Cell Biology
Developmental Biology

Shc1 cooperates with Frs2 and Shp2 to recruit Grb2 in FGF-induced lens development

Reviewed Preprint
v2 • February 20, 2025
Revised by authors

Reviewed Preprint
v1 • December 9, 2024

Qian Wang, Hongge Li, Yingyu Mao, Ankur Garg, Eun Sil Park, Yihua Wu, Alyssa Chow, John Peregrin, Xin Zhang

Department of Ophthalmology, Columbia University, New York, United States • Department of Pathology and Cell Biology, Columbia University, New York, United States

eLife Assessment

This **fundamental** article significantly advances our understanding of FGF signalling, and in particular, highlights the complex modifications affecting this pathway. The evidence for the authors' claims is **convincing**, combining state-of-the-art conditional gene deletion in the mouse lens with histological and molecular approaches. This work should be of great interest to molecular and developmental biologists beyond the lens community.

https://doi.org/10.7554/eLife.103615.2.sa4

Abstract

Fibroblast growth factor (FGF) signaling elicits multiple downstream pathways, most notably the Ras/MAPK cascade facilitated by the adaptor protein Grb2. However, the mechanism by which Grb2 is recruited to the FGF signaling complex remains unresolved. Here we showed that genetic ablation of FGF signaling prevented lens induction by disrupting transcriptional regulation and actin cytoskeletal arrangements, which could be reproduced by deleting the juxtamembrane region of the FGF receptor and rescued by Kras activation. Conversely, mutations affecting the Frs2-binding site on the FGF receptor or the deletion of Frs2 and Shp2 primarily impact later stages of lens vesicle development involving lens fiber cell differentiation. Our study further revealed that the loss of Grb2 abolished MAPK signaling, resulting in a profound arrest of lens development. However, removing Grb2's putative Shp2 dephosphorylation site (Y209) neither produced a detectable phenotype nor impaired MAPK signaling during lens development. Furthermore, the catalytically inactive Shp2 mutation (C459S) only modestly impaired FGF signaling, whereas replacing Shp2's C-terminal phosphorylation sites (Y542/Y580) previously implicated in Grb2 binding only caused placental defects, perinatal lethality, and reduced lacrimal gland branching without impacting lens development, suggesting that Shp2 only partially mediates Grb2 recruitment. In contrast, we observed that FGF signaling is required for the phosphorylation of the Grb2-binding sites on Shc1 and the deletion of Shc1 exacerbates the lens vesicle defect caused by

Frs2 and Shp2 deletion. These findings establish Shc1 as a critical collaborator with Frs2 and Shp2 in targeting Grb2 during FGF signaling.

Introduction

The lens is an exemplary model for studying signaling pathways (Cvekl and Zhang, 2017). In mice, the lens placode emerges as thickened epithelia within the lateral head ectoderm at E9.5 (**Fig. 1A**). It undergoes invagination to form the lens pit at E10.5, and upon separation from the surface ectoderm, progresses into the lens vesicle at E11.5. Following this, lens progenitor cells within the lens vesicle proliferate and migrate toward the equator of the lens, where they differentiate into lens fibers responsible for the lens's focusing power (Lovicu and McAvoy, 2005). Genetic modification of the FGF signaling cascade alters various lens developmental processes, including lens invagination (Carbe and Zhang, 2011; Pan et al., 2006), lens vesicle formation (Kuracha et al., 2011), the establishment of the transition zone (Li et al., 2019), lens epithelium proliferation and survival, as well as lens fiber differentiation and elongation (Lovicu and Overbeek, 1998; Qu et al., 2011b; Robinson et al., 1995; Zhao et al., 2008). Therefore, the lens provides valuable insights into the nuanced interplay of FGF signaling components during distinct stages of lens formation.

The current model of FGF signaling posits that its primary orchestration centers on the Frs2/Shp2/Grb2 complex (Beenken and Mohammadi, 2009; Brewer et al., 2016; Eswarakumar et al., 2005). According to this model, FGFR activation induces phosphorylation of the adaptor protein Frs2, creating a platform for recruiting Shp2 and Grb2. In conjunction with its constitutively bound partner Sos (a guanine nucleotide exchange factor), Grb2 subsequently initiates Ras/MAPK signaling (Hadari et al., 2001; Ong et al., 2000). Prior research in eye development supports this notion, revealing that Crk proteins augment Ras signaling by associating with the Frs2/Shp2/Grb2 complex (Collins et al., 2018; Li et al., 2014; Madakashira et al., 2012), while PI3K-AKT signaling is activated through direct Ras binding with the PI3K catalytic subunit p110 (Wang et al., 2021). However, unresolved questions persist regarding downstream mediators of FGF signaling. Earlier studies suggested that mice with Frs2 mutants lacking the Grb2 binding site can survive healthily, whereas those lacking the Shp2 binding site exhibit severe eye development defects and significantly reduced MAPK signaling (Gotoh et al., 2004). These results suggest the critical importance of Shp2 in Grb2-mediated Ras signaling, but the exact mechanism remains unclear. Additionally, Soriano and colleagues generated allelic series of *Fgfr1* and *Fgfr2* mutants disrupting the Frs2 binding sites and multiple tyrosine phosphorylation residues, both individually and in combination, yet their phenotypes proved less severe than those of the respective null mutants (Brewer et al., 2015; Clark and Soriano, 2024) These studies suggest that there may exist additional adaptor(s) other than Frs2 to mediate FGF signaling. One potential candidate for mediating FGF signaling is the Shc family of proteins, which exist in three isoforms: p66, p52, and p46 (Wills and Jones, 2012). These proteins contain a phosphotyrosine-binding (PTB) domain and an SH2 domain, both of which can interact with phosphorylated tyrosine residues. Additionally, Shc proteins possess multiple tyrosine residues that, upon phosphorylation, can be recognized by other molecules, including Grb2. Previous studies have demonstrated that FGF stimulation leads to increased Shc phosphorylation and its binding to Grb2, although the specific binding site remains unresolved (Dunican et al., 2001; Klint et al., 1995; Schuller et al., 2008). Furthermore, the functional significance of Shc's interaction with the FGF receptor in vivo has yet to be demonstrated.

In this study, we demonstrated that genetic alterations to FGFR— whether by deleting all isoforms, the juxtamembrane region, or the specific Frs2 binding site—disrupt the successive phases of lens development, encompassing lens induction, vesicle formation, and fiber differentiation. Surprisingly, while we established that Grb2-Ras signaling serves as the primary conduit of FGF signaling, interfering with Grb2 dephosphorylation and binding by Shp2 or even abolishing Shp2

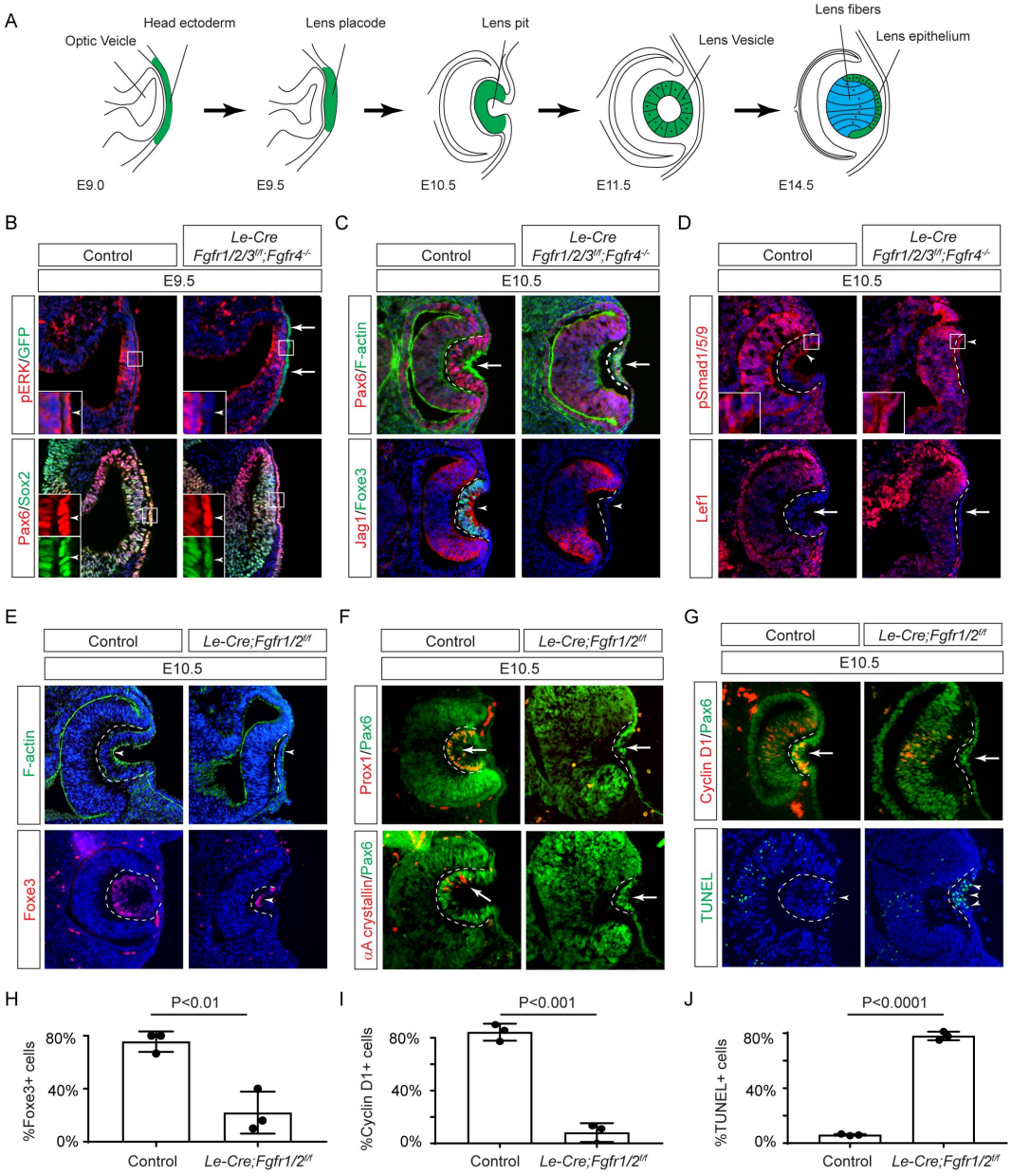


Figure 1.

FGF Signaling Regulates Lens Development in a Dose-Dependent Manner.

(A) Schematic diagram of murine lens development. The head ectoderm is induced by the underlying optic vesicle to become the lens placode, which subsequently folds inwards to become the lens pit. The closure of the lens vesicle sets the stage for the differentiation of the lens epithelium into the lens fibers. **(B)** Depletion of all four *Fgfr1/2/3/4*, driven by *Le-Cre* and traced with GFP (arrows), led to a thinner lens placode, evident from the absence of pERK signals and the failure to upregulate Sox2 like Pax6 (inserts, arrowheads). **(C)** *Fgfr1/2/3/4* mutants displayed disrupted apical constriction (F-actin accumulation, arrows) and lacked lens-specific expression of Foxe3 and Jag1 (arrowheads). Dotted lines outline the lens pit. **(D)** Despite *Fgfr1/2/3/4* mutations, BMP (pSmad1/5/9 staining, arrowheads) and Wnt signaling (Lef1 expression, arrows) remained unaffected. **(E)** The absence of *Fgfr1/2* alone did not impede the apical buildup of F-actin nor the expression of Foxe3, indicating partial retention of lens development processes. **(F)** Crucial lens markers, Prox1 and αA-crystallin, were absent in *Fgfr1/2* mutants, pointing to a significant developmental defect after the lens induction stage. **(G)** *Fgfr1/2* mutants exhibited loss of cell proliferation marker Cyclin D1 (arrows) and widespread apoptosis (TUNEL staining, arrowheads). **(H)** Quantification of Foxe3+ cells in *Fgfr1/2* mutants. Student's t-test, n=3, P<0.01. **(I)** Quantification of Cyclin D1+ cells in *Fgfr1/2* mutants. Student's t-test, n=3, P<0.001. **(J)** Quantification of TUNEL+ cells in *Fgfr1/2* mutants. Student's t-test, n=3, P<0.0001.

phosphatase activity did not eliminate either MAPK signaling or lens differentiation. Conversely, we observed that FGF-induced Shc phosphorylation hinges on the FGFR juxtamembrane domain rather than its Frs2 binding site. Although the deletion of Shc1 has only a modest impact on lens development and MAPK activity individually, its combination with Frs2 and Shp2 deletion results in a profound arrest of lens vesicle development. These results suggest that Shc functions independently of Frs2 and Shp2 to augment Grb2-Ras signaling within the FGF pathway.

Results

FGF signaling is required for lens induction

Previous studies have established the presence of all four FGF receptors in the surface ectoderm during lens induction (Garcia et al., 2011). However, despite the deletion of the primary FGFRs, *Fgfr1* and *Fgfr2*, only thinning of the lens placode occurred, with no discernible impact on lens determination transcription factors like Pax6, Sox2, and Foxe3. To explore whether the compensatory effects of remaining FGFRs obscure the role of FGF signaling in lens induction, we eliminated all four FGFRs using the *Le-Cre* deletor driven by the *Pax6* lens ectoderm (LE) enhancer (Ashery-Padan et al., 2000). The *Le-Cre* activity within the lens placode at E9.5, as indicated by the embedded GFP reporter (**Fig. 1B**, arrows), resulted in the complete loss of pERK in the ectoderm, confirming FGF signaling inactivation (**Fig. 1B**, inserts and arrowheads). Consequently, while the initial marker of lens induction, increased Pax6 expression as the surface ectoderm transitions into the lens placode, persisted in the mutant, Sox2 expression failed to be upregulated. Furthermore, subsequent lens placode invagination, driven by apical constriction evidenced by polarized F-actin localization on the apical side by E10.5 (Chauhan et al., 2011), was disrupted (**Fig. 1C** arrows). This occurred despite the proper localization of Fibronectin at the basal side of the lens placode (**supplementary Fig. 1A**), suggesting that overall cell polarity remained unaffected. The *Le-Cre;Fgfr1*$^{f/f}$*;Fgfr2*$^{f/f}$*;Fgfr3*$^{f/f}$*;Fgfr4*$^{-/-}$mutant also failed to exhibit the expected upregulation of Foxe3 and Jag1 in lens vesicle, further underscoring impaired lens induction (**Fig. 1C**, arrowheads). Previous studies have highlighted FGF and BMP interaction during lens formation (Garcia *et al*., 2011), yet the dorsal-to-ventral gradient of BMP signaling, as indicated by pSmad staining, persisted in *Le-Cre;Fgfr1*$^{f/f}$*;Fgfr2*$^{f/f}$*;Fgfr3*$^{f/f}$*;Fgfr4*$^{-/-}$knockouts (**Fig. 1D**, arrowheads). Furthermore, Wnt signaling, a known negative regulator of lens induction (Smith et al., 2005), showed no signs of abnormal activity, as indicated by the absence of Lef1 expression (**Fig. 1D**, arrows). These results demonstrated that FGF signaling is required independently of Bmp and Wnt signaling for lens induction.

We next focused on the two primary FGFRs, Fgfr1 and 2, to scrutinize the latter phases of lens development. In contrast to mutants with deletion of all four FGFRs, we observed apical confinement of F-actin and expression of Foxe3 in the *Le-Cre;Fgfr1*$^{f/f}$*;Fgfr2*$^{f/f}$mutant lens cells, although the number of Foxe3-expressing cells were reduced (**Fig. 1E and H**, arrows). However, there was a conspicuous reduction in phosphorylation of mTOR and its downstream target S6 in the lens vesicle, suggesting that deletion of *Fgfr1/2* disrupted mTOR signaling (**Supplementary Fig. 1B**, arrowheads). Underscoring the lens differentiation defect, Prox1, a crucial transcription factor for lens fiber development, along with the lens-specific protein αA crystallin expression, was also lost in the *Le-Cre;Fgfr1*$^{f/f}$*;Fgfr2*$^{f/f}$mutant lens vesicle (**Fig. 1F**, arrows) (Garg et al., 2020; Ochi et al., 2003; Xie et al., 2016). Additionally, there was a notable decrease in Cyclin D1 expression and increasing TUNEL staining within the lens vesicle, indicating cell proliferation and apoptosis defects ((**Fig. 1G, I** and J, arrows and arrowheads) (Garcia *et al*., 2011).

