocular tissue types. Therefore, in the absence of *Sufu*, ventral RPCs show aberrant expression of transcription factors that are required to maintain RPC multipotency and identity, and fail to initiate the expression of transcription factors for the specification and/or differentiation of all seven retinal cell lineages. Our data demonstrate a novel role for *Sufu* in the maintenance of RPC multipotency during early retinal development.

Sufu-null cells prematurely exit the cell cycle

We next sought to characterize the effect of *Sufu* on RPC proliferation. Acute *Sufu* deletion in perinatal retinal explants increased proliferation nearly threefold (Fig. 7*A*), indicating that Sufu is a potent negative regulator of RPC proliferation. However, quantitative analysis of the cycle kinetics *in vivo* revealed that the *in vivo* effects of Sufu loss on RPC proliferation are complex. *In vivo*, the *Sufu*-null region of the ventral, and to a lesser extent the dorsal, retina is hyperplastic (Fig. 7*L*). At E11.5, Sufu loss in both regions is associated with a significant increase in the BrdU labeling index (Fig. 7*L*), indicative of a faster cell cycle at this stage. By E13.5, however, we noted differences in cell-cycle kinetics between the ventral and dorsal RPCs. Sufu loss in ventral RPCs resulted in a slowing of the cell cycle and premature cell-cycle exit, as shown by the reduction in Ki67+ cells beginning at E14.5 (Fig. 7*C–E*,*L*). By E15.5 and onwards there were no cycling cells present in the ventral retina (Fig. 7*G*) (data not shown). The reduction in cell-cycle rate and premature cell-cycle exit was associated with ectopic expression of the cyclin-dependent kinase inhibitor $p57^{Kip2}$ in the *Sufu*-null region of the ventral retina at E12.5 and E14.5 (Fig. 7*I*,*K*) and a reduction in the anti-neurogenic factor, *Hes1* expression (Fig. 2*D*,*J*). In contrast, dorsal RPCs did not undergo premature cell-cycle exit (Fig. 7*L*). Thus, initially, dorsal and ventral RPCs respond to Sufu loss by faster cycling, which is followed by a slowing of the cell cycle and in the case of ventral progenitors, premature cell-cycle exit.

Aberrant Hh activity and loss of RPC identity in Sufu CKO retinae is mediated by Gli2

Sufu binds all three Gli proteins and has recently been shown to be an important regulator of *Gli2* and *Gli3* processing (Humke et al., 2010; Wang et al., 2010). However, the epistatic relationship between *Sufu* and *Gli2 in vivo* is unknown. As *Gli2* is the primary activator of Hh signaling *in vivo* (Bai et al., 2002) and is required for full Hh pathway activation in RPCs (Wall et al., 2009), we sought to determine whether genetic elimination of *Gli2* could rescue the effects of *Sufu* deletion in RPCs. In retinal explants, genetic elimination of *Gli2* was sufficient to prevent the increase

in *Gli*-Luc activity observed after acute *Sufu* deletion in a dose-dependent manner (Fig. 8*A*), indicating that transcriptional activation of the Hh pathway in the context of *Sufu* deletion is mediated by *Gli2*.

We next asked whether genetic elimination of *Gli2* was sufficient to rescue the effects of conditional *Sufu* deletion *in vivo* by generating *Sufu* CKO;*Gli2*$^{-/-}$ (double KO) mutant mice. Because homozygous deletion of *Gli2* is lethal at late stages in embryonic development (Mo et al., 1997), the retinae of *Sufu* CKO; *Gli2*$^{-/-}$ mice were examined at E14.5. *Gli2* deletion alone is not sufficient to dramatically alter the expression of *Sufu* (supplemental Fig. 2*E*, available at www.jneurosci.org as supplemental material), the proneural gene, *Math5* (supplemental Fig. 2, available at www.jneurosci.org as supplemental material), or *Pax6* and *Vsx2* (Furimsky and Wallace, 2006). Remarkably, removal of *Gli2* in *Sufu* CKO retinae restored RPC multipotentiality based on the upregulation of *Pax6* and *Rax* expression in double KO retinae compared with *Sufu* CKO retinae (Fig. 8*B–G*). In addition, the expression of *Foxn4*, *Math3*, *Math5*, and *Crx* were restored in double KO retinae (Fig. 8*H–S*), although not to the levels observed in WT retinae. Together, these data demonstrate that Sufu regulation of Gli2 activity is required for RPC multipotentiality.

