Sprouty2-modulated Kras signaling rescues Shp2 deficiency during lens and lacrimal gland development

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SUMMARY
Shp2/Ptpn11 tyrosine phosphatase is a general regulator of the RTK pathways. By genetic ablation, we demonstrate that Shp2 is required for lacrimal gland budding, lens cell proliferation, survival and differentiation. Shp2 deletion disrupted ERK signaling and cell cycle regulation, which could be partially compensated by activated Kras signaling, confirming that Ras signaling was the main downstream target of Shp2 in lens and lacrimal gland development. We also showed that Sprouty2, a general suppressor of Ras signaling, was regulated by Shp2 positively at the transcriptional level and negatively at the post-translational level. Only in the absence of Sprouty2 could activated Kras signaling robustly rescue the lens proliferation and lacrimal-gland-budding defects in the Shp2 mutants. We propose that the dynamic regulation of Sprouty by Shp2 might be important not only for modulating Ras signaling in lens and lacrimal gland development, but also for RTK signaling in general.

KEY WORDS: FGF, Ras, Shp2, Sprouty, Lacrimal gland

INTRODUCTION

Despite their morphological differences, both the lens and the lacrimal gland of the vertebrate eye originate from the surface head ectoderm during embryonic development. The presumptive lens ectoderm is first induced by the underlying optic vesicle to become a single layer of thickening cells, the lens placode, which invaginates at mouse embryonic day 10.5 (E10.5) to form a lens vesicle. The posterior lens epithelial cells next elongate into the primary lens fibers to fill up the lens vesicle cavity at E12.5. Further lens growth is driven by the anterior lens epithelial cells that proliferate and migrate inward at the equatorial region of the lens, eventually differentiating into orderly arrays of the secondary lens fibers. A few days after lens induction, at E12.5, the lacrimal gland anlage first appears as a thickening of conjunctival epithelium at the temporal side of the eye. This ectodermal placode also invaginates to form the lacrimal bud, invading the surrounding neural-crest-derived periorcular mesenchyme. Eventually, the elongating lacrimal gland undergoes branching morphogenesis to form dense glandular structures.

Development of the lens and lacrimal gland of the eye also share the requirement for RTK pathways, especially FGF signaling. Although several RTK signaling factors, including PDGF, IGF, EGF and HGF, can promote lens cell proliferation in vitro, only FGFs can induce lens cell differentiation in mammals (Choi et al., 2004; Hyatt and Beebe, 1993; Kok et al., 2002; Liu et al., 1996; McAvoy and Chamberlain, 1989; Reddan and Wilson-Dziedzic, 1983; Wormstone et al., 2000). In transgenic mice, misexpression of the secreted forms of Fgfl, 3, 4, 7, 8 or 9 led to premature differentiation of lens epithelial cells into fiber cells, whereas dominant-negative Fgfr1 suppressed lens epithelial cell proliferation (Faber et al., 2001; Lovicu and Overbeek, 1998; Robinson et al., 1998; Robinson et al., 1995). These studies were further corroborated by genetic deletion of Fgfr1/2/3 after lens induction, which resulted in an empty lens vesicle, completely devoid of lens fiber cells (Zhao et al., 2008). Similarly, Fgfl0 has been shown to induce ectopic lacrimal gland in explant cultures and transgenic animals, whereas ablation of Fgfl0 or Fgfr2 abolished mouse lacrimal gland development (Govindarajan et al., 2000; Makarenkova et al., 2000; Pan et al., 2008). Consistent with this, we have shown recently that defective biosynthesis of heparan sulfate, a crucial co-receptor of FGF, also resulted in loss of lens and lacrimal gland (Pan et al., 2008; Pan et al., 2006). These results firmly established the essential role of FGF signaling in lens and lacrimal gland development.

