Dlg-1 Interacts With and Regulates the Activities of Fibroblast Growth Factor Receptors and EphA2 in the Mouse Lens

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Purpose. We previously showed that *Discs large-1* (*Dlg-1*) regulates lens fiber cell structure and the fibroblast growth factor receptor (Fgfr) signaling pathway, a pathway required for fiber cell differentiation. Herein, we investigated the mechanism through which *Dlg-1* regulates Fgfr signaling.

Methods. Immunofluorescence was used to measure levels of Fgfr1, Fgfr2, and activated Fgfr signaling intermediates, pErk and pAkt, in control and *Dlg-1*–deficient lenses that were haplodeficient for *Fgfr1* or *Fgfr2*. Immunoblotting was used to measure levels of N-cadherin, EphA2, β-catenin, and tyrosine-phosphorylated EphA2, Fgfr1, Fgfr2, and Fgfr3 in cytoskeletal-associated and cytosolic fractions of control and *Dlg-1*–deficient lenses. Complex formation between Dlg-1, N-cadherin, β-catenin, Fgfr1, Fgfr2, Fgfr3, and EphA2 was assessed by coimmunoprecipitation.

Results. Lenses deficient for *Dlg-1* and haplodeficient for *Fgfr1* or *Fgfr2* showed increased levels of Fgfr2 or Fgfr1, respectively. Levels of pErk and pAkt correlated with the level of Fgfr2. N-cadherin was reduced in the cytoskeletal-associated fraction and increased in the cytosolic fraction of *Dlg-1*–deficient lenses. Dlg-1 complexed with β-catenin, EphA2, Fgfr1, Fgfr2, and Fgfr3. EphA2 complexed with N-cadherin, β-catenin, Fgfr1, Fgfr2, and Fgfr3. Levels of these interactions were altered in *Dlg-1*–deficient lenses. Loss of *Dlg-1* led to changes in Fgfr1, Fgfr2, Fgfr3, and EphA2 levels and to greater changes in the levels of their activation.

Conclusions. Dlg-1 complexes with and regulates the activities of EphA2, Fgfr1, Fgfr2, and Fgfr3. As EphA2 contains a Psd95/Dlg/ZO-1 (PDZ) binding motif, whereas Fgfrs do not, we propose that the PDZ protein, Dlg-1, modulates Fgfr signaling through regulation of EphA2.

Keywords: Dlg-1, fibroblast growth factor receptor signaling, Eph signaling, mouse, lens fiber cell differentiation

The formation and maintenance of the architecture of specialized organs are dependent on growth factor receptor signaling pathways that regulate cell proliferation, differentiation, cell–cell adhesion, cytoskeletal structure, apical–basal polarity, and planar cell polarity (PCP).[1–3] The ocular lens is an organ whose distinctive structure is known to be dependent on multiple growth factor receptor signaling pathways that regulate these diverse biologic processes. Identifying factors that coordinate these signaling pathways is crucial to our understanding of normal lens development and the lens pathology that forms when signaling pathways are disrupted. *Dlg-1* (*Discs large-1*), the mouse homolog of *Drosophila dlg*, is required for developmental processes in multiple organs[4–6] through its regulation of cell proliferation, cell–cell adhesion, cell shape, and apical–basal and PCP.[7–9] Previously, we showed that *Dlg-1* is required for lens fiber cell differentiation and maintenance of the architecture of the lens[8] and also is a modulator of the fibroblast growth factor receptor (Fgfr) signaling pathway in the mouse lens.[10] In this study, we address the possibility that *Dlg-1* is a factor that regulates the interaction of the ephrin/Eph[11–16] and Fgf/Fgfr[17–19] signaling pathways, two signaling pathway that are crucial for fiber cell structure and differentiation.

The lens is composed of a monolayer of epithelial cells that overlies a mass of derivative, differentiated, highly elongated cells, the fiber cells. After formation of the lens vesicle by day E10.5, cells in the anterior form the lens epithelial cells, whereas cells in the posterior undergo a terminal differentiation process to form the primary fiber cells. Subsequently, cells in the periphery of the epithelium undergo differentiation to form secondary fibers. Primary and secondary fiber cell differentiation is characterized by cell cycle withdrawal, cell elongation, and expression of differentiation-specific proteins. Notably, the secondary fibers take on a distinct hexagonal structure and are tightly packed into organized rows of cells that migrate along the capsule and epithelial cells until they meet their counterpart forming the lens sutures.[20,21]

Among the various growth factors that are expressed in the lens, Fgfs are the only known growth factors capable of inducing lens fiber cell differentiation.[2,22,23] In mice, simultaneous deletion of *Fgfrs* 1, 2, and 3 led to complete arrest of lens fiber differentiation, demonstrating that Fgfr signaling is required for lens development.[24] EphA2, a member of another family of receptor tyrosine kinases (RTKs), is expressed in the cortical lens fiber cells.[12–14] EphA2 and ephrin A-5, the ligand for EphA2 in the lens, have been shown to be required for the

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hexagonal structure of the lens fiber cells[11–16] and for linking the adherens junction protein, N-cadherin, to β-catenin, thereby promoting linkage to the cytoskeleton and cell–cell adhesion.[12] Interplay between the Eph/ephrin and Fgfr signaling pathways has been shown to regulate cell fate determination in *Ciona* embryos.[25] In mammalian cells, Fgfr and EphA4 have been shown to phosphorylate each other and costimulation of both receptors resulted in the potentiation of mitogen-activated protein kinase (MAPK) signaling.[26]

Recently, we discovered that *Dlg-1*, the mouse homolog of the *Drosophila* gene *discs-large* (*dlg),* is expressed in the lens and is required for cell adhesion, apical–basal polarity, and fiber cell structure and differentiation in the lens using lens-specific ablation of *Dlg-1*.[9,27] We also showed that loss of *Dlg-1* in the lens resulted in reduced levels of Fgfr2 and downstream signaling intermediates but increased levels of Fgfr1 and 3, indicating that *Dlg-1* is a modulator of the Fgfr signaling pathway.[10] Dlg-1 is a Psd95/Dlg/ZO-1 (PDZ) domain containing protein. These proteins are thought to act as scaffolding molecules that assemble large macromolecular complexes at the cell membrane.[28] PDZ proteins interact directly with proteins that contain a PDZ binding motif, usually positioned at the C terminus of the protein.[28] Because Fgfrs are not known to contain PDZ binding motifs, it is possible that the regulation of Fgfr signaling by *Dlg-1* is mediated by other proteins. Interestingly, the lenses of mice deficient for *Dlg-1,*[8] *Epha2,*[13,15,16] and *Efna5*[12] show similar disruption in the hexagonal architecture of the fiber cell and cell adhesion defects, suggesting that these proteins may mediate similar processes in the lens. EphA2 does contain a PDZ binding motif,[29] suggesting that Dlg-1 and EphA2 may interact to regulate fiber cell structure and cell–cell adhesion.

In this study, we first addressed the finding that Fgfr1 and Fgfr2 levels are oppositely affected in the *Dlg-1*–deficient state and the impact of this imbalance on Fgfr signaling. We found that levels of Fgfr1 negatively correlated with levels of Fgfr2 and that the levels of activated Fgfr signaling intermediates positively correlated with Fgfr2 levels. Second, we addressed the hypothesis that Dlg-1, EphA2, and Fgfrs interact, and Dlg-1 affects the activities of these RTKs. We found that ablation of *Dlg-1* led to reduced EphA2 levels and to disruption of the association of N-cadherin and β-catenin. We found that Dlg-1 complexes with EphA2, that EphA2 complexes with Fgfr1, Fgfr2, and Fgfr3 and that Dlg-1 complexes with Fgfr1, Fgfr2, and Fgfr3, albeit more weakly than does EphA2. Finally, we found that loss of *Dlg-1* led to reduced complexing of EphA2 with N-cadherin, β-catenin, and Fgfr2 but increased complexing of EphA2 with Fgfr1 and Fgfr3, and these changes correlated with the altered levels of activation of EphA2, Fgfr1, and Fgfr2. Taken together, these data suggest that Dlg-1 regulates EphA2 function and the interaction between Dlg-1 and EphA2 mediates, at least in part, the effect of *Dlg-1* on Fgfr signaling and adherens junction formation.

Materials and Methods

Animals and Tissue Preparation

The generation of mice carrying conditional alleles of *Dlg-1,*[8] *Fgfr1,*[30] and *Fgfr2*[31] and the *MLR10Cre*[32] mice have been described previously. *Dlg-1$^{f/f}$* mice were crossed to *MLR10Cre* mice to generate *Dlg-1$^{f/f}$;MLR10Cre* mice (referred to as *Dlg$^{f/f}$10Cre* mice). Mice carrying conditional null alleles of *Fgfr1* and *Fgfr2* (referred to as *Fgfr1$^{f/+}$* and *Fgfr2$^{f/+}$*) were obtained from Xin Sun (University of Wisconsin-Madison, Madison, WI, USA). *Fgfr1$^{f/+}$* and *Fgfr2$^{f/+}$* were crossed to *MLR10Cre* mice to generate *Fgfr1$^{f/+}$10Cre* and *Fgfr2$^{f/+}$10Cre* mice. To generate *Dlg$^{f/f}$;Fgfr1$^{f/+}$10Cre* and *Dlg$^{f/f}$;Fgfr2$^{f/+}$10Cre* mice, *Fgfr1$^{f/+}$* and *Fgfr2$^{f/+}$* mice were crossed to *Dlg-1$^{f/f}$;MLR10Cre* mice. Genotyping was carried out as described previously.[8,30–32] All procedures using mice conformed to the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health and the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Longitudinally oriented paraffin-embedded eye sections (5 μm) from postnatal day 2 (P2) mice of each control and mutant strain and transversely oriented cryogenic eye sections (10 μm) from P30 control mice were prepared as previously described.[10] Sections from the center of the lens were chosen for use in immunofluorescence experiments.

