Loss of *Dlg-1* in the Mouse Lens Impairs Fibroblast Growth Factor Receptor Signaling

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Abstract

Coordination of cell proliferation, differentiation and survival is essential for normal development and maintenance of tissues in the adult organism. Growth factor receptor tyrosine kinase signaling pathways and planar cell polarity pathways are two regulators of many developmental processes. We have previously shown through analysis of mice conditionally null in the lens for the planar cell polarity gene (PCP), *Dlg-1*, that *Dlg-1* is required for fiber differentiation. Herein, we asked if *Dlg-1* is a regulator of the Fibroblast growth factor receptor (Fgfr) signaling pathway, which is known to be required for fiber cell differentiation. Western blot analysis of whole fiber cell extracts from control and *Dlg-1* deficient lenses showed that levels of the Fgfr signaling intermediates pErk, pAkt, and pFrs2a, the Fgfr target, Erm, and the fiber cell specific protein, Mip26, were reduced in the *Dlg-1* deficient fiber cells. The levels of Fgfr2 were decreased in *Dlg-1* deficient lenses compared to controls. Conversely, levels of Fgfr1 in *Dlg-1* deficient lenses were increased compared to controls. The changes in Fgfr levels were found to be specifically in the triton insoluble, cytoskeletal associated fraction of *Dlg-1* deficient lenses. Immunofluorescent staining of lenses from E13.5 embryos showed that expression levels of pErk were reduced in the transition zone, a region of the lens that exhibits PCP, in the *Dlg-1* deficient lenses as compared to controls. In control lenses, immunofluorescent staining for Fgfr2 was observed in the epithelium, transition zone and fibers. By E13.5, the intensity of staining for Fgfr2 was reduced in these regions of the *Dlg-1* deficient lenses. Thus, loss of *Dlg-1* in the lens impairs Fgfr signaling and leads to altered levels of Fgfrs, suggesting that *Dlg-1* is a modulator of Fgfr signaling pathway at the level of the receptors and that *Dlg-1* regulates fiber cell differentiation through its role in PCP.

Introduction

Coordination of cell proliferation, differentiation and survival is essential for normal development and maintenance of tissues in the adult organism. This coordination involves growth factor receptor tyrosine kinase signaling pathways that they activate, as these pathways are critical regulators of cell fate [1,2]. Cell structure and the coordinated movement of cells during differentiation are also determinants of normal development. One property governing the coordinated movement of cells is referred to as Planar Cell Polarity (PCP), the polarized orientation of cells within a plane of a tissue perpendicular to the apical-basal axis [3,4,5]. In this study, we address the possibility that *Dlg-1*, a newly discovered PCP gene, is a regulator of the Fibroblast growth factor receptor (Fgfr) signaling pathway in the terminal differentiation of the epithelial cells in the mouse ocular lens.

The mouse ocular lens has been widely used as a system for studying mechanisms of mammalian development in part because of its spatially separated regions of cycling and differentiating cells. Terminal differentiation of lens epithelial cells into fiber cells involves cell cycle withdrawal, cytoskeletal reorganization, directed cell migration and organelle loss, all of which must be tightly coordinated [6]. In the mouse, lens fiber cell differentiation begins after the formation of the hollow lens vesicle at day E10.5. Cells in the posterior of the lens vesicle, closest to the developing retina, withdraw from the cell cycle, begin to express fiber cell specific proteins and elongate toward the anterior surface to form the primary fiber cells. Once the vesicle is occluded, around day E12.5, secondary fiber cells begin to differentiate from the periphery of the lens as epithelial cells withdraw from the cell cycle, elongate and migrate along the capsule and anterior epithelium until they meet up with their counter part from the opposite side of the lens to form the lens sutures [6].

The vitreous humor of the eye, which fills the space between the posterior of the lens and the retina, contains various growth factors including epidermal growth factor (EGF), fibroblast growth factor (FGF), insulin-like growth factor (IGF), platelet-derived growth factor (PDGF), hepatocyte growth factor (HGF), and vascular endothelial growth factor (VEGF) [1,7]. Among these various growth factors, FGF has been demonstrated to be the only growth factor capable of initiating lens fiber differentiation [7,8,9]. Studies using a rat lens explant system have demonstrated that a low dose of FGF can induce proliferation, a medium dose can lead to cell migration, and a high dose can elicit differentiation, as measured by cell elongation and expression of lens fiber cell specific proteins. FGFs affect cell behavior through the activation membrane bound tyrosine kinase receptors and the signaling pathways regulated by those receptors. Four Fgfrs are expressed in the embryonic mouse lens, Fgfr1, Fgfr2, Fgfr3, and Fgfr4 [2,10,11,12]. In *vivo* experiments using transgenic mice have shown that overexpression of...
numerous FGFs can elicit premature fiber cell differentiation whereas overexpressing a dominant-negative Fgfr inhibit lens fiber differentiation [13,14,15]. And, importantly, gene knockout experiments have shown that the simultaneous deletion of Fgfrs 1, 2 and 3 once the lens vesicle has formed results in complete inhibition of fiber cell differentiation [16], demonstrating that Fgfr signaling is required for fiber cell differentiation.