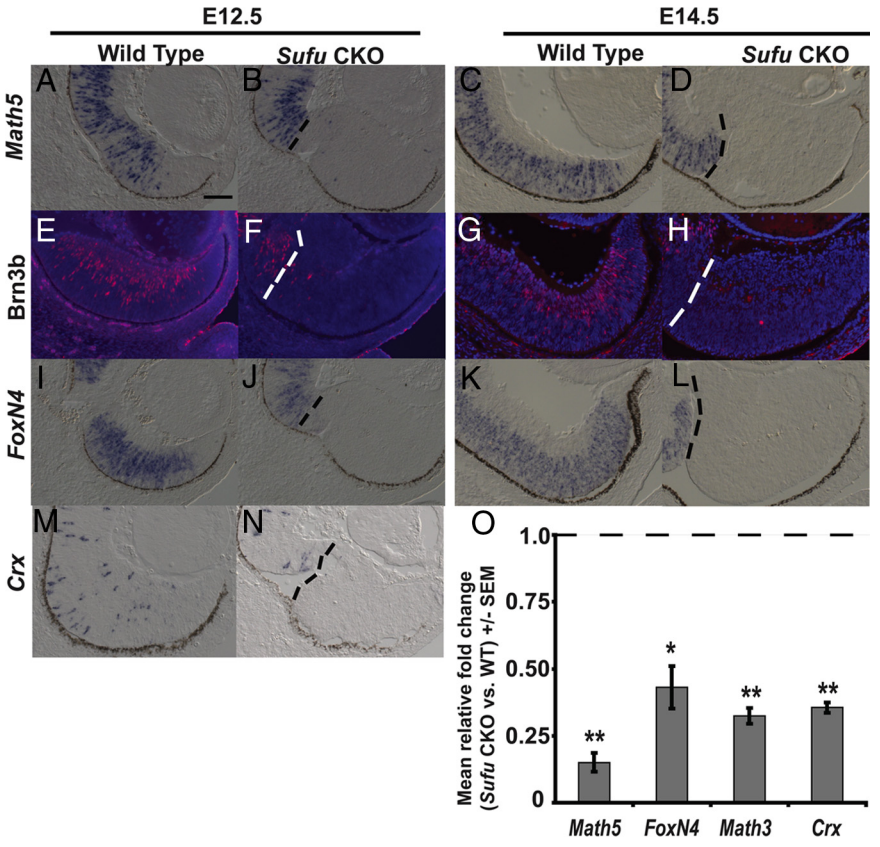

Figure 6. Multipotency is lost in Sufu-null RPCs. ***A–N***, *In situ* hybridization for *Math5* (***A–D***), *FoxN4* (***I–L***), and *Crx* (***M–N***) and fluorescent immunohistochemistry for Brn3b (***E–H***) with nuclei counterstained with DAPI in WT and *Sufu* CKO retinae at E12.5 and E14.5. Dashed lines delineate the border of the mutant region in the ventral peripheral retina. Note the absence of *Math5*, Brn3b, *FoxN4*, and *Crx* expression in the peripheral retinae of *Sufu* CKO mice. Signal in the mutant region in ***D*** is nonspecific staining that does not correspond to nuclei. Scale bar, 100 μm. ***O***, RT-qPCR was used to determine mean fold-changes of *Math5*, *FoxN4*, *Math3*, and *Crx* levels in whole E14.5 *Sufu* CKO retinae compared with whole WT retinae normalized to GAPDH. $n = 5$ retinae per genotype. Error bars represent SEM. *$p < 0.005$; **$p < 0.05$.