Immunofluorescence

Paraffin-embedded sections from P2 eyes were subjected to immunofluorescent staining for Fgfr2, pErk, pAkt, and Dlg-1 as previously described.[10] Immunofluorescent staining for Fgfr1 was carried out, as described previously for pAkt, using a rabbit anti-human Fgfr1 (Abcam, Cambridge, MA, USA, Cat# ab16046) at a 1:500 dilution. Fluorescence intensities were quantified by ImageJ (National Institutes of Health, Bethesda, MD, USA) as previously described using signal intensities in the corresponding retinae or cartilage of the snout (for pAkt) as an internal control.[10] At least three sections over two slides for at least three eyes were analyzed. The data reported are the mean ± SD across the samples for each.

Cryogenic sections from P30 control eyes were subjected to immunofluorescent staining with goat anti-mouse EphA2 (BD Biosciences, San Jose, CA, USA, Cat# AF639) and mouse anti-human N-cadherin (BD Biosciences, Cat# 610921) antibodies at a 1:500 dilution.

Western Blot

Lenses were dissected from P2 control and *Dlg$^{f/f}$10Cre* mice, and cytosolic- and cytoskeletal-associated protein lysates were prepared by Triton X-100 extraction, as previously described.[10] The lysates (50 μg each) were electrophoresed, the proteins were transferred to polyvinylidene difluoride (PVDF) membranes, and the membranes were blotted for with mouse anti–N-cadherin (BD Biosciences Cat# 610921), rabbit anti-human β-catenin (Sigma-Aldrich Corp., St. Louis, MO, USA, Cat# C2206), rabbit anti–human-active β-catenin (Millipore, Billerica, MA, USA, Cat# 05-665), or goat anti-mouse EphA2 (R&D Systems, Minneapolis, MN, USA, Cat# AF639) antibodies at a 1:100 dilution. The blots were reprobed with mouse anti-rabbit glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Millipore Cat# MAB374) as a loading control. Bands were visualized using the Enhanced Chemiluminescence Plus kit (ECL plus, ThermoScientific, Rockford, IL, USA), and protein levels were quantified by phosphorimager analysis on a Storm Scanner. At least three pools were generated, and each pool was analyzed in triplicate over one to three blots. Relative protein levels were calculated by setting the protein/Gapdh ratio for the controls at 1.0. The data reported are the mean ± SD across three to four pools.

PCR Array Profiling

Mouse Tyrosine Kinases RT^2 Profiler PCR Arrays (Qiagen, Germantown, MD, USA, Cast cat #PAMM-161Z) were used. RNA was prepared from 10 lenses from control and *Dlg10$^{f/f}$Cre* E17.5 embryos using a Qiagen RNeasy Mini Kit (Qiagen). RNA integrity and concentration was determined using a NanoDrop

spectrophotometer. RNA (500 ng) from each pool was used for each cDNA synthesis. Four control and four *Dlg$^{f/f}$10Cre* cDNA pools were synthesized using the RT^2 First Strand kit (Qiagen). The integrity of each cDNA pool was determined by real-time PCR with Gapdh and β-actin primers. The cDNA pools were amplified by real-time PCR using SYBR Green PCR master mix (Qiagen) as follows: (1) denaturation for 10 minutes at 95°C, (2) 40 cycles of 15-second denaturation at 95°C, and (3) annealing for 60 seconds at 60°C using a BioRad CFX real-time PCR machine. The ΔΔCT method (Qiagen) was used for averaging Ct values for each gene analysis. Relative gene expression values of control and *Dlg$^{f/f}$10Cre* samples were determined using web-based analysis software (available in the public domain at http://pcrdataanalysis.sabiosciences.com/pcr/arrayanalysis.php). The fold changes in gene expression were calculated as $2^{-\Delta\Delta CT}$.

Coimmunoprecipitation

Ten lenses from P10 control and *Dlg$^{f/f}$10Cre* mice were each pooled and extracted as described above in Triton-X 100 buffer. The Triton-X 100 insoluble fractions were resuspended in NP-40 buffer (1% NP-40, 150 mM NaCl, 2 mM EDTA, 50 mM Tris-HCl, pH 8.0). A total of 175 μg protein was precleared with Protein A Sepharose or Protein G Sepharose (Life Technologies, Carlsbad, CA, USA) for 1 hour at 4°C and then incubated with 10 μg rabbit anti-human β-catenin, mouse anti-rat SAP97 (Novus, Littleton, CO, USA, Cat# NBP1-48054), rabbit anti-human Fgfr1, 2, and 3 (Santa Cruz Biotechnology, Santa Cruz, CA, USA, Cat#s SC-7945, SC-122, and SC-123, respectively), and goat anti-mouse EphA2 antibodies or 10 μg corresponding IgGs (ThermoScientific) as controls, overnight at 4°C, followed by incubation with Protein A Sepharose or Protein G Sepharose beads for 2 hours at 4°C. Protein A Sepharose or Protein G Sepharose pellets were washed with NP-40 buffer and resuspended in urea buffer containing loading dye, and proteins were denatured at 95°C, fractionated on 7.5% SDS/PAGE, transferred to PVDF membranes, and immunoblotted for β-catenin, Dlg-1, Fgfr1, Fgfr2, Fgfr3, EphA2, and mouse anti-pTyr (Santa Cruz Biotechnology Cat# SC-508) antibody as described above. Levels of the immunoprecipitated protein were determined by reprobing the membrane with antibodies against those proteins. Three independent protein pools were prepared per mouse genotype. Three to four coimmunoprecipitation (co-IP) experiments were carried out. Quantification was carried out by phosphorimager analysis, as described above.

Statistical Analysis

The two-sided one-sample *t*-test was conducted using MSTAT software (available in the public domain at www.mcardle.wisc.edu/mstat). Because of the large number of experimental comparisons made in each figure,[33] we report the false discovery rate (FDR) rather than the unadjusted *P* values from the statistical tests. An FDR ≤ 0.05 was considered statistically significant.

Results

Cross-Regulation of Fgfrs in the Absence of Dlg-1

We previously showed that ablation of *Dlg-1* in the mouse lens led to decreased levels of Fgfr2; however, the levels of Fgfr1 were increased.[10] Therefore, we hypothesized that in the absence of *Dlg-1*, down-regulation of one Fgfr results in up-regulation of the other Fgfr. To test this hypothesis, we crossed *Dlg$^{f/f}$10Cre* mice to *Fgfr1$^{f/+}$* and *Fgf2$^{f/+}$* to generate *Fgfr1$^{f/+}$10Cre*, *Fgfr2$^{f/+}$10Cre, Dlg$^{f/f}$;Fgfr1$^{f/+}$10Cre,* and *Dlg$^{f/f}$;Fgfr2$^{f/+}$10Cre* mice. To verify that Dlg-1 was ablated, paraffin-embedded eye sections from P2 control, *Dlg$^{f/f}$10Cre*, *Dlg$^{f/f}$;Fgfr1$^{f/+}$10Cre,* and *Dlg$^{f/f}$;Fgfr2$^{f/+}$10Cre* mice were subjected to immunofluorescent staining for Dlg-1 using an anti–Dlg-1 antibody. Dlg-1 was not detected in the lenses of any mutant genotype; however, it was detected in other ocular tissues (Supplementary Fig. S1). Similarly, immunofluorescent staining for Fgfr1 and Fgfr2 using anti-Fgfr1 and anti-Fgfr2 antibodies was carried out to verify that their respective levels were reduced when one allele was mutated. Levels of Fgfr1 in the transition zone of *Fgfr1$^{f/+}$10Cre* lenses and levels of Fgfr2 in the transition zone of *Fgfr2$^{f/+}$10Cre* lenses were reduced approximately 50%, in keeping with the genetic disruption of one allele (Supplementary Fig. S2). Genetic disruption of one allele of *Fgfr1* in the *Dlg$^{f/f}$10Cre* lenses reduced the increase in Fgfr1 and genetic disruption of one allele of *Fgfr2* in *Dlg$^{f/f}$10Cre* lenses further reduced Fgfr2 levels (Supplementary Fig. S2). To determine the effect of genetic disruption of one allele of *Fgfr2* on Fgfr1 levels, eye sections from P2 control, *Fgfr2$^{f/+}$10Cre*, *Dlg$^{f/f}$10Cre*, and *Dlg$^{f/f}$;Fgfr2$^{f/+}$10Cre* mice were immunostained with anti-Fgfr1 antibodies, and the staining intensities were quantified. Interestingly, Fgfr1 levels were increased by 45% in *Dlg$^{f/f}$;Fgfr2$^{f/+}$10Cre* lenses compared with 25% in *Dlg$^{f/f}$10Cre* lenses. To determine the effect of genetic disruption of one allele of *Fgfr1* on Fgfr2 levels, eye sections from P2 control, *Fgfr1$^{f/+}$10Cre*, *Dlg$^{f/f}$10Cre*, and *Dlg$^{f/f}$;Fgfr1$^{f/+}$10Cre* mice were immunostained with anti-Fgfr2 antibodies, and the staining intensities were quantified. Fgfr2 levels were decreased by 19% in *Dlg$^{f/f}$;Fgfr1$^{f/+}$10Cre* compared with 53% in *Dlg$^{f/f}$10Cre* lenses (Fig. 1). Thus, deficiency of Fgfr1 in the absence of Dlg-1 reverses the effect on Fgfr2 levels. These results show that *Dlg-1* is required to maintain the normal balance between levels of Fgfr1 and Fgfr2 in the lens. Furthermore, unlike in the *Dlg-1*–sufficient state, in the absence of *Dlg-1*, down-regulation of one Fgfr leads to up-regulation of another Fgfr.

