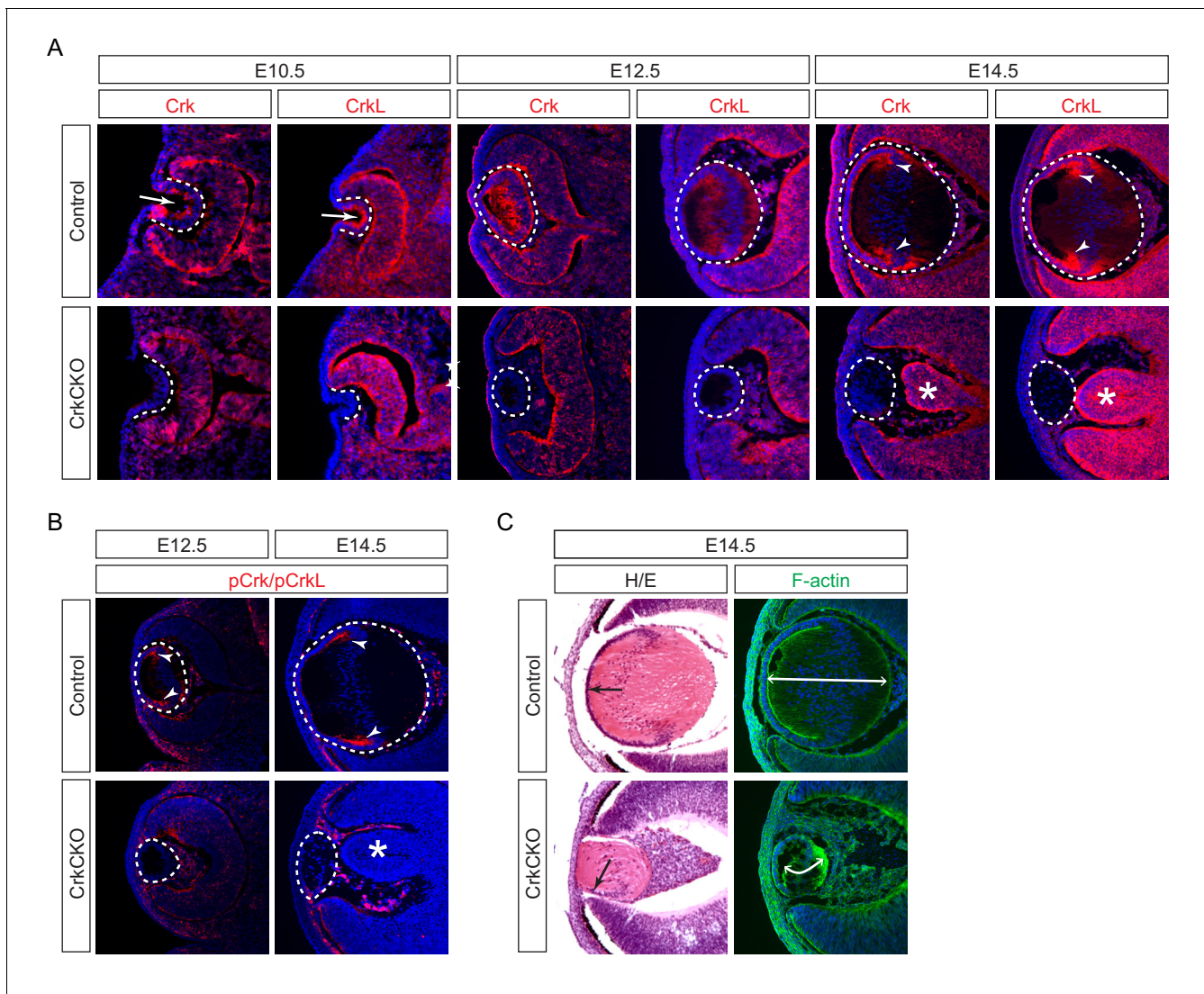


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## Figures and figure supplements

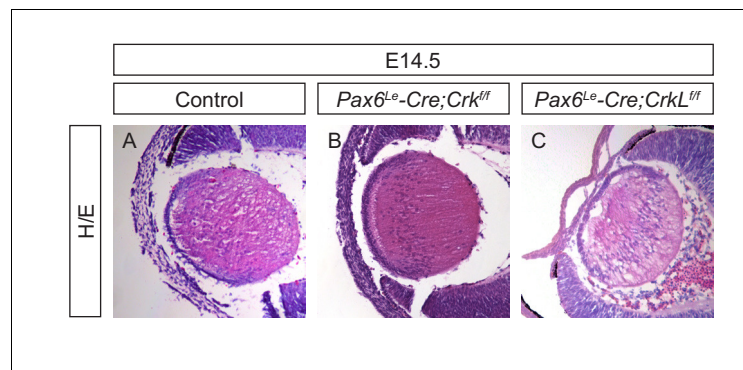
Crk proteins transduce FGF signaling to promote lens fiber cell elongation

