Temporal Requirement of the Protein Tyrosine Phosphatase Shp2 in Establishing the Neuronal Fate in Early Retinal Development

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FGF signaling is critical in the development of the vertebrate retina, which differentiates in a wave-like pattern similar to that found in the *Drosophila* eye. In this study, we investigated the mechanism of FGF signaling in vertebrate eye development by identifying Shp2, a protein tyrosine phosphatase, as a novel factor in orchestrating retinal morphogenesis. Using a series of *Shp2* conditional mutants, we have shown that *Shp2* is specifically required for the initiation of retinal neurogenesis but not for the maintenance of the retinal differentiation program. By mosaic deletion of *Shp2*, we further demonstrated that *Shp2* ablation did not prevent the spreading of the retinal differentiation wave. Shp2 instead controls the patterning of the optic vesicle by regulating the retinal progenitor factors and cell proliferation. In *ex vivo* culture models and genetic rescue experiments, we showed that *Shp2* acts downstream to FGF signaling in retinal development and that it can be functionally substituted by activated Ras signaling. Together, these results demonstrate that Shp2 mediates FGF–Ras signaling to control retinal progenitor cell fate.

Introduction

FGF signaling is required for both early patterning of the optic vesicle and the subsequent retinal differentiation wave. When the chick optic vesicle was stripped off the surface ectoderm (the source of Fgf1 and Fgf2), it developed into a mixture of neural and pigmented cells unless FGF-expressing fibroblasts or viruses were added to the optic vesicle culture (Pittack et al., 1997; Hyer et al., 1998). Numerous explant culture studies have also shown that ectopic FGF expression could transform the retinal pigmented epithelia (RPE) into a neural retina (Park and Hollenberg, 1989; Guillemot and Cepko, 1992; Zhao et al., 1995; Pittack et al., 1997). This appeared to be mediated by Ras–mitogen-activated protein kinase (MAPK) signaling, because similar RPE transformation could be induced by expression of constitutively MEK1 (activated MAP kinase kinase 1), an MAP kinase activator, or activated Ras (Zhao et al., 2001; Galy et al., 2002). Additional explant culture experiments using FGF1/2-soaked beads demonstrated that FGF signaling suppressed the expression of the RPE transcription factor Mitf but activated the neural retinal transcription factor Chx10, setting up the boundary of RPE and the

neural retina in the optic vesicle (Nguyen and Arnheiter, 2000; Horsford et al., 2005). FGF signaling may also drive the vertebrate retinal differentiation wave in eye development. Pharmacological inhibition of FGF signaling significantly retarded the movement of the retinal ganglion cell differentiation wave in chick embryos, whereas exogenous *Fgf8* appeared to substitute for midline signaling emanating from the optic stalk to initiate the retinal ganglion cell differentiation wave in zebrafish (McCabe et al., 1999; Martinez-Morales et al., 2005). Additional studies showed that zebrafish *Fgf3/Fgf8* were required for initiation of retinal neurogenesis, whereas *Fgf19* was required for its subsequent progression across the retina (Martinez-Morales et al., 2005; Vinothkumar et al., 2008).

Despite the above evidence in support of the role of FGF signaling in retinal development, other than the modest RPE expansion phenotype in *Fgf9* mutants, no other mouse mutations in either *Fgfs* or *Fgfrs* showed any retinal developmental defects (Dono et al., 1998; Ortega et al., 1998; Miller et al., 2000; Zhao et al., 2001; Wright et al., 2004). Although it is possible that FGF signaling may function in a species-dependent manner, the potential redundancy among 22 *Fgfs* and 4 *Fgfrs* certainly complicates the genetic analysis of FGF signaling in mammalian retinal development. One way to untangle the complexity of FGF signaling in retinal development is to study its downstream mediators. Many receptor tyrosine kinases, including *Fgfr*, require Shp2 protein-tyrosine phosphatase to attain full activation of MAPK signaling, although the exact mechanism is still unclear (Van Vactor et al., 1998; Feng, 1999). In this study, we have generated retinal specific ablations of *Shp2* to study its novel function in retinal development. Our results show that Shp2 signaling is required for the patterning of the neural retina, but this signaling is

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Figure 1. Lack of retinal defects in the *Six3-Cre;Shp2^{lox/lox}* and *α-Cre;Shp2^{lox/lox}* mutants. **A–C**, Expression of Shp2 and retinal differentiation genes *Math5* and *Brn3b* in wild-type retina at E14.5. **D–F**, Shp2 was abolished in the center of *Six3-Cre;Shp2^{lox/lox}* retina, but *Math5* and *Brn3b* expressions remained unaffected. **G–I**, Shp2 was efficiently depleted in the *α-Cre;Shp2^{lox/lox}* peripheral retina, but no changes were observed in *Math5* and *Brn3b* expressions. Scale bar, 100 μ m.

dispensable after the initiation of retinal differentiation. By *ex vivo* culture and genetic interaction experiments, we further demonstrate that Shp2 controls retinal development by mediating FGF–Ras signaling.