Effect of Fgfr1 and Fgfr2 on Activation of Downstream Signaling Intermediates

We previously showed that ablation of *Dlg-1* in the lens resulted in decreased levels of activated signaling intermediates of the Fgfr pathway, pFrs2α, pErk, and pAkt, and the Fgfr target, Erm.[10] From prior studies, it has been suggested that Fgfr2 is the primary Fgf receptor driving this pathway in the lens, at least during embryonic development.[34] If Fgfr2 is a driver of this pathway postnatally, reducing the level of Fgfr1 in *Dlg-1*–null lenses should result in increased levels of activated Fgfr signaling intermediates because Fgfr2 levels increase. On the other hand, reducing further the level of Fgfr2 should result in further decreased levels of these activated intermediates. To address these predictions, eye sections from control, *Fgfr1$^{f/+}$10Cre*, *Fgfr2$^{f/+}$10Cre*, *Dlg$^{f/f}$10Cre*, *Dlg$^{f/f}$;Fgfr1$^{f/+}$10Cre,* and *Dlg$^{f/f}$Fgfr2$^{f/+}$10Cre* mice were subjected to immunoflourescent staining using an anti-pErk and anti-pAkt antibodies and the staining intensities in the transition zone quantified (Materials and Methods). Levels of pErk in the transition zone of *Fgfr1$^{f/+}$10Cre* lenses were the same as in control lenses, whereas levels in *Fgfr2$^{f/+}$10Cre* lenses were marginally reduced (see regions in white dashed lines; Fig. 2). The intensity of pErk staining in the transition zones was decreased by 33% in *Dlg$^{f/f}$10Cre* lenses and by 58% in *Dlg$^{f/f}$;Fgfr2$^{f/+}$10Cre* lenses. In contrast, the intensity of pErk staining in *Dlg$^{f/f}$;Fgfr1$^{f/+}$10Cre* lenses was reduced by 22% compared with 33% in *Dlg$^{f/f}$10Cre* lenses (Fig. 2). Similarly, the intensity of pAkt staining was decreased by 39% in *Dlg$^{f/f}$10Cre* lenses and by 64% in *Dlg$^{f/f}$;Fgfr2$^{f/+}$10Cre* lenses, whereas the

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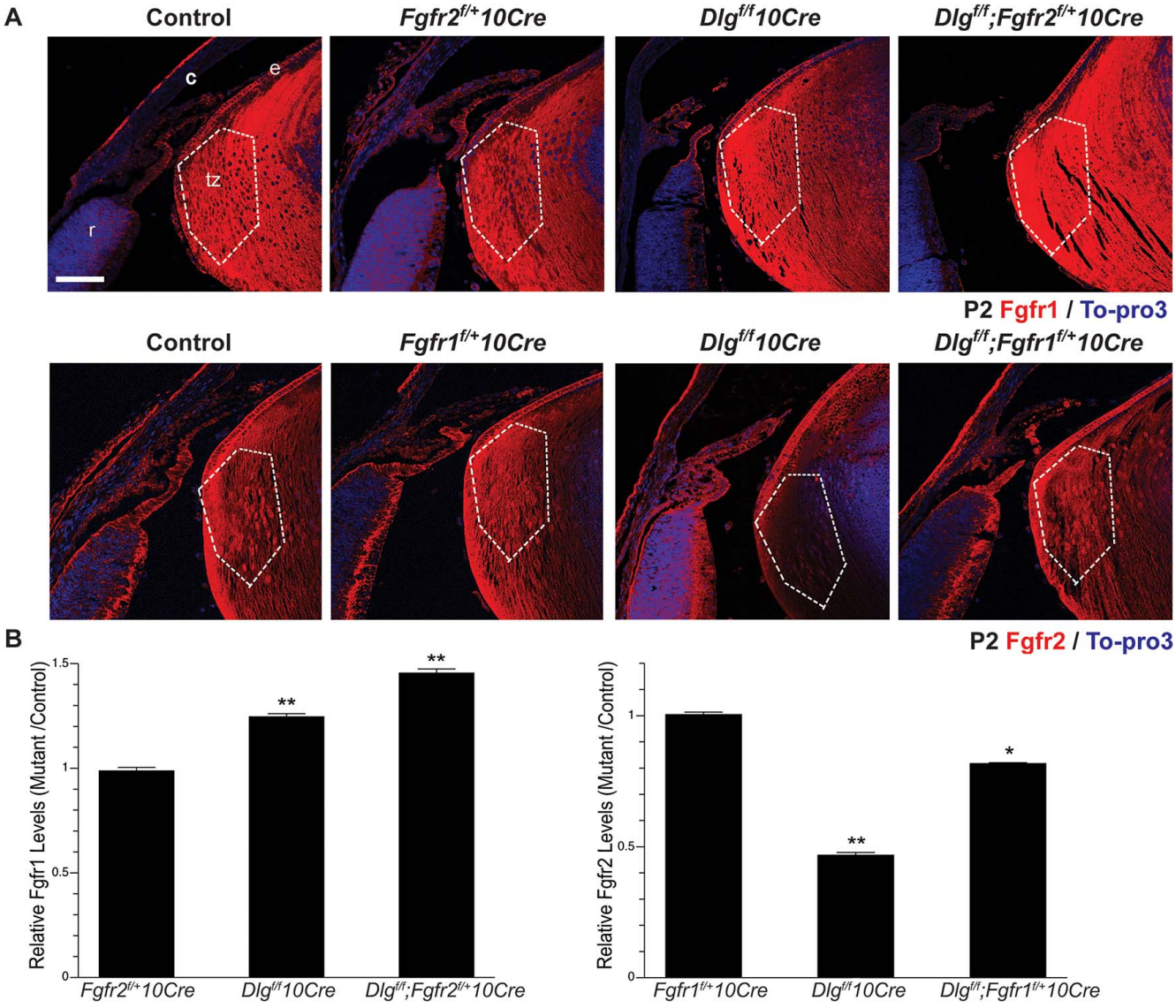

Figure 1. Relative Fgfr levels in *Dlg-1*, *Fgfr* compound mutant lenses. (**A**) Paraffin sections of eyes from P2 control, *Fgfr2*$^{f/+}$*1Cre*, *Dlg*$^{f/f}$*10Cre*, and *Dlg*$^{f/f}$;*Fgfr2*$^{f/+}$*10Cre* were subjected to immunofluorescence analysis using an anti-Fgfr1 antibody (*red*), and eye sections from P2 control, *Fgfr1*$^{f/+}$*10Cre*, *Dlg*$^{f/f}$*10Cre*, and *Dlg*$^{f/f}$;*Fgfr1*$^{f/+}$*10Cre* were subjected to immunofluorescence analysis using an anti-Fgfr2 antibody (*red*). The nuclei counterstained with To-Pro3 (*blue*). Representative images of the transition zone are shown for each genotype. c, cornea; e, lens epithelium; r, retina; tz, transition zone. *Scale bar*: 50 μm. (**B**) Quantification of Fgfr1 and Fgfr2 levels. Shown are the relative levels of Fgfr1 and Fgfr2 in the region within the *white dashed line* of the mutant lenses compared with levels in the corresponding regions of the control lenses (control levels set at 1.0). Quantification of signal intensities was carried out using ImageJ, and the data were subjected to statistical analysis as described in Materials and Methods. At least three different sections from at least three different lenses were evaluated. The Fgfr1 levels in *Fgfr2*$^{f/+}$*10Cre* lenses were the same as in controls. The Fgfr1 levels in *Dlg*$^{f/f}$*10Cre* lenses were increased compared with controls, and the Fgfr1 levels in *Dlg*$^{f/f}$;*Fgfr2*$^{f/+}$*10Cre* lenses were higher than in *Dlg*$^{f/f}$*10Cre* lenses. The Fgfr2 levels in *Fgfr1*$^{f/+}$*10Cre* lenses were the same as the control. The Fgfr2 levels were reduced in *Dlg*$^{f/f}$*10Cre* lenses compared with controls, and the Fgfr2 levels were higher in *Dlg*$^{f/f}$;*Fgfr1*$^{f/+}$*10Cre* lenses than in the *Dlg*$^{f/f}$*10Cre* lenses. *Error bars* denote SD. *FDR < 0.05; **FDR < 0.01.

intensity of pAkt staining in *Dlg*$^{f/f}$;*Fgfr1*$^{f/+}$*10Cre* was reduced by 22% compared with 39% in *Dlg*$^{f/f}$*10Cre* lenses (Fig. 3). Levels of pAkt in *Fgfr1*$^{f/+}$*10Cre* and *Fgfr2*$^{f/+}$*10Cre* lenses were the same as in control lenses. Thus, levels of activated Fgfr signaling intermediates correlate positively with levels of Fgfr2.