**Tamica N Collins et al**



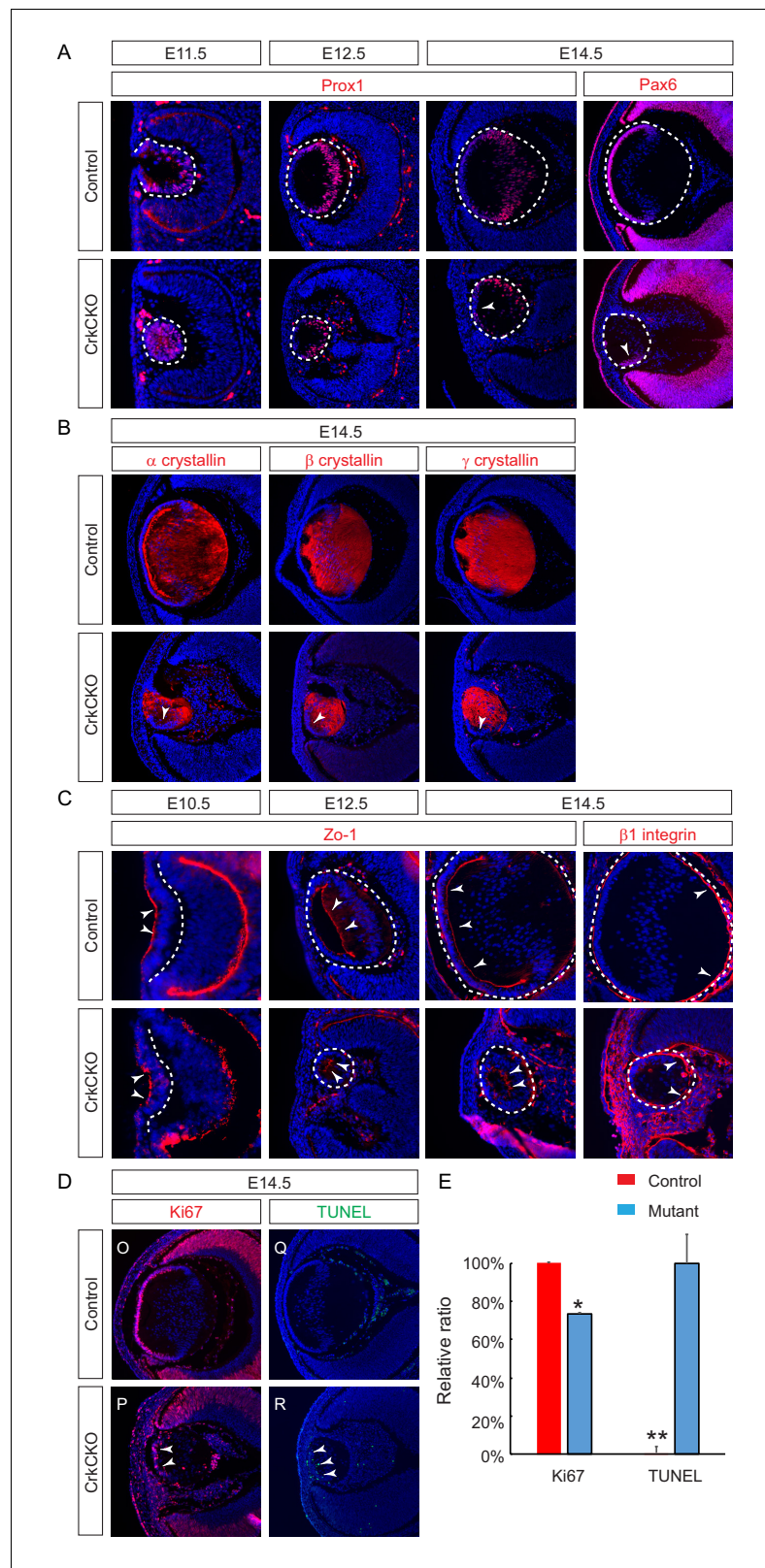
**Figure 1.** Crk and Crkl are essential for lens development. (A) Crk and Crkl immunostaining were localized to the invaginating lens vesicle at E10.5 (arrows) and to the elongating lens fiber cells near the transitional zone of the lens at E14.5 (arrowheads). These staining patterns were specifically lost in the CrkCKO lens. The dotted lines enclose the region of the lens and the disorganization of the retina was marked with asterisks (B) The phosphorylation of both Crk and Crkl was noticeably absent in the CrkCKO lens (arrowheads). (C) The CrkCKO lens size was significantly reduced with the anterior lens epithelium rotated sideways (arrows) and the disorganized lens fiber cells markedly shortened (double headed arrows).

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**Figure 1—figure supplement 1.** *Crk* and *Crkl* single mutants did not display lens phenotype. (A–C) H/E staining showed that the individual deletion of *Crk* and *Crkl* did not affect aspects of lens development.