Discussion

Shh signaling to progenitor cells in the developing CNS is required for fate specification, proliferation, and maintenance of the undifferentiated state. Here, we have investigated the requirement for Sufu, a downstream regulator of the Shh response, in RPCs. Our results indicate that Sufu acts downstream of Smo to suppress Gli2 activity and Hh pathway activation. Functionally, *Sufu* is required to antagonize RPC proliferation and, unexpectedly, it is also required for sustained expression of the retinal determination gene network and the maintenance of multipotentiality of RPCs. Finally, we show that the effect of *Sufu* on RPC multipotentiality is mediated primarily through *Gli2*. In summary, Sufu antagonism of Gli2 identifies a novel intrinsic component of RPC multipotentiality.

Consistent with previous studies that have characterized *Sufu* as a critical regulator of mammalian Hh signaling (Taylor et al., 2002; Cooper et al., 2005; Svard et al., 2006; Varjosalo et al., 2006), we show that conditional deletion of *Sufu* in RPCs *in vitro* and *in vivo* results in aberrant Hh pathway activity, increased proliferation, and hyperplasia. The transient increase of Hh signaling that we observe in the *Sufu* CKO retina could be caused by a failure to sustain the Gli2 expression and/or activity, which is consistent with labile nature of Gli proteins in Hh-activated cells (Humke et al., 2010) and a requirement for *Sufu* in promoting Gli stabilization (Chen et al., 2009). Interestingly, proliferation was also not maintained indefinitely in the retinae of *Sufu* CKO mice, and *Sufu*-null progenitors in the ventral retina exited the cell-cycle en mass between E14.5 and E15.5. There were differences in the severity of the phenotype between dorsal and ventral *Sufu* CKO RPC, the latter exhibiting a stronger phenotype. This could reflect dorsal–ventral differences in Sufu function or may be due to secondary effects related to incomplete deletion of Sufu in the dorsal retina.

The precocious cell cycle exit of ventral RPCs in the *Sufu* CKO mouse may be mediated by three nonmutually exclusive mechanisms. First, it could be secondary to the downregulation of Hh activation in this model. Second, deregulated expression of cell-cycle genes could contribute to early cell-cycle exit in *Sufu*-null progenitors. Conditional deletion of *Sufu in vivo* resulted in an increase in the cell-cycle rate followed by ectopic expression of the cell-cycle inhibitor $p57^{Kip2}$ at a stage prior to and during the timing of the downregulation of Ki67 expression. $p57^{Kip2}$ directs cell-cycle exit in a subpopulation of RPCs during retinogenesis (Dyer and Cepko, 2000), and Hh signaling is required for $p57^{Kip2}$ expression and cell-cycle exit in the zebrafish retina (Shkumatava and Neumann, 2005; Locker et al., 2006). While sufficient evidence supports the mitogenic role for *Shh* in mammalian RPCs (Jensen and Wallace, 1997, Yue et al., 2009, Wall et al., 2009), there is little evidence to suggest a role for *Shh* in driving cell-cycle exit in mammalian RPCs. Although future studies are required, our data indicate that *Hh* signaling may also control cell-cycle exit by modulating $p57^{Kip2}$ expression in mammalian RPCs, thus providing further evidence of Hh pathway conservation. Third, *Sufu* regulation of retinal determination genes could also affect RPC