How the Fgfr signaling pathway and the changes in cellular shape and organization that occur during fiber cell differentiation are coordinated is not well understood. However, recently, it has been shown that the newly forming fiber cells in the outermost regions of the fiber cell compartment exhibit planar cell polarity and lens fiber cells in mice carrying mutations in the core PCP gene, Vangl2, exhibit orientation, migration and suture defects [17,18]. Furthermore, transgenic expression of an inhibitor of Wnt signaling, secreted frizzled related protein 2 (Sfrp2) inhibited fiber cell differentiation [17]. Finally, it has been shown that FGF-induced fiber cell differentiation in rat explant cultures is dependent on Wnt signaling [19]. All these studies point toward a requirement for Wnt/PCP signaling in the transmission of Fgfr signaling to induce fiber cell differentiation.

Recently, we discovered that Drosophila Dlg-1 (Dlg-1), the mouse homolog of the Drosophila gene discs-large (dlg), is expressed in the lens [20,21] and, using lens specific deletion of Dlg-1, we demonstrated that Dlg-1 is required for cell adhesion, apical-basal polarity and fiber cell differentiation in the lens [22], consistent with the role documented for dlg in Drosophila [23]. However, in addition, the phenotype of the Dlg-1 deficient lens resembled that described for the Vangl2 heterozygous mutant lens. In histological section, the shape of the Dlg-1 mutant lens was flattened, fiber cell curvature remained concave rather than becoming convex and defects were observed in suture formation. The similarity in the phenotype of the Vangl2 heterozygous and Dlg-1 mutant lenses suggested that Dlg-1 might also play a role in PCP. Consistent with the prediction that Dlg-1 might play a role in PCP, we found that mice carrying germline mutation in Dlg-1 show characteristic phenotypes of mice deficient for core PCP genes [24], thereby identifying Dlg-1 as a new PCP gene.

In the course of our analysis of the phenotype of the Dlg-1 deficient lens, we observed that levels of activated Erk, a signaling intermediate of numerous growth factor signaling pathways, were reduced in the Dlg-1 deficient fiber cells [22]. Erk activation downstream of FGF stimulation is necessary for lens proliferation [25], and differentiation [26]. This led us to hypothesize that loss of Dlg-1 function in the lens impairs the Fgfr signaling pathway resulting in the observed fiber cell differentiation defects. In this study, we tested this hypothesis. Specifically, we assessed the impact of loss of Dlg-1 on components of the Fgfr signaling cascade, an Fgfr signaling target, and on Fgfrs 1, 2, and 3, themselves. We found that activation of signaling intermediates in the Fgfr pathway, along with levels of an Fgfr target, were reduced in Dlg-1 deficient lenses. Dlg-1 deficiency also led to changes in the relative levels of Fgfrs 1, 2 and 3. Finally, reduced levels of Fgfr2 and pErk were observed in the transition zone of embryonic lenses, a region of the lens that exhibits PCP. Taken together, these data show that Dlg-1 is required for the proper levels of Fgfrs and Fgfr signaling, and suggest that Dlg-1 acts as modulator of fiber differentiation in the lens through its role in PCP.

**Materials and Methods**

**Ethics Statement**

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. The protocol covering these studies (protocol #M00712) was approved by the Institutional Animal Care and Use Committee of the University of Wisconsin School of Medicine and Public Health (Animal Welfare Assurance #A3960-01).

**Animals and Tissue Preparation**

The generation of mice carrying the Dlg-1 conditional allele (Dlg-1f/f) mice [22] and the Cre driver transgenic mice, MLR10CRE and MLR39CRE [27] have been described previously. Dlg-1f/f mice were crossed to either MLR10CRE or MLR39CRE mice to generate Dlg-1f/f/MLR10CRE mice (here after referred to as Dlg10CRE mice) or Dlg-1f/f/MLR39CRE mice (here after referred to as Dlg39CRE mice). Genotyping was carried out as described previously [22]. Heads from embryonic day 12.5 (E12.5), E13.5, E13.5 and E17.5 control (Dlg-1f/f), Dlg10CRE, and Dlg39CRE embryos were fixed in 4% paraformaldehyde overnight at 4°C, washed in 1X phosphate buffered saline (PBS), dehydrated in increasing concentrations of ethanol, and embedded in paraffin oriented for longitudinal sectioning. Sections (5 μm) were cut and used for immunofluorescence. Embryos were staged by designating midday on the day of the vaginal plug as day E0.5. Postnatal mice were staged by designating the day of birth as neonate and subsequent days as postnatal day 1 (P1), P2, etc. Eyes from P30 control mice were fixed in 4% paraformaldehyde for 2 hours at 4°C, incubated in 10% and 20% sucrose for 1.5 hour each at 4°C and then in 30% sucrose overnight at 4°C and embedded in TissueTek oriented for transverse sectioning. Cryogenic sections (10 μm) were cut and used for immunofluorescence.