Materials and Methods

Mice. *Shp2^{lox}* mice have been described previously (Zhang et al., 2004). *Rx-Cre* mice were kindly provided by Dr. Milan Jamrich (Baylor College of Medicine, Houston, TX) (Swindell et al., 2006). *Six3-Cre* mice were kindly provided by Dr. Yasuhiko Furuta (M. D. Anderson Cancer Center, Houston, TX) (Furuta et al., 2000). *α-Cre* mice were kindly provided by Drs. Nadean Brown (Children's Hospital Research Foundation, Cincinnati, OH), Peter Gruss (Max Planck Institute for Biophysical Chemistry, Göttingen, Germany), and Ruth Ashery-Padan (Tel Aviv University, Tel Aviv, Israel) (Marquardt et al., 2001). *LSL-Kras^{G12D}* mice were obtained from the Mouse Models of Human Cancers Consortium Repository at National Cancer Institute (Tuveson et al., 2004). All mice are maintained in mixed genetic background. All experiments were performed in accordance with institutional guidelines.

Immunohistochemistry. Mouse embryos were staged according to the day the vaginal plug was observed, and somite number was counted for embryonic day 8.5 (E8.5) to E10.5 embryos. The samples were fixed in 4% paraformaldehyde (PFA) overnight for cryosections or paraffin sections before standard immunohistochemistry was performed as described previously (Pan et al., 2006, 2008). For Shp2 and phospho-extracellular signal-regulated kinase (ERK) staining, we performed the tyramide signal amplification (TSA) procedure as follows (TSA Plus Fluorescein System; PerkinElmer Life and Analytical Sciences). Briefly, cryo-

sections were incubated in sub-boiling citrate buffer for 10 min, quenching of peroxidase activity using a 3% H_2O_2 solution in methanol followed, and the sections were blocked in 5% goat serum in PBS at room temperature for 1 h and probed with primary antibodies at 4°C overnight. The sections were next blocked in TNB blocking buffer (0.1 M Tris-HCl, pH 7.5, 0.15 M NaCl, 0.5% blocking reagent) before an anti-rabbit HRP secondary antibody was added, and the signal was amplified and detected using TSA fluorescein. The antibodies used were anti-Shp2 (Sc-280; Santa Cruz Biotechnology), anti-phospho-ERK1/2 (catalog #9101; Cell Signaling Technology) and anti-Cyclin D1 (catalog #2926; Cell Signaling Technology), anti-Ki67 (catalog #550609; BD Pharmingen), anti-Islet1 (39.4D5; Developmental Studies Hybridoma Bank), anti-NF165 (2H3; Developmental Studies Hybridoma Bank), and anti-Pax6 (PRB-278P; Covance). Anti-Ptf1a was kindly provided by Dr. Jane E. Johnson (University of Texas Southwestern Medical Center, Dallas, TX) (Hori et al., 2008). Anti-TR β 2 was kindly provided by Dr. Douglas Forrest (National Institutes of Health, Bethesda, MD) (Ng et al., 2009).

RNA in situ hybridization. RNA *in situ* hybridization was performed as described previously, and the depletion of Shp2 was always confirmed by Shp2 immunohistochemistry on adjacent sections (Pan et al., 2008). For triple-fluorescent staining, frozen sections were first hybridized with digoxigenin-labeled *Fgf15* and fluorescein-labeled *Mitf* probes, incubated with anti-digoxigenin and anti-fluorescein peroxidase antibodies (Roche Diagnostics), and detected with FITC TSA-plus and cyanine 3 (Cy3) TSA-plus, respectively (PerkinElmer Life and Analytical Sciences). This was immediately followed by Islet1 antibody staining, detected with a Cy5-conjugated secondary antibody. The following probes were used: *Erm* (from Dr. Bridget Hogan, Duke University Medical Center, Durham, NC), *Brn3b*, *Ngn2*, and *Otx2* (from Dr. Lin Gan, University of Rochester, Rochester, NY), *Chx10* and *Mitf* (from Dr. Roderick R. McInnes, Hospital for Sick Children, Toronto, Ontario, Canada), *Crx* and *Math3* (from Valerie Wallace, Ottawa Health Research Institute, Ottawa, Ontario, Canada), *Fgf15* (from Minh-Thanh T. Nguyen, National Institutes of Health, Bethesda, MD), *Math5* (from Dr. Tom Glaser, University of Michigan, Ann Arbor, MI), and *Pax6* and *Hes1* (from Naoki Takahashi, Nara Institute of Science and Technology, Nara, Japan). At least three embryos of each genotype were analyzed for each probe.