Effect of Loss of *Dlg-1* on Fgfr RNA Levels

Our results showed that loss of *Dlg-1* affects the protein levels of Fgf receptors. To determine whether RNA levels of Fgf receptors were altered in *Dlg*$^{f/f}$*10Cre* lenses, we measured RNA levels of Fgfrs in E17.5 control and *Dlg*$^{f/f}$*10Cre* lenses using commercial receptor tyrosine kinase PCR arrays. No significant differences in the RNA levels of Fgfrs between control and *Dlg*$^{f/f}$*10Cre* lenses were observed (Table). Thus, the effect of *Dlg-1* deficiency on Fgf receptor levels is not at RNA level, but rather is at the protein level.

Effect of Loss of *Dlg-1* on N-Cadherin and EphA2

Previous studies in our laboratory and others have shown that N-cadherin is predominantly localized to the short sides of the hexagonally shaped fiber cells.[10] In the absence of *Dlg-1*, the normal shape of the cells is disrupted, and N-cadherin

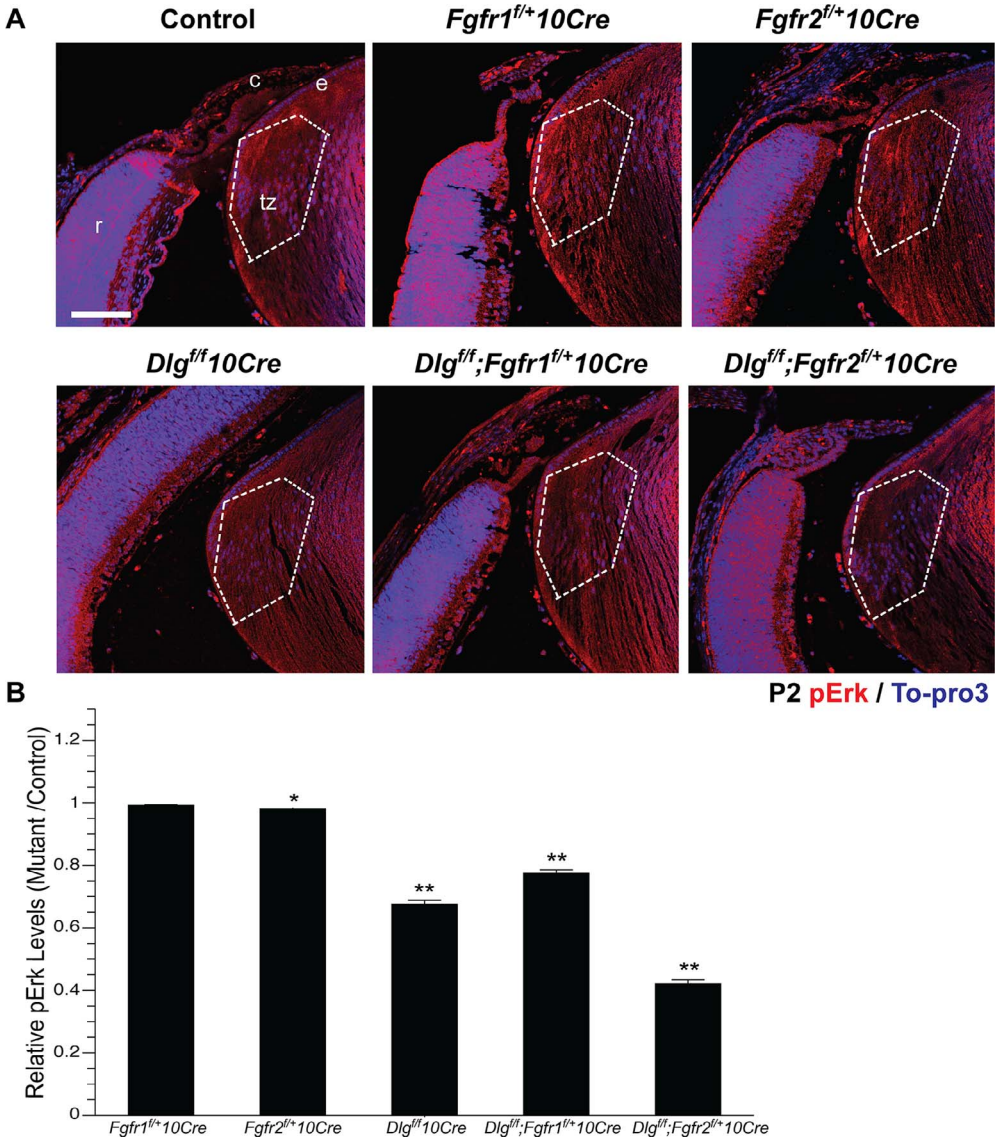

Figure 2. Levels of pErk are altered with the loss of *Dlg-1* and one allele of an *Fgfr*. (**A**) Paraffin-embedded sections of eyes from P2 control, *Fgfr1*$^{f/+}$*10Cre*, *Fgfr2*$^{f/+}$*10Cre*, *Dlg*$^{f/f}$*10Cre*, *Dlg*$^{f/f}$;*Fgfr1*$^{f/+}$*10Cre*, and *Dlg*$^{f/f}$;*Fgfr2*$^{f/+}$*10Cre* mice were subjected to immunofluorescence analysis using an anti-pErk antibody (*red*) and the nuclei counterstained with To-Pro3 (*blue*). Representative images of the transition zones of lenses from mice of the indicated genotype are shown. c, cornea; e, lens epithelium; r, retina; tz, transition zone. *Scale bar*: 50 μm. (**B**) Quantification of pErk levels. Shown are the relative levels of pErk in the region within the *white dashed line* of lenses (control levels set at 1.0). Quantification of signal intensities was carried out using ImageJ, and the data were subjected to statistical analysis as described in Materials and Methods. The relative level of pErk in the transition zone of the *Fgfr1*$^{f/+}$*10Cre* lenses was no different from the controls (0.994 ± 0.004, FDR > 0.05), whereas the relative level of pErk in the transition zone of the *Fgfr2*$^{f/+}$*10Cre* lenses differed slightly from controls (0.987 ± 0.004, FDR = 0.03). The relative pErk levels in *Dlg*$^{f/f}$*10Cre* lenses were reduced compared with levels in the control lenses. The relative pErk levels in the *Dlg*$^{f/f}$;*Fgfr1*$^{f/+}$*1Cre* lenses were higher than in the *Dlg*$^{f/f}$*10Cre* lenses, whereas the pErk levels in the *Dlg*$^{f/f}$;*Fgfr2*$^{f/+}$*10Cre* lenses were lower than in the *Dlg*$^{f/f}$*10Cre* lenses. *Error bars* denote SD. *FDR < 0.05; **FDR < 0.01.

appeared to be less tightly associated with the membrane.[10] In the absence of ephrin-A5, the ligand for EphA2 in the lens, N-cadherin was found redistributed to the cytoplasm, and the ephrin-A5-EphA2 interaction was shown to recruit N-cadherin to β-catenin.[12] As EphA2 has a PDZ binding motif at its C terminus, we hypothesized Dlg-1 might interact with EphA2 and that loss of *Dlg-1* might affect N-cadherin distribution in a manner similar to that of loss of *Efna5*. To begin to address this possibility, lenses from P2 control and *Dlg*$^{f/f}$*10Cre* mice were extracted with Triton-X 100, and the triton-soluble and triton-insoluble fractions were subjected to Western blot analysis with anti–N-cadherin antibodies. The levels of N-cadherin were increased more than 2-fold in the cytosolic fraction from the *Dlg*$^{f/f}$*10Cre* lenses relative to control lenses, whereas the levels of N-cadherin in the cytoskeletal-associated fractions of *Dlg*$^{f/f}$*10Cre* were decreased by 30% compared with controls (Fig. 4). As membrane-associated β-catenin normally is linked to N-cadherin, it was possible that levels and/or distribution of β-catenin may also be altered. Therefore, extracts were also blotted with anti-active (membrane associated) and anti-total β-catenin antibodies. Both active β-catenin and total β-catenin in the cytoskeletal fractions were reduced by 30% in the cytoskeletal-associated fraction of *Dlg*$^{f/f}$*10Cre* lenses, whereas total β-catenin in the soluble fraction was similar between extracts from control and *Dlg*$^{f/f}$*10Cre* lenses. Thus, the distribution of N-cadherin