**Western Blot**

Fiber cells were dissected from postnatal day 2 (P2) control, Dlg10CRE, Dlg39CRE mice and protein lysates were prepared by extraction in RIPA buffer with protease inhibitors. A total of 50–100 μg of each protein lysate was electrophoresed through 7.5% acrylamide gels, and the proteins were transferred to PVDF membranes. Membranes were blocked for one hour at room temperature (RT) in 5% nonfat dry milk dissolved in 1X phosphate buffered saline-tween 20 (PBST) or 5% bovine serum albumin (BSA) in Tris buffered saline-tween 20 (TBST). Blots were incubated with rabbit anti-human MIP26 (Alpha Diagnostics cat #AQP01-A) at 1:100 dilution, rabbit anti-human total ERK (Promega cat #V1141) at 1:100 dilution, rabbit anti-human pERK (Cell Signaling cat #4370) at 1:100 dilution, rabbit anti-human pAKT (Abcam cat #ab66138) at 1:1000 dilution, rabbit anti-human pFRS2α (Cell Signaling cat #3861) at 1:100 dilution, and rabbit anti-human ERM (Santa Cruz cat #SC-22807) at 1:1000 dilution were incubated overnight at 4°C. Membranes were washed in 1X PBST or 1X TBST and then were incubated for one hour at RT with goat anti-mouse horseradish peroxidase (HRP, Pierce) or donkey anti-rabbit HRP (GE Healthcare Life Sciences) diluted in blocking solution. To prepare soluble and cytoskeletal associated protein fractions, lenses from P2 control and Dlg10CRE lenses were lysed in Triton X-100 buffer (1% Triton X-100, 10 mM imidazole, 100 mM NaCl, 1 mM MgCl2, 5 mM EDTA) and centrifuged at 13,200 rpm for 15 minutes at 4°C to generate the detergent soluble supernatant (cytoplasmic) and detergent insoluble pellet (cytoskeletal associated) fractions. The pellets were resuspended in Urea buffer (7 M urea, 0.1 M Tris, pH 7.9, 5 mM EDTA) and centrifuged. A total of 30 μg of each soluble and 100 μg of each insoluble protein lysate was electrophoresed, transferred and immunoblotted for Fgfrs using...
rabbit anti-human FGF receptor 1, 2, or 3 antibodies (Santa Cruz Biotechnology cat #s SC-7945, SC-122, and SC-123, respectively) at 1:100 dilution as described above. All blots were reprobed with mouse anti-rabbit GAPDH antibody (Millipore cat #MAB374) as a loading control. Bands were visualized using Enhanced Chemiluminescence Plus kit (ECL plus, GE Healthcare Life-sciences) 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 1–3 blots. Relative protein levels were calculated by setting the protein/Gapdh ratio for the controls at 1.0. All data for an individual pool were combined to give a single value for the pool. The data reported are the mean ± standard deviation across 3–4 pools. For statistical analysis, the two-sided One Sample t-test was conducted using MSTAT software (www.mcardle.wisc.edu/mstat). Because of the large number of experimental comparisons made in these studies, we report the false discovery rates [28] rather than the unadjusted per comparison P-values from the statistical tests. A false discovery rate (FDR) ≤0.05 was considered to be statistically significant.

Immunofluorescence

Heads from day E12.5, E13.5, E15.5, and E17.5 control, Dlg10CRE or Dlg9CRE embryos were fixed, embedded in paraffin, and sectioned as described above. For immunofluorescence detection, sections were deparaffinized in xylene and rehydrated using graded ethanol. For Fgfr2 immunofluorescent staining, sections were trypsinized for 40 minutes at RT in a humidified chamber, washed in 1X PBS, and then incubated for 1 hour with a 0.5% Triton X-100 and 0.3 M glycine in 1X PBS in a humidified chamber. After antigen retrieval, sections were blocked in 0.5% nonfat dry milk, 10% horse serum, and 0.2% Triton X-100 diluted in 1X PBS for 3 hours at RT. For pErk and Dlg1 immunofluorescent staining, antigen retrieval was accomplished by boiling the sections in 0.1 M sodium citrate, pH 6.0, for 30 minutes in a rice cooker and then washed in 1X PBS. Sections were blocked with 5% horse serum in 1X PBS for 1 hour. The blocking solution was removed and sections were incubated with rabbit anti-human FGFR2 (Abcam cat#ab10648) which was raised against amino acids 362–374 in the extracellular domain of human FGFR2) diluted in blocking solution at 1:200 dilution, rabbit anti-human pERK (Cell Signaling cat#4370) diluted at 1:50, or mouse anti-rat SAP97 (Novus cat#NBP1-48054) at 1:500 overnight at 4°C. Sections were washed in 1X PBS and were incubated with the AlexaFluor 568 conjugated goat anti-rabbit (Molecular Probes) or FITC-conjugated horse anti-mouse (Vector Labs) for 1 hour at RT.

Results

Effect of Loss of Dlg-1 on Activation of the Fgfr Signaling Pathway

We previously have shown that ablation of Dlg-1 in the lens leads to defects in fiber cell differentiation including reduced levels of the fiber cell specific protein, Mip26. Additionally, levels of activated Erk (pErk), a signaling intermediate in the Ras/Raf/MEK/Erk pathway that is activated by various growth factor signaling pathways including the Fgfr pathway [1,2] appeared to be reduced in Dlg-1 deficient fiber cells [22]. Because Fgfr signaling has been shown to be essential for fiber cell differentiation [2,14], we hypothesized that Fgfr signaling is compromised in Dlg-1 deficient lenses. To test this hypothesis, Dlg10Cre/mice, which carry a conditional null allele of Dlg-1, were crossed to Mip10Cre mice [22] to generate Dlg10Cre/mice (controls) and Dlg10Cre/mice in which Dlg-1 is ablated throughout the lens beginning at day E10.5 in development. Whole cell lysates were prepared from lens fiber cells of postnatal day 2 (P2) mice control and Dlg10Cre/mice and the lysates subjected to western blot analysis using anti-MIP26 and anti-pERK antibodies. The blots were subsequently reprobed using anti-GAPDH antibody as a loading control and the levels quantified by phosphorimager analysis. Levels of Mip26 were reduced by 40% in Dlg10Cre lenses as compared to controls and levels of pErk were reduced by 30%, despite an increase in levels of total Erk (tErk; Fig. 1). Fgfr signaling is also known to activate another signaling intermediate, pAkt, via the PI3K pathway [1]. Levels of pAkt, were reduced by 25% in Dlg10Cre fiber cell extracts as compared to controls (Fig. 1).