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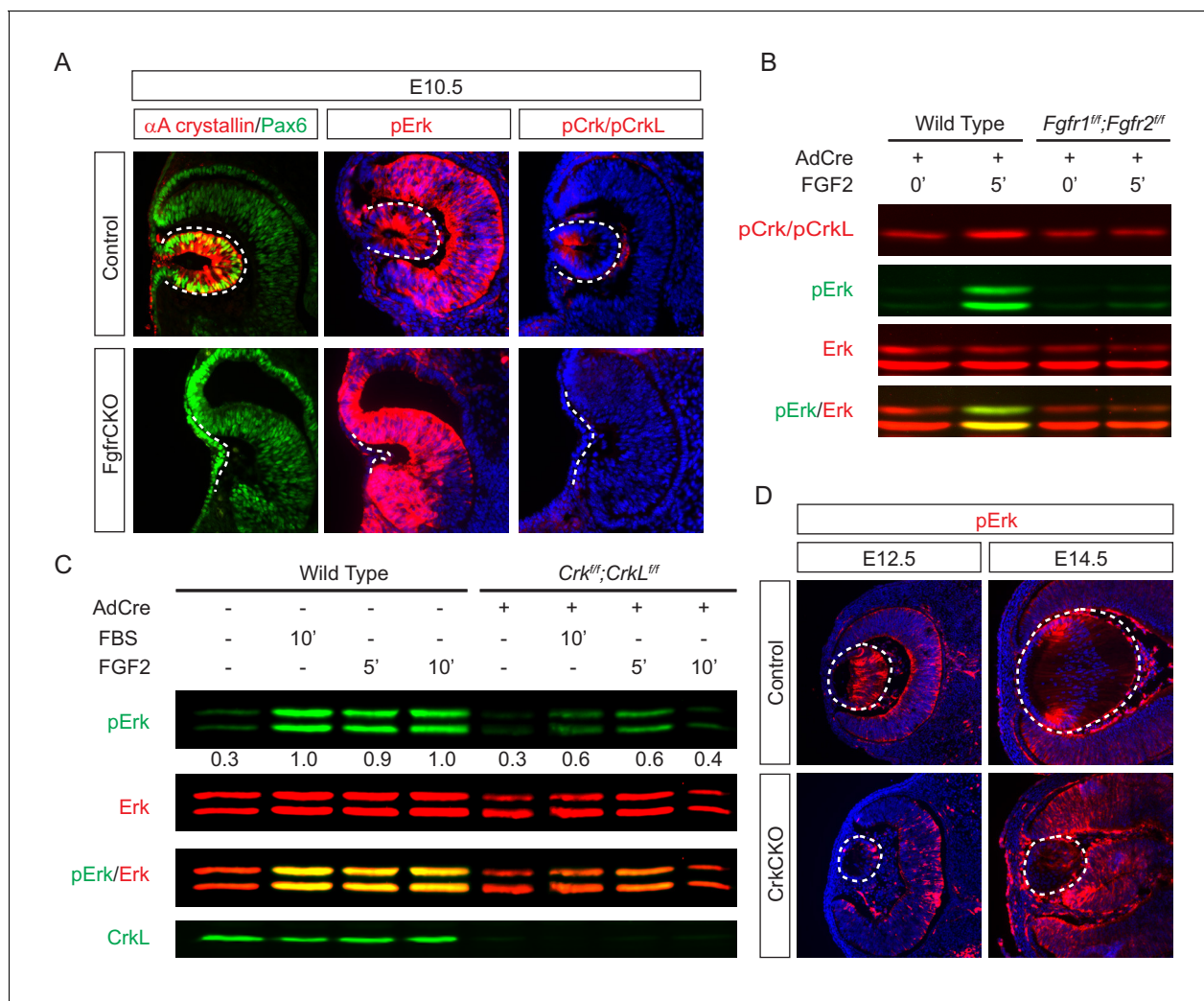
**Figure 2.** Molecular defects in the *Crk* and *Crkl* double mutant lens. (A) There were no significant changes in the staining intensity of the lens determinant markers Prox1 and Pax6. (B) Further, none of the three forms of Crystallins ( $\alpha$ ,  $\beta$ ,  $\gamma$ ) displayed any changes in staining intensity in the CrkCKO lens. (C) The polarity of the CrkCKO lens. (D) The proliferation and apoptosis of the CrkCKO lens. (E) The relative ratio of Ki67 and TUNEL staining. Figure 2 continued on next page