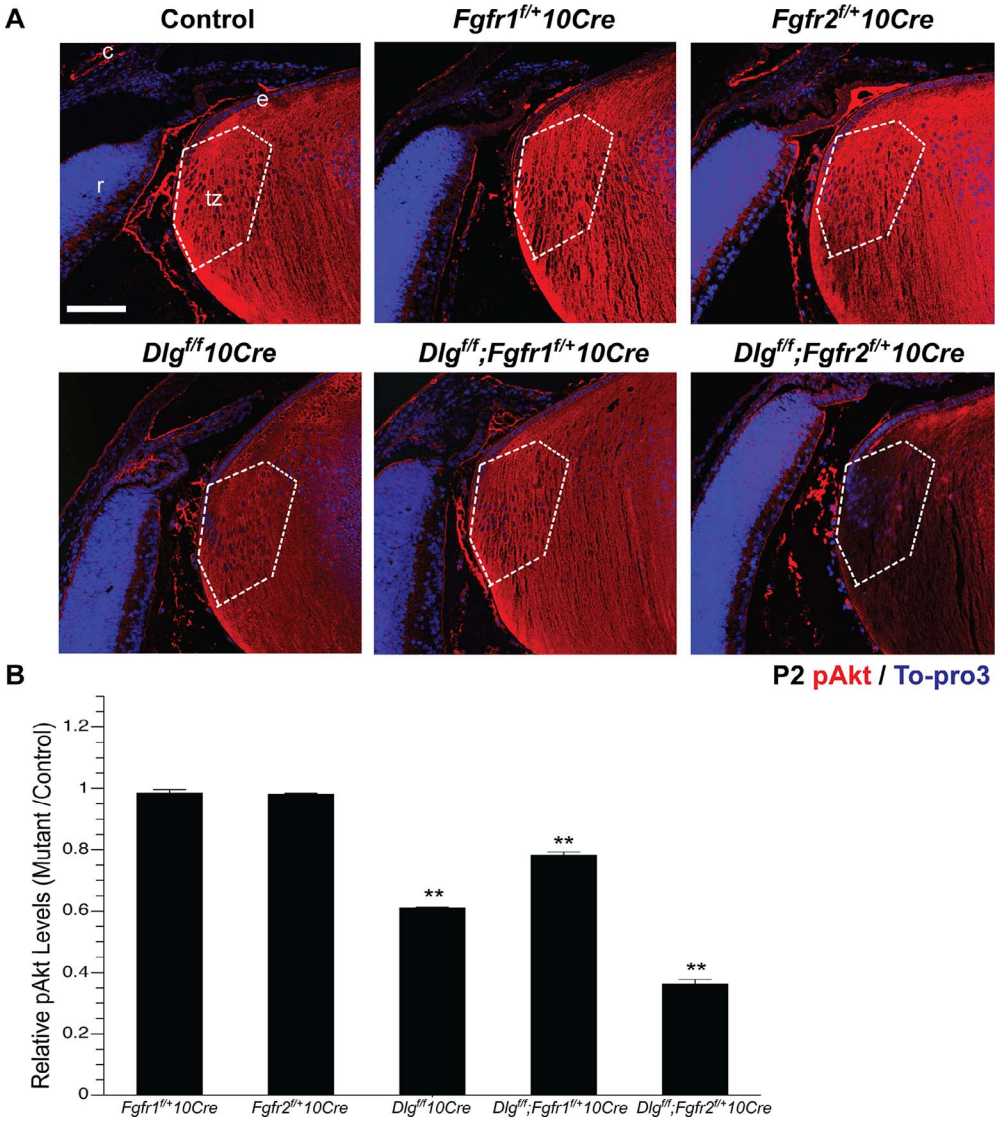

Figure 3. Levels of pAkt are altered with the loss of *Dlg-1* and one allele of an *Fgfr*. (**A**) Paraffin-embedded sections of eyes from P2 control, *Fgfr1*$^{f/+}$*10Cre*, *Fgfr2*$^{f/+}$*10Cre*, *Dlg*$^{f/f}$*10Cre*, *Dlg*$^{f/f}$;*Fgfr1*$^{f/+}$*10cre*, and *Dlg*$^{f/f}$;*Fgfr2*$^{f/+}$*10Cre* mice were subjected to immunofluorescence analysis using an anti-pAkt antibody (*red*) and the nuclei counterstained with To-Pro3 (*blue*). Representative images of the transition zones of lenses from mice of the indicated genotype are shown. c, cornea; e, lens epithelium; r, retina; tz, transition zone. *Scale bar*: 50 μm. (**B**) Quantification of pAkt levels. Shown are the relative levels of pAkt in the region within the *white dashed line* of lenses (control levels set at 1.0). Quantification of signal intensities was carried out using ImageJ, and the data were subjected to statistical analysis as described in Materials and Methods. The relative levels of pAkt in the transition zone of the *Fgfr1*$^{f/+}$*10Cre* and *Fgfr2*$^{f/+}$*10Cre* lens were not different than the controls (0.985 ± 0.012 and 0.982 ± 0.008, respectively; FDR > 0.05 for each). The relative pAkt levels in *Dlg*$^{f/f}$*10Cre* lenses were reduced compared with the levels in the control lenses. The relative pAkt levels in the *Dlg*$^{f/f}$;*Fgfr1*$^{f/+}$*1Cre* lenses were higher than in the *Dlg*$^{f/f}$*10Cre* lenses, whereas the pAkt levels in the *Dlg*$^{f/f}$;*Fgfr2*$^{f/+}$*10Cre* lenses were lower than in the *Dlg*$^{f/f}$*10Cre* lenses. *Error bars* denote SD. *FDR < 0.05; **FDR < 0.01.

Table. QPCR RTK Array on E17.5 mRNA From Control and *Dlg*$^{f/f}$*10Cre* Lenses

	$2^{\Delta C_T}$		Ratio	Fold Change	*t*-Test
Symbol	*Dlg*$^{f/f}$*10Cre*	Con	*Dlg*$^{f/f}$*10Cre*/Con	*Dlg*$^{f/f}$*10Cre*/Con	*P* Value
Fgfr1	0.02819	0.02815	1.00135	1.0013	0.97096
Fgfr2	0.01782	0.01849	0.96371	−1.0376	0.55996
Fgfr3	0.08635	0.08741	0.9878	−1.0123	0.72363
Fgfr4	8.2E-05	8.5E-05	0.96471	−1.0388	0.69293
EphA2	0.01935	0.01738	1.11324	1.1133	0.008601

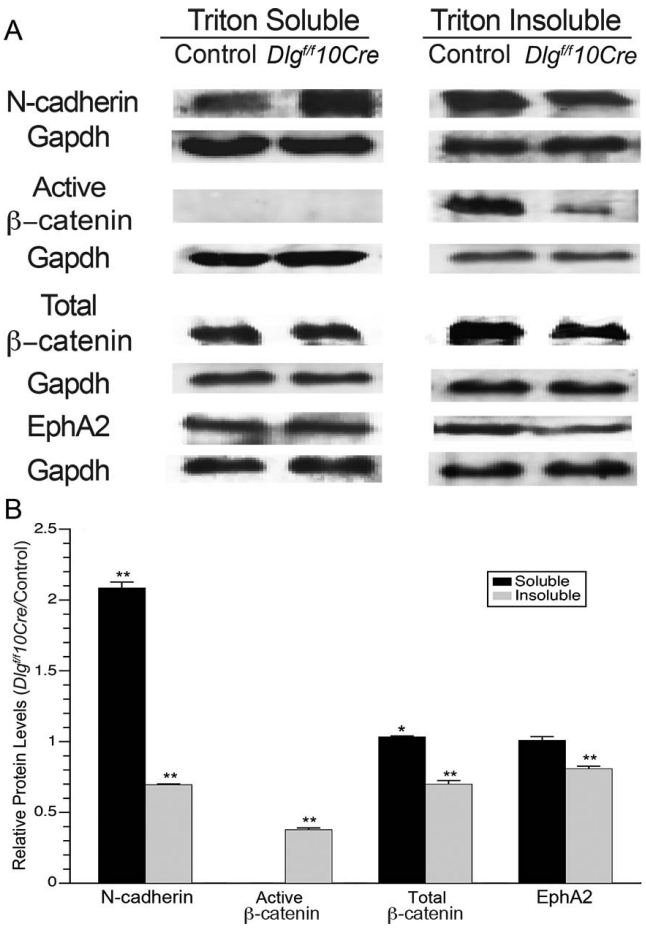

Figure 4. Levels of N-cadherin, β-catenin, and EphA2 are altered of *Dlg$^{f/f}$10Cre* lenses. (**A**) Cytosolic (Triton soluble) and cytoskeletal-associated (Triton X-100 insoluble) extracts from P2 control and *Dlg$^{f/f}$10Cre* lenses were subjected to Western blot analysis for the indicated proteins, and the blots were reprobed for Gapdh as a loading control. Representative blots are shown. (**B**) Quantification of protein levels. Shown are the levels of the indicated proteins in extracts from *Dlg$^{f/f}$10Cre* lenses relative to levels in the controls (control levels set a 1.0). Signal intensities were quantified by phosphorimager analysis, and the data were subjected to statistical analysis as described in Materials and Methods. At least three protein pools were blotted in triplicate over one to three blots. The relative levels of N-cadherin in the cytosolic fraction from *Dlg$^{f/f}$10Cre* lenses were increased compared with controls, whereas the levels of cytoskeletal associated N-cadherin were reduced. The levels of active β-catenin, total β-catenin, and EphA2 in the cytoskeletal associated fraction from *Dlg$^{f/f}$10Cre* lenses were reduced compared with controls. *Error bars* denote SD. *FDR < 0.05; **FDR < 0.01.

was altered in the absence of *Dlg-1*. Although the levels of membrane-associated β-catenin were altered, the distribution of active β-catenin was not.