Fgfr signaling stimulates the activation of signaling cascades starting with the phosphorylation of the docking protein, Frs2α [29,30], which binds directly to the receptor. Although Frs2α can be activated by receptors in addition to Fgfrs, these other receptors are not expressed in the lens [31]. Thus, activation of Frs2α in the lens is a read out specifically for Fgfr signaling. To determine if loss of Dlg-1 affected the levels of Frs2α activation, western blot analysis were carried out on P2 fiber cell extracts from control and Dlg10Cre lenses using anti-pFrs2α antibodies. Levels of pFrs2α were decreased by 30% in extracts from Dlg10Cre lenses as compared to controls. Finally, western blot analysis showed that protein levels of Erm, an Fgfr target, were decreased by 30% in extracts from Dlg10Cre fiber cells as compared to fiber cells from controls. Thus, levels of activated Fgfr signaling intermediates, including the proximal Frs2α, an Fgfr target, Erm, and a fiber cell marker, Mip26, were all significantly reduced in fiber cell extracts from Dlg10Cre mice, indicating that Dlg-1 is a modulator of Fgfr signaling and fiber cell differentiation in the lens.

In Dlg10Cre mice, Dlg-1 is ablated in both epithelial and fiber cells. Previously, by comparing the phenotype of the fiber cells in these mice to mice in which Dlg-1 is ablated only in the fiber cells
we showed that the epithelial and fiber cell defects were independent of each other [22]. To verify that the effects of Dlg-1 deficiency on Fgfr signaling in the fiber cells is a fiber cell autonomous effect, we quantified the levels of pErk and pFrs2α in the fibers of Dlg39CRE mice as compared to Dlg10CRE mice and controls by western blot analyses. As shown in Fig. 2, levels of pErk and pFrs2α were significantly reduced in the fiber cells from Dlg39CRE mice as compared to controls (20% reduction in each protein), although to a lesser extent than in the fiber cells from Dlg10CRE mice (30% reduction in pErk and 35% reduction in pFrs2α). That the reduction in pErk and pFrs2α in Dlg39CRE fibers is less than in Dlg10CRE fibers likely is due to a delay in the timing of Cre activity and/or in the turnover of existing Dlg-1 protein relative to the initiation of fiber cell differentiation [27]. These data indicate that the effect of Dlg-1 deficiency on Fgfr signaling is fiber cell autonomous.

**Effect of Loss of Dlg-1 on Levels of Fgfrs**

The reduced levels of activated Frs2α, which is phosphorylated directly by Fgfr, suggested that the levels of Fgfrs themselves might be altered in the lenses of Dlg10CRE mice. To address this possibility, whole cell extracts from P2 fiber cells of Dlg10CRE and control mice were subjected to western blot analyses using antibodies specific to Fgfr1, Fgfr2 or Fgfr3. Levels of Fgfr2 were significantly reduced by 40% in Dlg10CRE lenses compared to controls; however, levels of Fgfr1 and Fgfr3 trended toward being increased in the Dlg-1 deficient lenses (Figs. S2 and 3). To understand if the subcellular distribution of Fgfrs were affected, lenses from P2 control and Dlg10CRE mice were extracted in

![Figure 1. Components of the Fgfr signaling pathway are reduced in Dlg10CRE fiber cells.](http://www.plosone.org/figure1.png)

(A) RIPA lysates from P2 control and Dlg10CRE fiber cells were immunoblotted for the indicated proteins and the blots 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 Dlg10CRE fiber cells relative to levels in the controls (control levels set a 1.0). Signal intensities were quantified by phosphorimager analysis, as described in Materials and Methods, and the data subjected to statistical analysis using the two-sided One Sample t-test. At least 3 protein pools were blotted in triplicate over 1–3 blots. The relative levels of Fgfr signaling intermediates and target as well as the fiber cell specific protein, Mip26, were all reduced in Dlg10CRE lens fibers as compared to control. Error bars = standard deviation, * = FDR < 0.05, ** = FDR < 0.01. doi:10.1371/journal.pone.0097470.g001
Triton X-100 to generate Triton X-100 soluble and insoluble fractions. The triton soluble fraction contains mainly cytosolic proteins while the triton insoluble fraction contains cytoskeletal, membrane proteins, and cytosolic proteins that are tightly associated with the membrane. Immunoblotting the triton soluble and insoluble fractions for Mip26, an intrinsic membrane protein, confirmed that the fractionation was successful as the amount of Mip26 in the soluble fraction was less than 2% of that in the insoluble fraction (data not shown). Interestingly, the levels of Fgfrs were altered in the insoluble fraction from the Dlg10Cre lenses, relative to control lenses whereas the levels of soluble fractions of Dlg10Cre were similar to the levels in controls. The Fgfr2 levels were reduced by 60% in the cytoskeletal-associated fraction of Dlg10Cre lenses. Fgfr1 was significantly increased by 75% in Dlg10Cre insoluble fractions and levels of Fgfr3 were increased, although the increase was not statistically significant (Fig. 3). These results demonstrate that Dlg-1 is required to maintain the normal levels of Fgfrs in the lens and thereby regulate Fgfr signaling.