Ras mediates FGF signaling to suppress apoptosis in the developing lens

FGF signaling is known to stimulate the Ras-MAPK signaling pathway. To explore the significance of Ras signaling, we employed a genetic rescue strategy utilizing an inducible *Kras* allele capable of expressing the constitutively active $Kras^{G12D}$ upon Cre-mediated recombination (**Fig. 2A**) (Tuveson et al., 2004). When crossed with *Fgfr1/2* mutants, this allele effectively restored pERK expression and normalized lens vesicle invagination (**Fig. 2B**). By E13.5, the *Le- Cre;*$Fgfr1^{f/f}$*;*$Fgfr2^{f/f}$*;*$Kras^{G12D}$lens displayed robust phosphorylation of MEK, the upstream kinase of Erk, concomitant with the expression of lens fiber markers Prox1 and αA crystallin (**Fig. 2B and C**). Notably, the rescued lens reached about half the size of a control lens (**Fig. 2D**). These results suggest that Kras signaling acts as a primary conduit for FGF signaling during lens development.

To ascertain whether cell death underpins the pronounced lens vesicle defect in *Le-Cre;*$Fgfr1^{f/f}$*;*$Fgfr2^{f/f}$mutant, we targeted Bax and Bak, two core regulators of the intrinsic pathway of apoptosis, for deletion. This intervention notably reduced TUNEL signals within the lens placode (**Fig. 2E and F**) and resulted in a recovery of lens formation, as evidenced by the expression of Foxe3, Maf, and Jag1 (**Fig. 2G**), although the lens remained considerably smaller than the wild-type control (**Fig. 2H**). This underscores the critical role of FGF signaling in preventing excessive cell apoptosis during lens development.

The juxtamembrane domain and Frs2 binding site of FGFR regulate the consecutive steps of lens development

Previous studies have mapped the Frs2 binding site to the juxtamembrane domain of FGFR (Dhalluin et al., 2000; Ong *et al*., 2000). To probe the role of this region in lens development, we employed two distinct alleles: one entirely devoid of the juxtamembrane domain (amino acid 407-433 in Fgfr1, $Fgfr1^{\Delta Frs}$) (Hoch and Soriano, 2006), and another harboring mutations in two pivotal residues essential for Frs2 binding (L424A and R426A in Fgfr2, $Fgfr2^{LR}$) (**Fig. 3A**) (Eswarakumar et al., 2006). Intriguingly, while the *Fgfr1/2* compound mutant carrying $Fgfr1^{\Delta Frs}$ mirrored the null phenotype (**Fig. 3B**, arrowheads), the mutant featuring $Fgfr2^{LR}$ exhibited sustained expression of pERK, Cyclin D1, and αA crystallin, with no discernible increase in cell death, as confirmed by cleaved caspase 3 staining (see **Fig. 3B and C**). This suggests that the juxtamembrane domain of FGFR likely serves additional functions beyond Frs2 binding in lens induction.

The absence of a lens induction phenotype in the *Le-Cre;*$Fgfr1^{f/f}$*;*$Fgfr2^{f/LR}$ mutant raised the question regarding the role of Frs2 in lens development. Upon examining the iSyTE lens gene expression database (Kakrana et al., 2018), we observed a rapid increase in *Fgfr3* expression in the murine lens from E11.5 onwards, surpassing the levels of both *Fgfr1* and *Fgfr2* by E12.5 (**Fig. 3D**). This led us to consider whether heightened *Fgfr3* expression could potentially mask the effects of $Fgfr2^{LR}$ mutation during later stages of lens development. To explore this hypothesis, we further deleted *Fgfr3* in conjunction with the *Fgfr1* and $Fgfr2^{LR}$ mutant, which indeed impeded the differentiation and elongation of posterior lens epithelial cells, as evidenced by the absence of αA crystallin, Jag1, and Maf at E11.5 (**Fig. 3E**). By E12.5, unlike the *Le- Cre;*$Fgfr1^{f/f}$*;*$Fgfr2^{f/LR}$ mutant, which retained pERK staining and generated lens fibers to populate the lens vesicle similar to controls, the *Le-Cre;*$Fgfr1^{f/f}$*;*$Fgfr2^{f/LR}$*;*$Fgfr3^{f/f}$ triple mutant remained a hollow lens vesicle without any pERK expression (**Fig. 3F and G**). This observation bears a striking resemblance to the phenotype previously reported when all three FGFRs were deleted at this stage (Zhao *et al*., 2008). Thus, while the Frs2 binding site on FGFR is dispensable for lens induction and lens vesicle formation, it evidently emerges as crucial for the later stage of lens fiber differentiation.

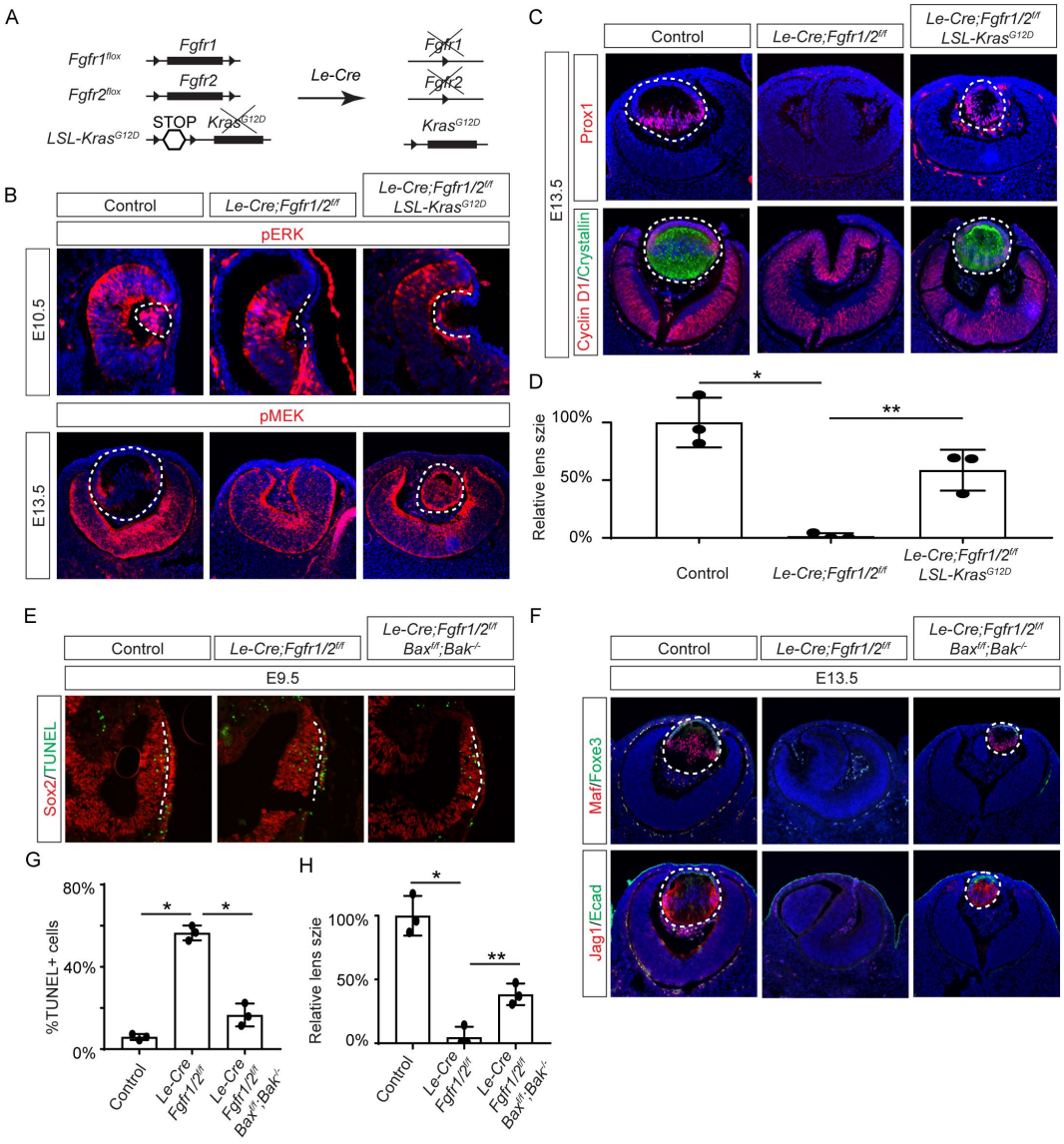


Figure 2.

Restoration of Lens Development in FGF Signaling Mutants via Kras Activation and Apoptosis Inhibition.

(A) The *Le-Cre* driver facilitated the excision of the floxed alleles of *Fgf1/2* along with the *LSL-STOP* cassette at the *Kras* locus, leading to the expression of the constitutively active $Kras^{G12D}$ allele within the *Fgf1/2* mutant background. **(B)** The activation of Kras signaling in the FGF signaling mutant lenses reinstated pERK activity at E10.5 and pMEK expression at E13.5, indicating restoration of MAPK signaling. **(C)** The lens-specific expression of Prox1 and αA-crystallin were also recovered, indicating successful lens development rescue. **(D)** Quantification of the lens size. One way ANOVA, n=3, *P<0.001, **P<0.02. **(E)** The deletion of pro-apoptotic genes *Bak* and *Bax* in *Fgf1/2* mutants suppressed apoptosis as shown by TUNEL staining. **(F)** Inhibiting apoptosis in *Fgfr1/2* mutants facilitated lens formation, as indicated by the expression of lens differentiation markers Prox1, Maf, and Jag1. **(G)** Quantification cell apoptosis at E9.5. One-way ANOVA, n=3, *P<0.001. **(H)** Quantification of the lens size at E13.5. One-way ANOVA, n=3, *P<0.0001, **P<0.05.

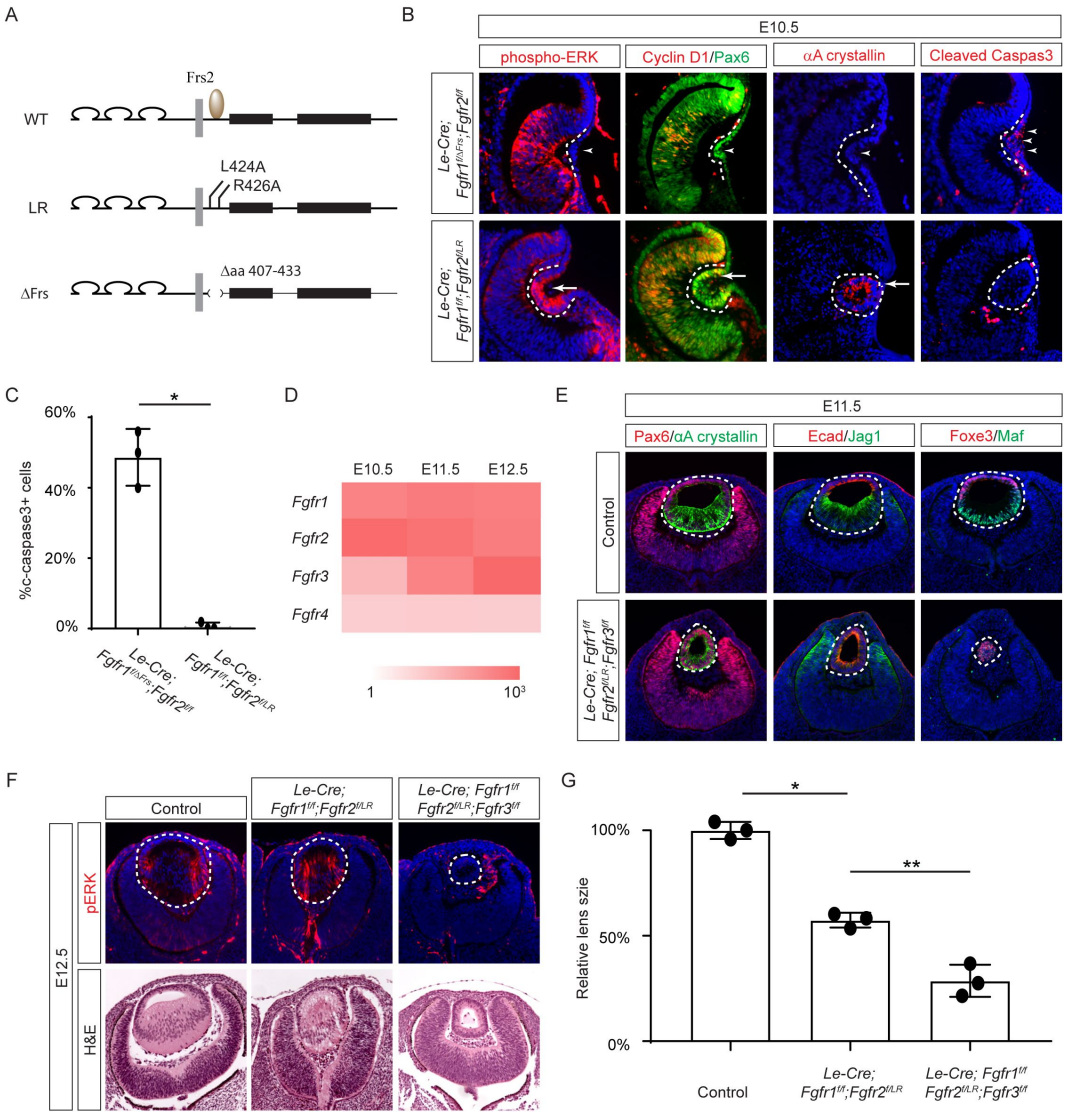


Figure 3.

The Frs2 binding site on FGFR is only required for lens vesicle differentiation.

(A) Overview of *Fgfr* mutant alleles. *Fgfr1*$^{\Delta Frs}$ lacks the Frs2 binding domain (amino acid 407- 433), and *Fgfr2*^{LR} has point mutations disrupting Frs2 binding (L424A and L426A). **(B)** In *Fgf1/2* compound mutants, loss of pERK, CyclinD1, αA-crystallin and increased cleaved caspase3 were observed with the *Fgfr1*$^{\Delta Frs}$ allele but not the *Fgfr2*^{LR} allele. **(C)** Quantification of cleaved caspase3 staining. Student's t-test, n=3, *P<0.001. **(D)** Heatmap depicts *Fgfr* expression levels during lens development. **(E)** *Fgfr2*^{LR}mutants in the *Fgfr1/2/3* genetic background showed impaired lens vesicle differentiation, with posterior lens epithelial cells failing to elongate and activate lens fiber cell markers Jag1 and Maf. **(F)** *Fgfr1/2/3* triple mutants with *Fgfr2*^{LR}lost pERK staining and displayed a shallow lens vesicle at E12.5. **(G)** Quantification of the lens size. One-way ANOVA n=3, *P<0.001, **P<0.05.

Grb2 mediates FGF-MAPK signaling in lens cell differentiation

If Frs2 is responsible for lens fiber differentiation rather than lens induction, we anticipate that its downstream target, Grb2, would exhibit a similar phenotype. To test this hypothesis, we genetically deleted *Grb2* using the *Le-Cre* driver. Indeed, the *Le-cre;Grb2$^{f/f}$* mutant formed a lens vesicle at E12.5 but lacked pERK staining, correlating with decreased Cyclin D3 expression and increased TUNEL staining (**Fig. 4A**). Notably, the initial lens determination gene Foxe3 was unaffected, but differentiation marker Jag1 was absent while Maf and crystallin were reduced. This lens differentiation defect is evident as early as E11.5, characterized by a significant reduction in the expression of cell cycle regulators Cyclin D1 and p57, as well as the pro- differentiation transcription factor Prox1, suggesting dysregulation of the cell cycle and failure to initiate the lens fiber cell differentiation program (**Fig. 4B**). By E13.5, the lens vesicle remained hollow and smaller compared to the control (**Fig. 4B and C**). The deletion of Grb2 affects lens differentiation without hindering the formation of the lens vesicle, mirroring the effect of Frs2 binding site mutation, thus supporting the notion that Grb2 is the primary downstream effector of Frs2 in promoting lens fiber differentiation.