Explant culture. Heads of E10 embryos (26–30 somites) were cut sagittally at the midline and laid flat on Millipore Corporation filters (Nitrocellulose Membrane Black Gridded, filter type 0.45 μ m) floating on DMEM/F-12 (1:1) (Invitrogen) culture medium. Heparin acrylic beads (Sigma) soaked with 0.1% BSA or 250 μ g/ml recombinant FGF2 (R & D Systems) were inserted around the eye region as described previously (Pan et al., 2008). After 3 d of incubation at 37°C with 5% CO_2 , the cultured head slices were photographed and fixed with 4% PFA before RNA *in situ* hybridization.

Results
***Six3-Cre* and *α-Cre*-mediated *Shp2* deletion did not disrupt retinal development**
To determine the role of *Shp2* during retinal development, we first crossed the *Shp2^{lox}* with *Six3-Cre*, which we confirmed to express Cre recombinase to induce R26R reporter activity in the

Figure 2. The time course of *Rx-Cre*- and *Six3-Cre*-mediated *Shp2* and phospho-ERK depletion. **A, B**, *Rx-Cre* but not *Six3-Cre* activated strong *R26R* reporter expression in E9.0 optic vesicle. **C–K**, Reduction of *Shp2* staining was first observed in the *Rx-Cre;Shp2^{lox/lox}* mutant optic vesicle at E9.0 and in the *Six3-Cre;Shp2^{lox/lox}* mutant at E10.5. In the *Rx-Cre;Shp2^{lox/lox}* mutant, notice the loss of *Shp2* staining in the future RPE (arrowhead in **G**) and mosaic pattern depletion of *Shp2* in retina (arrow in **H**). **L–T**, As a consequence, disruption of phospho-ERK staining was observed in the *Rx-Cre;Shp2^{lox/lox}* mutant optic vesicle at E10.5 but significantly

central neural retina (supplemental Fig. S1A,B, available at www.jneurosci.org as supplemental material) (Furuta et al., 2000). As expected, this also led to retinal-specific ablation of *Shp2* protein (Fig. 1A,D). We next analyzed the wave of retinal differentiation by focusing on the expressions of *Math5* and *Brn3b*, two transcription factors required for retinal ganglion cell development (Erkman et al., 1996; Gan et al., 1996; Brown et al., 2001; Wang et al., 2001). *Math5* expression initiates from the center of the mouse retina at E11.5, and gradually spreads toward the peripheral tip of the retina. This is immediately followed at E12.5 by a wave of *Brn3b* expression, which also progresses from the central retina to the peripheral retina. For both markers, we found the speed and extent of retinal ganglion cell differentiation was not changed between the wild-type and mutant retina ($n = 5$) (supplemental Fig. S1C–N, available at www.jneurosci.org as supplemental material) (Fig. 1A–F). Therefore, *Shp2* inactivation mediated by *Six3-Cre* did not affect retinal ganglion differentiation wave.

Because *Six3-Cre* transgene is primarily active in the central retina, it may not be able to inactivate *Shp2* efficiently in the peripheral retina. We therefore generated another *Shp2* conditional knock-out using the α -*Cre* transgene, which has been shown previously to be active in the distal retina (Marquardt et al., 2001). Indeed, we showed that the *Shp2* expression was completely abolished at the tip of the α -*Cre;Shp2^{lox/lox}* mutant retina (Fig. 1G). Nevertheless, the expressions of the *Math5* and *Brn3b* were again unaffected by the α -*Cre*-mediated *Shp2* deletion (Fig. 1H,I). Thus, retinal ganglion cell differentiation in the distal region of the retina also appeared to be insensitive to *Shp2* ablation. In the postnatal *Six3-Cre;Shp2^{lox/lox}* and α -*Cre;Shp2^{lox/lox}* mutants using the retinal neuron markers *Brn3a* and *Islet1* (ganglion cells), *calbindin* and *NF165* (horizontal cells), *VC1.1* and *syntrophin* (amacrine cells), *Chx10* (bipolar cells), *glutamine synthetase* (Müller cells), *recoverin* and *rhodopsin* (photoreceptors), we failed to observe any retinal differentiation defects (Z.C. and X.Z., unpublished observations).