Effect of Loss of Dlg-1 on Perk Levels during Embryogenesis

The western blot analyses demonstrating that Fgfr signaling is impaired in Dlg-1 deficient lenses were obtained from lens fibers from P2 mice. However, fiber cell differentiation begins during embryogenesis. Primary fiber cells begin to differentiate once the lens vesicle has detached from the surface ectoderm at day E10.5 and secondary fiber cell differentiation begins once the lens vesicle has been occluded with elongated primary fiber cells, at approximately day E12.5. Therefore, it is unclear from the western blot analyses at what point after ablation of Dlg-1 the changes in Fgfr signaling first occurred.

To determine when loss of Dlg-1 protein occurred in Dlg10Cre and Dlg39Cre lenses, immunofluorescence experiments were carried out on paraffin embedded sections of E12.5 and E13.5 Dlg10Cre and E13.5 and E14.5 Dlg39Cre embryos. In keeping with previous reports [27,32], immunoreactivity for Dlg-1 in Dlg10Cre lenses was reduced at E12.5 and absent at E13.5 (Fig. 4).

**Figure 2. Levels of Fgfr signaling intermediates are reduced in both Dlg10CRE and Dlg39CRE fiber cells.** (A) RIPA lysates from P2 control, Dlg10CRE, and Dlg39CRE fiber cells were immunoblotted for the pErk and pFrs2α and the blots 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 Dlg10CRE fiber cells relative to levels in the controls (control levels set at 1.0). Signal intensities were quantified by phosphorimager analysis, as described in Materials and Methods, and the data subjected to statistical analysis using the two-sided One Sample t-test. At least 3 protein pools were blotted in triplicate over 1–3 blots. The relative levels of Fgfr signaling intermediates were reduced in the fiber cells of Dlg39Cre mice as well as Dlg10Cre mice compared to controls, indicating that the effect was fiber cell autonomous. Error bars = standard deviations. ** = FDR<0.01.

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Triton X-100 to generate Triton X-100 soluble and insoluble fractions. The triton soluble fraction contains mainly cytosolic proteins while the triton insoluble fraction contains cytoskeletal proteins, membrane proteins, and cytosolic proteins that are tightly associated with the membrane. Immunoblotting the triton soluble and insoluble fractions for Mip26, an intrinsic membrane protein, confirmed that the fractionation was successful as the amount of Mip26 in the soluble fraction was less than 2% of that in the insoluble fraction (data not shown). Interestingly, the levels of Fgfrs were altered in the insoluble fraction from the Dlg10CRE lenses, relative to control lenses whereas the levels of soluble fractions of Dlg10CRE were similar to the levels in controls. The Fgfr2 levels were reduced by 60% in the cytoskeletal-associated fraction of Dlg10CRE lenses. Fgfr1 was significantly increased by 75% in Dlg10CRE insoluble fractions and levels of Fgfr3 were increased, although the increase was not statistically significant (Fig. 3). These results demonstrate that Dlg-1 is required to maintain the normal levels of Fgfrs in the lens and thereby regulate Fgfr signaling.

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To determine when loss of Dlg-1 protein occurred in Dlg10Cre and Dlg39Cre lenses, immunofluorescence experiments were carried out on paraffin embedded sections of E12.5 and E13.5 Dlg10Cre and E13.5 and E14.5 Dlg39Cre embryos. In keeping with previous reports [27,32], immunoreactivity for Dlg-1 in Dlg10Cre lenses was reduced at E12.5 and absent at E13.5 (Fig. 4).
Immunoreactivity for Dlg-1 in the fiber cell compartment of Dlg39CRE lenses was reduced at E13.5 and absent at E14.5 (Fig. 4). To determine if Fgfr signaling is compromised at the time Dlg-1 protein is lost from the Dlg10CRE lens, paraffin embedded sections from day E13.5 control and Dlg10CRE embryos were immuno-stained with anti-pERK antibodies. The intensities of staining in transition zone of control and Dlg10CRE lenses were measured by ImageJ (see Materials and Methods). In control lenses, pErk staining was observed throughout the fiber cell and was notably more concentrated toward the posterior region (Fig. 5). In contrast, the intensity of pErk staining was reduced in Dlg10CRE lenses by 64% (Fig. 5). These data show that Fgfr signaling is diminished at the time when Dlg-1 protein is lost from the lens and at a time in lens development much closer to the time when secondary fiber differentiation begins, suggesting that impaired Fgfr signaling does contribute to the observed defects in fiber cell differentiation. Furthermore, as reduced pErk was observed in the transition zone, a region of the lens that exhibits PCP, the data suggest that Dlg-1 may regulate fiber cell differentiation through its role in PCP.