The similarity of the effect of loss of *Dlg-1* on N-cadherin and β-catenin distribution to that of loss of *Efna5* raises the possibility that EphA2 is negatively affected by the loss of *Dlg-1*. Therefore, extracts from control and *Dlg$^{f/f}$10Cre* lenses were subjected to Western blot analysis with anti-EphA2 antibodies. Although EphA2 levels in the cytosolic fraction from *Dlg$^{f/f}$10*Cre lenses were not different from that of controls, levels in the cytoskeletal-associated fraction were decreased by 20% in *Dlg$^{f/f}$10Cre* lenses (Fig. 4). The effect of Dlg-1 on EphA2 protein levels was not due to an effect on RNA levels, as PCR array analysis showed that EphA2 RNA levels were, if anything, slightly increased (Table). Thus, loss of *Dlg-1* correlates with reduced levels of cytoskeletal-associated EphA2 and N-cadherin.

It is known that N-cadherin and EphA2 colocalize on the short sides of the fiber cells.[12] As the subcellular distribution of N-cadherin was altered in *Dlg$^{f/f}$10Cre* lenses, we asked if colocalization of N-cadherin and EphA2 localization was lost in the *Dlg$^{f/f}$10Cre* lenses. Double immunofluorescent staining was carried out on transversely oriented cryosections from the transition zone region of P30 control and *Dlg$^{f/f}$10Cre* lenses using anti–N-cadherin and anti-EphA2 antibodies. As shown previously,[12] immunostaining for EphA2 and N-cadherin was predominantly colocalized to the short sides of the fiber cells in the outer cortex of control lenses (Fig. 5). In *Dlg$^{f/f}$10Cre* lenses, the intensity of EphA2 and N-cadherin staining was reduced as was their colocalization (Fig. 5). Collectively, these data demonstrate that *Dlg-1* is required to maintain the normal levels and distribution of β-catenin, N-cadherin, and EphA2.

Effect of Loss of *Dlg-1* on Protein–Protein Interactions

Dlg-1 belongs to the PDZ domain-containing family of proteins, which are known to act as scaffolds to assemble large macromolecular complexes at the membrane. Interestingly, both β-catenin and EphA2 contain PDZ binding motifs at their C termini, suggesting that these three proteins may complex with each other. Fgfrs, on the other hand, have no known PDZ binding motif. However, a direct interaction between another Eph receptor, EphA4, and Fgfrs1, 2, and 3 has been documented.[26,35] Therefore, it is possible that Fgfrs could also be part of a complex with Dlg-1 through interaction with EphA2, as EphA4 is not known to be expressed in the lens,[12] and this could be the mechanism through which Dlg-1 status affects Fgfr signaling. Therefore, we hypothesized that Dlg-1, EphA2, and Fgfrs would be in a complex in the lens.

To test this hypothesis, coimmunoprecipitation experiments were carried out to determine which of these proteins complexed with each other in the control lenses and if these interactions were disrupted in the *Dlg-1*–deficient lenses. Lenses from P10 control and *Dlg$^{f/f}$10Cre* mice were extracted with Triton X-100 buffer, and then the cytoskeletal-associated fraction was resuspended in NP-40 buffer. Extracts were subjected to immunoprecipitation with antibodies against β-catenin or Dlg-1. The β-catenin immunoprecipitates were then Western blotted with antibodies against N-cadherin or EphA2, whereas the Dlg-1 immunoprecipitates were blotted with antibodies against β-catenin or EphA2 (Fig. 6). As expected, in control extracts, N-cadherin and EphA2 coimmunoprecipitated with β-catenin, and N-cadherin coimmunoprecipitated with EphA2. The levels of N-cadherin and EphA2 coimmunoprecipitating with β-catenin were reduced in extracts from *Dlg$^{f/f}$10Cre* lenses as were the levels of N-cadherin coimmunoprecipitating with EphA2. Dlg-1 coimmunoprecipitated with β-catenin and EphA2 in control extracts but not from *Dlg$^{f/f}$10Cre* extracts. Next, we asked whether EphA2 and Dlg-1 coimmunoprecipitate with N-cadherin, Fgfr1, Fgfr2, and/or Fgfr3. Extracts from control and *Dlg$^{f/f}$10Cre* lenses were subjected to immunoprecipitation with antibodies against EphA2, and the immunoprecipitates were blotted with antibodies for N-cadherin, Fgfr1, Fgfr2, or Fgfr3. Extracts were also subjected to immunoprecipitation with anti–Dlg-1 antibody, and the immunoprecipitates were blotted with antibodies for Fgfr1, Fgfr2, or Fgfr3. In control extracts, all three Fgfrs immunoprecipitated with EphA2. The levels of N-cadherin coimmunoprecipitating with EphA2 were reduced in extracts from *Dlg$^{f/f}$10Cre* lenses compared with control lenses. Interestingly, in extracts from *Dlg$^{f/f}$10Cre* lenses, the levels of Fgfr2 coimmunoprecipitating with EphA2 were

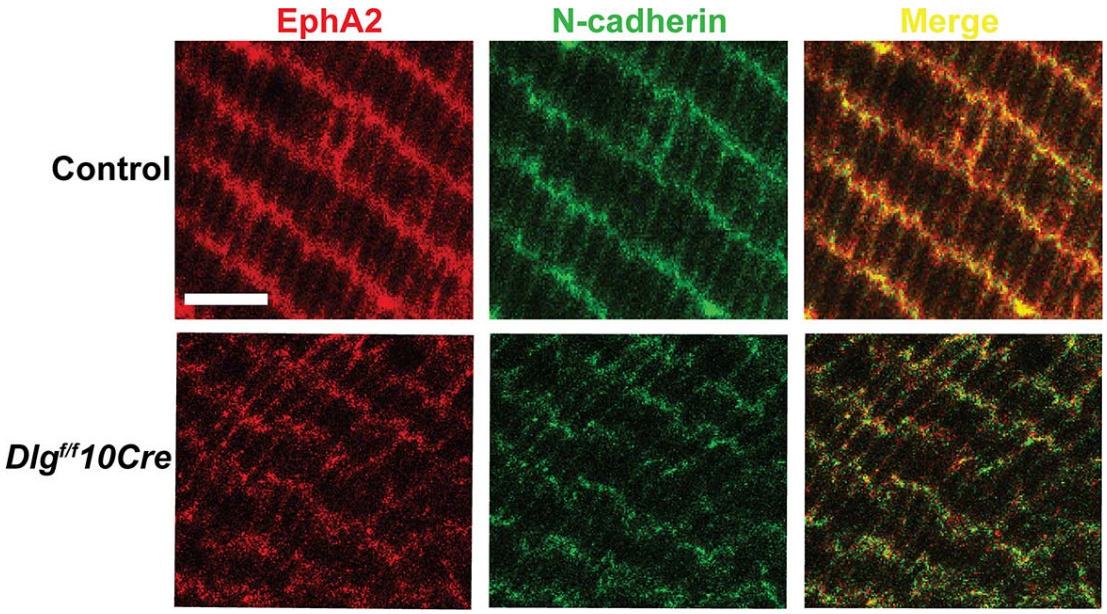

Figure 5. EphA2 and N-cadherin colocalization is disrupted in *Dlg$^{f/f}$10Cre* lenses. Cryogenic sections from control P30 lenses were subjected to immunofluorescent staining using anti-EphA2 anti–N-cadherin antibodies. Staining for EphA2 (*red*) was predominantly localized on the short sides of the fiber cells as was staining for N-cadherin (*green*) in control lenses. Overlap in staining (*yellow*) was observed. In *Dlg$^{f/f}$10Cre* lenses, the normal hexagonal shape of the fiber cells was disrupted. Staining for N-cadherin and EphA2 was diffuse, and colocalization was reduced. *Scale bar*: 50 μm.

reduced, whereas the levels of Fgfr1 and Fgfr3 coimmunoprecipitating with EphA2 were increased. In control extracts, all three Fgfrs also coimmunoprecipitated with Dlg-1. However, the amount of Fgfrs 1 and 2 coimmunoprecipitating with Dlg-1 was clearly less than the amount that coimmunoprecipitated with EphA2, suggesting that the interaction between EphA2 and the Fgfrs may be more direct than the interaction between Dlg-1 and the Fgfs. Together, these data demonstrate that Dlg-1 complexes with a network of proteins involved both in cell adhesion and cytoskeletal organization. In addition, Dlg-1 also complexes with proteins in signaling pathways that regulate fiber cell differentiation. Furthermore, in the absence of *Dlg-1*, the association between N-cadherin, β-catenin, EphA2, and the Fgfrs is altered, indicating that Dlg-1 is required for the proper levels of interactions between these proteins.

