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RESEARCH ARTICLE

Frs2α and Shp2 signal independently of Gab to mediate FGF signaling in lens development

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ABSTRACT

Fibroblast growth factor (FGF) signaling requires a plethora of adaptor proteins to elicit downstream responses, but the functional significances of these docking proteins remain controversial. In this study, we used lens development as a model to investigate Frs2α and its structurally related scaffolding proteins, Gab1 and Gab2, in FGF signaling. We show that genetic ablation of *Frs2α* alone has a modest effect, but additional deletion of tyrosine phosphatase *Shp2* causes a complete arrest of lens vesicle development. Biochemical evidence suggests that this Frs2α–Shp2 synergy reflects their epistatic relationship in the FGF signaling cascade, as opposed to compensatory or parallel functions of these two proteins. Genetic interaction experiments further demonstrate that direct binding of Shp2 to Frs2α is necessary for activation of ERK signaling, whereas constitutive activation of either Shp2 or Kras signaling can compensate for the absence of Frs2α in lens development. By contrast, knockout of *Gab1* and *Gab2* failed to disrupt FGF signaling *in vitro* and lens development *in vivo*. These results establish the Frs2α–Shp2 complex as the key mediator of FGF signaling in lens development.

KEY WORDS: FGF, Lens, Frs2, Shp2, Gab, Ras

INTRODUCTION

The fibroblast growth factor (FGF) signaling cascade is initiated by the binding of FGF and heparan sulfate proteoglycan (HSPG) to the FGF receptor (FGFR), which undergoes dimerization to activate its intrinsic tyrosine kinase activity (Schlessinger, 2004). This leads to phosphorylation of multiple tyrosine residues on FGFR, generating potential docking sites for signaling molecules containing SH2 (Src homology-2) or PTB (phosphotyrosine binding). However, only a handful of proteins, including Frs2 (Frs2α and Frs2β, encoded by the genes *Frs2* and *Frs3*, respectively), Crk (Crk and Crkl) and PLCγ have been identified to interact directly with activated FGFR (Mohammadi et al., 1991; Wang et al., 1996; Kouhara et al., 1997; Larsson et al., 1999). Among these factors, Crk proteins appear to function in a context-dependent manner in FGF signaling (Moon et al., 2006). PLCγ mainly triggers activation of PKC and intercellular calcium release. Mutation of the PLCγ binding residue on FGFR1 elevates FGF signaling *in vivo*, suggesting that the interaction between FGFR and PLCγ is unlikely to explain most of the FGF signaling effects (Partanen et al., 1998). Instead, biochemical studies show that FGFR signaling through Frs2 proteins is the primary pathway to activate downstream Ras–MAPK and PI3K–Akt cascades (Ong et al., 2000; Hadari et al., 2001).

Frs2 proteins are composed of N-terminal PTB domains that bind to the juxtamembrane domain of FGFR and C-terminal domains that can be phosphorylated at multiple tyrosine residues by activated FGFR (Gotoh, 2008). In Frs2α, for example, four of these phosphorylated tyrosine residues bind to the adaptor protein Grb2, which recruits its constitutively bound partner Sos, a guanine nucleotide exchange factor (GEF) that directly activates Ras signaling (Hadari et al., 2001). It is thought that another Grb2-associated protein, Gab1, can further engage the p85 protein, a regulatory subunit of PI3-kinase (PI3K) (Ong et al., 2001). This provides a direct route for the Frs2–Grb2–Gab1 complex to stimulate PI3K–Akt signaling. However, recent genetic studies have begun to question this Frs2-centric view of FGF signaling mechanism. Unlike Frs2β, which is restricted to nervous system, Frs2α is ubiquitously expressed during development. However, *Frs2α*-knockout embryos survive until mouse embryonic day 8 (E8), significantly late compared with some of the FGF-signaling knockouts such as Fgf4, which dies at E4 (Feldman et al., 1995; Gotoh et al., 2005). Deletion of the Frs2 binding site in Fgfr1 ($Fgfr^{\Delta Frs2}$) does not disrupt Fgfr1 signaling during gastrulation and somitogenesis, but results in neurulation, tail bud and pharyngeal arch defects at late embryogenesis (Hoch and Soriano, 2006). Furthermore, no major defects were reported in an *Fgfr2* mutant ($Fgfr2^{LR}$) deficient in Frs2 binding (Eswarakumar et al., 2006; Sims-Lucas et al., 2011). Therefore, the functional significance of Frs2 proteins in FGF signaling remains a major question in embryonic development.

The development of vertebrate lens is an excellent model for studying FGF signaling. At E10.5, the invaginating lens ectoderm detaches from the anterior head epithelium to give rise to the lens vesicle. This is followed by differentiation of the posterior lens vesicle, which generates primary lens fibers to occupy the lumen of lens vesicle by E12.5. The subsequent growth of the lens requires proliferation and migration of the anterior lens epithelial cells, which transition at the lens equator region into orderly arrays of secondary lens fibers. This establishes structural integrity and clarity of mature lens. Previous explants and transgenic studies have demonstrated that FGF signaling is necessary and sufficient for inducing lens fiber development

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(McAvoy and Chamberlain, 1989; Robinson et al., 1995; Lovicu and Overbeek, 1998). In support of this, deletion of the FGF receptors Fgfr1, Fgfr2 and Fgfr3 or their co-receptor heparan sulfates at various stages of lens development disrupts both lens vesicle formation and lens fiber differentiation (Pan et al., 2006; Zhao et al., 2008; Garcia et al., 2011; Qu et al., 2011a). In addition, a systemic *Frs2α* mutant (*Frs2α^{2F}*) lacking two tyrosine residues required for binding of Shp2 protein displays lens and retina developmental failure, whereas genetic ablation of the Ras signaling molecules Shp2, Nf1 and ERK disrupts lens development (Gotoh et al., 2004; Pan et al., 2010; Carbe and Zhang, 2011; Upadhya et al., 2013). However, it was recently reported that a lens-specific knockout of *Frs2α* results in a well-differentiated lens with only moderately reduced size (Madakashira et al., 2012). This result raises the question whether *Frs2α* is indeed essential for lens development.

In this study, we confirmed the relatively modest lens defect in an *Frs2α* conditional knockout mouse, but we observed that knockouts of both *Frs2α* and *Shp2* abrogated lens development by E14.5. Although genes involved in early differentiation of the lens were unaffected in the *Frs2α*, *Shp2* double mutant at E12.5, there was an arrest of proliferation and differentiation accompanied by a loss of ERK signaling. We further showed that the *Frs2α* mutant lacking the Shp2-binding site mimicked the *Frs2α*-null mutant in genetic interaction with *Shp2*, whereas gain-of-function mutations in either *Shp2* or *Kras* could ameliorate the *Frs2α* lens defect. Finally, we showed that Gab1 and Gab2, two potential substrates of Shp2 and binding partners of the Frs2–Grb2–Shp2 complex, were dispensable for lens development. Taken together, these results show that Frs2–Shp2–Ras signaling is essential in lens development.

RESULTS

Genetic interactions between *Frs2α* and *Shp2* in lens development

To determine downstream mediators of FGF signaling in lens development, we first generated a conditional knockout of *Frs2α* using *Le-Cre*, which is known to be active during lens development as early as E9.5. Although RNA *in situ* hybridization indicated that *Frs2α* transcripts had indeed disappeared in E12.5 *Le-Cre*; *Frs2α$^{flox/flox}$* mutant lens (Fig. 1A,B, arrows), immunohistochemistry revealed persistent Frs2α protein as late as E14.5, especially in the transition zone of