Effect of Loss of Dlg-1 on Fgfr2 Levels during Embryogenesis

Given that levels of pErk were reduced in the lenses of E13.5 Dlg10CRE embryos, we asked if levels of Fgfr2 were similarly reduced in Dlg10CRE lenses at earlier time points. To do so, immunofluorescence experiments were carried out on paraffin embedded sections from control and Dlg10CRE E13.5, E15.5, and E17.5 embryos using an anti-FGFR2 antibody. Representative images are shown in Fig. 6A. The intensities of staining in epithelium, transition zone and fiber cells in control and Dlg10CRE lenses were measured by ImageJ (see Materials and Methods). In

Figure 3. Levels of Fgfrs are altered in the lenses of Dlg10CRE mice. (A) RIPA, triton soluble (cytosolic) and triton insoluble (cytoskeletal-associated) extracts from P2 control and Dlg10CRE lenses were subjected to western blot analysis for Fgfr1, Fgfr2, and Fgfr3 and the blots reprobed for Gapdh as a loading control. Representative blots are shown. (B) Quantification of protein levels. Shown are the levels of each Fgfr in extracts from Dlg10CRE lenses relative to levels in the control (control levels set at 1.0). Signal intensities were quantified by phosphorimager analysis, as described in Materials and Methods, and the data subjected to statistical analysis using the two-sided One Sample t-test. At least 3 protein pools were analyzed in triplicate over 1–3 blots. The relative levels of Fgfr2 were reduced in the whole cell extract and cytoskeletal associated fraction compared to controls whereas the levels of Fgfr1 were increased as compared to controls. Error bars = standard deviations. * = FDR<0.05, ** = FDR<0.01. doi:10.1371/journal.pone.0097470.g003
control lenses, staining for Fgfr2 was seen in the epithelium, the transition zone, and the fiber cells (Fig. 6B). Also, Fgfr2 was detected in fiber cell nuclei. The intensity of staining for Fgfr2 in Dlg10CRE lenses was significantly reduced at E13.5 in all three areas of the lens (Fig. 6B). Levels of Fgfr2 were similarly reduced at E15.5 and were reduced in the transition zone and fibers at E17.5. The reduced intensity of staining for Fgfr2 in the transition zone and fiber cells appeared to be in both the cell membranes and the nuclei of the Dlg10CRE lenses. These results show that Fgfr2 levels begin to decrease at least by a time soon after the beginning of secondary fiber cell differentiation, suggesting that Dlg-1 deficiency affects Fgfr signaling at the level of the receptors and this contributes to the observed defects in fiber cell differentiation.

Effect of Loss of Dlg-1 on Fgfr2 Levels in Dlg39CRE Embryonic Lenses

In the Dlg10CRE lenses, reduced levels of Fgfr2 were found in the epithelium as well as in the fiber cells (Fig. 6). To determine if the effect of Dlg-1 deficiency on Fgfr2 levels in the fiber cells was independent of the effect in the epithelium, sections from day E15.5 control, Dlg10CRE and Dlg39CRE embryos were subjected to immunofluorescence experiments using an anti-Fgfr2 specific antibody. As shown in Fig. 7, the intensity of immunofluorescent staining for Fgfr2 in the transition zone of Dlg39CRE lenses was reduced as compared to that in the controls whereas the intensity of staining in the epithelium was similar. Therefore, effect of loss of Dlg-1 on Fgfr2 levels in the fiber cells is a fiber cell autonomous, suggesting that Dlg-1 plays a role in regulating Fgfr2 levels in the fiber cells independent of its role in the epithelium.

Localization of Dlg-1 and Fgfr2 in Lens Fiber Cells

PDZ domain containing proteins such as Dlg-1 are thought to function as scaffolding molecules capable of assembling large signaling complexes at the cell membrane [33]. Therefore, it is possible that Dlg-1 modulates Fgfr2 signaling because the two are associated in a large macromolecular complex. To begin to address this possibility, double immunofluorescence experiments were carried out using anti-Dlg-1 and anti-Fgfr2 antibodies on transversely oriented sections from the transition zone region of P90 control mice. Immunostaining for Fgfr2 was predominantly localized to the short sides of the fiber cells in the outer cortex (Fig. 8A), as was immunostaining for Dlg-1 (Fig. 8B). In merged images, overlap in staining was observed (Fig. 8C), suggesting that Dlg-1 and Fgfr2 colocalize on the short sides of the fiber cells.

Discussion

The development and maintenance of specialized organs is dependent on the ability of cells to coordinate their response to growth factors in the environment with cell structure and the overall architecture of the tissues. In this study, we examined the relationship between Fgfr signaling, which is known to be required for lens fiber cell differentiation [16], and the regulation of cell structure and tissue architecture by Dlg-1, a gene involved in both apical-basal polarity and planar cell polarity [24], which has also been shown to be required for fiber cell differentiation [22]. We found that loss of Dlg-1 led to reduced levels of Fgfr signaling intermediates and downstream targets. Loss of Dlg-1 also led to changes in the relative abundance of Fgfrs. Finally, loss of Dlg-1 led to reduced levels of Fgfr2 and pErk, an Fgfr signaling intermediate, in a region of the lens that exhibits PCP. Thus, Dlg-1 is the first PCP gene to be shown to modulate Fgfr signaling and fiber cell differentiation. That loss of Dlg-1 impairs Fgfr signaling suggests that the mechanism through which Dlg-1 regulates fiber cell differentiation is, at least in part, through regulation of Fgfr signaling.