Effect of Loss of *Dlg-1* on Activation of Fgfrs and EphA2

Based on the finding that Dlg-1 complexes with Fgfrs 1, 2, and 3 and EphA2 and that *Dlg-1* status affects not only the relative levels of these proteins but also the levels of interaction, it is possible that activation of these receptors also is modulated by *Dlg-1*. The level of tyrosine phosphorylation of the Fgfrs and EphA2 is a measure of their levels of activation. To determine if *Dlg-1* modulates the activation of Fgfrs and EphA2, the cytoskeletal-associated fractions of lenses from P10 control and *Dlg$^{f/f}$10Cre* mice were subjected to immunoprecipitation with antibodies against Fgfr1, Fgfr2, Fgfr3, and EphA2, and the immunoprecipitates were subjected to Western blotting using an anti-phosphotyrosine antibody (Fig. 7). In extracts from control lenses, all three receptors were tyrosine phosphorylated. In extracts from *Dlg$^{f/f}$10Cre* lenses, p-Tyr levels were 40% higher for Fgfr1 and 32% higher for Fgfr3 compared with the levels in control extracts. The level of tyrosine phosphorylation of Fgfr2 was reduced by 89%, and the level of tyrosine-phosphorylated EphA2 was reduced by 45%. Thus, in the absence of Dlg-1, receptor activation levels were altered, which is consistent with the reduced levels of activated Fgfr signaling intermediates (Figs. 2, 3). These results demonstrate that *Dlg-1* is required not only for maintaining normal levels of Fgfr1, Fgfr2, Fgfr3, and EphA2, but also for maintaining proper levels of activation of these receptors.

Discussion

The interplay between growth factors, RTKs, and adherens junction proteins is crucial for proper tissue development and the maintenance of tissue architecture. In this study, we examined the relationship between Dlg-1, a scaffolding protein involved in cell–cell adhesion, apical–basal polarity, and PCP,[7] EphA2, which plays a role in lens fiber cell structure and adherens junction formation,[12] and Fgfr signaling, which is required for lens fiber cell differentiation.[24] We found that in the *Dlg-1*–deficient state, the effects on pErk and pAkt correlated with levels of Fgfrs 2 and 3. Loss of *Dlg-1* also led to redistribution of N-cadherin to the cytosol. We found that Dlg-1 complexes with β-catenin, EphA2, and Fgfrs 1, 2, and 3, EphA2 complexes with N-cadherin, β-catenin, and Fgfrs1, 2, and 3, and the levels of these interactions are altered in the absence of *Dlg-1*. Finally, we found that loss of *Dlg-1* led to changes in the levels of activated Fgfr1, Fgfr2, Fgfr3, and EphA2. Based on our results, we propose that *Dlg-1* modulates lens fiber cell adhesion and Fgfr signaling through regulation of EphA2 (Fig. 8).

Dlg-1–Dependent Regulation of Fgfr1 and Fgfr2 Signaling

In this study, we showed that in the *Dlg-1*–deficient state, reduction in one of these Fgfrs through genetic manipulation resulted in increased levels of the other Fgfr (Fig. 1). The mechanism through which changes in Fgfr levels occur is not known. However, these changes in receptor levels occur at the protein level rather than at the RNA level (Table). In the *Dlg-1*–

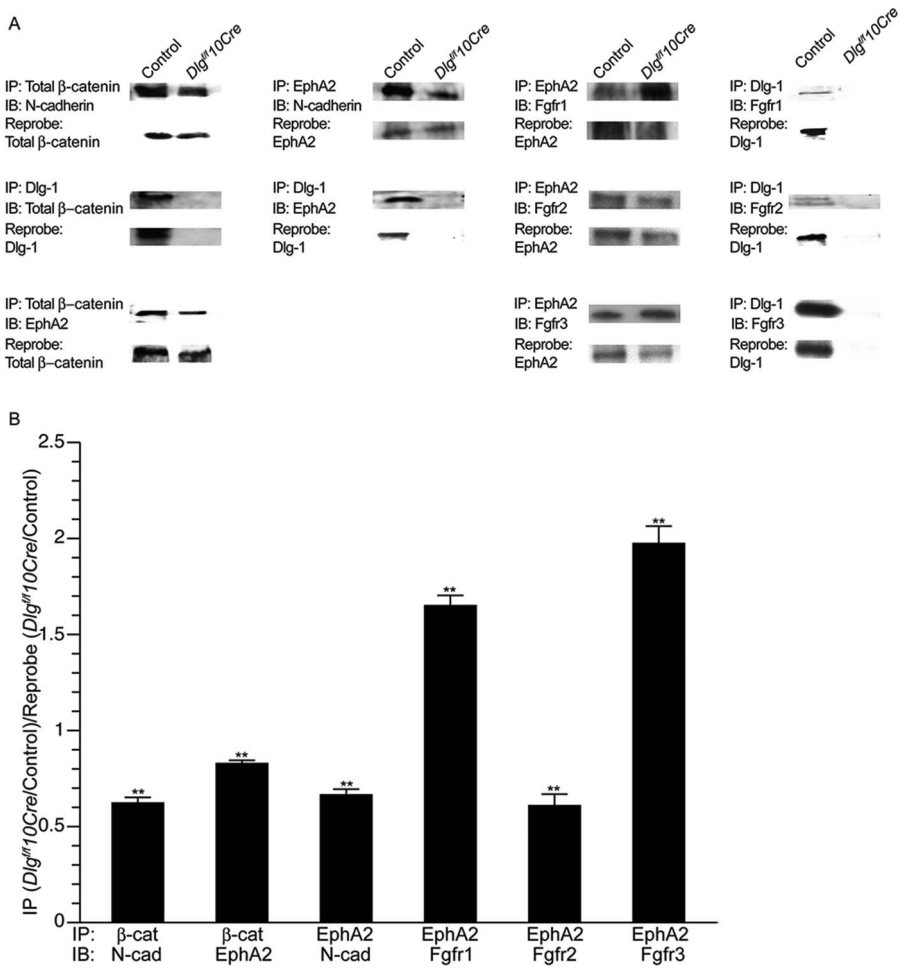

Figure 6. Levels of protein–protein interactions are altered in *Dlg$^{f/f}$10Cre* lenses. (**A**) Lenses from P10 control and *Dlg10$^{f/f}$Cre* mice were extracted with Triton X-100, and the pellets were resuspended in NP-40 buffer. Extracts were subjected to immunoprecipitation and immunoblot using antibodies against the indicated proteins. As a loading control, the blots were reprobed for the respective immunoprecipitated proteins. (**B**) Quantification of protein levels. Shown are the levels of the indicated coimmunoprecipitated proteins in *Dlg10$^{f/f}$Cre* extracts compared with control (normalized to the levels of reprobed protein in *Dlg10$^{f/f}$Cre* extracts compared with control). Signal intensities were quantified by phosphorimager analysis, and the data were subjected to statistical analysis as described in Materials and Methods. In control lenses, β-catenin interacts with N-cadherin, Dlg-1 interacts with β-catenin, EphA2, Fgfr1, Fgfr2, and Fgfr3, and EphA2 interacts with β-catenin, Fgfr1, Fgfr2, and Fgfr3. In *Dlg$^{f/f}$10Cre* lenses, the interactions between β-catenin and N-cadherin, β-catenin and EphA2, N-cadherin and EphA2, and EphA2 and Fgfr2 were reduced compared with controls, whereas the interaction between EphA2 and Fgfr1 and EphA2 and Fgfr3 were increased compared with controls. *Error bars* denote SD. *FDR $<$ 0.05; **FDR $<$ 0.01.

sufficient state, deletion of one allele of *Fgfr1* or *Fgfr2* did not result in increased levels of the other or significantly alter the levels of pAkt (Figs. 1, 3), and deletion of one allele of *Fgfr1* did not alter the levels of pErk (Fig. 2), suggesting that Fgfrs are largely functionally redundant and/or capable of compensating for reduced levels of one receptor. This view is consistent with prior studies showing that deletion of Fgfr 1, 2, or 3 in the lens after a lens vesicle has formed does not result in lens defects.[24,36] In these studies, one or more alleles of an Fgfr gene was ablated in the context of an otherwise wild-type background. However, the context is different in the *Dlg-1*–deficient background in which the function of an upstream regulator is disrupted. It is possible that the changes in cell shape that occur as a consequence of ablation of *Dlg-1* result in changes in the localization, stability, or trafficking of the protein complexes at the membrane, leading to differential effects on the levels of Fgfrs. Dlg-1 has been shown to play a role in vesicle trafficking.[37] In this scenario, despite the fact that the decrease in Fgfr2 is offset by increases in Fgfr1 and Fgfr3, there is not full compensation. Although conditional deletion of *Fgfr2* at the lens placode stage resulted in apoptosis, which implicates changes in cell structure,[38] no changes in fiber cell shape have been reported to occur in lenses where an Fgfr was deleted at the lens vesicle stage,[24] which is the stage at which *Dlg-1* was ablated. *Dlg-1* deficiency has additional effects on the lens that contribute to the altered context such as the reduced EphA2 activation, which not only altered levels of its association with individual Fgfrs (Fig. 6) but also reduced N-cadherin's localization and association with β-catenin (Figs. 4, 6), which is consistent with the disruption in cytoskeletal architecture and cell–cell adhesion in the *Dlg-1*–deficient lens.[8]

We showed that the levels of activated signaling intermediates, pErk and pAkt, correlated with levels of Fgfr2 and Fgfr2 activation (Figs. 2, 3, 7), suggesting that postnatally Fgfr2 is a driver of the MAPK and phosphoinositide 3-kinase (PI3K) pathways in the lens, as it was during embryonic stages.[39] However, the reduction in activated Fgfr2 was greater than the reduction in pErk and pAkt levels (Figs. 2, 3, 7). It is known that Fgfr3 is more abundant in the postnatal