*Figure 2 continued*

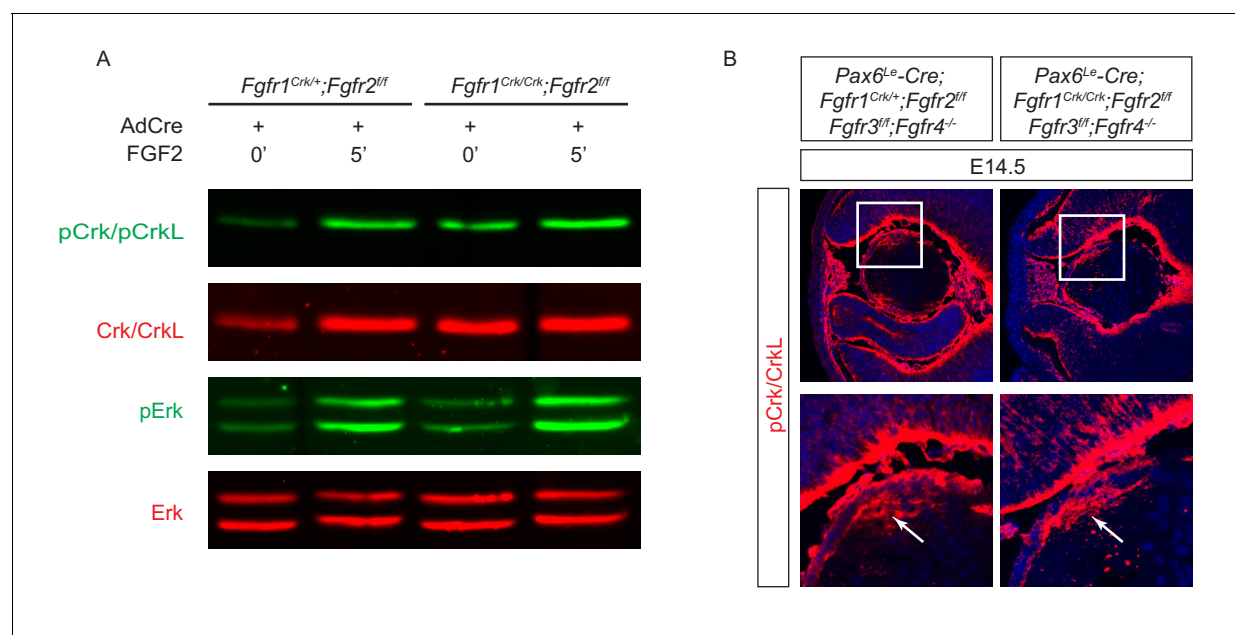
lens fiber cells was maintained as indicated by both the apical expression of Zo-1 and the basal expression of  $\beta 1$  integrin. (D) The number of Ki67-expressing proliferative cells was significantly decreased and the number of TUNEL-positive apoptotic cells was increased (arrowheads). (E) Quantification of proliferation and apoptosis in wild type and CrkCKO lens. Student's t test, \* $p < 0.01$ , \*\* $p < 0.001$ ,  $n = 4$ .

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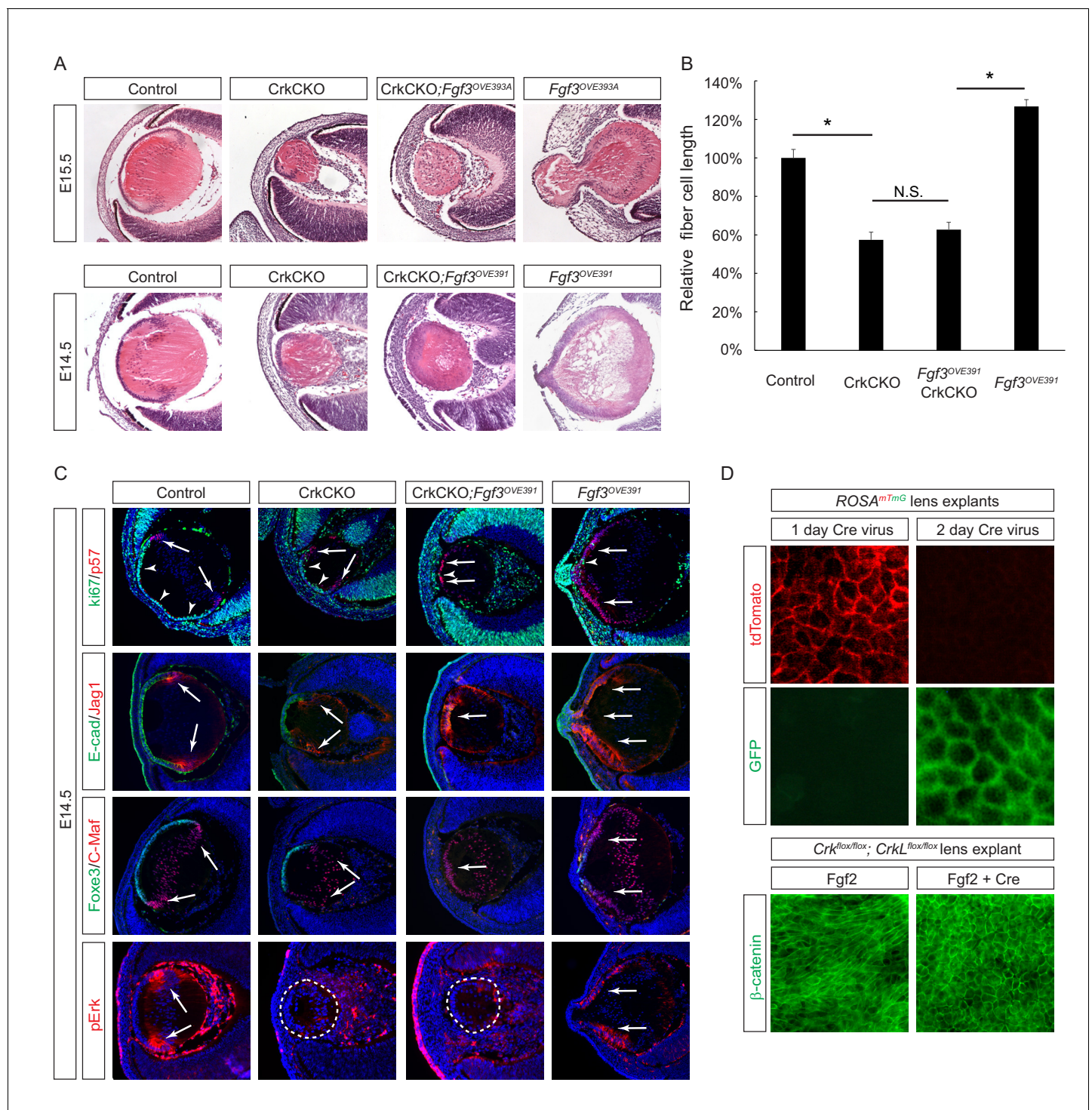
**Figure 3.** Crk proteins mediate FGF signaling in Erk phosphorylation. (A) Genetic ablation of *Fgfr1* and *Fgfr2* disrupted the proper formation of the lens vesicle with the phosphorylation of Erk and Crk/CrkL proteins being noticeably absent. (B) Mouse Embryonic fibroblast (MEF) cells treated with FGF2 displayed an increase in pCrk/CrkL and pErk levels, which were abrogated by the removal of *Fgfr1* and *Fgfr2* using a Cre-expressing adenovirus. (C) Ablation of Crk proteins in MEF cells reduced FGF2-induced Erk phosphorylation. The pErk/Erk ratios were noted below the pERK blot. (D) The CrkCKO lens displayed a significant decrease in pERK staining compared to the wild type lens.