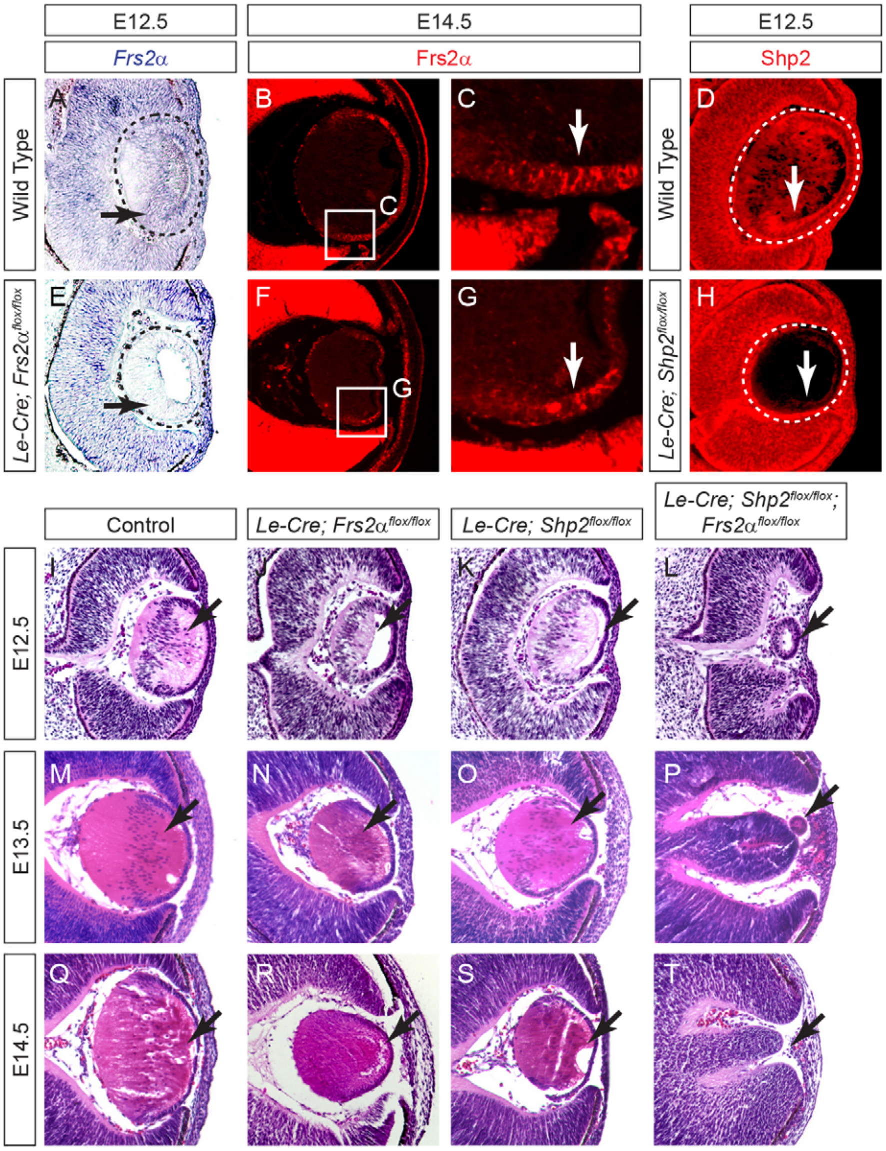

Fig. 1. Combined ablation of *Frs2α* and *Shp2* abolishes lens development. (A–H) *Frs2α* transcripts were depleted in E12.5 *Le-Cre*; *Frs2α$^{flox/flox}$* lens (A and E, arrows), but residual Frs2α protein can still be detected in E14.5 *Le-Cre*; *Frs2α$^{flox/flox}$* mutant lens (C and G, arrows). Shp2 immunostaining was markedly reduced in E12.5 *Le-Cre*; *Shp2$^{flox/flox}$* lens (D and H, arrows). Three embryos of each genotype were analyzed. (I–T) In *Le-Cre; Frs2α$^{flox/flox}$* embryos, elongation of primary lens fibers was delayed at E12.5 (arrow in J) but recovered at E13.5 (arrow in N). Single deletion of *Frs2α* (n=7) or *Shp2* (n=8) resulted in only modest reduction in lens sizes, but double knockout of both genes in *Le-Cre*; *Shp2$^{flox/flox}$*; *Frs2α$^{flox/flox}$* embryos (n=8) resulted in no lens development at E14.5 (arrow in T).

the lens (Fig. 1B,C,F,G, arrows). This is consistent with a previous study of *Le-Cre*; *Frs2α*$^{flox/flox}$ mutant, which found that Frs2α protein was significantly reduced but not eliminated in the E15.5 lens (Madakashira et al., 2012). We did not detect any overt phenotype in *Le-Cre*; *Frs2α*$^{flox/flox}$ mutants until E12.5, when the primary lens fibers failed to reach the anterior rim of mutant lens (Fig. 1I,J, arrows). However, this modest delay in elongation of lens fiber cells was no longer visible by E13.5, when the lens cavities were filled in both wild-type and mutant lenses (Fig. 1M,N, arrows). At E14.5, the *Frs2α* mutant lens was reduced by 50% in size compared with the wild type (n=8 controls and 7 mutants; P<0.01, Student's t test) (Fig. 1Q,R, arrows). As we previously reported, ablation of Shp2 protein in *Le-Cre*; *Shp2*$^{flox/flox}$ mutants also resulted in a similar lens phenotype (Fig. 1K,O,S, arrows) (Pan et al., 2010). Combined deletion of both *Frs2α* and *Shp2*, however, significantly disrupted lens development, leading to a complete loss of lens in E14.5 *Le-Cre*; *Shp2*$^{flox/flox}$; *Frs2α*$^{flox/flox}$ embryos (Fig. 1L,P,T, arrows, n=8). This genetic interaction suggests that *Frs2α* and *Shp2* together play important roles in lens development.

The *Frs2α* and *Shp2* double mutant has disrupted lens differentiation and ERK signaling

We next examined the molecular defects in *Frs2α* and *Shp2* double mutants. At E12.5, *Le-Cre*; *Shp2*$^{flox/flox}$; *Frs2α*$^{flox/flox}$ embryos still contained a rudimentary lens vesicle marked by expressions of Pax6 and *Foxe3*, two essential regulators of early lens development (Fig. 2A–A‴ and B–B‴). However, whereas these transcriptional factors were normally excluded from the differentiating cells in the posterior lens vesicle at this stage, they were maintained in the entire lens vesicle in *Frs2α* and *Shp2* double mutants. This suggests that combined deletion of *Frs2α* and *Shp2* allows proper specification of lens progenitor cells in the lens vesicle, but disrupts their subsequent differentiation into lens fibers. Consistent with this, the lens fiber differentiation factor Prox1 was expressed in *Frs2α* and *Shp2* double mutants at the low level typically found in wild-type lens epithelium, in contrast to the elevated level present in the posterior lens vesicle (Fig. 2C–C‴). By contrast, *Frs2α* and *Shp2* double mutants ectopically expressed the anterior lens epithelial marker E-cadherin in the posterior lens vesicle (Fig. 2D–D‴). Lens differentiation is characterized by sequential induction of α-, β- and γ-crystallins during development. Although α- and β-crystallins were still expressed, the late onset expression of γ-crystallin was absent in the posterior lens vesicle (Fig. 2E–E‴; Fig. 2F–F‴ and Fig. 2G–G‴). Because none of these genetic abnormalities were observed in *Frs2α* or *Shp2* single mutants, these results suggest that *Frs2α* and *Shp2* are synergistically required for proper differentiation of lens fibers.

The *Frs2α* and *Shp2* mutant phenotypes described above overlap with the lens defects previously observed when FGF receptors were ablated during lens vesicle development (Zhao et al., 2008). We thus investigated FGF downstream signaling in *Frs2α* and *Shp2* mutants. In contrast to *Frs2α* or *Shp2* single mutants, double deletion of *Frs2α* and *Shp2* abolished ERK phosphorylation and expression of the FGF signaling downstream response genes *Erm* and *Er81* (Fig. 3A–D; supplementary material Fig. S1A–D). D-type cyclins and their associated kinase CDK4 are required for G1–S transition, whereas p57 promotes cell cycle exit. In *Frs2α* and *Shp2* double mutant lenses, both cyclin D1 and p57 were lost and CDK4 failed to be localized to nucleus (Fig. 3E–L; supplementary material Fig. S2A,B). Although the cell cycle arrest marker p21 appeared to be unchanged, the *Frs2α* and *Shp2* mutant lens displayed significant reduction in cell proliferation marker phospho-histone H3 (pHH3) and increased cell death as shown by TUNEL staining (supplementary material Fig. S2D,E; Fig. 3M–Y). Therefore, *Frs2α* and *Shp2* cooperate in regulating ERK signaling and in promoting cell cycle progression and survival in lens development.

The Frs2α-Shp2 complex is required for lens development

Frs2α and *Shp2* double mutants clearly exhibited more severe lens defects than those of single mutants, suggesting that Frs2α and Shp2 mediate FGF–ERK signaling together. To test this notion, we generated primary mouse embryonic fibroblast (MEF) cells carrying *Frs2α* ^{flox} or *Shp2*^{flox} alleles. This allows us to ablate Frs2α and Shp2 *in vitro* by infecting these MEF cells with a Cre-expressing adenovirus for 5 days (Fig. 4A). Seemingly consistent with our *in vivo* finding in the lens, FGF2-stimulated ERK phosphorylation was either unaffected or reduced modestly in *Shp2* or *Frs2α* single mutant cells, but became further compromised in *Frs2α* and *Shp2* double mutant cells. However, we noticed that there was still residual Frs2α and Shp2 protein in these MEF cells, which might obscure the essential functions of these factors in FGF signaling. We thus extended the virus infection to 7 days, further reducing the level of Frs2α and Shp2 protein (Fig. 4B). Interestingly, *Frs2α* and *Shp2* single mutant cells now displayed downregulation of FGF2-induced ERK signaling similar to that of *Frs2α* and *Shp2* double mutant cells. Therefore, a more complete depletion of Frs2α and Shp2 protein in MEF cells abolished their apparent genetic synergy in FGF signaling.