Figure 4. Loss of Dlg-1 protein following cre-mediated excision of Dlg-1 sequences. Paraffin embedded sections of eyes from control, Dlg10CRE and Dlg39CRE day E12.5, E13.5, and E14.5 embryos were subjected to immunofluorescent staining for Dlg-1 (green). For Dlg10CRE lenses, the intensity of staining throughout the lens was greatly reduced at E12.5 and staining was undetectable at E13.5. For Dlg39CRE lenses, the intensity of staining in the fiber cell compartment was greatly reduced at E13.5 and staining in the fiber cell compartment was undetectable at E14.5. c, cornea; e, lens epithelium; f, lens fiber cells; r, retina. Bar = 50 µm.
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Dlg-1 in Mouse Lens Fiber Cell Differentiation

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Dlg-1 is a Modulator of Fgfr Levels and Fgfr Signaling in Fiber Cell Differentiation

In this study, we showed that Dlg-1 deficient lenses have reduced levels of numerous activated intermediates of the Fgfr signaling pathway as well as changes in the relative levels of Fgfrs 1, 2 and, to a lesser extent, Fgfr3 compared to control lenses. The changes in Fgfr intermediates may be responsible for some of the observed defects in the Dlg10CRE lenses. For example, the reduced levels of pErk may be related to the observed fiber cell elongation defects as this is known to be dependent on pErk [22,25]. Diminished levels of pAkt may be responsible for the apoptosis observed in the Dlg10CRE lenses [22], as it is known that pAkt is necessary for cell survival [34]. We also found reduced levels of pFrs2α, which is directly activated by the Fgfrs [29,30], and reduced levels of Fgfr2 that was similar to the reduction in levels of pFrs2α (40% vs. 30%), suggesting that the impairment of Fgfr signaling occurs at the level of the receptor. Levels of Fgfr2 and pErk were reduced in Dlg10CRE lenses at least by day E13.5, indicating that the effect of loss of Dlg-1 on Fgfr signaling occurred early in the course of secondary fiber cell differentiation. Interestingly, we found increased levels of Fgfr1, the significance of which is not known at this time. Additionally, the mechanism driving the shift in the relative levels of Fgfrs is not known. Further studies will be required to address these questions. However, in certain breast cancer cell lines, silencing Fgfr2 with siRNA results in increased Fgfr1 levels [35], providing a precedent for our observations.

Figure 5. Levels of the Fgfr signaling intermediate, pErk, are reduced in embryonic Dlg10CRE lenses. (A) Paraffin embedded sections of eyes from E13.5 control and Dlg10CRE embryos were subjected to immunofluorescence analysis using an anti-pErk antibody (red) and the nuclei counterstained with To-Pro3 (blue). Representative images of the transition zone are shown. c, cornea; e, lens epithelium; r, retina; tz, transition zone. Bar = 50 μm. (B) Quantification of pErk levels. Shown are the relative levels of pErk in the transition zone of DLG10CRE and control lenses (control levels set at 1.0) Quantification of signal intensities in the transition zone was carried out using ImageJ, as described in Materials and Methods, and the data subjected to statistical analysis using the two-sided One Sample t-test. At least 3 different sections from at least 3 different lenses were evaluated. The relative levels of pErk in the transition zone of the Dlg10CRE lens were reduced as compared to levels in the corresponding region of the control lenses. Error bars = standard deviations. * = FDR < 0.01.

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A

|        | Control | Dlg10CRE | Control | Dlg10CRE |
|--------|---------|----------|---------|----------|
| Epithelium | ![Image](E13.5) | ![Image](E13.5) | ![Image](E15.5) | ![Image](E15.5) |
| Transition Zone | ![Image](E13.5) | ![Image](E13.5) | ![Image](E15.5) | ![Image](E15.5) |
| Epithelium | ![Image](E17.5) | ![Image](E17.5) |
| Transition Zone | ![Image](E17.5) | ![Image](E17.5) |

B

| Relative Fgf2 levels (Dlg10CRE/Control) | Day E13.5 | Day E15.5 | Day E17.5 |
|----------------------------------------|-----------|-----------|-----------|
| Epithelium | ![Bar](E13.5) | ![Bar](E15.5) | ![Bar](E17.5) |
| Transition Zone | ![Bar](E13.5) | ![Bar](E15.5) | ![Bar](E17.5) |
| Fibers | ![Bar](E13.5) | ![Bar](E15.5) | ![Bar](E17.5) |
Figure 6. Levels of Fgfr2 are reduced in embryonic Dlg10CRE lenses. (A) Paraffin sections of eyes from day E13.5, E15.5 and E17.5 control and Dlg10CRE embryos were subjected to immunofluorescence analysis using an anti-FGFR2 antibody (red) and the nuclei counterstained with To-Pro3 (blue). Representative images of the epithelium and transition zone/fiber cell compartment from control and Dlg10CRE eyes are shown for each time point. c, cornea; e, lens epithelium; f, lens fibers; r, retina; tz, transition zone. Bar = 50 μm. (B) Quantification of Fgfr2 levels. Shown are the relative levels of FGFR2 in regions of the Dlg10CRE lens as compared to levels in the corresponding regions of the control lenses (control levels set at 1.0). Quantification of signal intensities in epithelium, transition zone and fibers was carried out using ImageJ, as described in Materials and Methods, and the data subjected to statistical analysis using the two-sided One Sample t-test. At least 3 different sections from at least 3 different lenses were evaluated for each time point. The Fgfr2 levels were reduced in transition zone and fiber cells of Dlg10CRE lenses compared to controls at all time points and in the epithelium of the Dlg10CRE lenses compared to controls at E13.5 and E15.5. Error bars = standard deviations. * = FDR<0.05, ** = FDR<0.01.