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**Figure 3—figure supplement 1.** The Y463F mutation in *Fgfr1* (*Fgfr1<sup>Crk</sup>*) did not affect the phosphorylation of Crk and Erk proteins that is essential for lens development. (A) FGF2 was still able to induce an upregulation of pCrk/CrkL and pERK in *Fgfr1<sup>Crk/Crk</sup>;Fgfr2<sup>fl/fl</sup>* MEF cells after treatment with a Cre-expressing adenovirus. (B) A homozygous *Fgfr1<sup>Crk/Crk</sup>* mutation did not affect pCrk/CrkL staining (arrows) in the lens even after the genetic removal of other Fgf receptors (*Fgfr2-4*).

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**Figure 4.** Crk/CrkI deletion prevented FGF-induced lens cell elongation without affecting differentiation. (A) Overexpression of *Fgf3* in *Fgf3*<sup>OVE391</sup> and *Fgf3*<sup>OVE393A</sup> strains resulted in increased lens fiber cell elongation and overall lens sizes. However, both of these phenotypes were suppressed after being crossed with the CrkCKO mutant, indicating a genetic epistasis interaction between the FGF and Crk signaling pathways. (B) Quantification of lens fiber cell length. One-way ANOVA test followed by Tukey's multiple comparisons test, \* $p < 0.01$ ,  $n = 3$ . (C) Deletion of the *Crk* genes did not prevent the premature differentiation phenotype observed in the *Fgf3* overexpressing lens, as indicated by a reduction of lens progenitor cell markers (Ki67, E-cad and Foxe3) and an increase of differentiation cell markers (p57, Jag1, and C-Maf) within the presumptive lens epithelial layer (arrows). Nonetheless, pERK staining was significantly reduced in the CrkCKO;*Fgf3*<sup>OVE391</sup> lens. (D) The Cre-expressing adenovirus induces efficient genetic