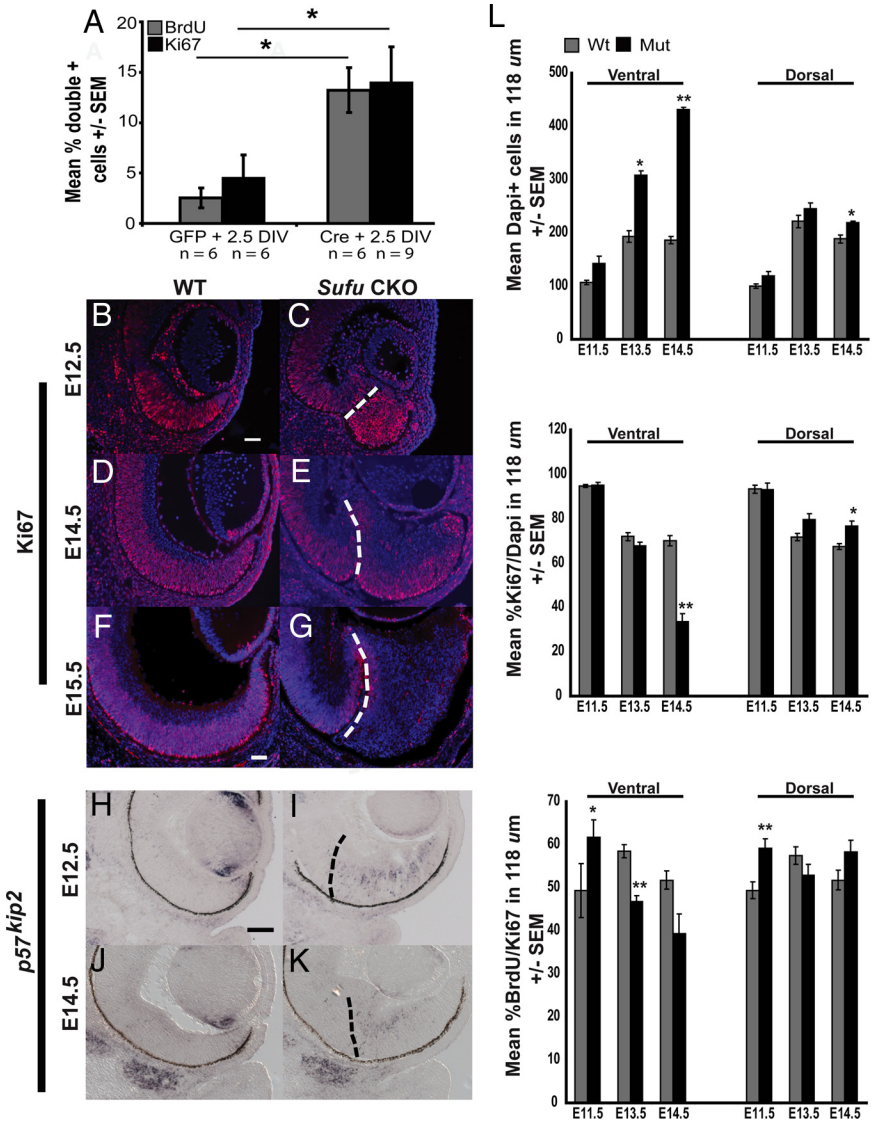

Figure 7. Sufu deletion results in aberrant RPC proliferation. ***A***, Acute deletion of *Sufu* induces proliferation in RPCs. P0 $Sufu^{F/F}$ retinas were coelectroporated with expression vectors for *eGFP* and *Cre* or *eGFP* and an empty vector and cultured as explants for 2.5 DIV. Explants were then pulsed with BrdU for 5 h, dissociated, and GFP+ cells were scored for the expression of Ki67 or BrdU incorporation. ***B–K***, Immunostaining for Ki67 (***B–G***) and *in situ* hybridization for $p57^{Kip2}$ (***H–K***) in the retinae of WT and *Sufu* CKO mice at the indicated ages. Nuclei are counterstained with DAPI. In the WT retina, RPCs are localized to the neuroblast layer and express Ki67 at E12.5 (***B***), E14.5 (***D***), and E15.5 (***F***). Cells in the mutant region of the retina express Ki67 at E12.5 (***C***) and E14.5 (***E***). However, few cells in the mutant region were Ki67+ at E15.5 (***G***). The cell-cycle inhibitor *p57(Kip2)* is upregulated in the ventral retina of *Sufu* CKO mice at E12.5 (***H***, ***I***) and E14.5 (***J***, ***K***). Dashed lines delineate the border of the mutant region in the ventral peripheral retina. Scale bars, 100 μm. ***L***, Quantitative analysis of cell number (DAPI), progenitor cells (%Ki67+), and BrdU labeling index (% BrdU+Ki67+/Ki67+) in the ventral and dorsal retina of wild-type and Sufu CKO mice at E11.5 (WT, $n = 6$; mutant, $n = 5$ eyes), E13.5 (WT, $n = 8$; mutant, $n = 7$ eyes), and E14.5 (WT, $n = 6$; mutant, $n = 4$ eyes). All error bars represent SEM. *$p < 0.05$; **$p < 0.005$. mut, Mutant.