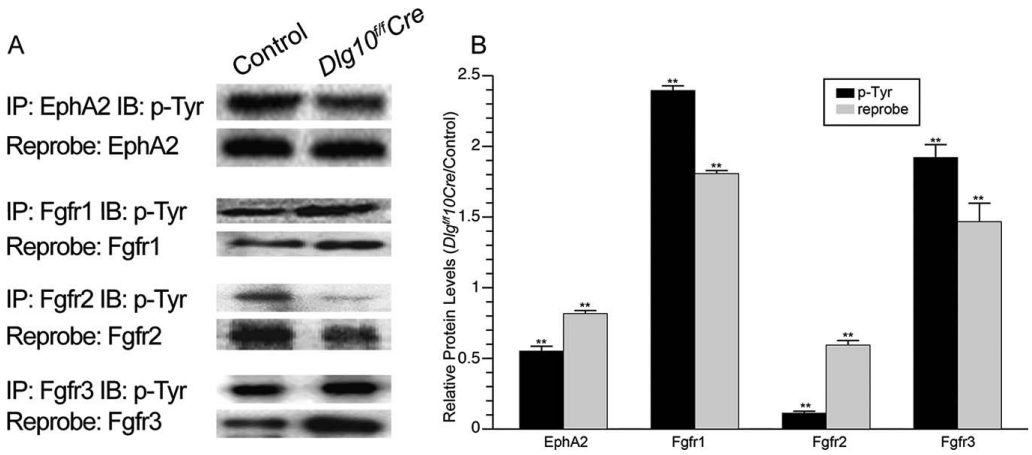

Figure 7. Levels of activated Fgfrs and activated EphA2 are altered in *Dlg$^{f/f}$10Cre* lenses. (**A**) Lenses from P10 control and *Dlg$^{f/f}$10Cre* lenses were extracted with Triton X-100, and the pellets were resuspended in NP-40 buffer. The extracts were subjected to immunoprecipitation (IP) with antibodies against the indicated proteins followed by Western blotting with antibody against phosphotyrosine (IB: p-Tyr). As a loading control, blots were reprobed for their respective immunoprecipitated receptor proteins. (**B**) Quantification of protein levels. Shown are the levels of the indicated proteins in extracts from *Dlg$^{f/f}$10Cre* lenses relative to levels in the controls (control levels set a 1.0). Signal intensities were quantified by phosphorimager analysis, and the data were subjected to statistical analysis as described in Materials and Methods. At least three protein pools were immunoprecipitated and immunoblotted in triplicate over one to three blots. *Error bars* denote SD. *FDR < 0.05; **FDR < 0.01.

lenses, at least at the RNA level, than Fgfrs 1 and 2.[40,41] We show that the level of Fgfr3 associating with EphA2 and the level of activated Fgfr3 increase in the *Dlg-1*–deficient lenses (Figs. 6, 7). Furthermore, preliminary analysis indicates that despite the increase in Fgfr3 in the *Dlg$^{f/f}$10Cre* lenses, when *Dlg-1* deficiency is combined with haplodeficiency for *Fgfr1* or *Fgfr2*, changes in Fgfr3 levels parallel the changes in Fgfr2 (not shown). We propose that the increased Fgfr3 activity accounts for the difference between the reduction in Fgfr2 activation and reduction in pErk and pAkt levels.

We also observed that Fgfr1 levels appear to negatively correlate with pErk and pAkt levels (Figs. 1–3, 7). Although it is thought that all Fgfrs behave similarly in the lens, there are contexts in which Fgfr1 and Fgfr2 have differing effects. For example, conditional activation of Fgfr1 in prostate cancer cells and mouse models has different effects on cell proliferation and Erk activation than conditional activation of Fgrfr2.[42,43] An alternative explanation for our results is that the increased level of Fgfr2 and Fgfr3 in the *Dlg$^{f/f}$;Fgfr1$^{f/+}$10Cre* lens leads to the increased levels of pErk and pAkt compared with *Dlg$^{f/f}$10Cre* lens and/or that the effect of the changing levels of activated Fgfr1 on pErk and pAkt was not distinguishable in our assays.

Dlg-1 Is a Regulator of EphA2 Activity

In this study, we demonstrated that loss of *Dlg-1* results in a redistribution of N-cadherin from the cytoskeletal-associated fraction to the cytosolic fraction (Fig. 4) and disrupts the interaction of N-cadherin and membrane-associated β-catenin (Fig. 6). We furthermore demonstrated loss of *Dlg-1* results in reduced levels of activated EphA2 (Fig. 7), suggesting that Dlg-1 is required for stimulating the appropriate level of EphA2 activation and function. Thus, we identified *Dlg-1* as an upstream regulator of EphA2. These results suggest that the mechanism through which Dlg-1 carries out its role in cell–cell adhesion is through its regulation of EphA2. However, the reduction in activation of EphA2 when *Dlg-1*

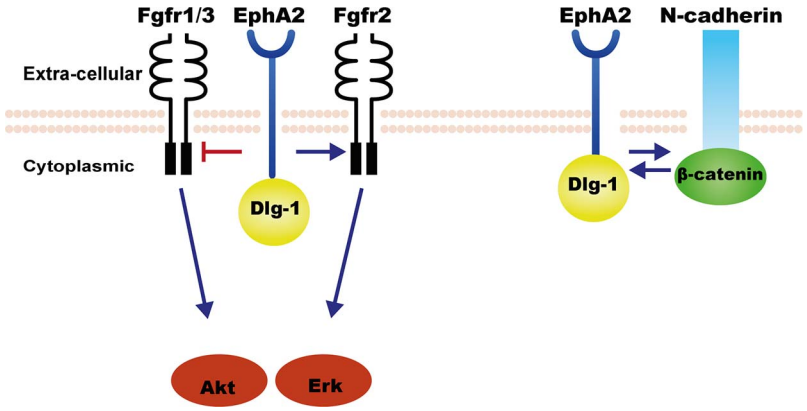

Figure 8. Proposed model of Dlg-1 function in the lens. The results obtained in this study support a model in which Dlg-1 affects lens fiber cell adhesion and differentiation at least in part by promoting the full activation of EphA2. EphA2, in turn, balances the activities of Fgf receptors by enhancing Fgfr2 activation while suppressing activation of Fgfr1 and Fgfr3, thus leading to the proper levels of activation of Erk and Akt. In addition, Dlg-1 promotes EphA2's regulation of the interaction between N-cadherin and β-catenin, thus stabilizing the cytoskeleton and membrane-anchored adherens junction complexes. Dlg-1 also potentially interacts directly with β-catenin.

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is ablated was only partial (Fig. 7), suggesting that there may be factors in addition to Dlg-1 that play a role in regulating EphA2 activity. Interestingly, the structural defects in the *Dlg-1, Epha2*, and *Efna5* mutant lenses resemble the lens phenotype of mutants in the core PCP genes, *Vangl2* and *Celsr*, suggesting that Dlg-1 and EphA2 may interact with core PCP factors in the lens to regulate fiber cell structure and growth factor signaling required for fiber cell differentiation.[44,45]

In neurons, some members of the Eph receptor family- have been shown to interact with PDZ proteins via their C-terminal PDZ binding motif.[46] However, neither EphA2 nor Dlg-1 was among the proteins identified in that screen. In this study, we showed by coimmunoprecipitation that Dlg-1 and EphA2 are found in the same cytoskeletal-associated complex in the lens (Fig. 6). These results suggest that Dlg-1, through its role as a scaffolding protein, assembles a complex that includes EphA2, N-cadherin, and β-catenin. The interaction between Dlg-1 and EphA2 may be direct via binding of a PDZ domain of Dlg-1 to the PDZ binding motif in EphA2. The level of association of Dlg-1 and EphA2 observed by coimmunoprecipitation was strong, suggesting that this might be the case. However, it is also possible that the interaction is indirect, as β-catenin is a PDZ binding protein,[47] and the association of Dlg-1 with β-catenin was strong (Fig. 6). Future studies will be needed to determine the exact nature of the interactions between these proteins.

Dlg-1: A Regulator of Fgfrs via EphA2?

In this study, we found that Dlg-1, EphA2, Fgfr1, Fgfr2, and Fgfr3 coimmunoprecipitate with each other (Fig. 6), suggesting that they complex with each other. Because the interaction between Dlg-1 and the Fgfrs 1 and 2 appeared to be weaker than the interaction between EphA2 and these Fgfrs, we suggest that EphA2 is a bridge between Dlg-1 and the Fgfrs. Thus, we propose a model in which Dlg-1 regulates EphA2 activity, which in turn regulates Fgfr activity. Cross-talk between Ephrin/Eph and Fgf/Fgfr signaling on cell development and downstream regulation has been shown previously.[25,26,48,49] In *Ciona,* it has been proposed that Eph and Fgfr signaling act antagonistically in Erk activation,[25] whereas in mammalian cells, agonistic interactions between Fgfr and EphA4 in MAPK signaling have been reported.[26,35] Based on our results, we suggest that EphA2 is a positive regulator of Fgfr2 activity, whereas it is a negative regulator of Fgfr1 and Fgfr3 activity. Future studies will be required to address this hypothesis and to understand how these signaling events are coupled in the lens.

Taken together, our study provides new insight into the role of *Dlg-1* in vertebrate development. We suggest that Dlg-1 at least in part regulates fiber cell structure and differentiation through coordinating the interaction between the Eph and Fgfr signaling pathways. Further studies will be needed to understand in detail the mechanisms involved in this regulation.

Acknowledgments

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