How is FGF signaling transduced in these two organs? Previous studies have shown that loss of FGF signaling in both lens and lacrimal gland disrupted ERK signaling, but the functional significance and regulatory mechanism of the FGF-ERK pathway remain to be explored. In this paper, we address these questions by studying Shp2 (Ptpn11 – Mouse Genome Informatics), a protein tyrosine phosphatase that controls RTK signaling through a yet unknown mechanism (Feng, 1999; Neel et al., 2003). Biochemical studies have indicated that Shp2 can be recruited to FGFIR by direct binding to the scaffold protein Frs2α, and together they provide docking sites for the adaptor protein Grb2 (Kouhara et al., 1997; Ong et al., 2000). Grb2 then activates Ras-ERK signaling via the nucleotide exchange factor Sos or stimulates PI3K signaling by its association with Gab1 protein (Hadari et al., 2001). Furthermore, Shp2 has been proposed to suppress the negative Ras regulators, Sprouty (Spry – Mouse Genome Informatics) and RasGAP (Rasal – Mouse Genome Informatics), or to activate Src family kinases, thus indirectly promoting Ras-ERK signaling (Cunnick et al., 2002; Hanafusa et al., 2004; Zhang et al., 2004b). By genetic analysis, we now show that Shp2 ablation results in ERK signaling failure and developmental defects in lacrimal gland and lens, which can be partially reversed by constitutively activated Kras signaling. However, loss of Shp2 function also exposes Sprouty2 (Spry2 – Mouse Genome Informatics) to excessive phosphorylation, which leads to downregulation of the overall Ras-ERK signaling. Only by
both deleting Sprouty2 and activating Kras can we restore robust lens proliferation and lacrimal gland budding. These results demonstrate that Shp2 controls Sprouty2 to regulate Ras-ERK signaling in lens and lacrimal gland development.

MATERIALS AND METHODS

Mice

Shp2flox mice have been previously described (Zhang et al., 2004a). Drs Ruth Ashery-Padan (Tel Aviv University, Tel Aviv, Israel) and Richard Lang (Children’s Hospital Research Foundation, Cincinnati, OH) kindly provided the Le-Cre mice, which exhibit normal lens and lacrimal gland development as heterozygotes (see Fig. S1 in the supplementary material) (Ashery-Padan et al., 2000; Pan et al., 2008). LSL-KrasG12D mice were obtained from the Mouse Models of Human Cancers Consortium (MMHCC) Repository at the National Cancer Institute (Tuveson et al., 2004). The genotyping primers used for detecting the loss of the LSL cassette in the Le-Cre;Shp2flox/flox;LSL-KrasG12D mice were: LSL (forward: 5'-CTAGCCACATGGCTGAGT-3'; reverse: 5'-GCTCCAACCAACAGATTT-3') and Kras (forward: 5'-GTCGCAAGGTATCGCGGTG3'-3'; reverse: 5'-CTTTCACAGCCGACGCAAGTCA-3'). Sprouty22/2 mice came from the Mutant Mouse Regional Resource Centers (MMRRC) (Shim et al., 2005) and were crossed with a germ cell active Cre line EIIa-Cre from the Jackson Laboratory (Bar Harbor, Maine) to generate the systemic Sprouty22/2 mice (Xu et al., 2001). All experiments were performed in accordance with institutional guidelines.

Immunohistochemistry

Brdu, TUNEL staining and immunohistochemistry were performed as previously described (Pan et al., 2008; Pan et al., 2006). The antibodies used were anti-Sprouty2 (Sc-280, Santa Cruz Biotechnology, Santa Cruz, CA), anti-phospho-ERK1/2 (#9101), anti-Cyclin D1 (#2926) and D3 (#2936) (all from Cell Signaling Technology, Beverly, MA), and band intensities were quantified using the Odyssey SA scanner (LICOR Biosciences, Lincoln, NE), and band intensities were quantified using the Odyssey software.