Rx-Cre but not *Six3-Cre* disrupted *Shp2*–ERK signaling in optic vesicle development

The lack of retinal developmental defects in the *Six3-Cre;Shp2^{lox/lox}* and α -*Cre;Shp2^{lox/lox}* mutants prompted us to consider the possibility that *Shp2* signaling is only required in early optic vesicle patterning. *Six3-Cre* and α -*Cre* were reported to be first active at E9.0 and E10.5, respectively (Furuta et al., 2000; Marquardt et al., 2001). Careful expression analysis has shown previously that the murine *Rx* gene becomes highly expressed in the prospective optic vesicle at E8.5, preceding *Six3* expression in the region of optic evaginations by a few hours (Furukawa et al., 1997b). We thus obtained from Dr. Milan Jamrich the *Rx-Cre* deleter (Swindell et al., 2006). Interestingly, compared with the abundant *R26R* Cre reporter expression induced by the *Rx-Cre* in the optic vesicle at E9.0 (16–20 somites), hardly any ocular Xgal (5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside) staining was observed in the *Six3-Cre;R26R* embryos (Fig. 2A,B, arrows). Consistent with this, although *Shp2* protein was ubiquitously expressed throughout retinal development in wild-type embryos (Fig. 2C–E), in the *Rx-Cre;Shp2^{lox/lox}* mutant, *Shp2* protein expression was reduced

←

delayed in *Six3-Cre;Shp2^{lox/lox}* mutant retina until E13.5. The embryos at the same stages share the scale bars (50 μ m). All sections are coronal with the dorsal retina at the top. Xgal, 5-Bromo-4-chloro-3-indolyl- β -D-galactopyranoside.

in the optic vesicle as early as E9.0 (Fig. 2*F*, arrow). At E10.5 when the optic vesicle invaginated to form the optic cup, the Shp2 protein was most significantly downregulated in the ventral retina and the retinal pigmented epithelium (Fig. 2*G*, arrow and arrowheads, respectively), although mosaic patches of Shp2-positive cells still existed in *Rx-Cre;Shp2^{flox/flox}* mutant retina at E13.5 (Fig. 2*H*, arrow). In contrast, Shp2 immunostaining only began to be reduced in the *Six3-Cre;Shp2^{flox/flox}* mutant optic vesicle at E10.5, confirming the relatively late ablation of Shp2 protein mediated by the *Six3-Cre* (Fig. 2*J*, arrow). Nevertheless, Shp2 protein was indeed depleted in the central region of the *Six3-Cre;Shp2^{flox/flox}* mutant retina at E13.5 (Fig. 2*K*, arrow). Importantly, there was also longer delay in the disruption of phospho-ERK, a downstream effector of FGF–Ras signaling, in the *Six3-Cre;Shp2^{flox/flox}* mutant. In the *Rx-Cre;Shp2^{flox/flox}* mutant, clear loss of phospho-ERK staining was detected particularly in ventral retina at E10.5 (Fig. 2*P*, arrow), but, in the *Six3-Cre;Shp2^{flox/flox}* mutant, phospho-ERK expression persisted in the retina until E13.5. Therefore, *Rx-Cre* activates earlier than *Six3-Cre* to disrupt Shp2 signaling during optic vesicle development.

Shp2 is required for retinal development but not the movement of neurogenic wave in the *Rx-Cre;Shp2^{flox/flox}* mutant

We next investigated the retinal development in the *Rx-Cre;Shp2^{flox/flox}* mutant. Compared with the wild-type embryos, the E14.5 *Rx-Cre;Shp2^{flox/flox}* mutants exhibited mosaic depletion of Shp2 mostly in the ventral neural retina (Fig. 3*A,A'*, arrow). This matched that of the loss of phospho-ERK staining on adjacent sections, demonstrating the requirement of Shp2 for ERK signaling (Fig. 3*B,B'*, arrow). In the rest of this study, we always carefully confirmed the Shp2 and phospho-ERK deficiency on adjacent sections before proceeding to examine the rest of retina because of the mosaic nature of Shp2 depletion in the *Rx-Cre;Shp2^{flox/flox}* mutants. By RNA *in situ* hybridization, we showed that the *Shp2* mutant cells expressed RPE marker *Mitf* at the expense of retinal neuronal marker *Chx10* (Fig. 3*C',D'*, arrows). Moreover, there was significant downregulation of Cyclin D1 expression, which led to a severe loss of Ki67 protein, a cell cycle marker (Fig. 3*E,E',F,F'*).

The profound cell proliferation defects in the *Rx-Cre;Shp2^{flox/flox}* mutants preclude a comprehensive survey of all retinal cell types in adult animals; we thus focused on the embryonic retinal development. *Pax6*, a key eye determination gene, was expressed in the *Rx-Cre;Shp2^{flox/flox}* mutant throughout eye development, suggesting that *Shp2* ablation did not affect the specification of the eye field (Fig. 3*G,G'*, arrow; and data not shown) (Hogan et al., 1986; Hill et al., 1991). *Math3*, *Mash1*, *Ngn2*, and *Hes1* are basic helix–loop–helix transcription factors important for retinal differentiation (Furukawa et al., 2000; Inoue et al., 2002). By RNA *in situ* hybridization, we showed that the expressions of these genes were all disrupted in E14.5 *Rx-Cre;Shp2^{flox/flox}* mutants (Fig. 3*H'–L'*, arrows). We next analyzed the retinal neurons that were born at E14.5 (retinal ganglion, amacrine, horizontal cells, and photoreceptors) to further investigate the retinal differentiation defects. Ptf1a, a transcription factor specifying retinal amacrine and horizontal cells, was significantly downregulated in the *Rx-Cre;Shp2^{flox/flox}* mutant retina (Fig. 3*L,L'*, arrow) (Fujitani et al., 2006; Nakhai et al., 2007). The expression of *Otx2* and *Crx*, two homeodomain transcription activators that control photoreceptor cell development, were abolished in the Shp2-deficient retina (Fig. 3*M',N'*, arrows) (Chen et al., 1997; Freund et al., 1997;