We have observed a similar delay in depletion of Frs2α protein *in vivo* (Fig. 1E–G), raising the possibility that residual Frs2α protein might be sufficient to sustain ERK signaling during the crucial period of lens development. In this scenario, because Frs2α and Shp2 operate in an epistatic cascade downstream to FGF, simultaneous depletion of both proteins in double mutants will synergistically destabilize the entire pathway, resulting in a drastic loss of ERK signaling and severe lens defects. To distinguish the epistatic and the parallel models of the function of *Frs2α* and *Shp2* in FGF signaling, we took advantage of an existing *Frs2α*^{2F} allele, which was mutated at two amino acids critical for Shp2 binding to Frs2α (Gotoh et al., 2004). We reasoned that, if the parallel model is correct, addition of the *Frs2α*^{2F} allele to the *Shp2* single mutant background should not aggravate its lens defects, because the Shp2-independent function of Frs2α remains intact in *Frs2α*^{2F} and *Shp2* compound mutants. However, the epistatic model predicts that combining disruption of the Shp2–Frs2α interaction upon depletion of Shp2 interferes with two consecutive nodes of FGF signaling, thus resulting in severe lens defects similar to combined depletion of Frs2α and Shp2.

To test these predictions, we first generated *Le-Cre*; *Frs2α*$^{flox/2F}$ mutants, which exhibited mild lens defects similar to *Le-Cre*; *Frs2α*$^{flox/flox}$ and *Le-Cre*; *Shp2*$^{flox/flox}$ single mutants (supplementary material Fig. S3A–N). *Le-Cre*; *Shp2*$^{flox/flox}$; *Frs2α*$^{flox/2F}$ compound mutants, however, closely resembled *Le-Cre*; *Shp2*$^{flox/flox}$; *Frs2α*$^{flox/flox}$ double mutants in their defective FGF signaling and lens differentiation, indicated by loss of ERK phosphorylation and reduced Prox1 expression at E12.5 (Fig. 4C–D″). Aberrant cell cycle controls were also evident in both compound mutants because cyclin D1 and p57 were abolished, CDK4 remained cytoplasmic and p21 was unaffected (Fig. 4E–F″;

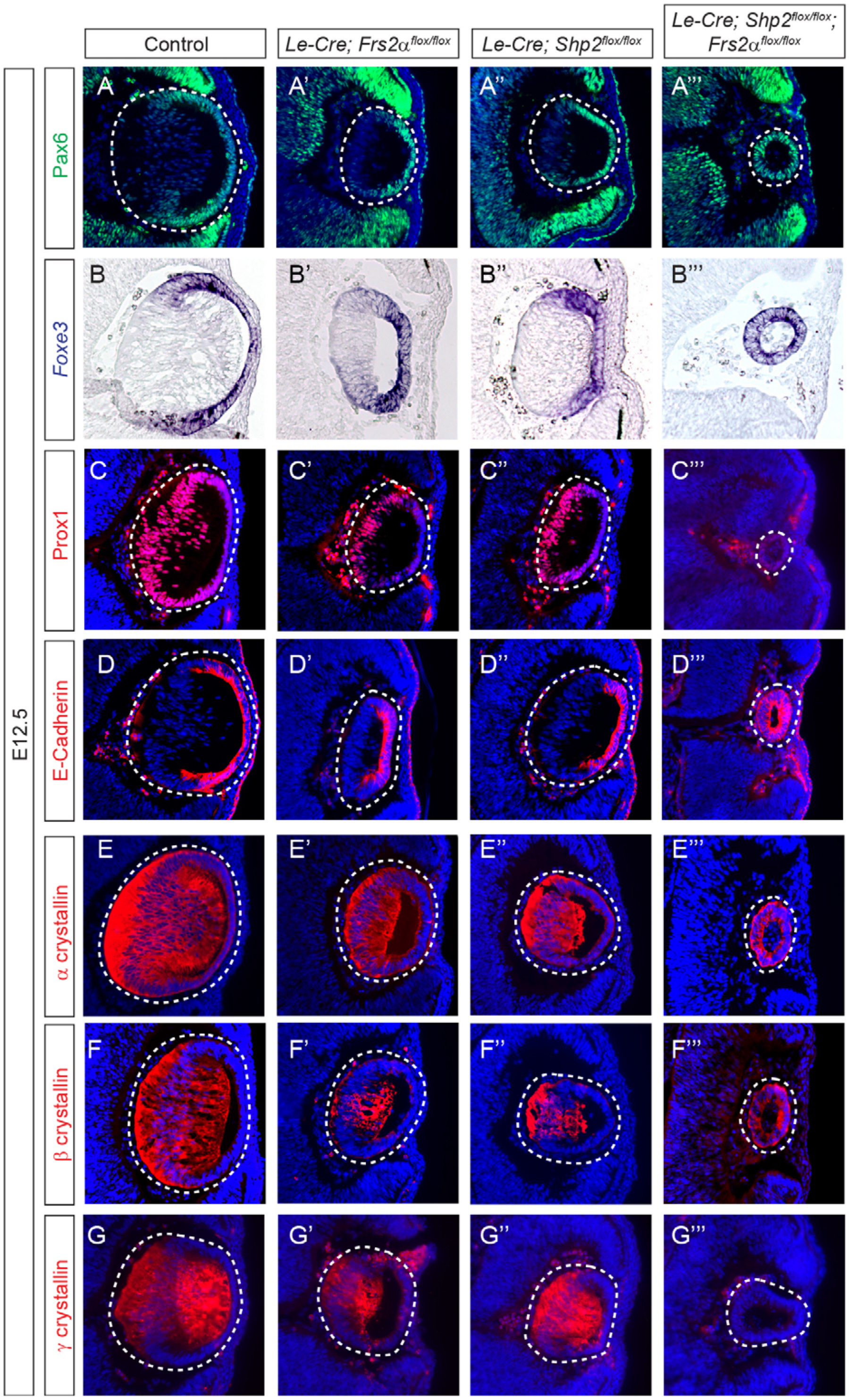

Fig. 2. Molecular defects in *Frs2α* and *Shp2* double mutant lens. (A–B′″) At E12.5, lens determination genes *Pax6* and *Foxe3* were restricted to the anterior epithelium in *Frs2α* and *Shp2* single mutants, but expanded to the entire lens vesicle in *Le-Cre*; *Shp2*$^{flox/flox}$; *Frs2α*$^{flox/flox}$ embryos. (C–D′″) Double knockout of *Frs2α* and *Shp2* resulted in a reduction of lens fiber differentiation gene *Prox1* and an expansion of lens epithelium marker E-cadherin. (E–G′″) α- and β-crystallin genes were preserved in *Frs2α* and *Shp2* double mutants, but γ-crystallin was drastically reduced. Three embryos of each genotype were analyzed for each marker. White dashed lines encircle lenses.

supplementary material Fig. S2C,F). This led to reduced cell proliferation and increased cell death as shown by Ki67 expression, BrdU incorporation and TUNEL staining in the hollow lens vesicles (Fig. 4E–J). At E14.5, neither *Le-Cre*; *Shp2*$^{flox/flox}$; *Frs2α*$^{flox/2F}$ (n=5) nor *Le-Cre*; *Shp2*$^{flox/flox}$; *Frs2α*$^{flox/flox}$ (n=8) mutants contained any obvious lens structure. Collectively, these results are consistent with the idea that the Shp2-interacting residues on Frs2α are essential for FGF–ERK signaling in lens development.