RESULTS

Shp2 ablation disrupts lens and lacrimal gland development

The Shp2 conditional knockout was generated by crossing the Shp2flox6 allele with the Le-Cre transgene, which is expressed as early as E9.5 in the surface ectoderm before it gives rise to the lens and the lacrimal gland. At E12.5, however, the Le-Cre;Shp2flox/flox mutant embryos were still indistinguishable from the wild-type controls, and histological analysis showed that the primary lens fiber cells had elongated anteriorly to fill up the lens vesicle (Fig. 1A,B). The lens phenotype became visible after E14.5, when the Le-Cre;Shp2flox/flox mutants exhibited reduced lens size and abnormal posterior shift of the lens epithelial cells (Fig. 1C-F, arrowheads). More severe defects, as we have shown previously, were observed in the development of lacrimal gland, which had clearly budded from the conjunctival epithelium in the wild-type embryos at E15.5, but never formed in the Shp2 mutants (Fig. 1G,H, arrows) (Pan et al., 2008). At postnatal day 0 (P0), the anterior lens epithelial cells, which normally migrate to the equatorial regions of the lens to differentiate, now completely surrounded the small Shp2 mutant lens, and the nuclei of the lens fiber cells inside the lens were also grossly disorganized amid numerous vacuoles (Fig. 1J, arrowheads). These results show that Shp2 is required for lens and lacrimal gland development.

Western blot and immunoprecipitation

The lenses were collected at E16.5 and homogenized in RIPA buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS) with protease and phosphatase inhibitors (Pierce, Rockford, IL). After heat denaturation, equal amounts of lens protein lysates, as previously determined by BCA protein assay (Pierce, Rockford, IL), were separated on 12% SDS-polyacrylamide gels and transferred to PVDF membranes (Millipore, Billerica, MA). The blots were blocked in 5% non-fat dry milk for 30 minutes and incubated overnight with anti-phospho-ERK1/2 (#9101), anti-ERK1/2 (#4695), anti-phospho-AKT (#3787) anti-AKT (#9272), (1:1000, all from Cell Signaling Technology, Beverly, MA) or anti-β-actin (A5411) (1:5000, Sigma) at 4°C. After incubation with the HRP-conjugated secondary antibody for 2 hours, the protein signals were revealed by enhanced chemiluminescence (Pierce, Rockford, IL) and exposed on X-ray films. For two-color quantitative western analysis, the blot was probed simultaneously with the mouse anti-ERK1/2 (sc-7383, Santa Cruz Biotechnology) and the rabbit anti-ERK1/2 (#4695, Cell Signaling Technology), followed by incubation with infrared-conjugated secondary antibodies. The membrane was scanned in an Odyssey SA scanner (LICOR Biosciences, Lincoln, NE), and band intensities were quantified using the Odyssey software.

For immunoprecipitation, lenses dissected from newborn pups were snap frozen in liquid nitrogen and stored at −80°C until use. Lens samples of 600 μg (about 20 pairs) were lysed in 300 μl of non-denaturing buffer (20 mM Tris-HCl (pH 8.0), 137 mM NaCl, 1% glycerol, 1% NP40, 2 mM EDTA) containing protease and phosphatase inhibitors (Pierce, Rockford, IL) and incubated with 2 μl normal goat serum for 1 hour, followed by 5 μl A/G protein agarose for 30 minutes to remove non-specific proteins. After centrifugation, the supernatant was incubated sequentially with 2 μg anti-Sprouty2 or control IgG antibodies and 20 μl A/G protein agarose overnight at 4°C, before being washed three times in 1 ml of lysis buffer. The pellet was collected by centrifugation and subjected to western blot as described above using the following antibodies: anti-Shp2 (Sc-280, Santa Cruz Biotechnology), anti-phosphotyrosine (clone 4G10, Upstate) and anti-Sprouty2 (S1444, Sigma).
now extended to the posterior rim of the lens (Fig. 2I-L). This is consistent with the histological analysis above that showed failure of the lens epithelial cells to migrate inside the lens to differentiate into secondary lens fibers. As shown by the BrdU incorporation assay and TUNEL staining, the Shp2 mutant lens epithelium also exhibited reduced cell proliferation and extensive apoptosis, which explained the significant reduction in lens size (Fig. 2M-R). Taken together, these data support the idea that Shp2 function is essential for lens epithelial cell proliferation, survival and differentiation.