The Grb-Shp2 binding plays a modest role in FGF signaling

While both Frs2 and Shp2 possess phosphotyrosine residues that can engage with Grb2 (**Fig. 5A**), it's noteworthy that only mutations in the Shp2 binding sites, rather than those in the Grb2 binding sites on Frs2, led to severe eye development defects (Gotoh *et al*., 2004). This intriguing observation spurred us to investigate the potential role of Shp2 as an adaptor in facilitating the interaction between Frs2 and Grb2. To this end, we engineered a mouse model with mutations in the Grb2 binding sites of Shp2 (Sun et al., 2013) by substituting the C-terminal tyrosine residues 542 and 580 with phenylalanine (*Shp2^{YF}*) (**Fig. 5B**), which was confirmed by southern blot analysis using both 5' and 3' probes and Sanger sequencing (**Fig. 5C**). However, homozygous mice carrying the *Shp2^{YF}* mutation died around E12.5 with visibly paler and smaller bodies (**Fig. 5D and E**). Histological analysis of mutants revealed a significantly thinner labyrinth zone in placentas, crucial for oxygen and nutrient supplies (**Fig. 5E**, dotted lines). To test whether this placental defect is responsible for the embryonic lethality, we combined the *Shp2^{YF}* mutation with the *Shp2* conditional allele using *Sox2Cre*, which is specifically active in the epiblast- derived embryonic tissues but not in the trophoblast-derived placenta (**Fig. 5F**)(Hayashi and McMahon, 2002). *Sox2Cre;Shp2$^{f/YF}$* embryos indeed survived past embryonic day 15.5 without evident morphological abnormalities, yet they succumbed shortly after birth for reasons yet unknown (**Fig. 5G**).

The survival of *Sox2Cre;Shp2$^{f/YF}$* mutant beyond embryonic development permitted the isolation of mouse embryonic fibroblast (MEF) cells for biochemical analysis. As anticipated, the phosphorylation of Shp2 (pShp2^{Y542}) induced by FGF was abolished in *Sox2Cre;Shp2$^{f/YF}$*MEF cells, yet the activation of pERK was only partially impaired (**Fig. 5H**). This contrasts with the more pronounced reduction in PDGF-induced ERK phosphorylation, underscoring a distinct requirement for Shp2-Grb2 binding across related receptor tyrosine kinase (RTK) pathways (Araki et al., 2003). In line with the subtle impact on FGF signaling, neither the pattern of pERK staining nor the size of the lens showed discernible alterations in *Sox2Cre;Shp2$^{f/YF}$* mutants (**Fig. 5I**, arrowheads). We further investigated lacrimal gland development due to its remarkable sensitivity to FGF signaling intensity, where even a heterozygous *Fgf10* mutation has been shown to stunt gland growth (Garg and Zhang, 2017; Qu et al., 2011a). Notably, we observed a slight reduction in pERK staining in the lacrimal gland primordia at E14.5 and fewer lacrimal gland buds at birth (**Fig. 5I**, arrows). These nuanced ocular phenotypes, alongside the overall normal morphology of *Sox2Cre;Shp2$^{f/YF}$* mutant embryos, collectively suggest that Shp2-Grb2 binding exerts a modest influence on FGF signaling.

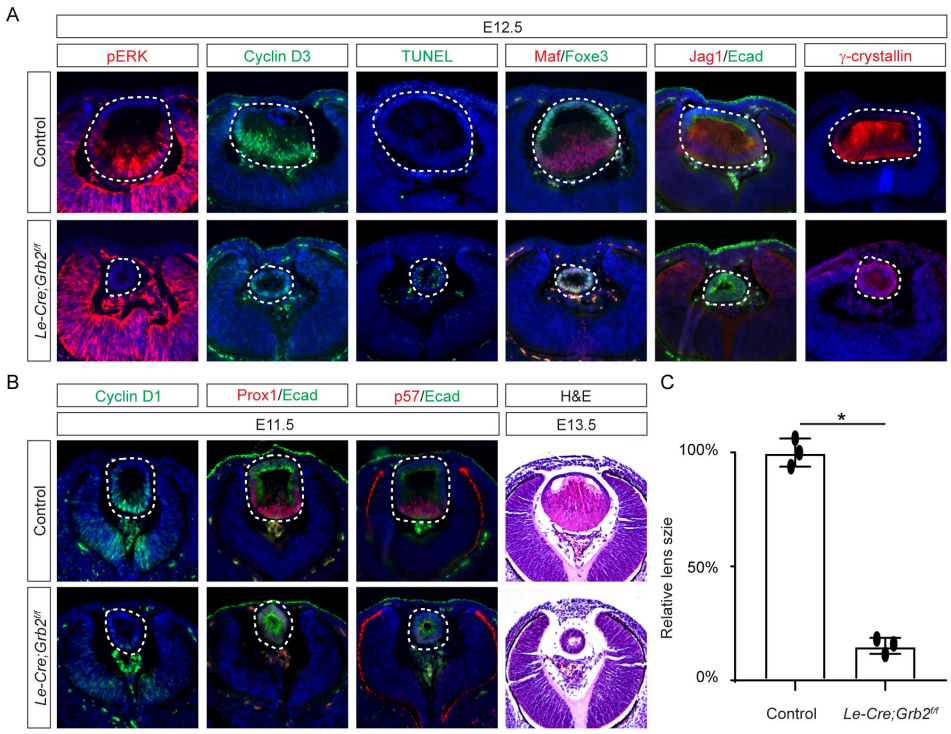


Figure 4.

Grb2 is essential for lens vesicle survival, proliferation, and differentiation.

(A) The targeted removal of Grb2 in the lens led to a loss of pERK signaling, reduced CyclinD3 expression, increased apoptosis (TUNEL staining), and disrupted expression of critical lens development genes Maf, Foxe3, Jag1 and γ-crystallin expression at E12.5. **(B)** Grb2 mutants displayed absent CyclinD1, Prox1, and p57 expression at E11.5 and remained an undifferentiated hollow vesicle at E13.5, failing to undergo normal lens fiber elongation. **(C)** Quantification of the lens size. Student's t-test, n=3, *P<0.0001.

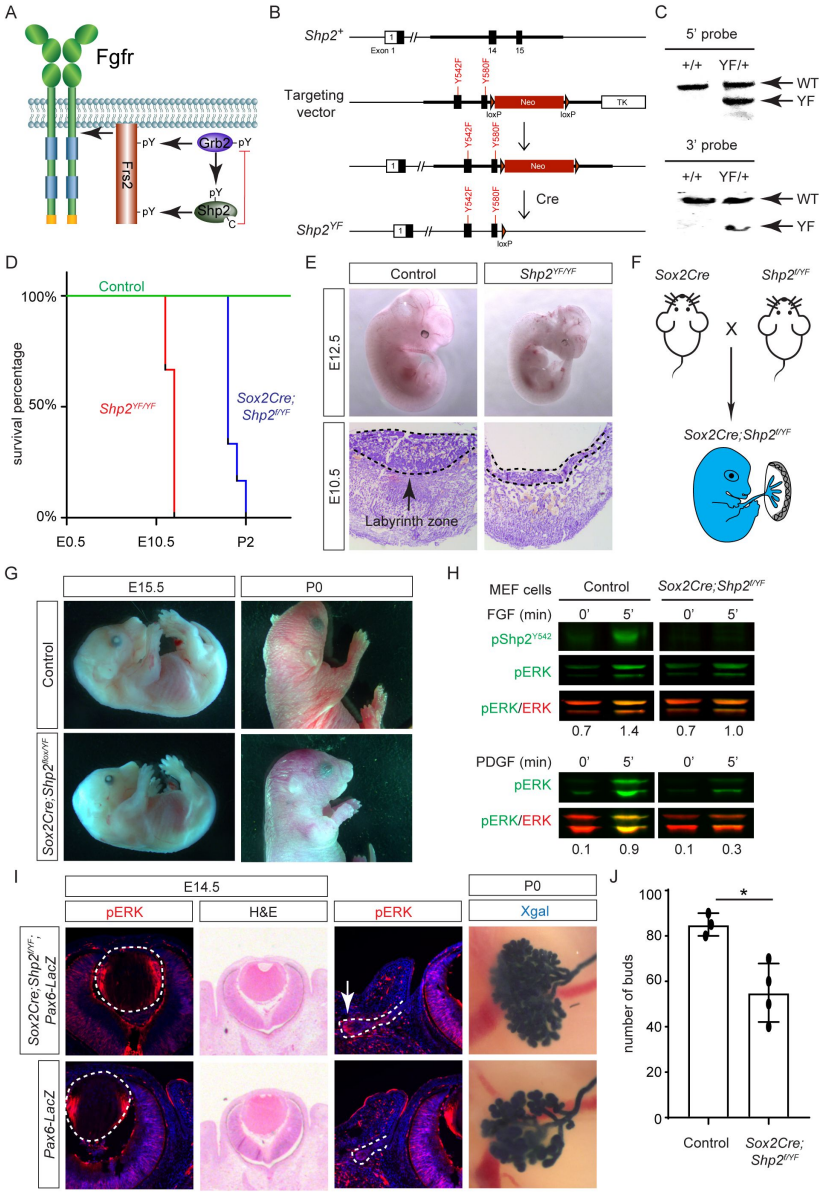


Figure 5.

Shp2 C-terminal tyrosine phosphorylation is required for embryonic survival but dispensable for lens development.

(A) Schematic of the core FGF signaling pathway. FGFR activation leads to phosphorylation of the adaptor Frs2 on N- and C-terminal tyrosines, recruiting Grb2 and Shp2 respectively. Shp2 can also bind Grb2 via its own C-terminal phosphotyrosines, and dephosphorylates Grb2 via its catalytic cysteine residue. **(B)** Generation of the *Shp2*^{YF} allele by homologous recombination to introduce *loxP*-flanked Neo and point mutations (Y542F and Y580F) disrupting the Shp2 C-terminal phosphotyrosine sites. The Neo cassette was subsequently excised by Cre-mediated recombination. **(C)** Validation of the *Shp2*^{YF} allele targeting was confirmed through Southern blot analysis with both 5′ and 3′ probes. **(D)** Kaplan-Meier survival curves demonstrate early lethality of *Shp2*^{YF} embryos (E12.5) and perinatal lethality of *Sox2Cre;Shp2*$^{f/YF}$ mutants. n=5 for *Shp2*$^{YF/YF}$and n=6 for *Sox2Cre;Shp2*$^{f/YF}$ mutants. **(E)** *Shp2*$^{YF/YF}$ embryos displayed reduced body size at E12.5 and thinner labyrinth zones in their placenta E10.5. **(F)** *Sox2Cre*-mediated targeting restricts Shp2 deficiency to the embryonic proper, circumventing placental abnormalities. **(G)** *Sox2Cre;Shp2*$^{f/YF}$ mutants appeared grossly normal at E15.5 but failed to survive after birth. **(H)** While Y542 phosphorylation in Shp2 was lost as expected, *Sox2Cre;Shp2*$^{f/YF}$MEFs exhibited a more pronounced reduction in pERK response to PDGF stimulation compared to FGF stimulation. **(I)** *Sox2Cre;Shp2*$^{f/YF}$mutant lens displayed normal pERK staining and morphology, but reduced pERK in lacrimal glands at E14.5 and decreased bud numbers at P0. **(J)** Quantification of the number of lacrimal gland buds. Student's t-test, n=3, *P<0.02.

Inactivation of Shp2 phosphatase activity failed to abrogate FGF-induced MAPK signaling

The results presented above indicate that the direct binding of Grb2 to the Shp2 C-terminus is not essential for FGF signaling. This led us to explore an alternative hypothesis that Shp2 might function by removing inhibitory tyrosine phosphorylation on Grb2, thereby promoting its interaction with Sos and subsequent Ras-MAPK activation (Ahmed et al., 2013; Vemulapalli et al., 2021). Based on the PhosphoSitePlus database, we identified Y209 as the most frequently phosphorylated tyrosine residue in Grb2 (**Fig. 6A**). Notably, previous studies have demonstrated that Shp2 dephosphorylates this specific site upon stimulation by various receptor tyrosine kinases (RTKs) (Ahmed *et al*., 2013; Haines et al., 2009; Li et al., 2001; Riera et al., 2010). To assess the functional significance of Y209 phosphorylation, we generated a mutant *Grb2* allele where Y209 was replaced with phenylalanine ($Grb2^{YF}$) using the ES cell-based gene targeting technique (**Fig. 6B and C**). Surprisingly, our findings revealed that $Grb2^{YF/YF}$ homozygous mutants exhibited normal viability and fertility without any obvious phenotype. Additionally, the intensity of pERK staining in mutant lenses remained unchanged in mutant lenses compared to controls, and markers of the cell cycle (Ki67 and p57) as well as differentiation (Jag1, Foxe3, and Maf) were unaffected (**Fig. 6D**). These results suggest that despite being a frequent target for phosphorylation, Y209 on Grb2 is dispensable for FGF signaling.

The PhosphoSitePlus database indicates that Grb2 still possesses a less frequently phosphorylated Y160 site, which has also been previously implicated in FGF signaling (Ahmed et al., 2015). To rigorously assess the potential impact of Shp2-mediated Grb2 dephosphorylation, we developed a $Shp2^{CS}$ mouse model by substituting the cysteine residue at position 463 (C459 in humans) with alanine in Shp2's catalytic domain, effectively abolishing its enzymatic activity (**Fig. 6E and F**). Unlike the earlier *Shp2* null mutants that perished by E7.5 (Yang et al., 2006), $Shp2^{CS/CS}$embryos exhibited stunted growth but survived until E9.5, indicating that the $Shp2^{CS}$ mutation doesn't entirely abrogate Shp2's function (**Fig. 6G**). Moreover, after removing the *flox* allele using Cre-expressing adenovirus, the $Shp2^{f/CS}$ MEF cells still retained considerable pERK activity in response to FGF stimulation (**Fig. 6H**). This was mirrored in vivo, with pERK detection in the *Le-Cre;*$Shp2^{f/CS}$lens but not in the *Le-Cre;*$Shp2^{f/f}$ mutants (**Fig. 6I**). Consequently, lens epithelial cells in *Le-Cre;*$Shp2^{f/f}$ mutants migrated to the posterior pole with reduced p57 and Jag1 expression, indicating impaired differentiation, but *Le- Cre;*$Shp2^{f/CS}$ lens epithelial cells showed proper p57-mediated cell cycle exit at the lens equator and initiated timely expression of Jag1 (**Fig. 6J**). However, these lenses showed normal proliferation (Ki67) but increased cell death (TUNEL), resulting in a smaller size (**Supplementary Fig. 2**). Moreover, the development of the FGF signaling-sensitive lacrimal gland was blocked in both *Le-Cre;*$Shp2^{f/f}$ and *Le-Cre;*$Shp2^{f/CS}$ mutants, a more pronounced effect than the modest reduction in lacrimal gland buds observed in $Shp2^{YF/YF}$ mutants (**Fig. 6I**). This suggests that inhibiting Shp2's phosphatase activity more significantly affects FGF signaling compared to obstructing its adaptor function, yet doesn't completely abolish FGF signaling.