Activated Shp2 signaling ameliorates *Frs2α* lens defects

The binding of Shp2 to Frs2 can generate additional docking sites for the downstream molecule Grb2, but it could also serve to activate Shp2 phosphatase activity. To test the significance of the latter possibility, we crossed *Le-Cre*; *Frs2α*$^{flox/flox}$ mutant with the *LSL-Shp2*^{D61Y} allele, which contains an oncogenic *Shp2*^{D61Y} knock-in mutation silenced by a floxed transcription stop cassette (Chan et al., 2009) (Fig. 5A). Expression of Cre recombinase from *Le-Cre* transgene will thus result in lens-specific induction

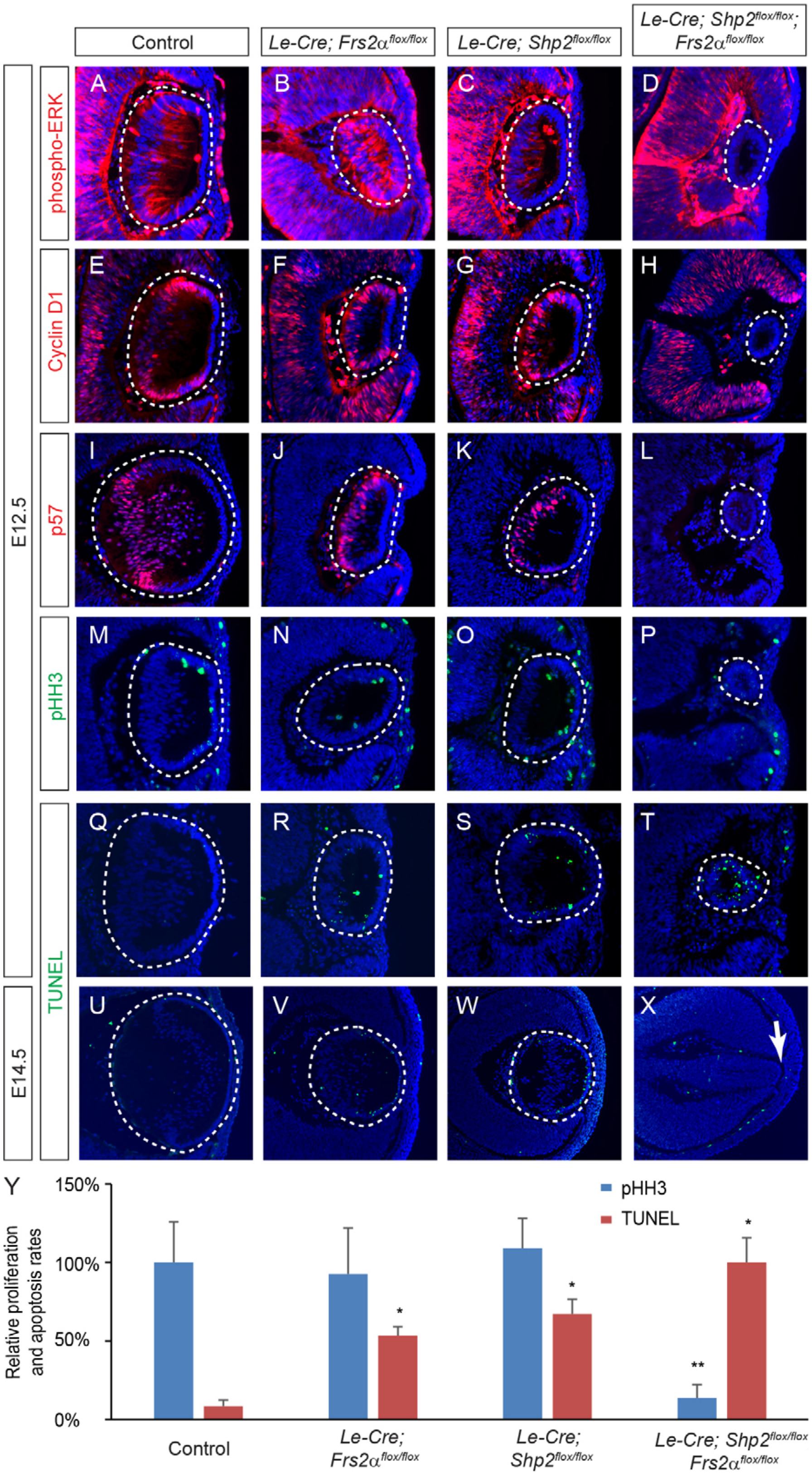

Fig. 3. Double knockout of *Frs2α* and *Shp2* disrupts ERK signaling and cell cycle control. (A–D) Phospho-ERK staining was abolished only in *Le-Cre*; *Shp2*$^{flox/flox}$; *Frs2α*$^{flox/flox}$ double mutants at E12.5. (E–L) Cell cycle regulators cyclin D1 and p57 were not detected in *Frs2α* and *Shp2* double mutant lenses. (M–P) In *Le-Cre*; *Shp2*$^{flox/flox}$; *Frs2α*$^{flox/flox}$ lenses, the M-phase marker phospho-histone H3 (pHH3) was significantly reduced. (Q–X) TUNEL staining was present in *Frs2α* and *Shp2* single mutants and further elevated in *Frs2α* and *Shp2* double mutant lenses at E12.5. Arrow indicates the absence of lens in E14.5 *Le-Cre*; *Shp2*$^{flox/flox}$; *Frs2α*$^{flox/flox}$ embryos. (Y) Quantification of relative proliferation and apoptosis rate. One way ANOVA test; *$P<0.01$ for the percentage of TUNEL positive cells in *Frs2α* and *Shp2* single and double mutants compared with those in wild-type controls; **$P<0.001$ for the percentage of pHH3-positive cells in *Frs2α* and *Shp2* double mutants compared with wild-type controls. $n=5$ for each genotype. Values are means ± s.e.m.

of Shp2^{D61Y}, which is known to exhibit constitutive phosphatase activity. The experiments presented in Fig. 5B first show that genetic depletion of *Frs2α* in MEF cells infected with Cre adenovirus effectively reduced FGF-induced phosphorylation of ERK and Gab1(Tyr627). Addition of *LSL-Shp2*^{D61Y} caused a notable increase in phospho-Gab1 and phospho-ERK levels. These results were next confirmed *in vivo*, where western blots and immunostaining showed that ERK phosphorylation was

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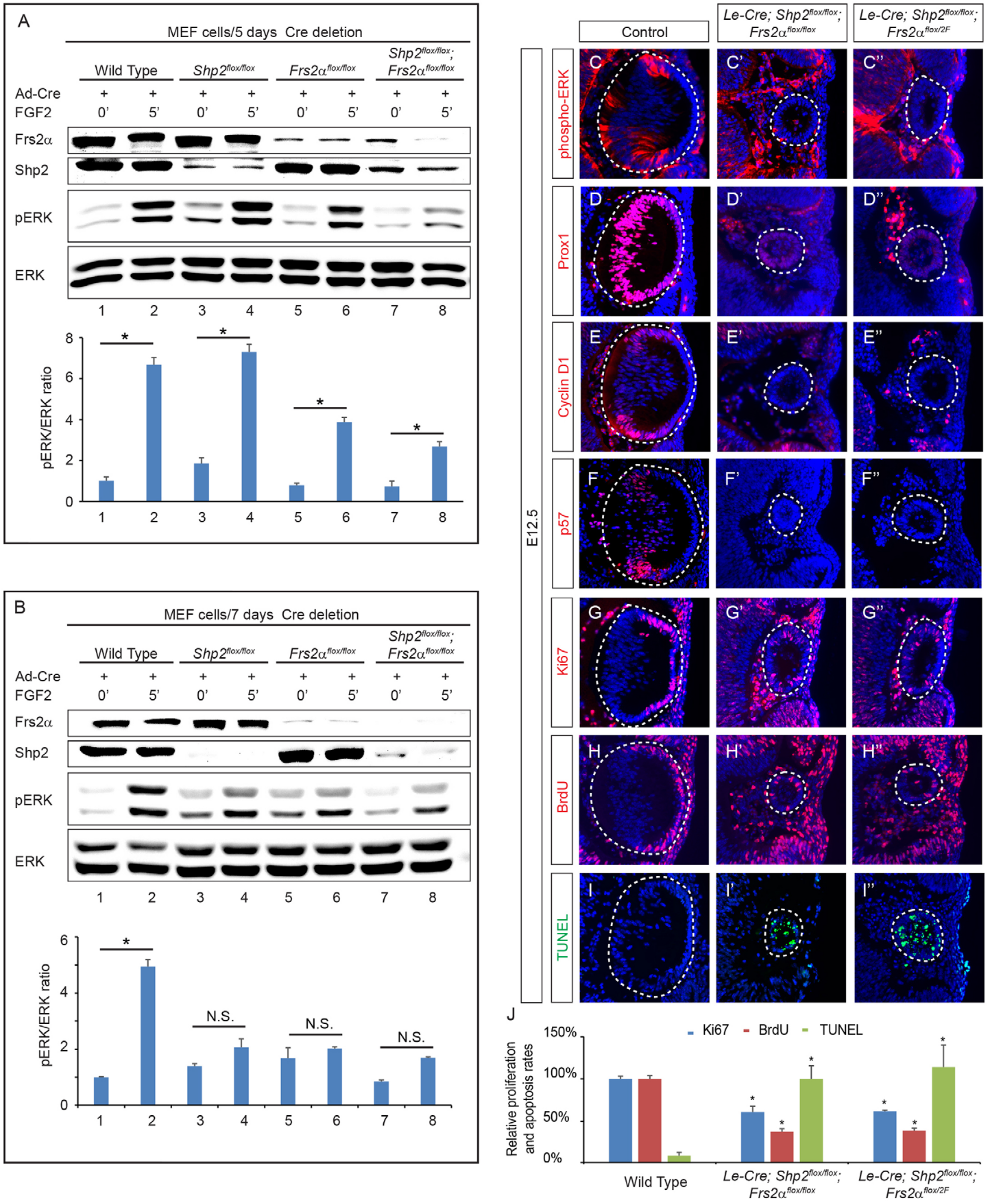

Fig. 4. See next page for legend.