**Shp2 regulates ERK signaling**

Considering that the Le-Cre transgene in the Le-Cre;Shp2fllox/fllox animals was active at E9.5, it is surprising that no lens abnormality was detected before E12.5. This prompted us to examine the timing of Shp2 ablation in the Le-Cre;Shp2fllox/fllox embryos. Using a probe specific to exon 4 of Shp2, which is flanked by LoxP sites in the Shp2fllox allele, we showed by RNA in situ hybridization that this crucial segment of Shp2 transcript was indeed lost in the mutant lens at E10.5 (Fig. 3A,B). Similarly, Shp2 protein was undetectable by immunohistochemistry in the E10.5 mutant lens vesicle and in the surface ectoderm, which was the precursor to the future lacrimal gland (Fig. 3C,D, arrows and arrowheads). We have previously shown that Shp2 is required for ERK signaling in lacrimal gland budding (Pan et al., 2008). However, using a phospho-specific

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**Fig. 1. Shp2 is required for lens and lacrimal gland development.** (A-D) The Le-Cre;Shp2fllox/fllox mutant lens exhibited normal primary lens fiber cell elongation at E12.5 but reduced lens size at E14.5. (E-H) The anterior lens epithelial cells shifted posteriorly in the E14.5 Le-Cre;Shp2fllox/fllox mutant lens (arrowheads), and no lacrimal gland was detected (arrows). (I-J) At P0, the Le-Cre;Shp2fllox/fllox mutant lens was completely surrounded by epithelial cells (arrowheads), indicating a failure of lens epithelial cell differentiation. Scale bars: 100 μm. LE, lens epithelium; LF, lens fiber; LG, lacrimal gland.

**Fig. 2. Defective lens epithelial cell development in the Shp2 mutant lens.** (A-H) Similar to wild type controls, the Le-Cre;Shp2fllox/fllox mutant lens expressed Pax6 and Prox1 at E10.5, and α-, β- and γ-crystallins at E16.5. (I-L) The Pax6 and E-Cadherin positive lens epithelium encircled the Le-Cre;Shp2fllox/fllox mutant lens, indicating the failure of lens epithelial cells to differentiate into secondary lens fibers. (M-P) Cell proliferation and survival defects in the Le-Cre;Shp2fllox/fllox mutant lens as shown by reduced BrdU incorporation and increased TUNEL staining. (Q) The ratio of BrdU-positive cells versus DAPI-positive cells decreased in the epithelial but increased in the fiber compartments of the E14.5 Le-Cre;Shp2fllox/fllox lens (n=3) compared with the wild type (n=4) (*P<0.001). The dashed lines in M and N encircled the lens cells that were counted. (R) The percentage of TUNEL-positive cells increased in the E14.5 Le-Cre;Shp2fllox/fllox mutant lens (n=5) compared with the wild type (n=3) (*P<0.0001). All lens cells within the dashed circle in O and P were counted. Scale bars: 100 μm.
antibody that recognizes the active form of ERK, we did not detect any reduction of phospho-ERK staining in the E10.5 lens (Fig. 3E,F). Phospho-ERK staining in the Shp2 mutant lens was eventually lost after E12.5, which coincided with the onset of the lens phenotype (Fig. 3G-J). This suggests that Shp2 controls ERK but not AKT signaling until after the lens induction phase.

We next assayed the known RTK downstream response genes, Erm (Etv5 – Mouse Genome Informatics), Er81 (Etv1 – Mouse Genome Informatics), and Pea3 (Etv4 – Mouse Genome Informatics), the expression of which is regulated by FGF and GDNF signaling in many developing tissues (Haase et al., 2002; Munchberg and Steinbeisser, 1999; Raible and Brand, 2001; Roehl and Nusslein-Volhard, 2001). At E14.5, the expression of Erm and Er81, which are normally present in the anterior lens epithelium and the lens equator, respectively, were abolished by Shp2 mutation (Fig. 3K-N). Similarly, Erm and Pea3 expression, which mark the budding lacrimal gland, were also completely lost in the Shp2 mutants (Fig. 3O,P, arrows; Fig. 4I-J’, arrowheads). Finally, we collected the lens from E16.5 wild-type and Shp2 mutant embryos and performed western blot experiments. Although ERK, AKT and phospho-AKT were present at comparable levels, phospho-ERK was lost in the Shp2 mutant lens (Fig. 3Q). Therefore, we conclude that Shp2 controls ERK but not AKT signaling in lens development.