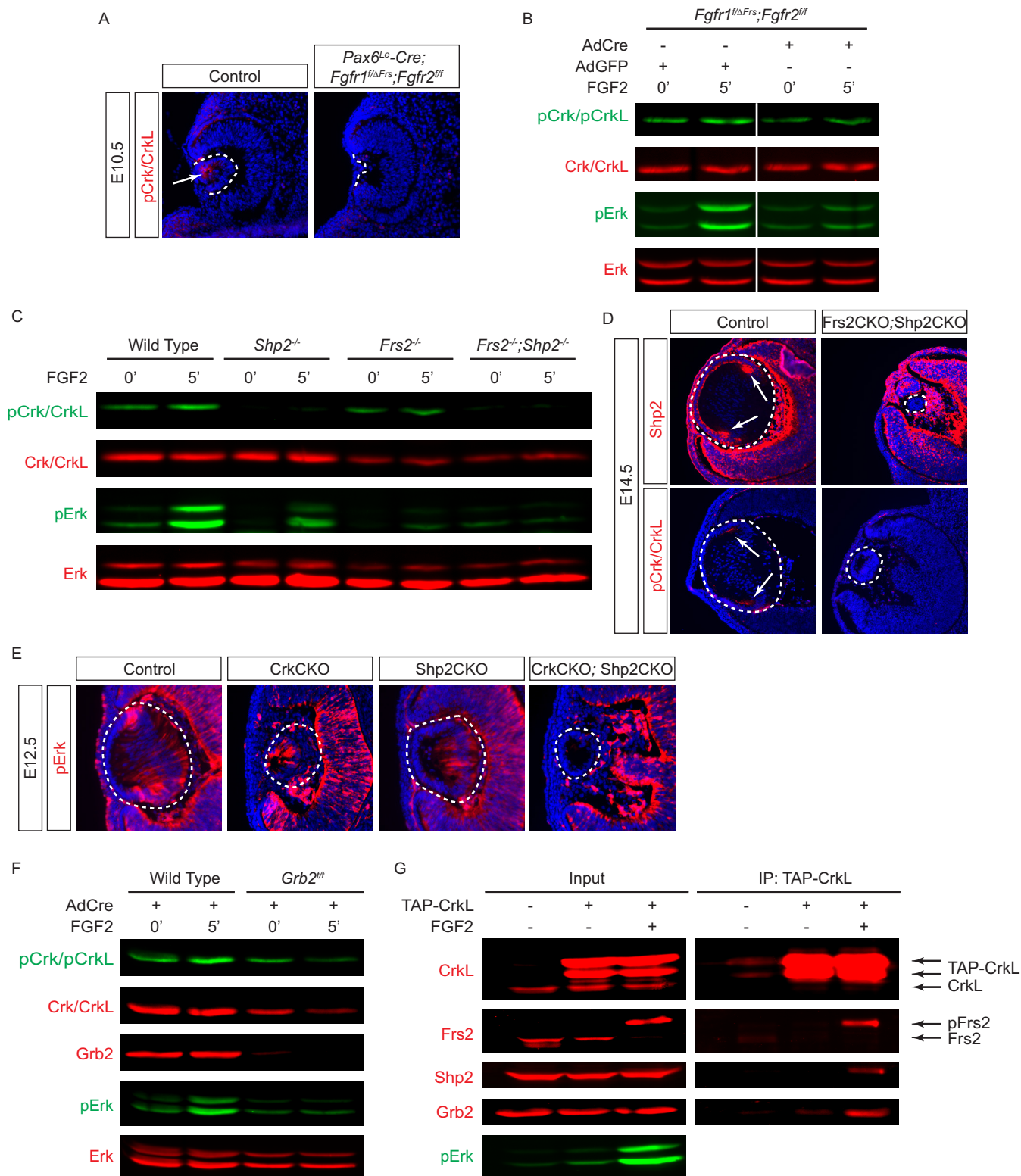
Figure 4 continued on next page

## Figure 4 continued

recombination in lens explant cultures as indicated by the *ROSA<sup>mTmG</sup>* reporter. In *Crk<sup>flox/flox</sup>;Crkl<sup>flox/flox</sup>* explants, the Cre-mediated deletion of Crk proteins prevented Fgf2 from inducing cell shape changes.