Shc1 cooperates with Frs2 and Shp2 to promote lens development

The mild lens defects observed in *Shp2* mutants lacking either adaptor or phosphatase function led us to investigate alternative mechanisms for Grb2 recruitment to the FGFR complex. Given the distinct lens phenotypes in *Le-Cre;*$Fgfr1^{f/f}$;$Fgfr2^{f/LR}$and *Le-Cre;*$Fgfr1^{f/\Delta Frs}$;$Fgfr2^{f/f}$mutants (**Fig. 7A**), we hypothesized that FGF might activate unidentified factor(s) in $Fgfr1^{f/f}$;$Fgfr2^{f/LR}$ MEF cells but not in $Fgfr1^{f/\Delta Frs}$;$Fgfr2^{f/f}$ cells following the excision of flox alleles by Cre-expressing adenovirus, mirroring the observed pattern of pERK activation. Interestingly, FGF stimulation was ineffective in raising pFrs2 and pShp2 levels in both sets of MEF cells and did not alter the phosphorylation states of Crk and Gab1, both recognized adaptors in FGF signaling (Collins *et al*., 2018; Hadari *et al*., 2001; Li *et al*., 2014). However, a key difference emerged – Shc phosphorylation was lost in $Fgfr1^{f/\Delta Frs}$;$Fgfr2^{f/f}$cells but persisted in $Fgfr1^{f/f}$;$Fgfr2^{f/LR}$ mutants (**Fig.**

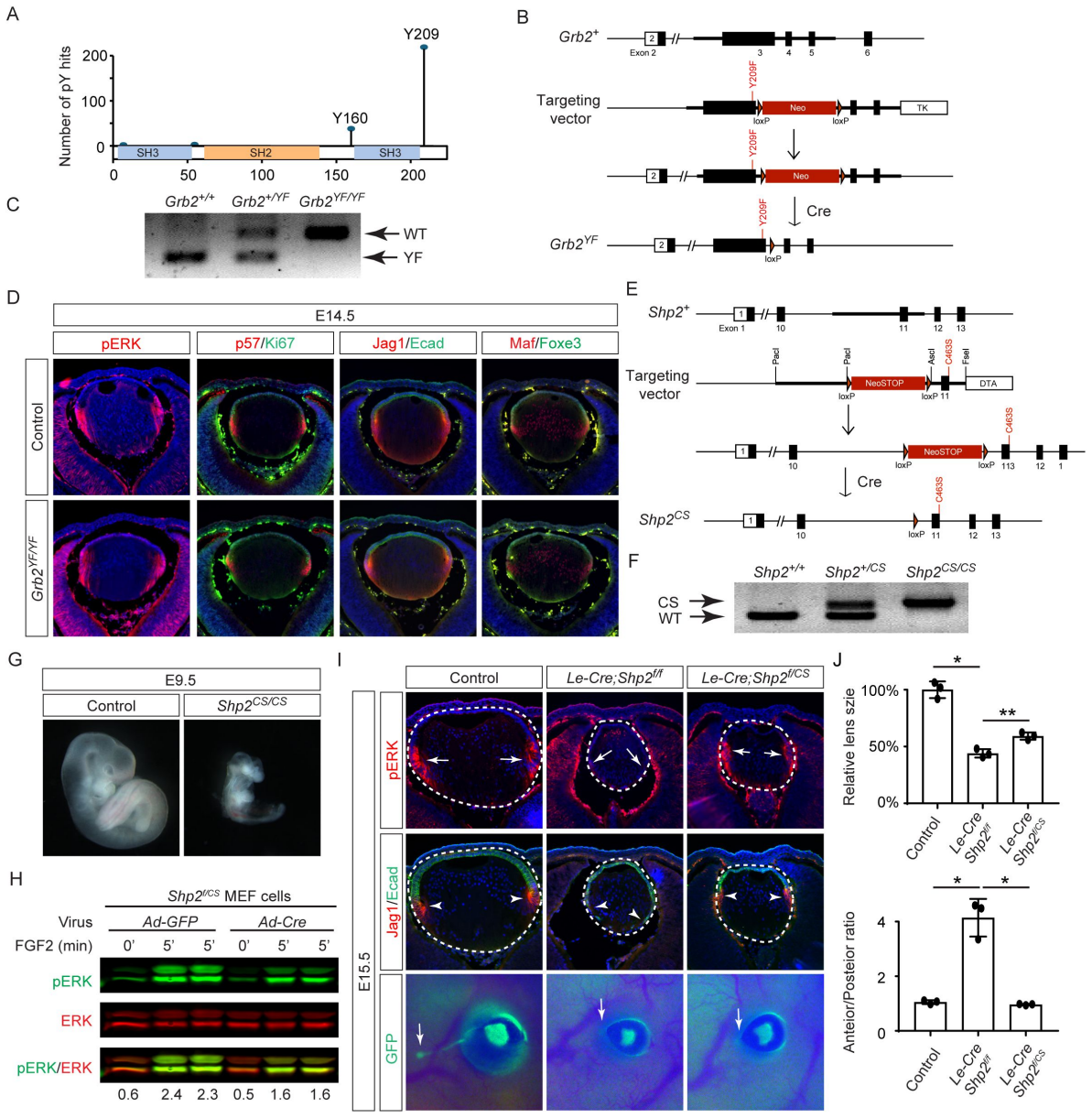


Figure 6.

Shp2 phosphatase activity is partially required for FGF signaling independently of Grb2 dephosphorylation.

(A) PhosphositePlus database indicates that Grb2 predominantly undergoes phosphorylation at Y209 and less frequently at Y160. **(B)** The *Grb2*^{YF} allele was constructed by homologous recombination to integrate a *loxP*-flanked *Neo* cassette and a Y209F point mutation into the *Grb2* locus. **(C)** PCR genotyping confirmed the presence of the *Grb2*^{YF} allele. **(D)** *Grb2* mutant lens typical expression patterns of pERK, cell cycle markers p57 and Ki67 and differentiation markers Foxe3, Maf and Jag1. **(E)** The *Shp2*^{CS} allele was generated by inserting the C459S mutation into the Shp2 locus by homologous recombination, followed by Cre-mediated removal of the *loxP*-flanked *Neo* cassette. **(F)** *Shp2*^{CS} allele validated by PCR genotyping. **(G)** *Shp2*$^{CS/CS}$ mutants exhibited growth retardation and died at E9.5. **(H)** *Shp2*$^{f/CS}$MEF cells retained a significant capacity to activate pERK upon FGF stimulation after Cre virus infection. **(I)** *Le-Cre;Shp2*$^{f/f}$ mutants exhibited loss of pERK and Jag1 staining at the lens transition zone (arrowheads), which also shifted posteriorly. *Le-Cre;Shp2*$^{f/CS}$ mutant lens, in contrast, maintained staining at the equatorial region. Notably, both mutant types lacked lacrimal gland buds (arrows). **(J)** Quantification of the lens size. One-way ANOVA n=3, *P<0.0001, **P<0.001. **(K)** Quantification of the lens perimeter spanning the anterior epithelium versus that of the posterior lens fiber. One-way ANOVA n=3, *P<0.001.

7A-B). This observation was further supported by in vivo data, which showed that pShc was detectable in both wild-type and *Le-Cre;Fgfr1*$^{f/f}$*;Fgfr2*$^{f/LR}$lens vesicles, but not in *Le-Cre;Fgfr1* $^{f/\Delta Frs}$*;Fgfr2*$^{f/f}$ mutants (**Fig. 7C**, arrowhead). These findings suggest that Shc can be activated by the FGFR independently of its Frs2 binding site, potentially serving as an alternate route for Grb2's engagement.

Among the four *Shc* genes present in the mammalian genome, *Shc1* is the most abundant in the lens (iSyTE lens gene expression database). This led us to ablate *Shc1* in the lens to determine its function in FGF signaling. However, *Le-Cre;Shc1*$^{f/f}$ lenses displayed only minor reductions in pERK staining and size, suggesting potential redundancy with other Shc proteins or compensatory mechanisms involving Frs2 and Shp2 (**Fig. 7D**). To investigate this further, we created compound mutants involving these genes. We have previously reported that the deletion of *Frs2* or *Shp2* alone led to a modest diminution in lens size, akin to the *Shc1* deletion effect. However, the combined knockout of *Frs2* and *Shp2* (*Le-Cre;Frs2*$^{f/f}$*;Shp2*$^{f/f}$) resulted in hollow lens vesicles, highlighting a synergistic interaction between these two genes (Li *et al*., 2014). While the *Le-Cre;Frs2*$^{f/f}$*;Shc1*$^{f/f}$compound mutant did not exhibit as severe abnormalities, introducing the *Shc1* knockout into the *Le-Cre;Frs2*$^{f/f}$*;Shp2*$^{f/f}$ background further diminished the lens size, suggesting that Shc1 contributes an additive role alongside Frs2 and Shp2 in modulating lens development (**Fig. 7E-F**). These findings collectively showed that Shc1 functions independently of Frs2 and Shp2 to transmit FGF signaling in lens development.

Discussion

This study used lens development as a model system to dissect the intricate mechanisms of FGF signaling. By systematically disrupting FGF signaling components, it unveiled a previously unappreciated dependency on precise FGF dosage for each developmental stage. Genetic rescue experiments and targeted manipulation of tyrosine phosphorylation further demonstrated that FGF signaling relies only partially on Frs2 and Shp2 for Grb2 recruitment, ultimately activating Ras and preventing cell death. Contrary to prevailing expectations, while both the adaptor function and the phosphatase activity of Shp2 are vital for embryonic survival, they play a surprisingly modest role in lens development, challenging the current understanding of Shp2 signaling mechanism. Notably, our research suggests that Shc may provide an alternative pathway for Grb2 recruitment and subsequent Ras activation. Although various adaptor proteins like Frs2, Crk, Shb, and Gab have been recognized for their roles in FGF signal transduction, recent findings suggest that mutations in their binding sites on FGF receptors have a significantly lesser impact compared to *Fgfr* null mutations (Brewer *et al*., 2015; Klint and Claesson-Welsh, 1999). Our data propose that Shc1 serves as an alternative route for FGF signal transmission, thereby adding a new dimension to our understanding of FGF signaling dynamics.

Lens induction, the pivotal event in eye development, has captivated developmental biologists since Hans Spemann's seminal discovery over a century ago, which identified the optic vesicle's role in triggering the overlying head ectoderm to differentiate into the lens (Spemann, 1901). However, the nature of the lens inductive signal has remained elusive (Makrides et al., 2022; Robinson, 2006). Previous studies suggested that FGF signaling might not be essential, as deleting Fgfr1/2 in the head ectoderm did not affect the expression of the early lens determination gene, Foxe3 (Garcia *et al*., 2011). Contrary to this notion, we have ablated all FGFRs present in the surface ectoderm, which led to a complete loss of pERK, confirming the absence of FGF signaling activity. While the mutant ectoderm still expressed Pax6, this observation may be confounded by the fact that the Le-Cre driver used for FGFR deletion depends on Pax6 activity. Importantly, the mutant tissue failed to upregulate Sox2 and Foxe3, the definitive markers of the lens induction. Moreover, the disruption of FGF signaling prevented the apical confinement of F-

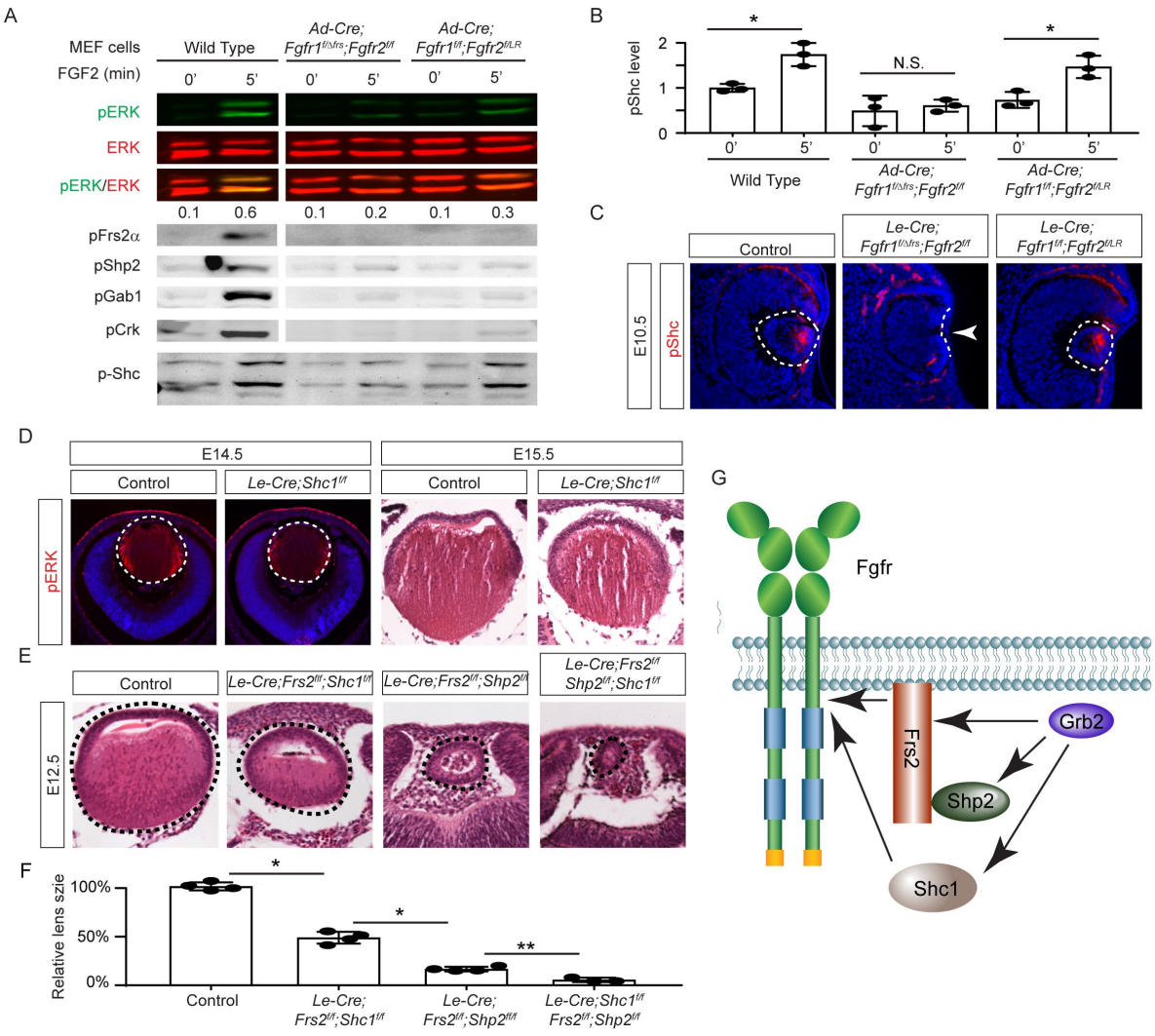


Figure 7.

Shc1 complements Frs2 and Shp2 in mediating FGF signaling in lens development.

(A) *Fgfr1*$^{f/f}$*;Fgfr2*$^{f/LR}$ infected with Cre virus showed stronger pERK and pShc activation than *Fgfr1* $^{f/\Delta Frs}$*;Fgfr2*$^{f/f}$ MEF cells, despite both losing Frs2, Shp2, Gab1, and Crk phosphorylation. **(B)** Quantification of pShc levels. One-way ANOVA, n=3, *P<0.02. N.S. Not significant. **(C)** pShc staining was lost in *Le-Cre;Fgfr1* $^{f/\Delta Frs}$*;Fgfr2*$^{f/f}$ mutant lens (arrowhead) but preserved in *Le-Cre;Fgfr1*$^{f/f}$*;Fgfr2*$^{f/LR}$ lens. **(D)** *Shc1*-deficient lenses showed a slight decrease in both pERK staining intensity and overall lens size. **(E)** E12.5 Frs2/Shc1 mutant lenses were smaller than controls, while lenses from Frs2/Shp2 mutants showed a pronounced hollow vesicle structure, a condition that worsened in Frs2/Shp2/Shc1 triple mutants. **(F)** Quantification of the lens size. One-way ANOVA n=4, *P<0.001, **P<0.05. **(G)** Model of FGF signaling network. Frs2 recruits Grb2 directly and indirectly through Shp2, while Shc1 provides an alternate Grb2 recruitment route independent of Frs2.

actin, impeding lens placode invagination driven by apical constriction (Chauhan *et al*., 2011). Considering the concomitant expression of FGF ligands in the optic vesicle, our findings suggest that FGF signaling is an indispensable factor in lens induction.

The role of Shp2 phosphatase in enhancing Receptor Tyrosine Kinase (RTK) signaling pathways is well established, yet its molecular mechanism remains largely unresolved (Neel et al., 2003). The prevailing model posits that Shp2 functions by dephosphorylating key tyrosine residues on target proteins, thereby activating Ras signaling. However, it has also been suggested that Shp2 itself can undergo C-terminal phosphorylation, potentially serving as a docking platform for other signaling molecules. Through targeted mutagenesis of these putative phosphorylation sites ($Shp2^{YF}$), we demonstrate the critical role of Shp2 phosphorylation in placental formation and neonatal survival. Biochemical analyses revealed distinct cellular responses to FGF and PDGF stimulation in $Shp2^{YF}$ mutants, suggesting context-dependent functions for these C-terminal modifications, which may explain the narrow phenotypic spectrum associated with the $Shp2^{YF}$ mutation. Intriguingly, the inactivation of Shp2 phosphatase activity via the $Shp2^{CS}$mutation also resulted in minimal disruption of FGF signaling in lens development, unlike the control *Le-Cre;* $Shp2^{f/f}$ mutant, which showed a significant reduction in pERK levels and subsequent lens differentiation defects. This lack of a robust phenotype cannot be attributed to residual protein activity following Cre-mediated gene deletion due to the systemic nature of the $Shp2^{CS}$ mutation. While Shp2 phosphatase deficiency did cause early embryonic lethality and abrogated the development of the more FGF-sensitive lacrimal gland, the absence of a pronounced lens phenotype calls into question the essentiality of Shp2 phosphatase activity for its overall function. Given the distinct phenotype observed in the $Shp2^{YF}$ mutant, it is plausible that Shp2 fulfills dual roles as both an adaptor and a phosphatase in certain signaling contexts, challenging existing paradigms and inviting further investigation into its multifaceted biological functions.