proliferation. Proliferation defects are observed in RPCs lacking *Pax6* (Marquardt et al., 2001), *Rax* (Zhang et al., 2000), *Vsx2* (Burmeister et al., 1996; Green et al., 2003), and *Hes1* (Lee et al., 2005). The marked downregulation of *Pax6*, *Rax*, and *Vsx2* in concert followed by the loss of *Hes1* may also contribute to the inability of *Sufu*-null cells to remain in cycle indefinitely.

We show that conditional deletion of *Sufu* resulted in the downregulation of *Pax6*, which is consistent with the antagonistic relationship between high levels of Shh signaling and *Pax6* expression in the neural tube (Ericson et al., 1997). Analysis of mutant retinae at P18 revealed that *Sufu*-null progenitors were restricted toward an interneuron lineage. This phenotype is consistent with that observed in the retinae of mice with a conditional deletion of *Pax6* (*Pax6 CKO*) (Marquardt et al., 2001). However, several observations suggest that the *Sufu* CKO phenotype is not exclusively the result of altered *Pax6* expression. *Sufu* CKO RPCs downregulate transcription factors, including Rax, NeuroD, Vsx2, and Crx that continue to be expressed in *Pax6* CKO retinae (Marquardt et al., 2001; Oron-Karni et al., 2008), indicating that *Sufu* regulates processes upstream of, and/or parallel to, *Pax6* (Oron-Karni et al., 2008). Although progenitors from both *Sufu* CKO and *Pax6* CKO mice adopt an interneuron fate, the compliment of neural-subtypes is markedly different. In the *Pax6* CKO retina, all neurotransmitter subtypes were made with the exception of GABAergic interneurons (Marquardt et al., 2001). Conversely, dopaminergic neurons, but not glycinergic, cholinergic, or GABAergic neurons were detected in *Sufu* CKO retinae. A subset of cells within the *Sufu*-null region is Lim1/2-positive, suggestive of horizontal cell differentiation. However, there is no evidence for horizontal cell differentiation in the *Pax6* CKO, although the expression pattern of Lim1 in this model has not been reported. Finally, the consequences of *Pax6* and *Sufu* deletions on RPC proliferation are markedly different. Proliferation is markedly reduced at embryonic stages in *Pax6* CKO retina (Marquardt et al., 2001) but not in the *Sufu* KO retina, which is hyperplastic. This observation also illustrates that regulation of the cell cycle can be separated from the loss of multipotency associated with Pax6 downregulation, which is consistent with previous studies demonstrating the uncoupling of cell fate and proliferation in Hh pathway mutants (Sakagami et al., 2009). Therefore, although the observed downregulation of *Pax6* certainly contributes to the overall *Sufu*-null phenotype, Sufu likely exerts some Pax6-independent effects on proliferation.

Sufu regulates the localization of all three *Gli* transcription factors (Barnfield et al., 2005) and the posttranslational processing of both *Gli2* (Chen et al., 2009) and *Gli3* (Chen et al., 2009; Jia et al., 2009). *Gli2* is the predominant activator of Hh signaling *in vivo*, including in RPCs (Bai et al., 2002; Wall et al., 2009); however, the functional importance of *Sufu*-mediated antagonism of *Gli2 in vivo* has not been reported. The inability of $Gli2^{-/-}$ explants to induce *Gli*-dependent transcriptional activation in the context of *Sufu* loss-of-function indicated that *Gli2* is required to induce Hh pathway activation in the absence of *Sufu*. Genetic deletion of *Gli2* in *Sufu* CKO retinae *in vivo* partially rescued the expression of transcription factors, such as *Pax6* and *Rax*. Moreover, subsets