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Reduced Fgfr Signaling in Dlg-1 Deficient Lenses is not Sufficient to Account for Differentiation Defects

Herein, we have determined that loss of Dlg-1 leads to reduced Fgfr signaling and changes in the relative abundance of the Fgfrs. As Fgfr signaling is known to be required for fiber cell differentiation [1,2,16], it is likely that the reduced level of Fgfr signaling in the Dlg-1 deficient lenses contributes to the observed defects in fiber cell differentiation in these mutant lenses. Fgfr2 has been shown to be required for maintaining normal rates of proliferation in the epithelium, cell cycle withdrawal and cell survival if deletion occurs at the lens placode stage [12]. This phenotype bears some similarity to the Dlg10CRE phenotype as Dlg10CRE lenses display small changes in rates of proliferation in the epithelium (unpublished data) and apoptosis in both the epithelium and fiber cell compartment of postnatal mice [22], suggesting that these aspects of the Dlg10CRE phenotype could be due at least in part to the reduced level of Fgfr2. However, the timing of gene disruption differs between these two models as disruption of Fgfr2 with LeCre occurs at the lens placode stage whereas disruption of Dlg-1 using MLR10CRE occurs only after lens vesicle formation. It is known that ablating one or two Fgfrs in the lens with MLR10CRE does not result in cell cycle or fiber cell differentiation defects [16], suggesting that in the lens the Fgfrs function redundantly or can compensate for each other. Ablating Fgfr 1, 2, and 3 with MLR10CRE results in a near complete failure of fiber cell differentiation [16], a pleiotropic phenotype that differs from that of the Dlg10CRE fiber cells. Taken together...

Figure 7. Fgfr2 levels are reduced in the transition zone and fiber cells of Dlg39CRE embryos. Paraffin embedded sections from day E15.5 control, Dlg10CRE and Dlg39CRE embryos were subjected to immunofluorescence analysis using an anti-FGFR2 antibody (red) and the nuclei counterstained with To-Pro3 (blue). Representative images of the epithelium and transition zone of each genotype are shown. The intensity of staining in the epithelium from the Dlg39CRE embryo is similar to that in control whereas intensity in the epithelium of Dlg10CRE embryos is less than in the control. The intensity of Fgfr2 staining was reduced in the transition zone of both Dlg10CRE and Dlg39CRE lenses as compared to controls, indicating that the effect is fiber cell autonomous. At least 3 different sections from at least 3 different lenses were evaluated. c, cornea; e, lens epithelium; f, lens fiber cells; r, retina; tz, transition zone. Bar = 50 μm.

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these data suggest that it is likely that loss of Dlg-1 disrupts signaling pathways in addition to Fgf signaling that contribute to the observed phenotype. A number of other growth factor signaling pathways have been reported to affect fiber cell differentiation including BMP [40,41], TGFβ superfamily [42,43], Notch [44,45] and Eph/Ephrin [46]. Additionally, signaling pathways via cell adhesion proteins such as N-cadherin [47] and integrins [48,49,50,51] are known to play roles in lens development. Impairment of any of these signaling pathways could cooperate with defects in Fgf signaling to elicit the defects in fiber cell differentiation in the Dlg-1 mutant lenses. Future studies are needed to determine the contribution of altered receptor levels to the Dlg-1 deficient lens phenotype and identify additional signaling pathways that are modulated by Dlg-1.

Supporting Information

Figure S1 Documentation of the specificity of the anti-Fgfr2 antibody. Paraffin embedded sections of day E17.5 control and Fgfr2f/f;LeCre embryos were immunostained with anti-Fgfr2 antibodies (red) and counterstained with To-pro3 (blue). Representative images are shown. Immunoreactivity for Fgfr2 in the lens of the Fgfr2f/f;LeCre embryo was nearly absent while immunoreactivity in the retina was similar between control and Fgfr2f/f;LeCre embryo. The signal intensity for Fgfr2 staining in the Fgfr2f/f;LeCre lens, quantified as described in Materials and Methods, was less than 10% of that in the control lens. c, cornea; e, lens epithelium; f, lens fibers; r, retina. Bar = 50 μm. (TIF)

Figure S2 Levels of Fgfr2 are reduced in Dlg10CRE lenses. RIPA lysates of lenses from control and Dlg10CRE P2 mice were subjected to western blot analysis for Fgfr2 and the blots reprobed for Gapdh as a loading control. Shown is a representative full western blot of lysates from control and Dlg10CRE lenses for Fgfr2. Two independently prepared pools of lysates from control and two independently prepared pools of lysates from Dlg10CRE lenses are included on this blot. Lanes: (1) control extract #1, (2) Dlg10CRE extract #1, (3) control extract #2, (4) Dlg10CRE extract #2, (5) molecular mass markers with sizes (in kD) indicated. (TIF)

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Author Contributions

Conceived and designed the experiments: SL. AEG. Performed the experiments: SL. Analyzed the data: SL. AEG. Wrote the paper: SL. AEG.
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