Previous studies have identified FGFR as a docking station for various signaling adaptor proteins, including Frs2, Plcg, Crk, Grb14 and Shb. In a herculean effort, Soriano and colleagues have eliminated these binding sites, both individually and collectively, but the outcomes were unexpectedly mild compared to the more severe phenotypes observed in the corresponding null mutants (Brewer *et al*., 2015; Clark and Soriano, 2024). For instance, whereas the *Fgfr1* null mutant is lethal by E6.5, mutants lacking the ability to bind Frs2, CrkL and Plcg/Shb//Grb14 survive until E10.5. More strikingly, *Fgfr2* null mutants typically succumb by E10.5 due to widespread organ development failures, yet mutants deficient in these specific binding sites can reach adulthood with minimal apparent defects. The fact that combining *Fgfr1/2* signaling mutations does not mimic the null phenotype further suggests that these mild mutant phenotypes are not simply a result of compensatory actions by other FGF receptors. This discrepancy highlights a crucial gap in our understanding of FGF signaling, implying the existence of unidentified factors that can compensate for the loss of Frs2 and other adaptors. Our study proposes Shc1 as a potential player in this complex signaling web, which is consistent with the observations that Shc1 can be phosphorylated in association with FGF receptors at sites known to facilitate Grb2 binding (Klint et al., 1995; Schuller et al., 2008), indicating an alternative route for signal propagation. Previous studies have shown that Shc proteins are predominantly expressed in lens epithelial cells and downregulated in lens fiber cells of the chick lens. Despite this downregulation, Shc becomes associated with α6A integrin in the lens fiber zone (Walker et al., 2002). However, the functional significance of Shc's abundant expression in the lens epithelial zone remained unclear. In our study, we found that *Shc1* knockout models exhibited relatively mild lens phenotypes at E15.5 during lens differentiation, possibly due to functional redundancy among Shc family proteins. Importantly, we discovered that Shc phosphorylation is dependent on FGF signaling during lens vesicle development. Furthermore, simultaneous deletion of *Shc1*, *Frs2*, and *Shp2* exacerbated lens development defects, resulting in a hollow lens vesicle significantly smaller than that observed in Frs2/Shp2 double mutants. These results demonstrate that Shc participates in FGF signaling in lens epithelial cells during lens vesicle development. These findings point towards a robust and adaptable FGF signaling network, capable of engaging alternative pathways through Frs2, Shc, and

other adaptor proteins, thereby maintaining its function despite significant genetic disruptions. These insights underscore the complexity and resilience of cellular signaling networks, understanding which is important for developing strategies to manipulate them in developmental and disease contexts.

Methods and materials

Mice

All procedures related to animal care and experimentation were conducted in adherence to the protocols and guidelines approved by the Institutional Animal Care and Use Committee at Columbia University. We obtained *Fgfr1*$^{\Delta Frs}$ from Dr. Raj Ladher (RIKEN Kobe Institute-Center for Developmental Biology, Kobe, Japan) (Hoch and Soriano, 2006), *Fgfr2*^{LR} from Dr. Jacob V.P. Eswarakumara (Yale University School of Medicine, New Haven, CT) (Eswarakumar *et al*., 2006) and *Fgfr2*^{flox} from Dr. David Ornitz (Washington University Medical School, St Louis, MO) (Yu et al., 2003). *Fgfr3*^{flox} from Dr. Xin Sun (University of California San Diego, La Jolla, CA) (Su et al., 2010), *Fgfr4*$^{-/-}$ from Dr. Chu-Xia Deng (National Institute of Health, Bethesda, MD) (Weinstein et al., 1998), *Frs2α*^{flox} from Fen Wang (Texas A&M, Houston, TX) (Lin et al., 2007), *Grb2*^{flox}from Dr. Lars Nitschke (University of Erlangen-Nürnberg, Erlangen, Germany) (Ackermann et al., 2011), *Le-Cre* from Richard Lang (Children's Hospital Research Foundation, Cincinnati, OH) (Ashery-Padan *et al*., 2000), P6 5.0 *lacZ* (*Pax6-LacZ*) reporter transgenic mice from Dr. Paul A. Overbeek (Baylor College of Medicine, Houston, TX) (Makarenkova et al., 2000), *Shc1*^{flox} from Tony Pawson (University of Toronto, Ontario, Canada) (Hardy et al., 2007), *Shp2*^{flox} from Gen-sheng Feng (UCSD, Sad Diego, CA) (Zhang et al., 2004), *LSL-Kras*^{G12D} mice was obtained from the Mouse Models of Human Cancers Consortium (MMHCC) Repository at National Cancer Institute (Tuveson *et al*., 2004). *Bax*$^{flox/flox}$;*Bak*$^{KO/KO}$(Stock No: 006329), *Fgfr1*^{flox} (Stock No: 007671), *Sox2Cre* (Stock No: 008454) mice were obtained from Jackson Laboratory. Animals were maintained on mixed genetic backgrounds. In all conditional knockout experiments, mice were maintained on a mixed genetic background and *Le-Cre* only or *Le-Cre* and heterozygous flox mice were used as controls. Mouse maintenance and experimentation were performed according to protocols approved by Columbia University Institutional Animal Care and Use Committee.

Shp2^{YF}, *Shp2*^{CS} and *Grb2*^{YF} targeting vectors were constructed using the recombineering method from C57BL/6 Bac clones (RP23-257E17 for *Shp2*, P23-2814 for *Grb2*, BACPAC Resources Center at Children's Hospital Oakland Research Institute) (Carbe et al., 2012). The *Shp2*^{YF}vector includes a neomycin resistance (*Neo*) cassette bordered by *loxP* sites, along with exon 14 of the *Shp2* gene harboring Y542F mutations and exon 15 with Y580F mutations. The *Shp2*^{CS} vector comprises a *NeoSTOP* cassette encased by *loxP* sites and exon 11 of the *Shp2* gene with the C483S mutation. Similarly, the *Grb2*^{YF} vector contains a *NeoSTOP* cassette flanked by *loxP* sites and exon 3 of the *Grb2* gene with the Y209F mutation. These targeting constructs, once linearized, were introduced into C57BL/6 and 129 hybrid ES cells via electroporation. *Shp2*^{YF} recombinant clones were screened by Southern blot analysis with 5' and 3' external probes after restriction digestion with *EvoR V*, while *Grb2*^{YF} and *Shp2*^{CS}clones were identified by long-range PCR before being injected into C57BL/6 blastocysts. Chimeras were further bred with C57BL/6 mice for germline transmission, verified through PCR genotyping with specific primers for each mutation: *Grb2*^{YF} *F*: 5'- TGGGGGTCAAAGTCAAAGAG -3'; *R*: 5'- CGGAGGGAGTGAGGTATGAG -3' (wild type: 179 bp, mutant: 270 bp), *Shp2*^{YF} *F*: 5'- AAAAAGAGGCTGCTCTGCAC -3'; *R*: 5'- TCTGCAGAATGAGGGAGGAC -3' (wild type: 195 bp, mutant: 250 bp) and *Shp2*^{YF} *F*: 5'- TGGGAAGACAGACTGCAGTC-3'; *R*: 5'-GAAGGAGCACCTGCCTGTTA-3' (wild type: 180 bp, mutant: 210 bp). The Neo cassette was subsequently excised by breeding with an *EIIa-cre* transgenic line (stock number 003724, Jackson Laboratory, Bar Harbor, ME).

Database Analysis

The cumulative references for each phosphorylation site, derived from both low-throughput (LTP) and high-throughput (HTP) experiments, were sourced from the PhosphositePlus database (*phosphosite.org*) and graphically represented along the amino acid sequence. The expression data for lens genes from embryonic days 10.5 to 12.5 was extracted from the iSyTE database (*https://research.bioinformatics.udel.edu/iSyTE/ppi/expression.php*) and visualized as heatmaps to illustrate the variations in expression levels over time.

Histology and immunohistochemistry

Histology and immunohistochemistry were performed on the paraffin and cryosections as previously described (Carbe *et al*., 2012; Carbe and Zhang, 2011). For hematoxylin and eosin (H&E) staining, 10 μM paraffin sections underwent deparaffinization with histosol wash, rehydration through decreasing concentrations of ethanol solutions, and final washing in water. The slides were immersed in hematoxylin for 3 minutes, followed by a 10-15 minute wash with tap water. Subsequently, they were decolorized with 1% acid alcohol for 30 seconds before treatment with eosin for 1 minute. Samples were dehydrated through increasing ethanol concentrations, transferred to histosol, and mounted using a Permount mounting medium. For X- gal staining, the mouse lacrimal gland was exposed by dissecting away the periocular skin and fixed in 4% paraformaldehyde at 4 °C overnight.

The antibodies used are phospho-ERK1/2 (#4370), phospho-mTOR (#2971), phospho-S6 (#5364), phospho-Shc (#2434), phospho-MEK1/2 (#2338), phospho-Smad1/5/9 (#13820), LEF1 (#2230), Cleaved caspase3 (#9662), cyclin D1 (#2926, discontinued), cyclin-D1 (#2978), cyclin- D3(#2936), N-cadherin (#13116) (all from Cell Signaling Technology), Fibronectin (AB2033) (from Millipore), Foxe3 (#377465), Jag1(#6011), Maf (#7866) (all from Santa Cruz), E- cadherin (#610181) and Ki67 (#550609) (both from BD Pharmingen), GFP (GFP-1010) (from Aves Labs), p57 (#75947) (from Abcam), Prox1 (PRB-238C) and Pax6 (PRB-278P) (both from Covance), Sox2 (14-9811-82) (from Thermo Fisher). Antibodies against α- and γ-crystallins were kindly provided by Sam Zigler (National Eye Institute, Bethesda, MD).

Phospho-ERK, phopho-MEK, phospho-mTOR, phosphor-Shc and phospho-Akt staining was amplified using a Tyramide Signal Amplification kit (TSATM Plus System, PerkinElmer Life Sciences, Waltham, MA). Alexa Fluor secondary antibodies and Alexa Fluor 488 phalloidin (A-12379) were ordered from Invitrogen. TUNEL staining was performed following the in situ cell death detection kit (Roche Applied Science, Indianapolis, IN). All commercial antibodies were validated by vendors. At least three embryos of each genotype were stained for each marker.

Cell culture and western blot

Primary mouse embryonic fibroblast (MEF) cells were generated according to a previously published protocol and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS and 1% penicillin-streptomycin. Adenovirus expressing Cre recombinase (Ad-cre) (Gene Transfer Vector Core, University of Iowa, IA.) were applied to MEF cells carrying flox alleles for 5 days to achieve gene deletion, while adenovirus expressing GFP (Ad-GFP) served as a control treatment. To assess growth factor responses, MEF cells were starved overnight and then treated with either FGF2 (50 ng/μl) or PDGFA (20 ng/μl) (from R&D systems) for 5 mins. Cells were immediately washed with cold PBS and harvested in ice-cold CelLytic buffer (C2978, Sigma-Aldrich, St.Louis, MO) supplemented with protease and phosphatase inhibitor cocktails (Pierce, Rockford, IL). Extracted proteins were subjected to standard western blot analysis. The antibodies used include ERK1/2 (#4695), phospho-Shp2 (#15543), phospho-Crk(#3491), phospho-Gab1(#3234), phopho-Frs2 (#3861), phospho-Shc (#2434) (all from Cell Signaling Technology), along with phospho-ERK1/2 (#7383) from Santa Cruz Biotechnology.

Quantification and Statistical Analysis

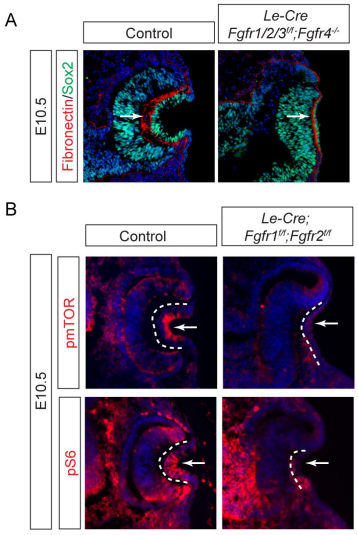
The relative lens sizes were measured using Image J and normalized against the control. The anterior/posterior lens ratio was determined by comparing the length of the anterior epithelium, as measured in Image J, to the length of the posterior lens boundary. The percentage of TUNEL and cleaved-caspase3 positive cells were normalized against the total number of DAPI positive cells. Statistical analysis was performed using GraphPad Prism 7. Sample sizes were not predetermined. Data represent mean ± s.d. Statistical differences between two groups were assessed using an unpaired, two-tailed t-test, while comparisons among three or more groups employed one-way ANOVA followed by Tukey's multiple comparison test.

Supplementary figures

Supplementary Figure 1.

Lens development in Fgf receptor mutants.

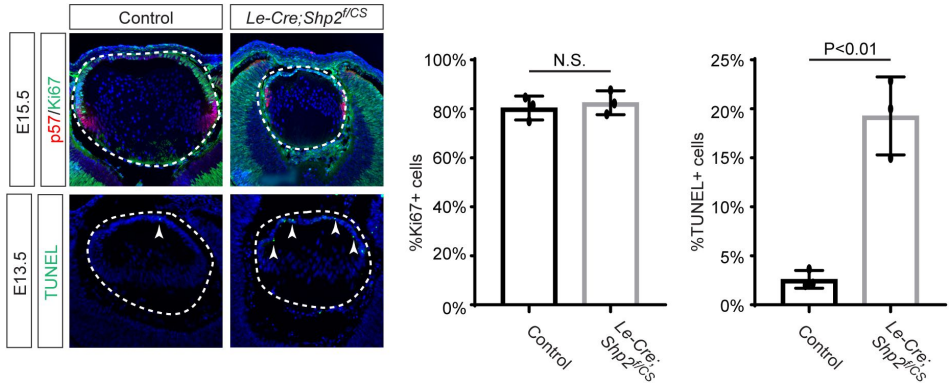
(A) Depletion of all four *Fgfr1/2/3/4* did not disrupt the Fibronectin expression at the basal side of the lens placode (arrows). **(B)** pmTOR and pS6 staining were lost in *Le-Cre;Fgfr1*$^{f/f}$*;Fgfr2*$^{f/LR}$lens.



Supplementary Figure 2.

Cell proliferation and apoptosis in *Shp2*^{CS}mutants.

Le- Cre;Shp2$^{f/CS}$ lens exhibits normal expression of proliferation marker Ki67, but there is a significant increase in TUNLE+ cells (arrowheads). Student's t-test, N.S. (not significant) for %Ki67+ cells and P<0.01 for %TUNEL+ cells in the lens epithelium.



Acknowledgements

The authors thank Drs. Ruth Ashery-Padan, Chu-Xia Deng, Jacob V.P. Eswarakumara, Gen- sheng Feng, Raj Ladher, Richard Lang, Lars Nitschke, David Ornitz, Paul A. Overbeek, Tony Pawson, Philippe Soriano, Xin Sun, Fen Wang for mice. The work was supported by grants from NIH (R01EY017061, EY018868 and R01EY025933 to X.Z.). Q.W. is supported by a Pathway to Independence Award (K99EY032171). The Columbia Ophthalmology Core Facility is supported by NIH Core grant 5P30EY019007 and unrestricted funds from Research to Prevent Blindness (RPB).