Fig. 4. Interaction of Frs2α and Shp2 is necessary for lens development. (A,B) In MEF cells, 5 days after Cre-mediated genetic deletion, residual Frs2α and Shp2 proteins were still present and ERK phosphorylation was reduced only in *Frs2α* and *Shp2* double mutants. In Cre-virus-infected MEF cells after 7 days, further depletion of Frs2α and Shp2 proteins correlated with downregulation of ERK signaling in both single and double mutants. The intensity of the pERK and ERK protein bands was measured using Odyssey SA scanner and similar results were obtained in three independent experiments. One way ANOVA test, $^*P<0.02$. N.S., not significant. (C–F‴) Similar to *Frs2α* and *Shp2* double conditional null mutants, *Le-Cre*; *Shp2*$^{flox/flox}$; *Frs2α*$^{flox/2F}$ lenses showed downregulated ERK phosphorylation and expression of Prox1, cyclin D1 and p57. (G–J) Compared with the wild type, there was reduced cell proliferation as indicated by Ki67 and BrdU staining and increased cell death as shown by TUNEL in both *Le-Cre*; *Shp2*$^{flox/flox}$; *Frs2α*$^{flox/2F}$ and *Le-Cre*; *Shp2*$^{flox/flox}$; *Frs2α*$^{flox/flox}$ mutant lenses. $n=10$ for each genotype, One-way ANOVA test, $^*P<0.01$. All values are means ± s.e.m.

reduced in *Le-Cre*; *Frs2α*$^{flox/flox}$ mutant lens, but recovered in *Le-Cre*; *Frs2α*$^{flox/flox}$; *LSL-Shp2*^{D61Y} mutants (Fig. 5C–F). As a result, a statistically significant increase in lens size was observed in *Le-Cre*; *Frs2α*$^{flox/flox}$; *LSL-Shp2*^{D61Y} mutants compared with *Le-Cre*; *Frs2α*$^{flox/flox}$ mutants (Fig. 5G–J). These results demonstrate that *Shp2* gain-of-function mutation can compensate for the loss of *Frs2α* in lens development.

Gab1 and Gab2 are dispensable for FGF signaling

Our above results show that phosphorylation of Gab1 at Tyr627, a known Shp2 binding site, is strictly dependent on Frs2α during FGF signaling, suggesting that Gab1 is a partner of the Frs2α–Shp2 complex (Schaeper et al., 2000). Indeed, a previous study has suggested that recruitment of Gab1 by Frs2α leads to activation of PI3K–Akt signaling (Hadari et al., 2001). We thus investigated function of Gab1 and its close homologue Gab2 using genetically modified MEF cells. In this experiment, *Gab1*$^{flox/flox}$; *Gab2*$^{-/-}$ MEF cells were infected with Cre-expressing adenovirus and then subject to FGF stimulation. The samples were separated by SDS-PAGE followed by immunoblotting with phospho-specific antibodies. The experiment presented in Fig. 6A shows that simultaneous depletion of Gab1 and Gab2 in MEF cells failed to disrupt FGF-induced phosphorylation of Shp2, ERK and Akt, indicating that Gab1 and Gab2 are dispensable for FGF signaling *in vitro*.

The function of Gab proteins has never been directly investigated in lens development. We thus generated *Le-Cre*; *Gab1*$^{flox/flox}$ mice and confirmed the loss of Gab1 protein in E12.5 and E14.5 lens (Fig. 6B–E, arrows). *Le-Cre* activity is known to also be present in the ocular surface and lacrimal gland (Pan et al., 2008; Qu et al., 2011b). Interestingly, ablation of Gab1 did not perturb development of lacrimal gland, an Fgf10 signaling-dependent process, but resulted in an open eyelid at birth, consistent with the previously reported role of *Gab1* in EGF-signaling-dependent eyelid closure (supplementary material Fig. S4A–J, arrows) (Makarenkova et al., 2000; Schaeper et al., 2007; Pan et al., 2008; Qu et al., 2011b). Immunoblotting of E16 lenses showed that ERK and Akt phosphorylation in Gab1-depleted mutants remained unchanged (Fig. 6H). Consistent with this, there were no reductions in either lens size or immunostaining intensities for phospho-Akt and phospho-ERK (Fig. 6I,J,L,M,O,P). Because there is a detectable level of Gab2 in the lens upon immunostaining (Fig. 6F,G), we also generated *Le-Cre*; *Gab1*$^{flox/flox}$; *Gab2*$^{-/-}$ mice, which again failed to display any reduction in lens size or Akt and ERK phosphorylation (Fig. 6K,N,Q). Finally, examination of *Le-Cre*; *Gab1*$^{flox/flox}$; *Frs2α*$^{flox/flox}$ and *Le-Cre*; *Gab1*$^{flox/flox}$; *Shp2*$^{flox/flox}$ mutants showed that *Gab1* did not genetically interact with *Frs2α* or *Shp2* (data not shown). These results show that *Gab1* and *Gab2* are not required for FGF signaling either *in vitro* or *in vivo*.

Constitutive Kras signaling rescues *Frs2α* and *Shp2* deficiency in lens development

We next considered Ras signaling as the main target of the Frs2α–Shp2 complex in lens development. In a genetic rescue experiment, we crossed *Le-Cre*; *Shp2*$^{flox/flox}$; *Frs2α*$^{flox/flox}$ mutants with the *LSL-Kras*^{G12D} allele, which can be induced to express a constitutively active *Kras*^{G12D} mutant by Cre-mediated recombination (Fig. 7A). The results presented in Fig. 7B–G show that, despite a loss of Shp2 expression in *Le-Cre*; *Shp2*$^{flox/flox}$; *Frs2α*$^{flox/flox}$; *LSL-Kras*^{G12D} mutant lens at E12.5, ERK phosphorylation was induced to 52±6% (mean ± s.e.m) of wild-type level (Student's *t*-test: $n=4$; $P=0.0231$). This was accompanied by a recovery of the lens development markers Prox1, cyclin D1 and p57, cell proliferation marker Ki67 and a substantial increase in lens size (Fig. 7H–Q; supplementary material Fig. S5A,B). Consistent with our previous report (Pan et al., 2010), abnormal cell apoptosis, as indicated by TUNEL staining, was not rescued by activated Kras signaling (supplementary material Fig. S5C,D). Nevertheless, the *Le-Cre*; *Shp2*$^{flox/flox}$; *Frs2α*$^{flox/flox}$; *LSL-Kras*^{G12D} mutant lens at E16.5 exhibited elongated lens fibers capped anteriorly by a single layer of epithelial cells, closely resembling wild-type controls (supplementary material Fig. S5E,F). Taken together, these results show that activated Ras signaling can significantly subvert the requirement of both Frs2α and Shp2 in lens development.

DISCUSSION

In this study, we investigated the function of Frs2α and its downstream targets in FGF signaling during lens development. Combining biochemical analysis and mouse genetics, we demonstrate that simultaneous depletion of Frs2α and Shp2 results in a more severe FGF signaling defect than either single mutant. This synergy between Frs2α and Shp2 is not a result of overlapping or compensatory functions of these two factors. Instead, we show that Frs2α acts directly upstream to Shp2 in a linear cascade to mediate FGF signaling. We provide further evidence that Gab, a commonly believed downstream target of Frs2α, is in fact dispensable for FGF signaling. However, both constitutively activated Shp2 and Kras signaling can ameliorate lens defects in *Frs2α* mutants. These results show that Frs2α is a crucial link in the FGFR–Shp2–Ras–ERK signaling cascade in lens development.