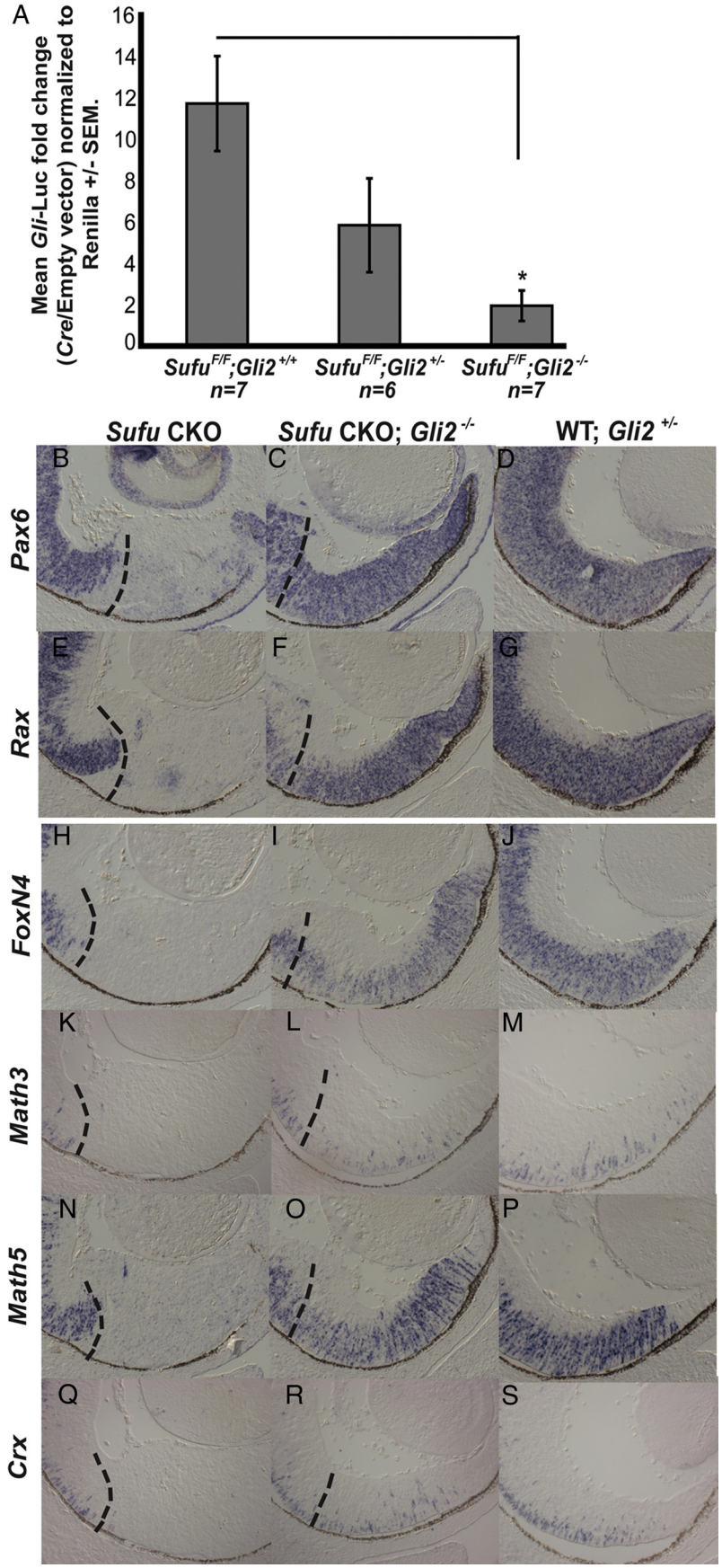

Figure 8. Deletion of *Gli2* partially recues the aberrant *Hh* activity and multipotency in *Sufu* CKO retinae. ***A***, Genetic deletion of *Gli2* inhibits *Gli*-dependent transcription in the context of conditional *Sufu* deletion. Retinas from P0 $Sufu^{F/F};Gli2^{+/+}$, $Sufu^{F/F};Gli2^{+/-}$, or $Sufu^{F/F};Gli2^{-/-}$ mice were coelectroporated with expression vectors for *Gli-luc*, *Renilla luiferase*, *Cre* or *Gli-luc*, and *Renilla* and an empty vector and cultured as explants for 2.5 DIV and 5 DIV, and luciferase activity was measured. Error bars represent SEM. *$p < 0.05$. ***B–S***, Genetic deletion of *Gli2* rescues multipotency in *Sufu*-null RPCs *in vivo*. *In situ* hybridization for *Pax6* (***B–D***), *Rax* (***E–G***), *FoxN4* (***H–J***), *Math3* (***K–M***), *Math5* (***N–P***), and *Crx* (***Q–S***) in the retinae of Sufu CKO; $Gli2^{+/+}$, Sufu CKO;$Gli2^{-/-}$, and $Gli2^{+/-}$ mice at E14.5. There was no difference in gene expression between $Gli2^{+/+}$ and $Gli2^{+/-}$ mice (data not shown). Dashed lines delineate the border of the mutant region in the ventral peripheral retina.

of *Sufu* CKO;$Gli2^{-/-}$ RPCs expressed *Math5*, *Math3*, and *Crx*, which are markers of postmitotic retinal precursors (Ohsawa and Kageyama, 2008).Together, these data demonstrate that *Sufu*-mediated antagonism of *Gli2* is required to maintain multipotency in early RPCs. *Sufu* deletion increases the ratio of Gli3 activator:Gli3 repressor (Jia et al., 2009), raising the possibility that deregulated Gli3 activity contributes to the Sufu CKO phenotype in the retina. However, we observed no difference in the phenotypes of *Sufu* CKO;$Gli3^{+/-}$ retinae compared with *Sufu* CKO retinae (Cwinn and Wallace, unpublished observations). Additionally, the RPCs of $Gli3^{-/-}$ mice, which also exhibit a Hh gain-of-function, presumably due to a loss of Gli3-repressor activity (Wang et al., 2000), express *Pax6* and *Vsx2* (Furimsky and Wallace, 2006), indicating that