**Activated Kras attenuated the Shp2 lens and lacrimal gland phenotype**

To investigate the molecular mechanism of Shp2 in ERK signaling, we next asked whether constitutively activated Ras signaling, which is upstream to ERK, could rescue the Shp2 mutant phenotype in vivo. This was accomplished by crossing Le-Cre;Shp2$^{flox/flox}$ with the LSL-Kras$^{G12D}$ allele, which contains a floxed transcriptional STOP cassette (LSL) in front of a constitutively active G12D mutation in the Kras gene (Tuveson et al., 2004). Cre-mediated recombination driven by the Le-Cre transgene could thus simultaneously disable Shp2 and activate Kras signaling by removing the STOP cassette, which had prevented the expression of Kras$^{G12D}$ (Fig. 4A). Furthermore, as a genetic knock-in, the LSL-Kras$^{G12D}$ allele was under the same transcriptional regulation as the endogenous Kras locus, which ensures the expression of Kras$^{G12D}$ at the normal physiological level. Indeed, allele-specific genotyping showed that the LSL cassette was lost in the lens but not the tail of the Le-Cre;Shp2$^{flox/flox}$;LSL-Kras$^{G12D}$ double mutants, whereas the Kras allele was unaffected (Fig. 4B). We further showed that the Le-Cre;Shp2$^{flox/flox}$;LSL-Kras$^{G12D}$ double mutants exhibited a statistically significant increase in lens size (Fig. 4C-E,6W). Importantly, whereas the Le-Cre;Shp2$^{flox/flox}$;LSL-Kras$^{G12D}$ double mutants now showed a slight bulging at the fornix of conjunctival epithelium at E14.5 and a more obvious extrusion at E15.5, although the lacrimal gland budding was still not comparable to the wild type when quantified (Fig. 4C’-E’, arrowheads, R-W, dashed lines; Fig. 6W). Therefore, at least morphologically, activated Kras signaling partially compensated for the loss of Shp2 in lens and lacrimal gland development.

We next examined the Le-Cre;Shp2$^{flox/flox}$;LSL-Kras$^{G12D}$ double mutants in molecular detail. Consistent with the role of Kras in ERK signaling, phospho-ERK staining was now clearly observed in the double mutant lens and became weakly detectable at the conjunctival fornix (Fig. 4F-H, arrows; 4F’-H’, arrowheads). The
relatively low level of ERK activation was further supported by the expression of Erm and Pea3, which was also weakly induced in the Le-Cre;Shp2fl/o;LSL-KrasG12D lacrimal gland primordium (Fig. 4I-K’, arrowheads). This would explain the very modest initiation of lacrimal gland budding in the double mutants compared with that of the wild types. By immunostaining, we showed that Shp2 ablation downregulated the expression of the cell cycle regulators Cyclin D1 and Cyclin D3 in the Le-
Cre;Shp2\textsuperscript{flox/flox} single mutant lens, as expected from the cell proliferation defects observed earlier. In the Le-Cre;Shp2\textsuperscript{flox/flox};LSL-Kras\textsuperscript{G12D} double mutants, however, both Cyclin D1 and Cyclin D3 expression was elevated (Fig. 4L-N), which also led to increased levels of the general cell cycle markers Ki67 and M-phase-specific marker phospho-Histone H3 in the lens (Fig. 4O-Q,X). Cyclin D1, Ki67 and phospho-Histone H3 expressions were also increased in the Le-Cre;Shp2\textsuperscript{flox/flox};LSL-Kras\textsuperscript{G12D} lacrimal gland, although the percentage of Ki67 or phospho-Histone H3 positive cells was still significantly lower than that of the wild-type lacrimal gland (Fig. 4R-W, dashed lines). Direct activation of Kras could thus partially restore ERK signaling and cell proliferation in the Shp2 lens and lacrimal gland.