The functional significance of Frs2α remains a controversial issue in the field of FGF signaling research. Although earlier biochemical studies have indicated that Frs2α mediates most, if not all, FGF signaling effects, more recent genetic studies have begun to cast doubt on this central role of Frs2α. Not only do knockouts of several *Fgf* and *Fgfr* family members cause embryonic lethality earlier than *Frs2α* null animals, mutation of the Frs2-binding sites on either Fgfr1 or Fgfr2 generally results in far less severe phenotypes than their null counterparts (Feldman et al., 1995; Gotoh et al., 2005; Eswarakumar et al., 2006; Hoch and Soriano, 2006). However, one should exercise caution when interpreting these genetic results because conformational changes in *Fgfr* mutants might cause fortuitous heterodimerization with other Fgfr family members, which could act in trans to rescue Frs2α-binding deficiency (Bellot et al., 1991; Ueno et al., 1992). In the case of systemic knockouts, deposition of long-lived

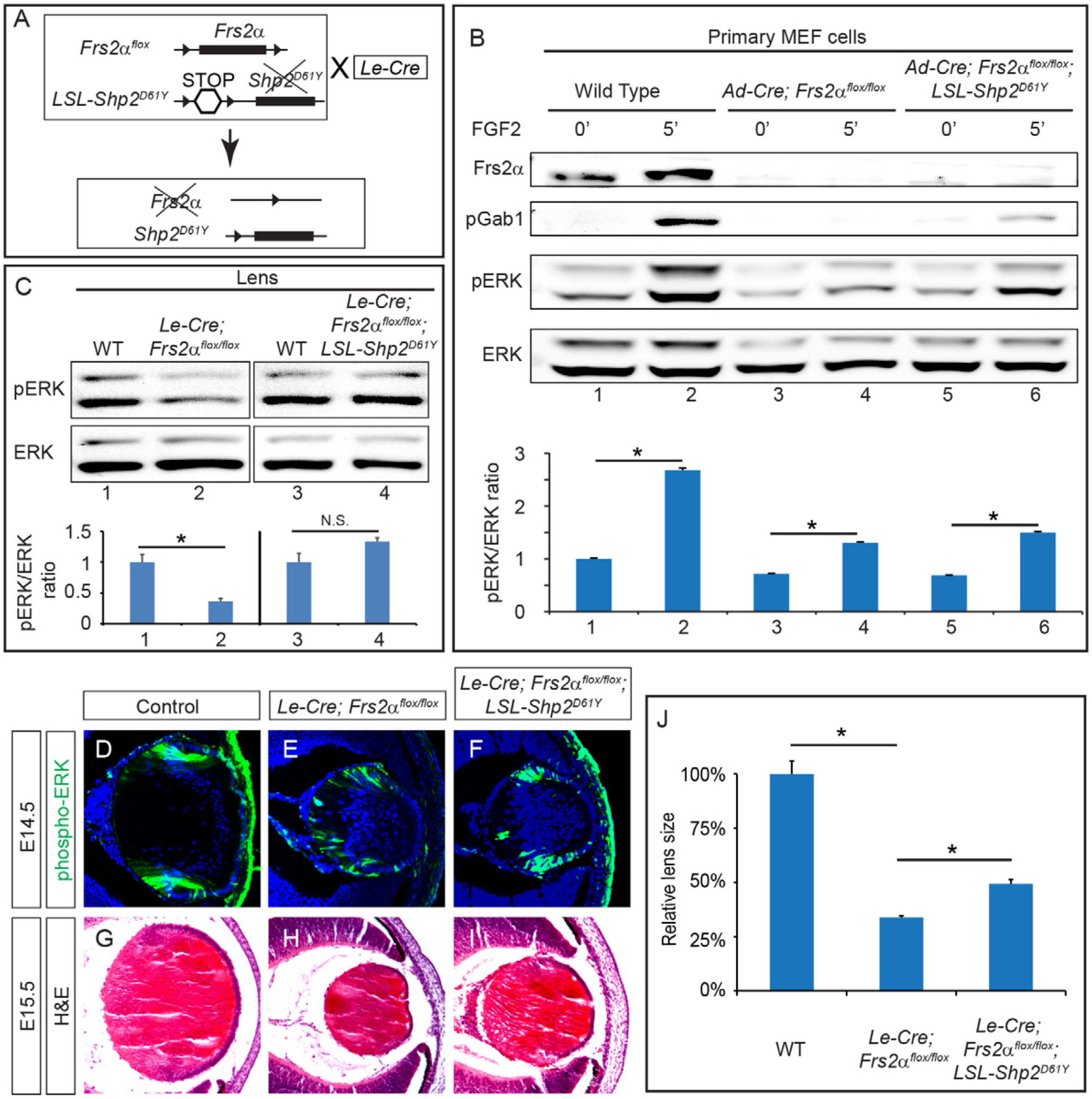

Fig. 5. Genetic rescue of *Frs2α* deletion by a gain-of-function *Shp2* mutant. (A) Schematic diagram of *Frs2α* rescue by *Shp2* activation. The constitutively active *Shp2*^{D61Y} is normally silenced by a transcriptional STOP cassette in the *LSL-Shp2*^{D61Y} allele, which can be relieved by Cre-mediated recombination that simultaneously ablates *Frs2α*^{flox} within the same cells. (B) Depletion of Frs2α in MEF cells led to downregulation of Gab1 and ERK phosphorylation, which was reversed by *Shp2*^{D61Y} expression. pERK/ERK ratios were quantified from three western blot experiments. One-way ANOVA test, *$P<0.05$. (C–F) Western blots and immunohistochemistry showed that activation of *LSL-Shp2*^{D61Y} allele results in increased ERK phosphorylation in E16.5 *Le-Cre*; *Frs2α*$^{flox/flox}$; *LSL-Shp2*^{D61Y} lenses compared with *Le-Cre*; *Frs2α*$^{flox/flox}$ mutants. Student's *t* test, *$P<0.05$; N.S., not significant. (G–J) Quantification of lens sizes in wild type ($n=4$), *Le-Cre*; *Frs2α*$^{flox/flox}$ ($n=6$) and *Le-Cre*; *Frs2α*$^{flox/flox}$; *LSL-Shp2*^{D61Y} ($n=4$) mutants. One-way ANOVA test, *$P<0.01$. Values are means ± s.e.m.

maternal proteins in zygotes could distort the timing and severity of early embryonic defects. The complication of long-lasting proteins especially confounds conditional knockouts, where Cre-mediated genetic ablation will inevitably leave behind residual protein that persists with variable half-life dictated by the individual protein degradation rate. Indeed, by modeling protein degradation kinetics using MEF cells infected with Cre virus, we showed that trace amounts of Frs2α and Shp2 proteins existed several days after genetic deletion to sustain a significant FGF signaling response, which agrees with the extremely slow turnover rate of Shp2 previously reported (Siewert et al., 1999). Further protein depletion after prolonged exposure to Cre virus eventually revealed that both Frs2α and Shp2 are essential for FGF signaling. Therefore, the gradual depletion of Frs2α and Shp2 proteins expected in conditional knockouts might have significantly masked the true function of these two proteins in embryonic development.

The conditional knockouts of *Frs2a* and *Shp2* described in this study should thus be construed as hypomorphic alleles, which can instead be valuable tools to probe protein–protein interaction. This is because hypomorphic mutations in two interacting factors are known to cause synergistic rather than additive effects,