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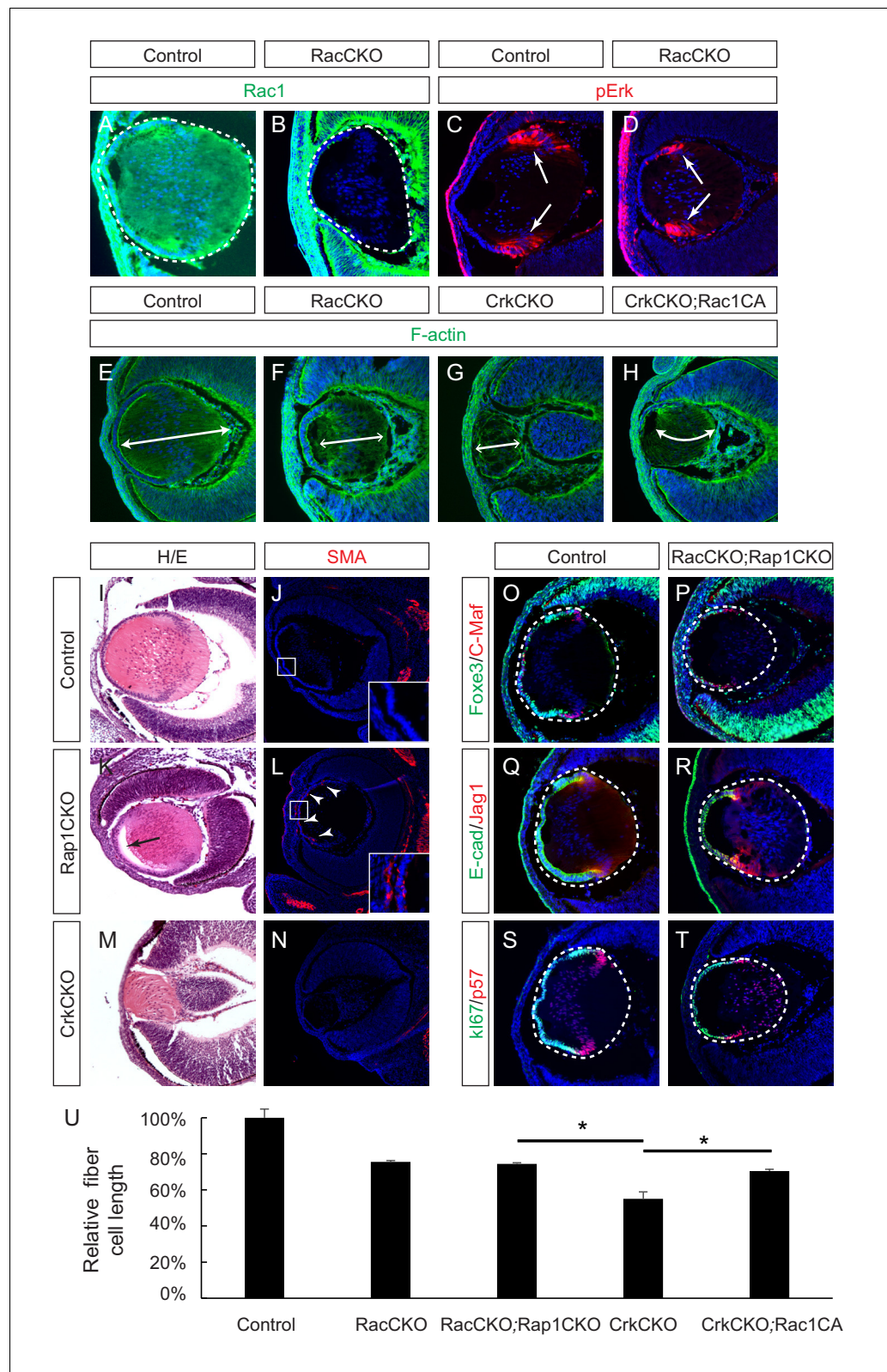


**Figure 5.** Crk proteins are recruited to the Frs2-Shp2-Grb2 complex in FGF signaling. (A–B) Mutating the Frs2-binding site in *Fgfr1<sup>ΔFrs</sup>* resulted in the loss of pCrk/CrkL in the *Pax6<sup>Lo</sup>-Cre;Fgfr1<sup>fl/ox/ΔFrs</sup>;Fgfr2<sup>fl/ox/fl</sup>* mutant lens. (B) FGF2 was unable to induce the phosphorylation of Crk proteins in *Fgfr1<sup>fl/ox/ΔFrs</sup>*. Figure 5 continued on next page

## Figure 5 continued

$\Delta^{Frs2};Fgfr2^{flox/flox}$  MEF cells after treatment with the Cre expressing adenovirus. (C) FGF2-induced pCrk/CrkI and pErk were significantly downregulated in both *Frs2* and *Shp2* null MEF cells. (D) *Shp2* was successfully depleted in the *Frs2*CKO;*Shp2*CKO lens, which resulted in the loss of pCrk/CrkI staining. (E) pERK was downregulated in both *Crk*CKO and *Shp2*CKO lenses and was further reduced in *Crk*CKO;*Shp2*CKO mutants. (F) FGF2-induced pCrk/CrkI and pErk were down regulated in *Grb2* deficient MEF cells. (G) TAP-tagged CrkI pulled down *Frs2*, *Shp2* and *Grb2* after FGF2 stimulation. Note that only the slower moving phosphorylated form of *Frs2* successfully interacted with CrkI.