Gli3 repressor is not required to maintain RPC identity. Although the partial rescue in *Sufu* CKO;$Gli2^{-/-}$ retinae could be due to an increase in Gli3 activator (Jia et al., 2009) or compensation by Gli1 (Kogerman et al., 1999; Taylor et al., 2002; Barnfield et al., 2005), our data strongly suggest that *Gli2* is the principal mediator of Hh signaling in the absence of *Sufu* in RPCs.

At early stages in retinal development, induction of Hh signaling lags behind the central to peripheral wave of GC differentiation (Wang et al., 2005). Our findings suggest that *Sufu* is required in RPCs located ahead of the differentiation wave to maintain RPC multipotency. At later stages in retinal development, Sufu function is still required to control Hh activation and RPC proliferation. In the developing spinal cord, the intensity of Hh signaling in progenitors is determined by their position within the ventral to dorsal gradient of the Shh protein from its source in the ventral floor plate, with the most ventral cells having the highest level of signaling (Jacob and Briscoe, 2003). In this context, Sufu deletion mimics a high Shh signal and causes cells in the neural tube to adopt the most ventral cell

fates (Svard et al., 2006). Because RPCs and *Shh*-expressing GCs are equidistant in the retina, it is less obvious how graded responses to Shh are achieved in this tissue. Nonetheless, it is tempting to speculate that the consequences of *Sufu* deletion in embryonic RPC, namely amacrine cell development, mimic the highest level of Shh signaling. In summary, the data presented here implicates *Sufu* as a novel regulator of the Hh pathway in RPCs and further confirms that *Sufu* is a critical antagonist of the *Hh* pathway in mammalian systems. The unexpected requirement for *Sufu* in the maintenance of early RPC multipotency suggests that *Sufu*-mediated antagonism of *Gli2* is required to mediate the intrinsic behavior of RPCs in response to extrinsic cues.

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