References

Ackermann J.A., Radtke D., Maurberger A., Winkler T.H., Nitschke L 2011) **Grb2 regulates B-cell maturation, B-cell memory responses and inhibits B-cell Ca2+ signalling** *EMBO J* **30**:1621–1633 https://doi.org/10.1038/emboj.2011.74

Ahmed Z., Lin C.C., Suen K.M., Melo F.A., Levitt J.A., Suhling K., Ladbury J.E 2013) **Grb2 controls phosphorylation of FGFR2 by inhibiting receptor kinase and Shp2 phosphatase activity** *J Cell Biol* **200**:493–504 https://doi.org/10.1083/jcb.201204106

Ahmed Z., Timsah Z., Suen K.M., Cook N.P., Lee G.R.t., Lin C.C., Gagea M., Marti A.A., Ladbury J.E. 2015) **Grb2 monomer-dimer equilibrium determines normal versus oncogenic function** *Nat Commun* **6**:7354 https://doi.org/10.1038/ncomms8354

Araki T., Nawa H., Neel B.G 2003) **Tyrosyl phosphorylation of Shp2 is required for normal ERK activation in response to some, but not all, growth factors** *J Biol Chem* **278**:41677–41684 https://doi.org/10.1074/jbc.M306461200

Ashery-Padan R., Marquardt T., Zhou X., Gruss P 2000) **Pax6 activity in the lens primordium is required for lens formation and for correct placement of a single retina in the eye** *Genes Dev* **14**:2701–2711

Beenken A., Mohammadi M 2009) **The FGF family: biology, pathophysiology and therapy** *Nature reviews. Drug discovery* **8**:235–253 https://doi.org/10.1038/nrd2792

Brewer J.R., Mazot P., Soriano P 2016) **Genetic insights into the mechanisms of Fgf signaling** *Genes Dev* **30**:751–771 https://doi.org/10.1101/gad.277137.115

Brewer J.R., Molotkov A., Mazot P., Hoch R.V., Soriano P 2015) **Fgfr1 regulates development through the combinatorial use of signaling proteins** *Genes Dev* **29**:1863–1874 https://doi.org/10.1101/gad.264994.115

Carbe C., Hertzler-Schaefer K., Zhang X 2012) **The functional role of the Meis/Prep- binding elements in Pax6 locus during pancreas and eye development** *Dev Biol* **363**:320–329 https://doi.org/10.1016/j.ydbio.2011.12.038

Carbe C., Zhang X 2011) **Lens induction requires attenuation of ERK signaling by Nf1** *Hum Mol Genet* **20**:1315–1323 https://doi.org/10.1093/hmg/ddr014

Chauhan B.K., Lou M., Zheng Y., Lang R.A 2011) **Balanced Rac1 and RhoA activities regulate cell shape and drive invagination morphogenesis in epithelia** *Proc Natl Acad Sci U S A* **108**:18289–18294 https://doi.org/10.1073/pnas.1108993108

Clark J.F., Soriano P. 2024) **Diverse Fgfr1 signaling pathways and endocytic trafficking regulate early mesoderm development** *bioRxiv* https://doi.org/10.1101/2024.02.16.580629

Collins T.N., Mao Y., Li H., Bouaziz M., Hong A., Feng G.S., Wang F., Quilliam L.A., Chen L., Park T., et al. 2018) **Crk proteins transduce FGF signaling to promote lens fiber cell elongation** *eLife* **7** https://doi.org/10.7554/eLife.32586

Cvekl A., Zhang X 2017) **Signaling and Gene Regulatory Networks in Mammalian Lens Development** *Trends Genet* **33**:677–702 https://doi.org/10.1016/j.tig.2017.08.001

Dhalluin C., Yan K.S., Plotnikova O., Lee K.W., Zeng L., Kuti M., Mujtaba S., Goldfarb M.P., Zhou M.M 2000) **Structural basis of SNT PTB domain interactions with distinct neurotrophic receptors** *Mol Cell* **6**:921–929 https://doi.org/10.1016/s1097-2765(05)00087-0

Eswarakumar V.P., Lax I., Schlessinger J 2005) **Cellular signaling by fibroblast growth factor receptors** *Cytokine Growth Factor Rev* **16**:139–149

Eswarakumar V.P., Ozcan F., Lew E.D., Bae J.H., Tome F., Booth C.J., Adams D.J., Lax I., Schlessinger J 2006) **Attenuation of signaling pathways stimulated by pathologically activated FGF-receptor 2 mutants prevents craniosynostosis** *Proc Natl Acad Sci U S A* **103**:18603–18608 https://doi.org/10.1073/pnas.0609157103

Garcia C.M., Huang J., Madakashira B.P., Liu Y., Rajagopal R., Dattilo L., Robinson M.L., Beebe D.C 2011) **The function of FGF signaling in the lens placode** *Dev Biol* **351**:176–185 https://doi.org/10.1016/j.ydbio.2011.01.001

Garg A., Hannan A., Wang Q., Makrides N., Zhong J., Li H., Yoon S., Mao Y., Zhang X 2020) **Etv transcription factors functionally diverge from their upstream FGF signaling in lens development** *eLife* **9** https://doi.org/10.7554/eLife.51915

Garg A., Zhang X 2017) **Lacrimal gland development: From signaling interactions to regenerative medicine** *Dev Dyn* **246**:970–980 https://doi.org/10.1002/dvdy.24551

Gotoh N., Ito M., Yamamoto S., Yoshino I., Song N., Wang Y., Lax I., Schlessinger J., Shibuya M., Lang R.A 2004) **Tyrosine phosphorylation sites on FRS2alpha responsible for Shp2 recruitment are critical for induction of lens and retina** *Proc Natl Acad Sci U S A* **101**:17144–17149

Hadari Y.R., Gotoh N., Kouhara H., Lax I., Schlessinger J 2001) **Critical role for the docking-protein FRS2 alpha in FGF receptor-mediated signal transduction pathways** *Proc Natl Acad Sci U S A* **98**:8578–8583 https://doi.org/10.1073/pnas.161259898

Haines E., Minoo P., Feng Z., Resalatpanah N., Nie X.M., Campiglio M., Alvarez L., Cocolakis E., Ridha M., Di Fulvio M., et al. 2009) **Tyrosine phosphorylation of Grb2: role in prolactin/epidermal growth factor cross talk in mammary epithelial cell growth and differentiation** *Mol Cell Biol* **29**:2505–2520 https://doi.org/10.1128/MCB.00034-09

Hardy W.R., Li L., Wang Z., Sedy J., Fawcett J., Frank E., Kucera J., Pawson T 2007) **Combinatorial ShcA docking interactions support diversity in tissue morphogenesis** *Science* **317**:251–256 https://doi.org/10.1126/science.1140114

Hayashi S., McMahon A.P 2002) **Efficient recombination in diverse tissues by a tamoxifen-inducible form of Cre: a tool for temporally regulated gene activation/inactivation in the mouse** *Dev Biol* **244**:305–318

Hoch R.V., Soriano P 2006) **Context-specific requirements for Fgfr1 signaling through Frs2 and Frs3 during mouse development** *Development* **133**:663–673 https://doi.org/10.1242/dev.02242

Kakrana A., Yang A., Anand D., Djordjevic D., Ramachandruni D., Singh A., Huang H., Ho J.W.K., Lachke S.A 2018) **iSyTE 2.0: a database for expression-based gene discovery in the eye**

Nucleic Acids Res **46**:D875–D885 https://doi.org/10.1093/nar/gkx837

Klint P., Claesson-Welsh L 1999) **Signal transduction by fibroblast growth factor receptors** *Front Biosci* **4**:D165–177

Klint P., Kanda S., Claesson-Welsh L 1995) **Shc and a novel 89-kDa component couple to the Grb2-Sos complex in fibroblast growth factor-2-stimulated cells** *J Biol Chem* **270**:23337–23344 https://doi.org/10.1074/jbc.270.40.23337

Kuracha M.R., Burgess D., Siefker E., Cooper J.T., Licht J.D., Robinson M.L., Govindarajan V 2011) **Spry1 and Spry2 are necessary for lens vesicle separation and corneal differentiation** *Invest Ophthalmol Vis Sci* **52**:6887–6897 https://doi.org/10.1167/iovs.11-7531

Li H., Mao Y., Bouaziz M., Yu H., Qu X., Wang F., Feng G.S., Shawber C., Zhang X 2019) **Lens differentiation is controlled by the balance between PDGF and FGF signaling** *PLoS biology* **17**:e3000133 https://doi.org/10.1371/journal.pbio.3000133

Li H., Tao C., Cai Z., Hertzler-Schaefer K., Collins T.N., Wang F., Feng G.S., Gotoh N., Zhang X 2014) **Frs2alpha and Shp2 signal independently of Gab to mediate FGF signaling in lens development** *Journal of cell science* **127**:571–582 https://doi.org/10.1242/jcs.134478

Li S., Couvillon A.D., Brasher B.B., Van Etten R.A. 2001) **Tyrosine phosphorylation of Grb2 by Bcr/Abl and epidermal growth factor receptor: a novel regulatory mechanism for tyrosine kinase signaling** *EMBO J* **20**:6793–6804 https://doi.org/10.1093/emboj/20.23.6793

Lin Y., Zhang J., Zhang Y., Wang F 2007) **Generation of an Frs2alpha conditional null allele** *Genesis* **45**:554–559 https://doi.org/10.1002/dvg.20327

Lovicu F.J., McAvoy J.W 2005) **Growth factor regulation of lens development** *Dev Biol* **280**:1–14 https://doi.org/10.1016/j.ydbio.2005.01.020

Lovicu F.J., Overbeek P.A 1998) **Overlapping effects of different members of the FGF family on lens fiber differentiation in transgenic mice** *Development* **125**:3365–3377

Madakashira B.P., Kobrinski D.A., Hancher A.D., Arneman E.C., Wagner B.D., Wang F., Shin H., Lovicu F.J., Reneker L.W., Robinson M.L 2012) **Frs2alpha enhances fibroblast growth factor-mediated survival and differentiation in lens development** *Development* **139**:4601–4612 https://doi.org/10.1242/dev.081737

Makarenkova H.P., Ito M., Govindarajan V., Faber S.C., Sun L., McMahon G., Overbeek P.A., Lang R.A 2000) **FGF10 is an inducer and Pax6 a competence factor for lacrimal gland development** *Development* **127**:2563–2572

Makrides N., Wang Q., Tao C., Schwartz S., Zhang X 2022) **Jack of all trades, master of each: the diversity of fibroblast growth factor signalling in eye development** *Open Biol* **12**:210265 https://doi.org/10.1098/rsob.210265

Neel B.G., Gu H., Pao L 2003) **The 'Shp'ing news: SH2 domain-containing tyrosine phosphatases in cell signaling** *Trends in biochemical sciences* **28**:284–293

Ochi H., Ogino H., Kageyama Y., Yasuda K 2003) **The stability of the lens-specific Maf protein is regulated by fibroblast growth factor (FGF)/ERK signaling in lens fiber differentiation** *J Biol Chem* **278**:537–544 https://doi.org/10.1074/jbc.M208380200

Ong S.H., Guy G.R., Hadari Y.R., Laks S., Gotoh N., Schlessinger J., Lax I 2000) **FRS2 proteins recruit intracellular signaling pathways by binding to diverse targets on fibroblast growth factor and nerve growth factor receptors** *Mol Cell Biol* **20**:979–989

Pan Y., Woodbury A., Esko J.D., Grobe K., Zhang X 2006) **Heparan sulfate biosynthetic gene Ndst1 is required for FGF signaling in early lens development** *Development* **133**:4933–4944

Qu X., Carbe C., Tao C., Powers A., Lawrence R., van Kuppevelt T.H., Cardoso W.V., Grobe K., Esko J.D., Zhang X. 2011a) **Lacrimal Gland Development and Fgf10-Fgfr2b Signaling Are Controlled by 2-O- and 6-O-sulfated Heparan Sulfate** *J Biol Chem* **286**:14435–14444 https://doi.org/10.1074/jbc.M111.225003

Qu X., Hertzler K., Pan Y., Grobe K., Robinson M.L., Zhang X 2011b) **Genetic epistasis between heparan sulfate and FGF-Ras signaling controls lens development** *Dev Biol* **355**:12–20 https://doi.org/10.1016/j.ydbio.2011.04.007

Riera L., Lasorsa E., Ambrogio C., Surrenti N., Voena C., Chiarle R 2010) **Involvement of Grb2 adaptor protein in nucleophosmin-anaplastic lymphoma kinase (NPM- ALK)-mediated signaling and anaplastic large cell lymphoma growth** *J Biol Chem* **285**:26441–26450 https://doi.org/10.1074/jbc.M110.116327

Robinson M.L 2006) **An essential role for FGF receptor signaling in lens development** *Semin Cell Dev Biol* **17**:726–740 https://doi.org/10.1016/j.semcdb.2006.10.002

Robinson M.L., MacMillan-Crow L.A., Thompson J.A., Overbeek P.A 1995) **Expression of a truncated FGF receptor results in defective lens development in transgenic mice** *Development* **121**:3959–3967

Schuller A.C., Ahmed Z., Levitt J.A., Suen K.M., Suhling K., Ladbury J.E 2008) **Indirect recruitment of the signalling adaptor Shc to the fibroblast growth factor receptor 2 (FGFR2)** *Biochem J* **416**:189–199 https://doi.org/10.1042/BJ20080887

Smith A.N., Miller L.A., Song N., Taketo M.M., Lang R.A 2005) **The duality of beta- catenin function: a requirement in lens morphogenesis and signaling suppression of lens fate in periocular ectoderm** *Dev Biol* **285**:477–489

Spemann H 1901) **Uber Korrelationen in die Entwickelung des Auges** *AVerh. Anat. Ges* **15**:61–79

Su N., Xu X., Li C., He Q., Zhao L., Li C., Chen S., Luo F., Yi L., Du X., et al. 2010) **Generation of Fgfr3 conditional knockout mice** *Int J Biol Sci* **6**:327–332

Sun J., Lu S., Ouyang M., Lin L.J., Zhuo Y., Liu B., Chien S., Neel B.G., Wang Y 2013) **Antagonism between binding site affinity and conformational dynamics tunes alternative cis-interactions within Shp2** *Nat Commun* **4**:2037 https://doi.org/10.1038/ncomms3037

Tuveson D.A., Shaw A.T., Willis N.A., Silver D.P., Jackson E.L., Chang S., Mercer K.L., Grochow R., Hock H., Crowley D., et al. 2004) **Endogenous oncogenic K-ras(G12D) stimulates proliferation and widespread neoplastic and developmental defects** *Cancer Cell* **5**:375–387

Vemulapalli V., Chylek L.A., Erickson A., Pfeiffer A., Gabriel K.H., LaRochelle J., Subramanian K., Cao R., Stegmaier K., Mohseni M., et al. 2021) **Time-resolved phosphoproteomics reveals scaffolding and catalysis-responsive patterns of SHP2-dependent signaling** *eLife* **10** https://doi.org/10.7554/eLife.64251

Wang Q., Tao C., Hannan A., Yoon S., Min X., Peregrin J., Qu X., Li H., Yu H., Zhao J., Zhang X 2021) **Lacrimal gland budding requires PI3K-dependent suppression of EGF signaling** *Sci Adv* **7** https://doi.org/10.1126/sciadv.abf1068

Weinstein M., Xu X., Ohyama K., Deng C.X 1998) **FGFR-3 and FGFR-4 function cooperatively to direct alveogenesis in the murine lung** *Development* **125**:3615–3623

Xie Q., McGreal R., Harris R., Gao C.Y., Liu W., Reneker L.W., Musil L.S., Cvekl A 2016) **Regulation of c-Maf and alphaA-Crystallin in Ocular Lens by Fibroblast Growth Factor Signaling** *J Biol Chem* **291**:3947–3958 https://doi.org/10.1074/jbc.M115.705103

Yang W., Klaman L.D., Chen B., Araki T., Harada H., Thomas S.M., George E.L., Neel B.G 2006) **An Shp2/SFK/Ras/Erk signaling pathway controls trophoblast stem cell survival** *Dev Cell* **10**:317–327

Yu K., Xu J., Liu Z., Sosic D., Shao J., Olson E.N., Towler D.A., Ornitz D.M 2003) **Conditional inactivation of FGF receptor 2 reveals an essential role for FGF signaling in the regulation of osteoblast function and bone growth** *Development* **130**:3063–3074

Zhang E.E., Chapeau E., Hagihara K., Feng G.S 2004) **Neuronal Shp2 tyrosine phosphatase controls energy balance and metabolism** *Proc Natl Acad Sci U S A* **101**:16064–16069

Zhao H., Yang T., Madakashira B.P., Thiels C.A., Bechtle C.A., Garcia C.M., Zhang H., Yu K., Ornitz D.M., Beebe D.C., Robinson M.L 2008) **Fibroblast growth factor receptor signaling is essential for lens fiber cell differentiation** *Dev Biol* **318**:276–288 https://doi.org/10.1016/j.ydbio.2008.03.028

Author information

Qian Wang[§#]
Department of Ophthalmology, Columbia University, New York, United States

[§]These authors contributed equally to this work

[#]Present address: Department of Ophthalmology and Visual Sciences, Washington University School of Medicine, St. Louis, MO 63110, USA

Hongge Li[§]
Department of Ophthalmology, Columbia University, New York, United States

[§]These authors contributed equally to this work

Yingyu Mao[§]
Department of Ophthalmology, Columbia University, New York, United States

[§]These authors contributed equally to this work

Ankur Garg
Department of Ophthalmology, Columbia University, New York, United States

Eun Sil Park
Department of Ophthalmology, Columbia University, New York, United States

Yihua Wu
Department of Ophthalmology, Columbia University, New York, United States

Alyssa Chow
Department of Ophthalmology, Columbia University, New York, United States

John Peregrin
Department of Ophthalmology, Columbia University, New York, United States

Xin Zhang
Department of Ophthalmology, Columbia University, New York, United States, Department of Pathology and Cell Biology, Columbia University, New York, United States
ORCID iD: 0000-0001-5555-0825

For correspondence: xz2369@columbia.edu

Editors

Reviewing Editor
David Reboutier

Senior Editor
Lori Sussel
University of Colorado Anschutz Medical Campus, Aurora, United States of America

Reviewer #1 (Public review):

Summary:

This manuscript uses the eye lens as a model to investigate basic mechanisms in the Fgf signaling pathway. Understanding Fgf signaling is of broad importance to biologists as it is involved in the regulation of various developmental processes in different tissues/organs and is often misregulated in disease states. The Fgf pathway has been studied in embryonic lens development, namely with regards to its involvement in controlling events such as tissue invagination, vesicle formation, epithelium proliferation and cellular differentiation, thus making the lens a good system to uncover the mechanistic basis of how the modulation of this pathway drives specific outcomes. Previous work has suggested that proteins, other than the ones currently known (e.g., the adaptor protein Frs2), are likely involved in Fgfr signaling. The present study focuses on the role of Shp2 and Shc1 proteins in the recruitment of Grb2 in the events downstream of Fgfr activation.