Shp2 controls Sprouty2 tyrosine phosphorylation in the lens

The incomplete rescue in the Le-Cre;Shp2\textsuperscript{flox/flox};LSL-Kras\textsuperscript{G12D} double mutants suggested that there existed additional Shp2 signaling defects not compensated by the direct Kras activation. Although the direct targets of Shp2 tyrosine phosphatase are still uncertain, biochemical studies have shown that Shp2 can dephosphorylate Sprouty family proteins, which are general suppressors of RTK signaling (Hanafusa et al., 2004; Jarvis et al., 2006; Tefft et al., 2005). This will negatively regulate the Sprouty activities and eventually lead to increased ERK signaling. Interestingly, we noticed that Sprouty2 was expressed in the wild-type but not in the Shp2 mutant lens and lacrimal gland primordium, demonstrating that Shp2 signaling also positively regulated Sprouty2 expression (Fig. 5A,B,D,E, arrows and arrowheads). Introduction of the LSL-Kras\textsuperscript{G12D} allele also induced Sprouty2 expression in both lens and lacrimal gland (Fig. 5C,F, arrow and arrowhead), but this did not necessarily indicate that Sprouty2 protein was appropriately phosphorylated in the Le-Cre;Shp2\textsuperscript{flox/flox};LSL-Kras\textsuperscript{G12D} double mutants. We therefore immunoprecipitated Sprouty2 protein from the lens extracts collected from newborn animals. The antibody we used is highly specific to Sprouty2 protein, as demonstrated in the western blot (Fig. 5H) (Hanafusa et al., 2004; Jarvis et al., 2006; Tefft et al., 2005). As expected from the Sprouty2 mRNA expression above, western blot analysis showed that Sprouty2 protein was present in both wild type and the Le-Cre;Shp2\textsuperscript{flox/flox};LSL-Kras\textsuperscript{G12D} double mutants but not in the Le-Cre;Shp2\textsuperscript{flox/flox} single mutant (Fig. 5H). Importantly, Shp2 protein also co-immunoprecipitated with Sprouty2 in the wild-type lens extracts, consistent with the previous reports of direct protein-protein interaction between Shp2 and Sprouty (Fig. 5H) (Hanafusa et al., 2004; Jarvis et al., 2006; Tefft et al., 2005). Finally, we showed by phosphotyrosine immunoblotting that Sprouty2 was hyperphosphorylated in the Le-Cre;Shp2\textsuperscript{flox/flox};LSL-Kras\textsuperscript{G12D} double mutants (Fig. 5H). These results indicate that loss of Shp2 phosphatase activity leads to unchecked tyrosine phosphorylation in Sprouty2 protein.

Combined Sprouty2 ablation and Kras activation rescued Shp2 deletion in lens and lacrimal gland development

Sprouty2 phosphorylation is known to augment its inhibitory activity, which would dampen the ERK activation in the Le-Cre;Shp2\textsuperscript{flox/flox};LSL-Kras\textsuperscript{G12D} double mutants. We thus hypothesized that the removal of Sprouty2 would relieve this excessive inhibitory loop, allowing full activation of Kras-ERK signaling to rescue the Shp2 mutant phenotype. Indeed, quantitative western blot analysis showed that, although ERK phosphorylation in the Le-Cre;Shp2\textsuperscript{flox/flox};LSL-Kras\textsuperscript{G12D} lens remained significantly lower than that in the wild-type controls, additional ablation of Sprouty2 further elevated the phospho-ERK level (Fig. 6A,B). This was also demonstrated by the direct immunodetection of phospho-
ERK on lens sections, which was weak and diffuse in the Le-Cre;Shp2flox/flox,LSL-KrasG12D mutant, but strongly activated in the equatorial regions of the wild type and the Le-Cre;Shp2flox/flox,LSL-KrasG12D,Spry2flox/flox triple mutants (Fig. 6C-G, arrows). Similarly, although phospho-ERK staining was only modestly induced in the Le-Cre;Shp2flox/flox,LSL-KrasG12D conjunctival epithelium, it appeared to reach the same intensity level as the wild type in the Le-Cre;Shp2flox/flox,LSL-KrasG12D,Spry2flox/flox triple mutant lacrimal gland bud (Fig. 6M-Q). Therefore, in both lens and lacrimal gland, combined activation of Kras and ablation of Sprouty2 fully restored ERK signaling in the Shp2 mutants.