Furukawa et al., 1997a). Consistent with this, the early cone cell marker TRβ2 was also lost (Fig. 3*O,O'*, arrow) (Roberts et al., 2006; Ng et al., 2009). Finally, the *Rx-Cre;Shp2^{flox/flox}* mutant retina exhibited significant loss of *Math5* and *Brn3b* expression, which are essential for retinal ganglion cell differentiation (Fig. 3*P',Q'*, arrows). As a result, the neural filament marker NF165 was abolished (Fig. 3*R,R'*, arrow). Together, these results demonstrated that the mosaic depletion of Shp2 in the *Rx-Cre;Shp2^{flox/flox}* mutant disrupted retinal differentiation.

The mosaic pattern of *Shp2* ablation in the *Rx-Cre;Shp2^{flox/flox}* embryos also allowed us to test the sequential-induction model for mammalian retinal development, which predicts that the early differentiated proximal retina is required for the development of the distal retina. We reasoned that, if FGF signaling participates in such a mode of tissue induction, disruption of FGF signaling by *Shp2* ablation should prevent the progression of the retinal differentiation wave. *Erm* is an ETS domain transcription factor inducible by FGF signaling during embryonic development (Munchberg and Steinbeisser, 1999; Raible and Brand, 2001; Roehl and Nüsslein-Volhard, 2001). In the *Rx-Cre;Shp2^{flox/flox}* mutants in which Shp2 was only depleted in a small patch of the retina (Fig. 4*A,D*, brackets), the *Shp2* ablation area fit exactly the region deficient in *Erm* expression on an adjacent section, suggesting a loss of FGF signaling in this area (Fig. 4*B,E*, brackets). Within the *Shp2* mutant domain in the *Rx-Cre;Shp2^{flox/flox}* retina, the expression of *Math5*, the retinal neurogenic wave marker, was lost in the cells closer to the distal retina (Fig. 4*C,F*, black arrowheads) but preserved in the cells closer to the proximal retina that had differentiated earlier, demonstrating that *Shp2* is not required for maintenance of retinal differentiation (Fig. 4*C,F*, black arrows). Furthermore, the wild-type cells distal to the *Shp2* mutant cells also expressed *Math5*, suggesting that wild-type retinal cells can differentiate cell autonomously adjacent to *Shp2* mutant cells (Fig. 4*C,F*, red arrow). To determine whether isolated wild-type clones completely surrounded by *Shp2* mutant cells could still differentiate independently, we performed whole-mount *in situ* hybridization and found islands of *Fgf15*-expressing retinal progenitor cells close to the periphery at E15.5 (Fig. 4*G*, arrow). By triple-fluorescent staining on serial sections, we then mapped the three-dimensional structure of the *Shp2* mutant retina. Although the immunohistochemistry condition for the Shp2 antibody is not optimum for other markers, because our above analysis has shown that retinal *Mitf* expression was induced only in the *Shp2* mutant cells, we were able to identify the *Shp2* mutant domain by its ectopic expression of *Mitf*. The wild-type retinal cells were identified by *Fgf15*, which marks the retinal progenitor cells in the ventricular zone, and *Islet1*, which predominantly labels newly differentiated retinal ganglion cell at this stage (Rachel et al., 2002; Elshatory et al., 2007). At the passage of the neurogenic wave, *Islet1*-positive cells emerged from the *Fgf15*-expressing neuroblasts in the proximal retina (Fig. 4*H–L*, arrows and arrowheads), but no *Islet1*-positive cells were found in the *Mitf*-expressing zone (Fig. 4*H–L*, white brackets), consistent with our above results that *Shp2* is required for retinal differentiation. However, reconstruction of the serial images showed that a cluster of *Fgf15*-expressing wild-type cells in the distal retinal, although completely enclosed in an *Mitf*-expressing mutant domain, were still able to commence *Islet1* expression (Fig. 4*H–L*, yellow brackets). Therefore, these distal retinal cells can apparently initiate neuronal differentiation program without direct physical contact with the proximal wild-type retina, demonstrating the *Shp2* mutation clones were unable to block the movement of the retinal neurogenic wave.