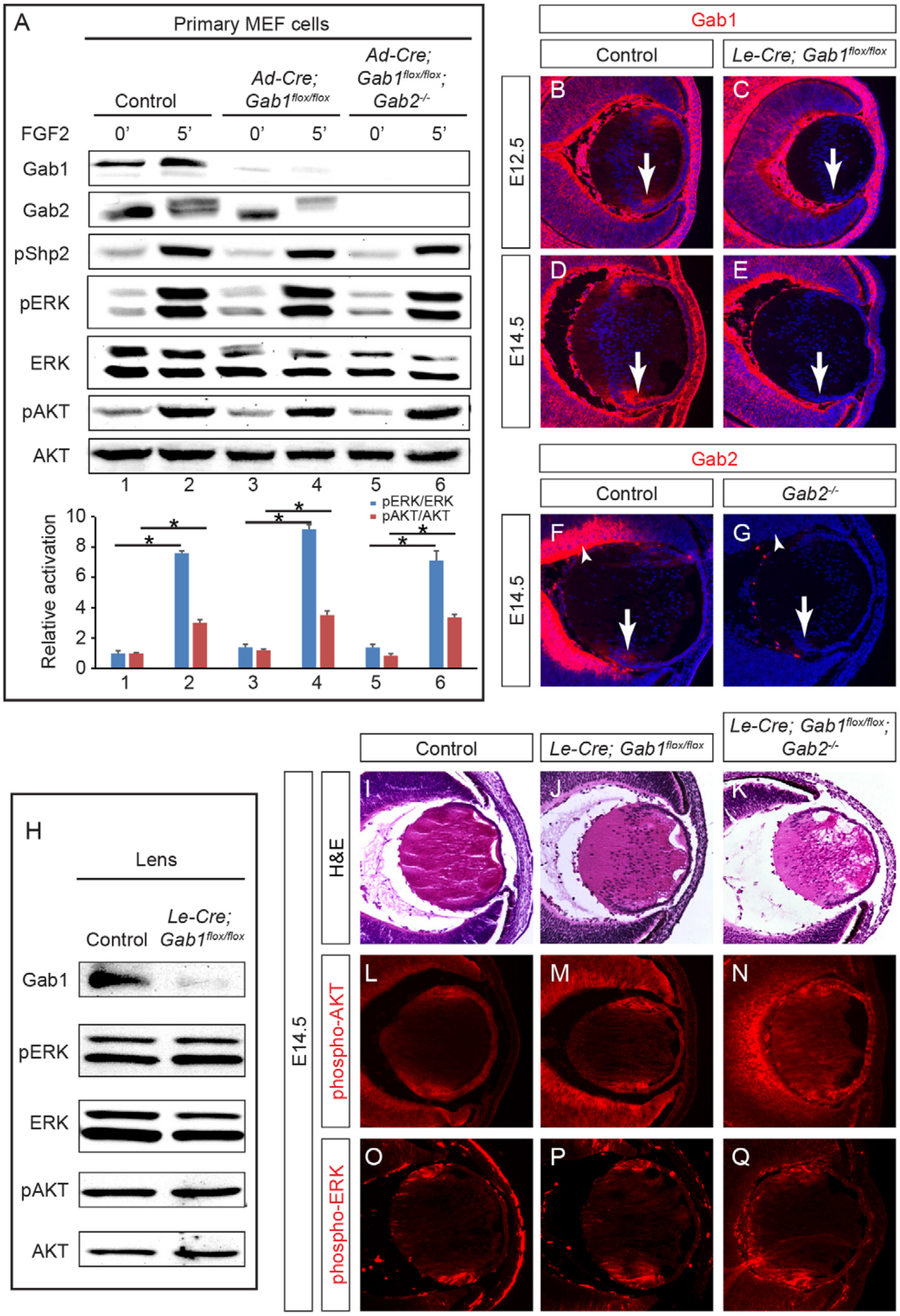

Fig. 6. *Gab1* and *Gab2* are not required for FGF signaling in lens development. (A) Adenovirus-mediated depletion of Gab1 and knockout of Gab2 in MEF cells failed to disrupt FGF-induced phosphorylation of Shp2, ERK and Akt. The intensities of pERK/ERK and pAkt/Akt bands were quantified from three independent experiments. One-way ANOVA test, $^*P<0.01$. Values are means ± s.e.m. (B–E) Gab1 was efficiently ablated in E12.5 and E14.5 *Le-Cre*; *Gab1$^{flox/flox}$* lenses (arrows). (F,G) Gab2 was expressed strongly in wild-type control retina (arrowheads) and weakly in lens (arrows). Both expressions were abolished in *Gab2$^{-/-}$* embryos. (H) ERK and Akt signaling was unaffected in lens lysates from E16.5 Gab1 conditional mutants. (I–Q) Normal phospho-ERK and phospho-Akt staining in lens-specific knockout of *Gab1* and *Gab2*. Three embryos of each genotype were analyzed.

manifesting as genetic interactions *in vivo*. Indeed, we showed that conditional knockouts of *Frs2α* and *Shp2* displayed mild phenotypes individually, but abolished lens development when combined. Lens induction failure has been previously reported in *Frs2α^{2F}* mutants that lack a Shp2-binding site, but these observations are confounded by the crucial role of FGF signaling in retinal development (Gotoh et al., 2004; Cai et al., 2010; Cai et al., 2013). We thus combined *Frs2α^{2F}* and *Frs2α^{flox}* alleles to generate a lens-specific disruption of Shp2 binding to Frs2α, showing that *Frs2α^{2F}* can substitute for *Frs2α^{flox}* to reproduce the genetic interaction between *Frs2α* and *Shp2* in lens development. This result not only confirms the physical recruitment of Shp2 by Frs2α *in vivo*, but also suggests that the Frs2α–Shp2 complex is the signaling center of the FGF pathway during lens development. In this model, Frs2α acts directly upstream of Shp2 in a signaling cascade and cannot be compensated by other factors that mediate parallel signaling.

Although it has been generally assumed that Gab proteins play important roles in FGF signaling, this notion has never been tested *in vivo* (reviewed by Eswarakumar et al., 2006; Goetz and

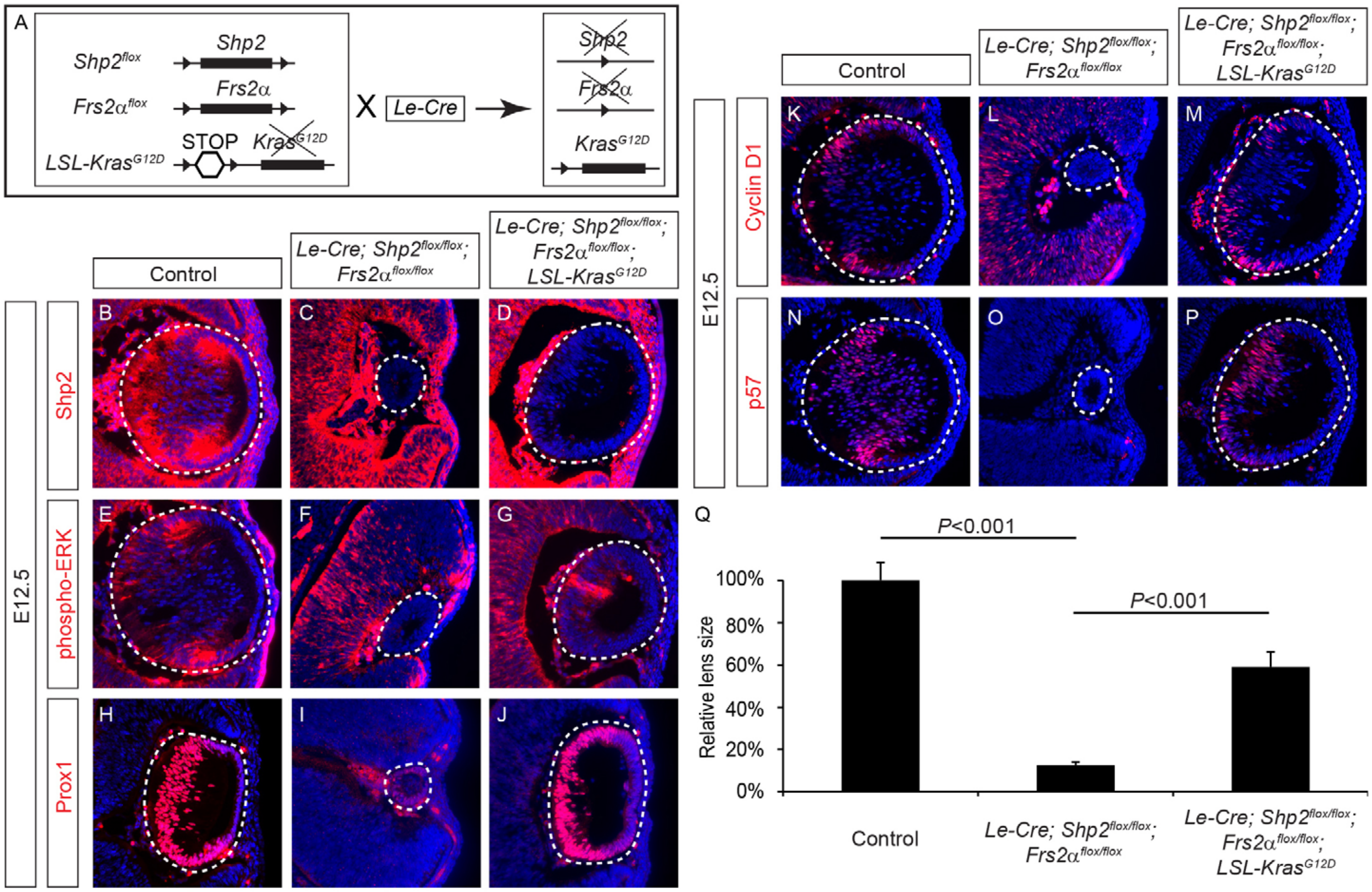

Fig. 7. Kras signaling can compensate for the loss of *Frs2α* and *Shp2* in lens development. (A) Cre-mediated recombination from *Le-Cre* leads to genetic deletion of *Frs2α* and *Shp2*, accompanied by constitutive activation of *LSL-Kras*^{G12D} allele. (B–P) Despite the loss of Shp2, ERK phosphorylation recovered in *Le-Cre*; *Shp2*$^{flox/flox}$; *Frs2α*$^{flox/flox}$; *LSL-Kras*^{G12D} mutant lens. As a result, Prox1, cyclin D1 and p57 were upregulated. Three embryos of each genotype were analyzed. (Q) Quantification of lens sizes in E12.5 wild type ($n=3$), *Le-Cre*; *Shp2*$^{flox/flox}$; *Frs2α*$^{flox/flox}$ ($n=10$) and *Le-Cre*; *Shp2*$^{flox/flox}$; *Frs2α*$^{flox/flox}$; *LSL-Kras*^{G12D} ($n=4$) mutants. One-way ANOVA test, $P<0.01$. Values are means ± s.e.m.