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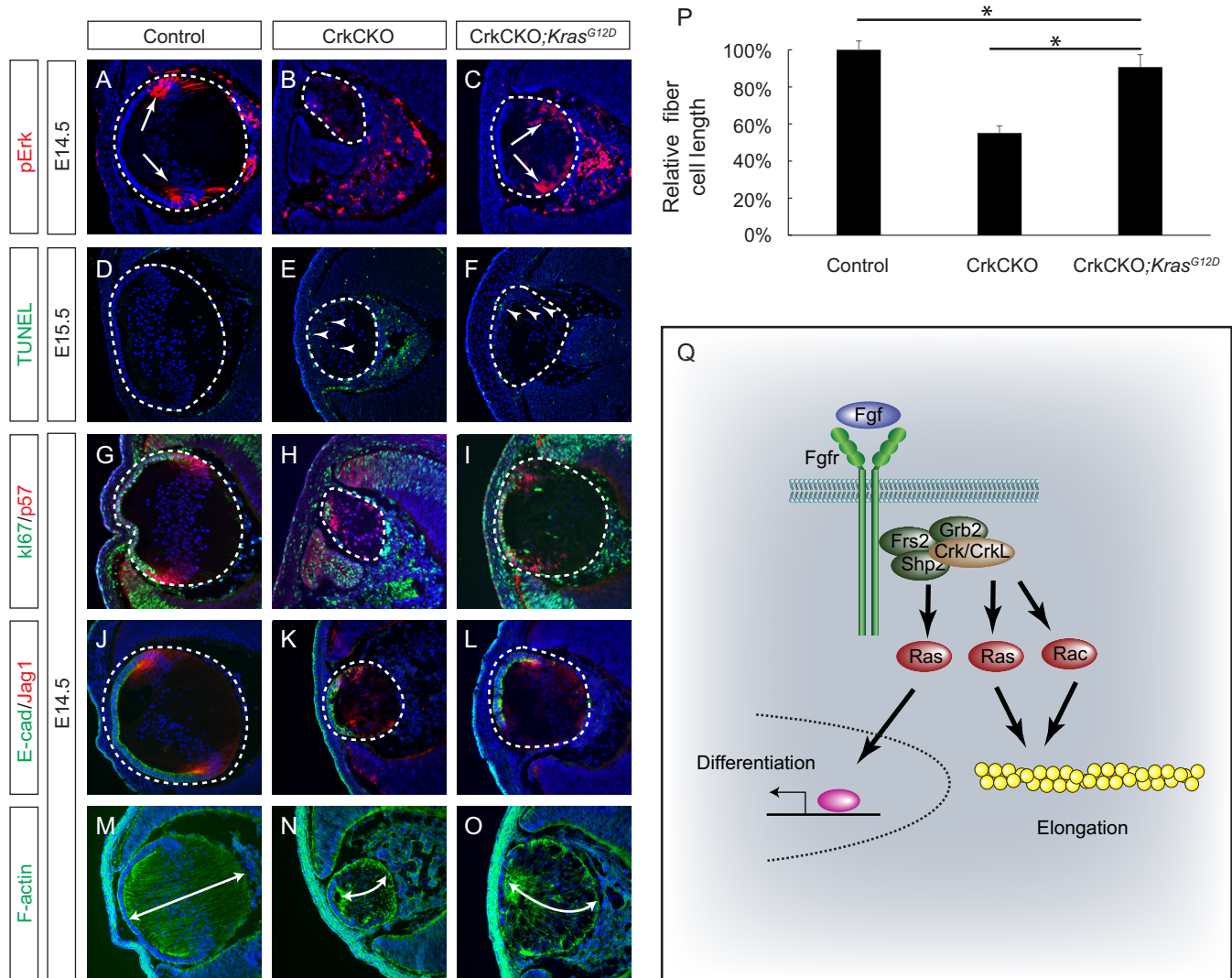
**Figure 6.** Rac proteins are downstream effectors of Crk signaling. (A–D) Staining of Rac-depleted lenses with a pErk antibody showed no significant difference in staining intensity. (E–H) Phalloidin (F-actin) staining revealed that the length of lens fiber cells (indicated by arrows) was significantly reduced in CrkCKO and RacCKO lenses, which was partially reversed after the activation of Rac signaling in CrkCKO;Rac1CA lenses. (I–N) The Rap1 depleted

Figure 6 continued on next page

*Figure 6 continued*

mutants displayed a detachment of the lens fiber cells from the anterior side of the lens epithelial cells (arrow) and Smooth Muscle Actin (SMA) staining within the lens epithelial layer itself (arrowheads). These phenotypes were absent both in the control and the Crk/Crkl depleted lenses. (O–T) Immunostaining of the Rac and Rap1 depleted lenses with progenitor (Foxe3, E-cad, Ki67) and differentiation (C-Maf, Jag1, p57) markers did not reveal any defects in differentiation or any further shortening of the fiber cells. (I) Quantification of fiber cell lengths. One-way ANOVA test followed by Tukey's multiple comparisons test,  $*p < 0.01$ ,  $n = 3$ .

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**Figure 7.** Constitutive Kras signaling can compensate for the loss of Crk and Crkl in lens development. (A–C) Despite the loss of Crk and Crkl, Erk phosphorylation was partially recovered in CrkCKO;*Kras*<sup>G12D</sup> lenses. (D–F) A significant amount of TUNEL positive cells remained in both CrkCKO and CrkCKO;*Kras*<sup>G12D</sup> lenses. (G–L) Cell proliferation indicated by Ki57 increased in the CrkCKO;*Kras*<sup>G12D</sup> lens as compared to the CrkCKO lens, but there was no significant difference in staining intensity for the differentiation markers E-cad and Jag1. (M–P) Lens fiber cell length increased significantly in CrkCKO;*Kras*<sup>G12D</sup> lenses as compared to CrkCKO ones. Fiber cell length was measured based on F-actin staining and statistical analysis was performed using the one-way ANOVA test followed by Tukey's multiple comparisons test (\**p* < 0.01, *n* = 3). (Q) Model of Crk function in FGF signaling. The binding of FGF to its receptor induces the assembly of the Frs2-Shp2-Grb2 complex, which subsequently activates Ras signaling to promote lens differentiation. When FGF signaling is further elevated at the transitional zone of the lens, Crk proteins were additionally recruited by the Frs2-Shp2-Grb2 complex to further promote Ras and Rac signaling, resulting in actin cytoskeletal rearrangement and cell shape changes.

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