We have also confirmed that the Sprouty2 systemic knockout (Spry2KO/ko) animals are viable and fertile, without any obvious ocular defects (Shim et al., 2005). Similarly, the conditional Sprouty2 knockout (Le-Cre; Spry2fl/fl) had no effect by itself or in the context of the Shp2 single mutant during lens and lacrimal gland development (Fig. 6I,J,T,W; see Fig. S1 in the supplementary material). However, when combined with the KrasG12D allele, Sprouty2 ablation restored the lens to the wild-type size in the Le-Cre;Shp2flox/flox,LSL-KrasG12D,Spry2flox/flox triple mutants, and completely reversed the abnormal posterior shift of the lens epithelium cells (Fig. 6H-L,W, arrows). Of note, although the lens cell proliferation and differentiation defects were rescued, there still exist extensive TUNEL-positive cells in the triple mutant lens was approximately that of the E15.5 wild-type controls (Fig. 6R-W).

Nevertheless, although the KrasG12D allele could only induce at most a minuscule outgrowth of lacrimal gland in the context of Shp2 mutation, the combination of Sprouty2 ablation and Kras activation in the Le-Cre;Shp2flox/flox,LSL-KrasG12D,Spry2flox/flox triple mutants was able to promote lacrimal gland growth to approximately that of the E15.5 wild-type controls (Fig. 6R-W). Therefore, inhibition of Sprouty2 and activation of Kras signaling both contributed to the genetic rescue of the Shp2 phenotype in lens and lacrimal gland development.
DISCUSSION

In summary, we have shown that Shp2 tyrosine phosphatase is a key factor in regulating the growth, survival and differentiation of lens epithelial cells and lacrimal gland epithelial budding. Shp2 ablation disrupted ERK signaling, its downstream response gene expressions, and cell cycle regulator activities. Although the lens and lacrimal gland phenotype can be partially rescued by activated Kras signaling, the absence of Shp2 led to uncontrolled tyrosine phosphorylation of Sprouty2 protein, which ultimately prevented the full activation of ERK pathway. Only the removal of Sprouty2 allowed Kras activation to fully restore lens growth and lacrimal gland budding. These results suggest that Sprouty2 suppression is an integral part of Shp2 function in modulating Ras-ERK signaling.

Previous studies have uncovered many potential substrates of Shp2, but how these factors are integrated into a coherent signaling pathway and whether such biochemical interactions are functionally relevant in vivo remain unclear. It is especially perplexing that a protein such as Ras-MAPK (Dance et al., 2008). It has been proposed that Shp2 dephosphorylates Sprouty to suppress its negative regulation of such as Ras-MAPK (Dance et al., 2008). It has been proposed that Shp2 dephosphorylates Sprouty to suppress its negative regulation of Ras activity, thus indirectly promoting Ras-MAPK signaling. This is supported by studies in Drosophila in which removing one copy of the Sprouty allele suppressed the photoreceptor defect caused by the inactivation of the Shp2 homolog Corkscrew. However, it should be noted that this genetic interaction was based on the expression of a dominant-negative Shp2 homolog Corkscrew, which was unlikely to fully suppress the endogenous Corkscrew function. Using a complete loss-of-function allele of Shp2, we have now shown that at least in lens and lacrimal gland development, Sprouty2 expression was abrogated by Shp2 ablation. If Sprouty2 was never expressed in the Shp2 null background, further deletion of the Sprouty2 allele would certainly not elicit any effect. Indeed, we showed that Sprouty2 genetic ablation did not ameliorate the Shp2 knockout ocular phenotype, and ERK signaling remained downregulated. Therefore, the loss of Sprouty does not preclude the need for Shp2 in activating Ras signaling, suggesting that there exist Sprouty-independent function(s) of Shp2 in RTK signaling.