Figure 3. *Rx-Cre*-mediated *Shp2* ablation disrupted retinal development. **A–F, A'–F'**, At E14.5, mosaic depletion of *Shp2* in the *Rx-Cre;Shp2^{lox/lox}* mutant retinas not only disrupted phospho-ERK staining but also resulted in the loss of Cyclin D1, Ki67, and *Chx10* expression. In contrast, *Mitf* expression was upregulated (arrows). **G–K, G'–K'**, The eye field specification gene *Pax6* is unchanged, but the retinal differentiation genes *Math3*, *Math1*, *Ngn2*, and *Hes1* are all lost in the *Rx-Cre;Shp2^{lox/lox}* mutant retinas (arrows). **L–R, L'–R'**, Loss of *Ptfla* indicated the disruption of retinal amacrine and horizontal cell differentiation. Loss of *Otx2*, *Crx*, and *TRβ2* indicated photoreceptor cell defects. Loss of *Math5*, *Brn3b*, and *NF165* indicated retinal ganglion cells defects. Scale bars, 100 μ m.

Shp2 controls FGF–Kras signaling during retinal development

To investigate the mechanism of *Shp2* function in retinal development, we next examined the *Rx-Cre;Shp2^{lox/lox}* mutant retina at E12.5 before cell proliferation defects had significantly distorted retinal morphogenesis. After confirming the loss of *Shp2*

and phospho-ERK expression, we found that the retinal progenitor cell markers *Sox2* and *Chx10* were also abolished (Fig. 5A–D, G–J, arrows) (Burmeister et al., 1996; Taranova et al., 2006). We also noticed that RPE-like pigmented cells were frequently present at the tip of the *Rx-Cre;Shp2^{lox/lox}* retina (Fig. 5L, arrowhead), and the RPE determination gene *Mitf* was ectopically ex-

Figure 4. Shp2 ablation did not affect the neurogenic wave progression. **A–F**, On adjacent sections, although the Shp2 depletion pattern region exactly matched the *Erm*-deficient zone (red brackets), only the distal portion of the Shp2-negative zone lost *Math5* expression (black arrow and arrowheads). Moreover, the wild-type cells distal to the Shp2-deficient zone still expressed *Math5* (red arrows). **G**, Whole-mount *in situ* hybridization identified an island of *Fgf15*-expressing retinal progenitor cells isolated from proximal retina (arrow). The red lines indicated the serial sections used in **H–L** to fully map the *Fgf15*-positive domains. **H–L**, By combining fluorescent RNA *in situ* hybridization (*Fgf15* and *Mitf*) and immunofluorescence (Islet1), we identified the *Fgf15*/Islet1-positive cell clusters (arrows and arrowheads within the yellow brackets) enclosed in the *Mitf*-positive domain (white brackets), demonstrating that neural differentiation can occur in the isolated wild-type cells in distal retina without direct contact with the proximal retina. Scale bars, 100 μ m.

pressed in distal retina, demonstrating that the *Shp2* mutant cells had shifted from a neural retinal fate to RPE fate (Fig. 5*E,K*, arrow). Finally, we observed that *Fgf15* expression was abolished in the *Mitf*-expressing domain, indicating that Shp2 signaling controlled FGF ligand expression (Fig. 5*F,L*, arrow).

We next examined whether *Shp2* could also act downstream to FGF signaling to induce the optic vesicle into the neural retina.

This was tested by *in vitro* explant culture experiments, given that *Shp2* is also deleted in presumptive RPE region of *Rx-Cre; Shp2^{flox/flox}* embryos at E10.5 (Fig. 2*G*, arrowhead). As a control, we first implanted BSA-soaked beads adjacent to the eye rudiments collected from E10.5 wild-type embryos. As expected, BSA beads did not perturb the development of the optic vesicle into *Chx10*-expressing neural retina and *Mitf*-expressing RPE (Fig.

Figure 5. Shp2 signaling promotes the neural retinal fate against retinal pigmented epithelium fate. At E12.5, the *Shp2* null cells in *Rx-Cre;Shp2^{lox/lox}* mutants maintained the expression of the eye field specification gene *Pax6* but lost the retinal progenitor markers *Sox2* and *Chx10* (**A–D, G–J**, arrows). There was a corresponding invasion of *Mitf* expression into the presumptive neural retinal domain and loss of *Fgf15* expression (**F, L**, arrow). Notice the presence of pigmented RPE cells in the tip of the mutant retina (**L**, arrowhead). Scale bar, 100 μ m.