Strengths:

The findings reveal that the juxtamembrane region of the Fgf receptor is necessary for proper control of downstream events such as facilitating key changes in transcription and cytoskeleton during tissue morphogenesis. The authors conditionally deleted all four Fgfrs in the mouse lens that resulted in molecular and morphological lens defects, most importantly,

preventing the upregulation of the lens induction markers Sox2 and Foxe3 and the apical localization of F-actin, thus demonstrating the importance of Fgfrs in early lens development, i.e. during lens induction. They also examined the impact of deleting Fgfr1 and 2, on the following stage, i.e. lens vesicle development, which could be rescued by expressing constitutively active KrasG12D. By using specific mutations (e.g. Fgfr1ΔFrs lacking the Frs2 binding domain and Fgfr2LR harboring mutations that prevent binding of Frs2), it is demonstrated that the Frs2 binding site on Fgfr is necessary for specific events such as morphogenesis of lens vesicle. Further, by studying Shp2 mutations and deletions, the authors present a case for Shp2 protein to function in a context-specific manner in the role of an adaptor protein and a phosphatase enzyme. Finally, the key surprising finding from this study is that downstream of Fgfr signaling, Shc1 is an important alternative pathway - in addition to Shp2 - involved in the recruitment of Grb2 and in the subsequent activation of Ras. The methodologies, namely, mouse genetics and state-of-the-art cell/molecular/biochemical assays are appropriately used to collect the data, which are soundly interpreted to reach these important conclusions. Overall, these findings reveal the flexibility of the Fgf signaling pathway and it downstream mediators in regulating cellular events. This work is expected to be of broad interest to molecular and developmental biologists.

Weaknesses:

A weakness that needs to be discussed is that Le-Cre depends on Pax6 activation, and hence its use in specific gene deletion will not allow evaluation of the requirement of Fgfrs in the expression of Pax6 itself. But since this is the earliest Cre available for deletion in the lens, mentioning this in the discussion would make the readers aware of this issue.

https://doi.org/10.7554/eLife.103615.2.sa3

Reviewer #2 (Public review):

Summary

I have reviewed the revised manuscript submitted by Wang et al., which is entitled "Shc1 cooperates with Frs2 and Shp2 to recruit Grb2 in FGF-induced lens development". In this paper, the authors first examined lens phenotypes in mice with Le-Cre-mediated knockdown (KD) of all four FGFR (FGFR1-4), and found that pERK signals, Jag1 and foxe3 expression are absent or drastically reduced, indicating that FGF signaling is essential for lens induction. Next, the authors examined lens phenotypes of FGFR1/2-KD mice and found that lens fiber differentiation is compromised and that proliferative activity and cell survival are also compromised in lens epithelium. Interestingly, Kras activation rescues defects in lens growth and lens fiber differentiation in FGFR1/2-KD mice, indicating that Ras activation is a key step for lens development, downstream of FGF signaling. Next, the authors examined the role of Frs2, Shp2 and Grb2 in FGF signaling for lens development. They confirmed that lens fiber differentiation is compromised in FGFR1/3-KD mice combined with Frs2-dysfunctional FGFR2 mutants, which is similar to lens phenotypes of Grb2-KD mice. However, lens defects are milder in mice with Shp2YF/YF and Shp2CS mutant alleles, indicating that involvement of Shp2 is limited for the Grb2 recruitment for lens fiber differentiation. Lastly, the authors showed new evidence on the possibility that another adapter protein, Shc1, promotes Grb2 recruitment independent of Frs2/Shp2-mediated Grb2 recruitment.

Strength

Overall, the manuscript provides valuable data on how FGFR activation leads to Ras activation through the adapter platform of Frs2/Shp2/Grb2, which advances our understanding on complex modification of FGF signaling pathway. The authors applied a

genetic approach using mice, whose methods and results are valid to support the conclusion. The discussion also well summarizes the significance of their findings.

Weakness

The authors found that the new adaptor protein Shc1 is involved in Grb2 recruitments in response to FGF receptor activation. However, the main data on Shc1 are only histological sections and statistical evaluation of lens size. In the revised manuscript, the authors did not answer my major concern that cellular-level data are missing, which is not fully enough to support their main conclusion on the involvement of Shc1 in Grb2 recruitment of FGF signaling for lens development. Since the title of this manuscript is that Shc1 cooperates with Frs2 and Shp2 to recruit Grb2 in FGF-induced lens development, it is important to provide the cellular-level evidence on Shc1.

https://doi.org/10.7554/eLife.103615.2.sa2

Reviewer #3 (Public review):

Summary:

The manuscript entitled "Shc1 cooperates with Frs2 and Shp2 to recruit Grb2 in FGF-induced lens development" by Wang et al., investigates the molecular mechanism used by FGFR signaling to support lens development. The lens has long been known to depend on FGFR-signaling for proper development. Previous investigations have demonstrated the FGFR signaling is required for embryonic lens cell survival and for lens fiber cell differentiation. The requirement of FGFR signaling for lens induction has remained more controversial as deletion of both Fgfr1 and Fgfr2 during lens placode formation does not prevent the induction of definitive lens markers such as FOXE3 or αA-crystallin. Here the authors have used the Le-Cre driver to delete all four FGFR genes from the developing lens placode demonstrating a definitive failure of lens induction in the absence of FGFR-signaling. The authors focused on FGFR1 and FGFR2, the two primary FGFRs present during early lens development and demonstrated that lens development could be significantly rescued in lenses lacking both FGFR1 and FGFR2 by expressing a constitutively active allele of KRAS. They also showed that the removal of pro-apoptotic genes Bax and Bak could also lead to a substantial rescue of lens development in lenses lacking both FGFR1 and FGFR2. In both cases, the lens rescue included both increased lens size and the expression of genes characteristic of lens cells.

Significantly the authors concentrated on the juxtamembrane domain, a portion of the FGFRs associated with FRS2. Previous investigations have demonstrated the importance of FRS2 activation for mediating a sustained level of ERK activation. FRS2 is known to associate both with GRB2 and SHP2 to activate RAS. The authors utilized a mutant allele of Fgfr1, lacking the entire juxtamembrane domain (Fgfr1ΔFrs) and an allele of Fgfr2 containing two-point mutations essential for Frs2 binding (Fgfr2LR). When combining three floxed alleles and leaving only one functional allele (Fgfr1ΔFrs or Fgfr2LR) the authors got strikingly different phenotypes. When only the Fgfr1ΔFrs allele was retained, the lens phenotype matched that of deleting both Fgfr1 and Fgfr2. However, when only the Fgfr2LR allele was retained the phenotype was significantly milder, primarily affecting lens fiber cell differentiation, suggesting that something other than FRS2 might be interacting with the juxtamembrane domain to support FGFR signaling in the lens. The authors also deleted Grb2 in the lens and showed that the phenotype was similar to that of the lenses only retaining the Fgfr2LR allele, resulting a failure of lens fiber cell differentiation and decreased lens cell survival. However, mutating the major tyrosine phosphorylation site of GRB2 did not affect lens development. The authors additionally investigated the role of SHP2 in lens development by either deleting SHP2 or by making mutations in the SHP2 catalytic domain. The deletion of the SHP2

phosphatase activity did not affect lens development as severely as total loss of SHP2 protein, suggesting a function for SHP2 outside of its catalytic activity. Although the loss of Shc1 alone has only a slight effect on lens size and pERK activation in the lens, the authors showed that the loss of Shc1 exacerbated the lens phenotype in lenses lacking both Frs2 and Shp2. The authors suggest that SHC1 binds to the FGFR juxtamembrane domain allowing for the recruitment of GRB2 in independently of FRS2.

Strengths:

(1) The authors used a variety of genetic tools to carefully dissect the essential signals downstream of FGFR signaling during lens development.

(2) The authors made a convincing case that something other than FRS2 binding mediates FGFR signaling in the juxtamembrane domain.

(3) The authors demonstrated that despite the requirement of both the adaptor function and phosphatase activity of SHP2 are required for embryonic survival, neither of these activities is absolutely required for lens development.

(4) The authors provide more information as to why FGFR loss has a phenotype much more severe than the loss of FRS2 alone during lens development.

(5) The authors followed up their work analyzing various signaling molecules in the context of lens development with biochemical analyses of FGF-induced phosphorylation in murine embryonic fibroblasts (MEFs).

(6) In general, this manuscript represents a Herculean effort to dissect FGFR signaling in vivo with biochemical backing with cell culture experiments in vitro.

Weaknesses:

(1) The authors demonstrate that the loss of FGFR1 and FGFR2 can be compensated by a constitutive active KRAS allele in the lens and suggest that FGFRs largely support lens development only by driving ERK activation. However, the authors also saw that lens development was substantially rescued by preventing apoptosis through the deletion of BAK and BAX. To my knowledge, the deletion of BAK and BAX should not independently activate ERK. The authors do not show whether ERK activation is restored in the BAK/BAX deficient lenses. Do the authors suggest the FGFR3 and/or FGFR4 provide sufficient RAS and ERK activation for lens development when apoptosis is suppressed? Alternatively, is it the survival function of FGFR-signaling as much as a direct effect on lens differentiation?

(2) Do the authors suggest that GRB2 is required for RAS activation and ultimately ERK activation? If so, do the authors suggest that ERK activation is not required for FGFR-signaling to mediate lens induction? This would follow considering that the GRB2 deficient lenses lack a problem with lens induction.

(3) The increase in p-Shc is only slightly higher in the Cre FGFR1f/f FGFR2r/LR than in the FGFR1f/Δfrs FGFR2f/f. Can the authors provide quantification?

(4) The authors have not shown directly that Shc1 binds to the juxtamembrane region of either Fgfr1 or Fgfr2.

https://doi.org/10.7554/eLife.103615.2.sa1

Author response:

The following is the authors' response to the original reviews.

Public Reviews:

Reviewer #1 (Public review):

Summary:

This manuscript uses the eye lens as a model to investigate basic mechanisms in the Fgf signaling pathway. Understanding Fgf signaling is of broad importance to biologists as it is involved in the regulation of various developmental processes in different tissues/organs and is often misregulated in disease states. The Fgf pathway has been studied in embryonic lens development, namely with regards to its involvement in controlling events such as tissue invagination, vesicle formation, epithelium proliferation, and cellular differentiation, thus making the lens a good system to uncover the mechanistic basis of how the modulation of this pathway drives specific outcomes. Previous work has suggested that proteins, other than the ones currently known (e.g., the adaptor protein Frs2), are likely involved in Fgfr signaling. The present study focuses on the role of Shp2 and Shc1 proteins in the recruitment of Grb2 in the events downstream of Fgfr activation.

Strengths:

The findings reveal that the juxtamembrane region of the Fgf receptor is necessary for proper control of downstream events such as facilitating key changes in transcription and cytoskeleton during tissue morphogenesis. The authors conditionally deleted all four Fgfrs in the mouse lens that resulted in molecular and morphological lens defects, most importantly, preventing the upregulation of the lens induction markers Sox2 and Foxe3 and the apical localization of F-actin, thus demonstrating the importance of Fgfrs in early lens development, i.e. during lens induction. They also examined the impact of deleting Fgfr1 and 2, on the following stage, i.e. lens vesicle development, which could be rescued by expressing constitutively active KrasG12D. By using specific mutations (e.g. Fgfr1ΔFrs lacking the Frs2 binding domain and Fgfr2LR harboring mutations that prevent binding of Frs2), it is demonstrated that the Frs2 binding site on Fgfr is necessary for specific events such as morphogenesis of lens vesicle. Further, by studying Shp2 mutations and deletions, the authors present a case for Shp2 protein to function in a context-specific manner in the role of an adaptor protein and a phosphatase enzyme. Finally, the key surprising finding from this study is that downstream of Fgfr signaling, Shc1 is an important alternative pathway - in addition to Shp2 - involved in the recruitment of Grb2 and in the subsequent activation of Ras. The methodologies, namely, mouse genetics and state-of-the-art cell/molecular/biochemical assays are appropriately used to collect the data, which are soundly interpreted to reach these important conclusions. Overall, these findings reveal the flexibility of the Fgf signaling pathway and its downstream mediators in regulating cellular events. This work is expected to be of broad interest to molecular and developmental biologists.

Weaknesses:

A weakness that needs to be discussed is that Le-Cre depends on Pax6 activation, and hence its use in specific gene deletion will not allow evaluation of the requirement of Fgfrs in the expression of Pax6 itself. But since this is the earliest Cre available for deletion in the lens, mentioning this in the discussion would make the readers aware of this issue. Referring to Jag1 among "lens-specific markers" (page 5) is debatable, suggesting changing to the lines of "the expected upregulation of Jag1 in lens vesicle". The Abstract could be modified to clearly convey the existing knowledge gap and the key findings of the present study. As it stands now, it is a bit all over the place. Some typos in the manuscript need to be fixed, e.g. "...yet its molecular mechanism remains largely

> *resolved" - unresolved? "...in the development lens" - in the developing lens? In Figure 4 legend, "(B) Grb2 mutants Grb2 mutants displayed...", etc.*

We thank the reviewer for the thoughtful and constructive feedback. We have added the caveat regarding the Le-Cre dependency on *Pax6* expression to the discussion, removed the reference to Jag1 as a "lens-specific marker" and corrected the typographical errors noted by the reviewer.

> ***Reviewer #2 (Public review):***
>
> *Summary:*
>
> *I have reviewed a manuscript submitted by Wang et al., which is entitled "Shc1 cooperates with Frs2 and Shp2 to recruit Grb2 in FGF-induced lens development". In this paper, the authors first examined lens phenotypes in mice with Le-Cre-mediated knockdown (KD) of all four FGFR (FGFR1-4), and found that pERK signals, Jag1, and foxe3 expression are absent or drastically reduced, indicating that FGF signaling is essential for lens induction. Next, the authors examined lens phenotypes of FGFR1/2-KD mice and found that lens fiber differentiation is compromised and that proliferative activity and cell survival are also compromised in lens epithelium. Interestingly, Kras activation rescues defects in lens growth and lens fiber differentiation in FGFR1/2-KD mice, indicating that Ras activation is a key step for lens development. Next, the authors examined the role of Frs2, Shp2, and Grb2 in FGF signaling for lens development. They confirmed that lens fiber differentiation is compromised in FGFR1/3-KD mice combined with Frs2-dysfunctional FGFR2 mutants, which is similar to lens phenotypes of Grb2-KD mice. However, lens defects are milder in mice with Shp2YF/YF and Shp2CS mutant alleles, indicating that the involvement of Shp2 is limited for the Grb2 recruitment for lens fiber differentiation. Lastly, the authors showed new evidence on the possibility that another adapter protein, Shc1, promotes Grb2 recruitment independent of Frs2/Shp2-mediated Grb2 recruitment.*
>
> *Strengths:*
>
> *Overall, the manuscript provides valuable data on how FGFR activation leads to Ras activation through the adapter platform of Frs2/Shp2/Grb2, which advances our understanding of complex modification of the FGF signaling pathway. The authors applied a genetic approach using mice, whose methods and results are valid to support the conclusion. The discussion also well summarizes the significance of their findings.*
>
> *Weaknesses:*
>
> *The authors eventually found that the new adaptor protein Shc1 is involved in Grb2 recruitments in response to FGF receptor activation. however, the main data for Shc1 are histological sections and statistical evaluation of lens size. So, my major concern is that the authors need to provide more detailed data to support the involvement of Shc1 in Grb2 recruitment of FGF signaling for lens development.*

We thank the reviewer for the positive comments and valuable suggestions. We have addressed the concerns in detail in the response to the recommendation outlined below.