Mohammadi, 2013). Biochemical studies have previously suggested that FGF signaling induces an indirect coupling of Frs2 and Gab1 mediated by the Grb2 adaptor protein, leading to phosphorylation of Gab1 on multiple tyrosine residues (Hadari et al., 2001). These phosphorylation sites can attract binding of PI3K for activation of Akt signaling, RasGAP for suppression of Ras–MAPK signaling and protein tyrosine phosphatase Shp2 (Maroun et al., 2000; Rodrigues et al., 2000; Montagner et al., 2005). It has been proposed that Shp2 dephosphorylates Gab1 to eliminate the specific binding site for the SH2 domain of RasGAP, resulting in ultimate activation of Ras–MAPK signaling (Montagner et al., 2005). Therefore, Gab1 is not only an attractive mediator of PI3K–Akt signaling downstream of Frs2, but also serves as a phosphatase substrate enabling Shp2 to play a positive role in Ras–MAPK signaling. In primary MEF cells, we indeed observed FGF-induced phosphorylation of Gab1 on a tyrosine residue that engages Shp2. Genetic ablation of Gab1 and its homologue Gab2, however, did not affect either Akt or ERK signaling *in vitro* or *in vivo*. Consistent with this, we failed to observe any defects in *Gab1*- and *Gab2*-deficient lens and lacrimal gland, both of which require FGF signaling during embryonic development. To our knowledge, these studies provide the first genetic tests of Gab function in FGF signaling *in vivo* and they argue against a functional role of Gab in FGF–Frs2–Shp2 signaling in general.

Our study thus reveals that the Frs2α–Shp2 complex is crucial for mediating FGF signaling in lens development. It is notable that our *Frs2α Shp2* mutant at E12.5 closely resembles a previously reported *Fgfr1*, *Fgfr2* and *Fgfr3* triple mutant in forming a hollow lens vesicle with aberrant expressions of Pax6, Prox1, E-cadherin and γ-crystallin (Zhao et al., 2008). However, our mutant also displayed a more severe phenotype in its abrogation of Cyclin D1 and P57 expression, resulting in a loss of lens structure after E14.5. It has previously been shown that *Fgfr4* is present at low levels as early as E9.5 in lens placode, and one of its ligands *Fgf15* is also abundantly expressed in the eye (Garcia et al., 2011; Cai et al., 2013). It would be interesting to test whether Fgfr4 signaling partially compensates for the loss of Fgfr1, Fgfr2 and Fgfr3, allowing these mutant lens cells to retain a limited proliferative capacity. The FGF receptor is the only known receptor tyrosine kinase that can induce mammalian lens fiber differentiation, which has been proposed to depend on the unique capacity of *Frs2α* in orchestrating downstream signaling (Madakashira et al., 2012). By showing that constitutive Ras signaling is sufficient to obviate the requirement of the Frs2α–Shp2 complex in lens development, we demonstrate that the ultimate target of Frs2α is Ras signaling. Ras signaling is known to have multiple downstream effector pathways, including RAF–MEK–ERK, PI3K and RalGDS.

Importantly, Upadhya and colleagues (Upadhya et al., 2013) recently showed that genetic deletion of *Erk1* and *Erk2* in the lens also caused significant reduction in cell proliferation and aberrant apoptosis, but unlike the eventual loss of lens structure in our *Frs2α* and *Shp2* mutants, embryos lacking *Erk1* and *Erk2* appear to retain a rudimentary lens at E14.5 (Upadhya et al., 2013). Whether additional Ras downstream effector pathways complement ERK signaling in lens development will be an important topic for future study.

MATERIALS AND METHODS

Mice

Mice carrying $Frs2\alpha^{flox}$, $Frs\alpha^{2F}$, $Gab1^{flox}$, $Gab2^{+/-}$ and $Shp2^{flox}$ alleles were bred and genotyped as described (Gotoh et al., 2004; Zhang et al., 2004; Bard-Chapeau et al., 2005; Teal et al., 2006; Lin et al., 2007). *LSL-*$Shp2^{D61Y}$ mice were gifts from Rebecca Chan, Indiana University School of Medicine, Indianapolis, IN and Benjamin G. Neel, Ontario Cancer Institute, Ontario, Canada (Chan et al., 2009). *Le-Cre* mice were kindly provided by Ruth Ashery-Padan, Tel Aviv University, Tel Aviv, Israel and Richard Lang, Children's Hospital Research Foundation, Cincinnati, OH (Ashery-Padan et al., 2000). *LSL-*$Kras^{G12D}$ mice were obtained from the Mouse Models of Human Cancers Consortium (MMHCC) Repository at National Cancer Institute (Tuveson et al., 2004). In all conditional knockout experiments, mice were maintained in mixed genetic background and *Le-Cre* only mice in the same litters were used as wild type controls. All experiments were performed in accordance with institutional guidelines.

Histology, immunohistochemistry and RNA *in situ* hybridization

Mouse embryos were fixed with 4% paraformaldehyde (PFA) in PBS overnight and paraffin embedded. The sections (10 µm) were rehydrated and stained with hematoxylin and eosin (H&E) for histological analysis. Lens sizes were measured as previously described (Pan et al., 2010). TUNEL staining and immunostaining were performed on the cryosections (8 µm) as previously described (Pan et al., 2006; Pan et al., 2008). For phospho-ERK, phospho-Akt, p21, Frs2α and Shp2 staining, the signal was amplified using a Tyramide Signal Amplification kit (TSA^{TM} Plus System, PerkinElmer Life Sciences, Waltham, MA). Antibodies used were: anti-BrdU (G3G4, Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA), anti-p21 (sc-397), anti-Shp2 (Sc-280), anti-γ-crystallin (SC-22415) (from Santa Cruz biotechnology, Santa Cruz, CA), anti-phospho-ERK1/2 (#4370), anti-phospho-AKT (#4060), anti-CDK4 (#2906), anti-cyclin-D1 (#2926), anti-Gab1 (#3232) and anti-Gab2 (#3239) (all from Cell Signaling Technology, Beverly, MA), anti-P57 (ab75974) and anti-Frs2α (ab10425) (both from Abcam, Cambridge, MA), anti-E-cadherin (U3254, Sigma, St Louis, Missouri), anti-Ki67 (#550609, from BD Pharmingen San Diego, CA), anti-Prox1 (PRB-238C) and anti-Pax6 (PRB-278P) (both from Covance, Berkeley, CA). Antibodies against α- and β-crystallins were kindly provided by Sam Zigler (National Eye Institute, Bethesda, MD). Cell proliferation and apoptosis were measured as the ratio of BrdU, pHH3, Ki67 or TUNEL-positive cells versus DAPI-positive cells, and analyzed by one-way ANOVA analysis. For the quantification of phospho-ERK staining, the fluorescent pixel intensity in the lens was measured by the ImageJ program. This value was further divided by the averaged phospho-ERK pixel intensity at the nearby retinal region to obtain the normalized lens phospho-ERK level.

RNA *in situ* hybridizations were performed on cryosections (8 µm) as previously described (Pan et al., 2006; Cai et al., 2010). *Foxe3* probe was generously provided by Milan Jamrich (Baylor College of Medicine, Houston, TX). *Erm* and *Er81* were both from Bridget Hogan (Duke University Medical Center, Durham, NC). The *Frs2α* probe was generated from a genomic fragment containing the last exon flanked by *loxP* sites in the $Frs2\alpha^{flox}$ allele (Cai et al., 2013). For all immunohistochemistry and RNA *in situ* hybridization procedures, at least three embryos of each genotype were analyzed.

MEF cells

Primary MEF cells were isolated from embryos at E12.5–E14.5 stages and cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% FBS as described (Qu et al., 2011b). MEFs from the second passage were infected with Ad5CMVCre or Ad5CMVCre-eGFP (Gene Transfer Vector Core, University of Iowa, IA) in DMEM containing 2% FBS overnight at multiplicity of infection 500 plaque-forming units/cell and cultured for 5 or 7 days.

Western blots

Lens tissues were collected at E15–16 stages and homogenized in RIPA buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, 1 µg/ml aprotinin, 1 µg/ml pepstatin, 10 mM sodium pyrophosphate, 1 mM PMSF, 0.2 mM Na_3VO_4, 50 mM NaF). 4.24–6.36×10^5 MEF cells were seeded in 60 mm dishes and serum starved (0.5% FBS in DMEM) for 36–48 hours before stimulated by 50 ng/ml FGF2 (R&D Systems, Minneapolis, MN) for 5 minutes at 37°C. After washed twice in cold PBS, MEF cells were lysed in 160 µl RIPA buffer and processed for infrared-based western blot analysis using an Odyssey SA scanner (LICOR Biosciences, Lincoln, NE) (Qu et al., 2011b). The signal intensity was quantified using the Odyssey software. The antibodies used were mouse anti-phospho-ERK1/2 (sc-7383), rabbit anti-Frs2α (sc-8318) (both from Santa Cruz Biotechnology, Santa Cruz, CA) and mouse anti-AKT (#4060), rabbit anti-phospho-AKT (#4060), anti-ERK1/2 (#4695), anti-Gab1 (#3232), anti-phospho-Gab1(#3233), anti-Shp2 (#3752) and anti-phospho-Shp2 (#3751) (all from Cell Signaling Technology).

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Competing interests

The authors declare no competing financial interests.

Author contributions

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

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Supplementary material

Supplementary material available online at
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