6A,D,G, arrows). FGF2-soaked beads, however, induced obvious loss of pigmentation in the adjacent eye tissue (Fig. 6B, arrow) (13 of 16 wild-type explants). By RNA *in situ* hybridization, we further confirmed that exogenous FGF2 induced the duplication of *Chx10*-positive neural retina and corresponding loss of *Mitf*-positive RPE in wild-type eye rudiments (Fig. 6E,H, arrows). Finally, FGF2 beads failed to transform RPE into neural retina in six of nine *Rx-Cre;Shp2^{lox/lox}* embryos (Fig. 5C, arrow) ($p = 0.0308$, Fisher's exact test). In these mutant embryos, *Mitf* expression was not only preserved in RPE tissue adjacent to the FGF2 beads but also extended into the presumptive retinal region (Fig. 6I, arrow and arrowhead, respectively). Therefore, FGF signaling depends on Shp2 in directing neural retinal fate.

To define the downstream target of Shp2 in retinal-specific FGF signaling, we next asked whether activated Ras could ameliorate the *Rx-Cre;Shp2^{lox/lox}* mutant phenotype. To conditionally express an activated form of Ras at a physiologically relevant level, we used the *LSL-Kras^{G12D}* mouse, which carries a missense G to D mutation at codon 12 in the endogenous *Kras* gene, resulting in constitutive Ras signaling by disruption of the GTPase activity of Kras (Tuveson et al., 2004). Normally silenced by an insertion of a transcription stop cassette flanked by two *LoxP* sites, the *LSL-Kras^{G12D}* allele was able to be activated by the same *Rx-Cre* transgene while *Shp2* was being ablated in the *Rx-Cre;Shp2^{lox/lox}* retina. Interestingly, we did not observe any overt ocular phenotype in the *Rx-Cre;LSL-Kras^{G12D}* animals, suggesting that activated *Kras* expressed at a

Figure 6. FGF signaling depended on Shp2 to induce neural retina in optic vesicle. **A, D, G**, Wild-type retinal culture developed with proper demarcation of *Chx10* and *Mitf* expressions in the presence of BSA beads (arrows). **B, E, H**, In wild-type cultures, FGF2 beads transformed the *Mitf*-expressing RPE into *Chx10*-expressing the neural retina (arrows). **C, F, I**, In *Rx-Cre;Shp2^{lox/lox}* mutants, FGF2 beads were unable to induce RPE into the neural retina (arrowheads). Asterisks denote implanted beads. Scale bars, 200 μ m.

moderate level from its endogenous locus did not perturb regular eye development. The retinæ in the *Rx-Cre;Shp2^{lox/lox}* embryos, however, showed mosaic depletion of Shp2 and an identical pattern of phospho-ERK downregulation (Fig. 7F,G, arrows), which

Figure 7. Activated Ras signaling rescued *Shp2* mutant retinal development. **A–E**, In the E14.5 wild-type embryos, *Shp2*, phospho-ERK, *Math5*, and *Brn3b* were expressed throughout the retina, whereas *Mitf* expression was restricted to the tip of the retina. **F–J**, Mosaic depletion of *Shp2* in the *Rx-Cre;Shp2^{flox/flox}* mutants led to a loss of phospho-ERK, *Math5*, and *Brn3b* expressions, whereas *Mitf* expression extended into the neural retinal region (arrows). **K–O**, In the *Rx-Cre;Shp2^{flox/flox};LSL-Kras^{G12D}* compound mutants, phospho-ERK, *Math5*, and *Brn3b* expression was restored in the *Shp2*-negative cells, whereas *Mitf* was properly expressed in the tip of the retina (arrowheads). Scale bar, 100 μ m.

led to invasion of *Mitf* and corresponding loss of *Math5* and *Brn3b* expression in the distal retinae (Fig. 7H–J, arrows). Consistent with the activation of Ras signaling by the *Kras^{G12D}* allele, the *Rx-Cre;Shp2^{flox/flox};LSL-Kras^{G12D}* compound mutants exhibited random patches of *Shp2* depletion but normal level of phospho-ERK throughout the retinae (Fig. 7K, L). More importantly, *Mitf* expression was confined to the retinal tip, whereas the expression of *Math5* and *Brn3b* extended uninterruptedly into the distal retina (Fig. 7M–O, arrows). The genetic rescue of the *Shp2* mutant phenotype could be fully explained by the loss of Ras signaling during retinal development.