> ***Reviewer #3 (Public review):***
>
> *Summary:*
>
> *The manuscript entitled "Shc1 cooperates with Frs2 and Shp2 to recruit Grb2 in FGF-induced lens development" by Wang et al., investigates the molecular mechanism used by FGFR signaling to support lens development. The lens has long been known to depend*

on FGFR signaling for proper development. Previous investigations have demonstrated that FGFR signaling is required for embryonic lens cell survival and for lens fiber cell differentiation. The requirement of FGFR signaling for lens induction has remained more controversial as deletion of both Fgfr1 and Fgfr2 during lens placode formation does not prevent the induction of definitive lens markers such as FOXE3 or αA-crystallin. Here the authors have used the Le-Cre driver to delete all four FGFR genes from the developing lens placode demonstrating a definitive failure of lens induction in the absence of FGFR signaling. The authors focused on FGFR1 and FGFR2, the two primary FGFRs present during early lens development, and demonstrated that lens development could be significantly rescued in lenses lacking both FGFR1 and FGFR2 by expressing a constitutively active allele of KRAS. They also showed that the removal of pro-apoptotic genes Bax and Bak could also lead to a substantial rescue of lens development in lenses lacking both FGFR1 and FGFR2. In both cases, the lens rescue included both increased lens size and the expression of genes characteristic of lens cells.

Significantly the authors concentrated on the juxtamembrane domain, a portion of the FGFRs associated with FRS2. Previous investigations have demonstrated the importance of FRS2 activation for mediating a sustained level of ERK activation. FRS2 is known to associate both with GRB2 and SHP2 to activate RAS. The authors utilized a mutant allele of Fgfr1, lacking the entire juxtamembrane domain (Fgfr1ΔFrs), and an allele of Fgfr2 containing two-point mutations essential for Frs2 binding (Fgfr2LR). When combining three floxed alleles and leaving only one functional allele (Fgfr1ΔFrs or Fgfr2LR) the authors got strikingly different phenotypes. When only the Fgfr1ΔFrs allele was retained, the lens phenotype matched that of deleting both Fgfr1 and Fgfr2. However, when only the Fgfr2LR allele was retained the phenotype was significantly milder, primarily affecting lens fiber cell differentiation, suggesting that something other than FRS2 might be interacting with the juxtamembrane domain to support FGFR signaling in the lens. The authors also deleted Grb2 in the lens and showed that the phenotype was similar to that of the lenses only retaining the Fgfr2LR allele, resulting in a failure of lens fiber cell differentiation and decreased lens cell survival. However, mutating the major tyrosine phosphorylation site of GRB2 did not affect lens development. The author additionally investigated the role of SHP2 lens development by making by either deleting SHP2 or by making mutations in the SHP2 catalytic domain. The deletion of the SHP2 phosphatase activity did not affect lens development as severely as the total loss of SHP2 protein, suggesting a function for SHP2 outside of its catalytic activity. Although the loss of Shc1 alone has only a slight effect on lens size and pERK activation in the lens, the authors showed that the loss of Shc1 exacerbated the lens phenotype in lenses lacking both Frs2 and Shp2. The authors suggest that SHC1 binds to the FGFR juxtamembrane domain allowing for the recruitment of GRB2 independently of FRS2.

Strengths:

(1) The authors used a variety of genetic tools to carefully dissect the essential signals downstream of FGFR signaling during lens development.

(2) The authors made a convincing case that something other than FRS2 binding mediates FGFR signaling in the juxtamembrane domain.

(3) The authors demonstrated that despite the requirement of both the adaptor function and phosphatase activity of SHP2 are required for embryonic survival, neither of these activities is absolutely required for lens development.

(4) The authors provide more information as to why FGFR loss has a phenotype much more severe than the loss of FRS2 alone during lens development.

> *(5) The authors followed up their work analyzing various signaling molecules in the context of lens development with biochemical analyses of FGF-induced phosphorylation in murine embryonic fibroblasts (MEFs).*
>
> *(6) In general, this manuscript represents a Herculean effort to dissect FGFR signaling in vivo with biochemical backing with cell culture experiments in vitro.*

We thank the reviewer for the thorough review of our paper and positive comments.

> *Weaknesses:*
>
> *(1) The authors demonstrate that the loss of FGFR1 and FGFR2 can be compensated by a constitutive active KRAS allele in the lens and suggest that FGFRs largely support lens development only by driving ERK activation. However, the authors also saw that lens development was substantially rescued by preventing apoptosis through the deletion of BAK and BAX. To my knowledge, the deletion of BAK and BAX should not independently activate ERK. The authors do not show whether ERK activation is restored in the BAK/BAX deficient lenses. Do the authors suggest the FGFR3 and/or FGFR4 provide sufficient RAS and ERK activation for lens development when apoptosis is suppressed? Alternatively, is it the survival function of FGFR-signaling as much as a direct effect on lens differentiation?*

Our interpretation is that at the lens induction stage, where FGFR1 and FGFR2 are crucial, their primary function operates through Ras signaling to promote cell survival. Thus, either constitutively active *KRAS* or the direct suppression of apoptosis by deleting *Bak* and *Bax* is sufficient to rescue lens induction. This rescue enables the subsequent differentiation of lens progenitor cells, a process for which FGFR3 and FGFR4 are sufficient to support.

> *(2) The authors make the argument that deleting all four FGFRs prevented lens induction but that the deletion of only FGFR1 and FGFR2 did not. Part of this argument is the retention of FOXE3 expression, αA-crystallin expression, and PROX1 expression in the FGFR1/2 double mutants. However, in Figure 1E, and Figure 1F, the staining of the double mutant lens tissue with FOXE3, αA-crystallin, and PROX1 is unconvincing. However, the retention of FOXE3 expression in the FGFR1/FGFR2 double mutants was previously demonstrated in Garcia et al 2011. Also, there needs to be an enlargement or inset to demonstrate the retention of pSMAD in the quadruple FGFR mutants in Figure 1D.*

We have updated Figure 1E with a clearer image of FOXE3 staining to better illustrate FOXE3 expression in the FGFR1/2 double mutants. It seems there may have been a misunderstanding regarding our claims about αA-crystallin and PROX1. To clarify, our observation is that both αA-crystallin and PROX1 are lost in the FGFR1/2 double mutants, which we believe is clearly demonstrated in Figure 1F. Additionally, we have added inserts to Figure 1D to highlight the retention of pSMAD.

> *(3) Do the authors suggest that GRB2 is required for RAS activation and ultimately ERK activation? If so, do the authors suggest that ERK activation is not required for FGFR-signaling to mediate lens induction? This would follow considering that the GRB2 deficient lenses lack a problem with lens induction.*

We do believe that GRB2 is required for RAS-ERK signaling activation; however, ERK activation is not absolutely required for lens induction. This conclusion is consistent with our previous study, which showed that deletion of ERK1/2 did not prevent lens induction (Garg et al. eLife 2020;9:e51915), as well as with our current findings demonstrating that the GRB2-deficient mutant is still capable of supporting lens induction.

> *(4) The increase in p-Shc is only slightly higher in the Cre FGFR1f/f FGFR2r/LR than in the FGFR1f/Δfrs FGFR2f/f. Can the authors provide quantification?*

pShc quantification is now provided in Fig. 7B.

> *(5) The authors have not shown directly that Shc1 binds to the juxtamembrane region of either Fgfr1 or Fgfr2.*

It is not yet clear whether Shc1 directly binds to the juxtamembrane region of FGFR1 or FGFR2, as it may also be recruited indirectly. We acknowledge this as an important question that warrants further investigation in future studies.

> *(6) The authors have used the Le-Cre strain for all of their lens deletion experiments. Previous work has documented that the Le-Cre transgene can cause lens defects independent of any floxed alleles in both homozygous and hemizygous states on some genetic backgrounds (Dora et al., 2014 PLoS One 9:e109193 and Lam et al., Human Genomics 2019 13(1):10. Are the controls used in these experiments Le-Cre hemizygotes?*

As stated in the Method section, Le-Cre only or Le-Cre and heterozygous flox mice were used as controls.

> ***Recommendations for the authors:***
>
> ***Reviewer #1 (Recommendations for the authors):***
>
> *Weaknesses*
>
> *There are only a few minor weaknesses that need to be addressed.*
>
> *(1) The point could be made in the Discussion that since Le-Cre depends on Pax6 placodal expression, it is challenging to evaluate the impact of deletion of the four Fgfrs on the expression of Pax6 (since Pax6 needs to be activated prior to achieving Fgfr deletion). A different Cre line (e.g. a Cre which is expressed in the surface ectoderm prior to lens placode formation) could help partially address this question, although it may not be able to comment on the requirement of the Fgfrs specifically in the lens ectoderm. Thus, it will be prudent to mention this in the discussion.*

We have added the caveat regarding the Le-Cre dependency on *Pax6* expression to the discussion.

> *(2) Referring to Jag1 among "lens-specific markers" (page 5) is debatable, I suggest changing it along the lines of "the expected upregulation of Jag1 in lens vesicle".*

The wording has been changed as suggested.

> *(3) The Abstract could be modified to clearly convey the existing knowledge gap and the key findings of the present study. As it stands now, it is a bit all over the place.*

The abstract has been revised.

> *(4) Some typos in the manuscript need to be fixed.*
>
> *e.g. "...yet its molecular mechanism remains largely resolved" - unresolved?, "...in the development lens" - in the developing lens?, In Fig. 4 legend, "(B) Grb2 mutants Grb2 mutants displayed...", etc.*

These typos have been corrected.

> ***Reviewer #2 (Recommendations for the authors):***
>
> *My specific suggestions are shown below.*
>
> *(1) The authors need to describe the role of Shc1 in FGF signaling and vertebrate lens development, by citing previous publications in the introduction.*

We have detailed previous studies on the role of Shc in FGF signaling in the Introduction and discussed its function in the vertebrate lens in the Discussion section.

> *(2) Figure 1B bottom panels: Inset images seem to be missing, although frames and arrowheads are there. Please check them.*

The inset images were correctly placed.

> *(3) Results (page 5, line 13): The authors mentioned "Sox2 expression remained at basal levels". Since Figure 1B indicates that Sox2 expression fails to be upregulated in FGFR1/2 mutant lens placode in contrast to Pax6, it is better to clearly mention the failure in upregulation of Sox2 expression in the FGFR1/2 mutants.*

This sentence has been rewritten as suggested.

> *(4) Results (page 6, line 8): The authors mentioned "we observed .... expression of Foxe3 in ...mutant lens cells (Figure 1E, arrows). However, Foxe3-expressing lens cells are a very small population in Figure 1E. It is important to state the decreased number of Foxe3-expressing lens cells in FGFR1/2 mutants. In addition, I would like to request the authors to show histograms indicating sample size and statistical analysis for marker expression: Foxe3 (Figure 1E), Prox1 and αA-crystallin (Fig. 1F), cyclin D1 and TUNEL (Fig. 1G) and pmTOR and pS6 (Supplementary figure 1B).*

We added a statement indicating that the number of *Foxe3*-expressing cells is reduced in FGFR1/2 mutants, which is now quantified in Fig. 1H. Quantifications for Cyclin D1 and TUNEL are now shown in Fig. 1I and J, respectively. However, we chose not to quantify Prox1, αA-crystallin, pmTOR, and pS6, as the FGFR1/2 mutants showed no staining for these markers.

> *(5) Results (page 6, line 19- page 7, line 6): The authors showed that inducible expression of constitutive active Kras, KrasG12D, using Le-Cre, recovered lens size to the half level of wild-type control. However, in the lens of mice with Le-Cre; FGFR1/2f/f; LSL-KrasG12D, pERK was detected in the most posterior edge of the lens fiber core, whereas pERK was detected in the broader area of the lens in control. Furthermore, pMEK was detected in the whole lens of mice with Le-Cre; FGFR1/2f/f; and LSL-KrasG12D, whereas pMEK was detected only in the lens epithelial cells at the equator. So, the spatial profile of pERK and pMEK expression was different from those of wild-type, although the authors observed that Prox1 and Crystallin expression are normally induced in the lens of mice with Le-Cre; FGFR1/2f/f; LSL-KrasG12D. I wonder whether the lens normally develops in mice with Le-Cre; LSL-KrasG12D? Is the lens growth enhanced in mice with Le-Cre; LSL-KrasG12D? Please add the panels of mice with Le-Cre; LSL-KrasG12D in Figure 2B and 2C. In addition, I wonder whether apoptosis is suppressed in the lens of mice with Le-Cre; FGFR1/2f/f; LSL-KrasG12D?*

As we previously reported (Developmental Biology 355, 2011, 12–20), *Le-Cre; LSL-KrasG12D* did not lead to enhanced lens growth. While we agree that including images of *Le-Cre; LSL-*

KrasG12D as controls in Fig. 2B and C and evaluating apoptosis in *Le-Cre; FGFR1/2f/f; LSL-KrasG12D* mutants would be appropriate, we regretfully no longer have these animals available to conduct these experiments.

> *(6) Results (page 11, line 15): the PCR genotyping image of Fig. 6C seems to be missing.*

The PCR genotyping image was correctly placed below Fig. 6B.

> *(7) Results (page 11, lines 15-20): there is no citation of Figure 6D in the results section.*

The citation for Fig. 6D is added in the results section.

> *(8) Figures 5H, 6H, and 7A: Western blotting of some of the pERK, ERK lanes is missing.*

These western blots all have pERK/ERK overlay images.

> *(9) Figure 7A, western blotting data on pShc levels are important to suggest the involvement of Shc1 in Frs2-independent Grb2 activation by FGF stimulation. Please provide the histogram for statistical analysis.*

pShc quantification is now provided in Fig. 7B.

> *(10) There is no citation of Figure 7D, E, and F in the results section. Please add them.*

These citations have been added.

> *(11) Figures 7E, and 7F: The authors showed that lens morphology and lens size evaluation in genetic combinations: control, Frs2/Shc1 KD, Frs2/Shp2 KD, and Frs2/Shp2/Shc1 KD. However, I would like to request the authors to show more detailed data in these genetic combinations, for example, pERK, foxe3, Maf, Prox1, Jag1, p57, cyclin D3, g-crystallin, and TUNEL.*

Unfortunately, we no longer have these mutant mice to perform these detailed staining.

> ***Reviewer #3 (Recommendations for the authors):***
>
> *(1) The figure legend for Figure 2 lists (G) twice. The second (G) should be (H). Also, in Figures 2G and H there is no indication as to what stage lenses were used for the TUNEL and size analyses. I assume that it was E13.5, but it should be explicitly stated.*

The figure labeling has been corrected and the stage added to the figure legend.

> *(2) In Figure 4 A the label should be gamma-crystallin rather than r-crystallin.*

The figure labeling has been corrected.

> *(3) In Figure 6 D, I believe that the immunolabeling for Maf and Foxe3 are reversed. The Maf should be red as it is in the fibers and the Foxe3 should be green as it is epithelial.*

The figure labeling has been corrected.

> *(4) In Figure 6C I believe that the labels for the WT and YF alleles on the western blot are reversed.*

The YF PCR band was designed to be larger than WT, so the labeling was correct as is.

> *(5) In Figure 6F I believe that the labels for WT and CS on the western blot are reversed.*

The figure labeling has been corrected.

> *(6) In Supplemental figure 2 there are no genotype labels for the TUNEL bar graph.*

The figure labeling has been added.

https://doi.org/10.7554/eLife.103615.2.sa0