Our results thus support the idea that Shp2 induces Sprouty2 expression indirectly via Ras signaling, while directly downregulating Sprouty2 activity by controlling its phosphorylation state (Fig. 7). It might appear paradoxical that Shp2 evolves to be both a positive and a negative regulator of Sprouty2; however, it should be noted that Sprouty2 inhibition via direct Shp2 dephosphorylation is likely to be much faster than Sprouty2 induction via Ras signaling, because the latter requires new protein synthesis. The kinetic difference between these two pathways could potentially sharpen the dynamic response of Ras signaling to the change of extracellular stimulus, thus tightly controlling the progenitor cells to rapidly enter/exit the proliferation and differentiation states. This biphasic regulation of Sprouty by Shp2 is also consistent with the previous observations that Shp2 appears to employ distinct mechanisms to control both the immediate-early and late signaling responses to growth factors (Bennett et al., 1996). Finally, our model might further explain why Kras activation alone achieved much more robust rescue of the lens than the lacrimal gland in the Shp2 mutant background. Here we need to take into account the fact that the lacrimal gland tip cells must sustain intense RTK signaling to lead the continuous outgrowth of the lacrimal gland, whereas the lens epithelial cells only transiently experience a more moderate RTK activation during their differentiation, as evident by the differential intensity of phospho-ERK and Erm staining in these two tissues (Fig. 4F,1). This would lead to continuously high Sprouty2 expression, and as a consequence, a greater need for Shp2 to restrict its inhibitory effect in the lacrimal gland. It is tempting to speculate that, in the presence of unconstrained Sprouty2 inhibition, Kras activation might be sufficient to induce RTK downstream signaling to the moderate threshold required for lens development, but not enough to maintain the peak level required for the lacrimal gland budding. Numerous cell culture studies have shown that, in the absence of Shp2, most RTK signaling can still induce ERK activation, but its intensity and duration is much diminished (Feng, 1999; Neel et al., 2003). Our comparative analysis of the lens and the lacrimal gland not only show that this intriguing biochemical observation is applicable in vivo, but also suggests that at least one of its underlying mechanisms might be the dynamic regulation of Sprouty by Shp2.

How important, then, is the Shp2-Ras-ERK pathway in mediating RTK signaling, including FGFR signaling, in lens and lacrimal gland? In lacrimal gland development, either Shp2 or Fgfr2 ablation abolished lacrimal gland budding. In lens development, however, the Shp2 phenotype is surprisingly milder than that of the reported FGFR signaling defects. Using a Cre driver that acted even later than Le-Cre, Zhao and colleagues showed that deletion of the Fgfr1/2/3 completely abrogated lens fiber cell elongation, resulting in a hollow lens structure without any γ-crystallin expression (Zhao et al., 2008). Moreover, Gotoh and colleagues showed in a systemic knockout model that mutations in the key FGFR adaptor protein Ffrs2ζ to disrupt its interaction with Shp2 resulted in lens induction failure (Gotoh et al., 2004). These phenotypes are in striking contrast to our Le-Cre;Shp2<sup>flx/flx</sup> mutant lens, which exhibited cell proliferation...
and apoptosis defects but maintained α, β and γ-crystallin expressions. The phenotypic discrepancy between the Shp2 and the Frs2α mutants is almost certainly due to the timing of knockouts, as we have shown that the Le-Cre mediated Shp2 knockout did not appreciably affect ERK signaling at E10.5. Although we cannot rule out the possibility that undetectable levels of Shp2 protein persisted after the lens induction phase to support further lens development, it should be noted that the Shp2 and Fgfr1/2/3 mutants both appeared to reduce phospho-ERK expression to a similar extent in the E12.5 lens, but only the Fgfr1/2/3 mutant exhibited primary lens fiber cell elongation and differentiation defects. This raises an important question about whether Shp2-Ras-ERK pathway can sufficiently account for all of the RTK signaling effects in the lens. Considering that RTK signaling is known to promote other downstream pathways, such as P13K- AKT and PLCγ signaling, further studies are needed to investigate whether these pathways might cooperate with Shp2-Ras-ERK signaling to control lens development.

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Competing interests statement
The authors declare no competing financial interests.

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