Discussion

In this study, we have generated three temporally distinct models of *Shp2* ablation during retinal development. Interestingly, unlike the severe retinal defects in the *Rx-Cre;Shp2^{flox/flox}* mutants, the *Six3-Cre* and α -*Cre*-mediated knock-outs of *Shp2* had no discernable effect on embryonic retinal development. For example, although the expressions of the retinal progenitor marker such as *Sox2* and *Chx10* or retinal differentiation genes such as *Brn3b* and *Crx* were lost in the *Rx-Cre;Shp2^{flox/flox}* mutant retina, none of these retinal-specific factors were disrupted in the *Six3-Cre;Shp2^{flox/flox}* or the α -*Cre;Shp2^{flox/flox}* mutant retina. As a result, only the *Rx-Cre;Shp2^{flox/flox}* mutant embryos exhibited the loss of the early ganglion marker NF165 and cone cell marker TR β 2. These phenotypic differences are unlikely attributable to the lack of Cre expression, because both *Six3-Cre* and the α -*Cre* are efficient Cre drivers widely used in studying retinal develop-

ment, and we also showed that *Shp2* protein were indeed depleted in the *Six3-Cre;Shp2^{flox/flox}* and the α -*Cre;Shp2^{flox/flox}* mutant retina at E14.5. Conversely, our Cre reporter assay and immunohistochemistry clearly showed that *Rx-Cre* acted earlier than *Six3-Cre*, depleting *Shp2* at E9.0 and E10.5, respectively, and previous study has also shown that α -*Cre* becomes first active at E10.5. These results suggest that *Shp2* signaling is only required in a narrow time window during early retinal development. Indeed, we have further showed that the *Rx-Cre;Shp2^{flox/flox}* mutant retina ectopically expressed RPE marker *Mitf* but suppressed the neural progenitor marker *Chx10* and *Sox2*, suggesting a general fate change from neural retinal fate into RPE fate. This disruption of early ocular tissue specification thus provides the simplest explanation for the pleiotropic loss of retinal neurogenesis in the *Rx-Cre;Shp2^{flox/flox}* mutants. *Shp2* signaling in the brain is also known to act as a critical switch in biasing cortical precursor cells and neural stem cells toward a neuronal fate rather than a glial fate (Gauthier et al., 2007; Ke et al., 2007). Therefore, *Shp2*-mediated growth factor signaling may play a general role in promoting neural cell fate during nervous system development.

If the retinal defects in the *Rx-Cre;Shp2^{flox/flox}* mutants can be readily explained by the general failure of retinal fate determination, does *Shp2* signaling still play a role in retinal differentiation? In species ranging from *Drosophila* to mammals, retinal differentiation spreads in a fan-like manner from the root of the optic stalk to the edge of the retina. The most elegant hypothesis to explain this strikingly conserved pattern of the retinal differentiation wave is the sequential-induction model, in which earlier differentiated retinal neurons secrete diffusible factors to induce neighboring retinal progenitor cells to differentiate (Ma et al.,

1993; Greenwood and Struhl, 1999; Neumann and Nusslein-Volhard, 2000). In addition to Sonic hedgehog, FGF has emerged as a potential signal for both the initiation and the progression of the neurogenic wave in vertebrates (Martinez-Morales et al., 2005; Vinothkumar et al., 2008). However, previous results have also shown that isolation of retinal fragments from the rest of eye did not abolish their neuronal differentiation (McCabe et al., 1999; Kay et al., 2005). In our current study, we showed that the retinal differentiation wave in the *Rx-Cre;Shp2^{flox/flox}* mutants could move across the *Shp2* mutant clones to reach more distal retinal cells. Furthermore, retinal differentiation proceeded normally in the *Six3-Cre;Shp2^{flox/flox}* and *α -Cre;Shp2^{flox/flox}* mutants in which the Shp2 ablation occurred during retinal differentiation. These results suggest that Shp2 is not required for progression and maintenance of neurogenic wave.

Our results thus support that Shp2 signaling is required for establishing the neuronal versus RPE fate in mammalian retinal development, consistent with its role in mediating FGF signaling. Indeed, *Shp2* mutation prevented the transformation of RPE into neuronal tissue by ectopic FGF in organ cultures, whereas *in vivo*, activated Kras can fully rescue the ERK phosphorylation and retinal defects in the *Rx-Cre;Shp2^{flox/flox}* mutants. These results support the FGF–Shp2–Ras–ERK signaling cascade in retinal development. Nevertheless, inhibition of FGF signaling in *Xenopus* embryos by dominant-negative Fgfr1 expression did not disrupt the demarcation of the neural retina versus RPE or the production of retinal ganglion cells; instead, it reduced photoreceptor cell numbers (McFarlane et al., 1998). Conversely, in *Xenopus*-expressing dominant-negative Fgfr4, the loss of photoreceptor cells and defects in retinal lamination were observed, whereas retinal ganglion cell development was unaffected (Zhang et al., 2003). The phenotypic discrepancies between these and our studies could be attributable to the species and/or the technical difference, but it is also possible that Shp2 may mediate other receptor tyrosine kinase signaling, whereas FGF signaling may function through other Shp2-independent downstream pathways. Additional studies are required to determine the exact molecular interaction connection between FGF and Shp2 signaling and the full spectrum of FGF signaling in retinal development.

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