The PDZ proteins, Dlg-1 and Scribble, and core PCP protein,Vangl2 regulate Fgfr Signaling during Lens Development in the Mouse

By

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A dissertation submitted in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

(Molecular Environmental Toxicology)

at the

University of Wisconsin-Madison

2015

Date of final oral examination: 6/30/2015

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Abstract

The PDZ proteins, Dlg-1 and Scribble, and core PCP protein,Vangl2 regulate Fgfr Signaling during Lens Development in the Mouse

SungKyoung Lee

Under the supervision of Professor Anne E. Griep

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Studies in the Griep lab have shown that the PDZ (**P**SD95/**D**lg/**Z**O-1) domain-containing proteins, Discs large-1 (Dlg-1) and Scribble (Scrib), are required to maintain proliferation, cell-cell adhesion, apico-basal polarity and fiber cell differentiation in the mouse ocular lens. The Griep lab also has identified *Dlg-1* as a Planar Cell Polarity (PCP) gene in the mouse. My thesis project addressed the hypothesis that *Dlg-1* regulates fiber cell differentiation, at least in part, by modulating the Fibroblast growth factor receptor (Fgfr) signaling pathway, a pathway that is required for fiber cell differentiation. Using cre-lox technology, I conditionally inactivated *Dlg-1*, *Scrib*, *Fgfr1*, *Fgfr2*, or *Vangl2* or combinations thereof in the lens and assessed the effects of these deficiencies on Fgfr signaling. First, I asked if *Dlg-1* is a regulator of the Fgfr signaling in the lens. I showed that loss of *Dlg-1* in the lens leads to altered levels of Fgfrs and impairs Fgfr signaling. Thus, *Dlg-1* is the first PCP gene reported to modulate Fgfr signaling in the lens.

Second, I asked if there is another factor that links Dlg-1 to Fgfrs. Dlg-1 and Fgfrs may not interact directly because there is no known PDZ binding motif in Fgfrs. I showed that Dlg-1 interacts with the Eph receptor, EphA2, which also interacts with Fgfr1 and Fgfr2. I also showed that *Dlg-1* promotes EphA2 activation and EphA2's interaction with N-cadherin and β-catenin, which stabilizes the adherens junction complex. These results are the first to report molecular interactions through which Dlg-1 affects cell adhesion and fiber cell differentiation. Third, I asked if the genetic interactions between PCP genes *Dlg-1*, *Scrib*, and *Van Gogh-like*2 (*Vangl2*), which affect lens cell shape, similarly affect Fgfr signaling. My results show that *Dlg-1* and *Scrib* act in opposing ways with respect to *Vangl2* in modulating Fgfr signaling in ocular lens. In sum, through my thesis work, I have made the discovery that Dlg-1 regulates lens development through interactions with other PCP factors and the Eph and Fgfr signaling pathways. Overall, the results in my thesis provide new insights into the mechanisms driving lens development.

Acknowledgements

Firstly, I would like to thank my PI. Dr. Anne Griep for everything she has done for me in the last six years. I would also like to thank my committee members (Drs. Ahmad, Bushman Sheibani, and Ikeda) for their helpful advice and time. Also, I would like to thank individuals who provided mice for these studies including the Transgenic Animal Facility for generating the *Dlg-1* mice, Dr. Michael Robinson for *MLR10Cre* and *MLR39Cre* mice, Dr. Xin Sun for *Fgfr1* and *Fgfr2* mice, and Dr. Rivka A. Rachel for *Vangl2* mice. There are several people who provided assistance in protocols and experimental technique including Satoshi Kinoshita for cryosection, Lance Rodenkirch of Keck Confocal Facility, and Jinwoo Lee for assistance of RNA work.

I want to thank my former and present lab members, Idella Yamben, Regan Borchardt, Ellen Hebron, Patience Oladeinde, Tony Paese, Jeremy Riekena, Ivy Bong, and Shalini Shatadal for their help and contribution to this thesis work. I also want to thank to my parents and wife for their support and prayer. Without their help, I may not have been able to make it. Most of all, I want to thank God who knows my life path. To God be the Glory.

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List of Abbreviations

BMP	**B**one **M**orphogenetic **P**rotein
Crb	**C**rum**b**s
Cre	**C**auses **Re**combination
Dlg	**D**iscs-**l**ar**g**e
EMT	**E**pithelial-to-**M**esenchymal **T**ransition
EGF	**E**pidermal **G**rowth **F**actor
FGF	**F**ibroblast **G**rowth **F**actor
FGFR	**F**ibroblast **G**rowth **F**actor **R**eceptor
FRS	**F**ibroblast **R**eceptor **S**ubstrate
GPI	**G**lycosyl-**p**hosphatidyl**i**nositol
GK	**G**uanylate **K**inase
HGF	**H**epatocyte **G**rowth **F**actor
IGF	**I**nsulin-like **G**rowth **F**actor
LAP	**L**eucine Rich Repeats **a**nd **P**DZ Domains
LGL	**L**ethal-**g**iant **L**arvae
LRR	**L**eucine **R**ich-**R**epeats
MAGUK	**M**embrane-**a**ssociated **gu**anylate **k**inases
MAPK	**M**itogen **A**ctivated **P**rotein **K**inase
MIP	**M**embrane **I**ntrinsic **P**rotein

PCP	**P**lanar **C**ell **P**olarity
PDGF	**P**latelet **D**erived **G**rowth **F**actor
PDZ	**P**SD-95, **D**lg-1, **Z**O-1
PSD	**P**ost **S**ynaptic **D**ensity
RTK	**R**eceptor **T**yrosine **K**inase
Scrib	**Scrib**ble
SAM	**S**terile **A**lpha **M**otif
SAP97	**S**ynapse-**A**ssociated **P**rotein 97
STBM	Strabismus
SH3	**S**rc **H**omology**3**
TGF	**T**ransforming **G**rowth **F**actor
TZ	**T**ight **J**unction
Vang	**Van G**ogh
Vanlg2	**Van G**ogh-**l**ike2
WNT	**W**ingless and I**nt**
ZO-1	**Z**ona **O**ccludens **1**

CHAPTER I: INTRODUCTION

This chapter provides general background of lens development, Fgfr signaling, Eph signaling, and the function of PDZ domain-containing proteins and their potential roles in the lens.

Preface

Eyes are the organs that are responsible for vision. The eye has been known as one of the most complex organ in vertebrates. This distinct organ plays an essential role in vision, which is the ability to catch light from the surrounding environment, convert this light into nerve impulses and transmit these nerve impulses out of the eye to the brain where these impulses are transformed into visual perception we refer to as sight or vision. Figure I.1 shows the general anatomy of the human eye. Incident light is refracted by the cornea, the front surface of the eye. The iris controls the amount of light reaching the posterior of the eye by automatically adjusting the size of the pupil. The crystalline lens is located behind the iris and focuses the light on the retina. Through a process of accommodation, the lens helps the eye automatically focus on objects both near and far away. The retina converts light into electrochemical signals. The optic nerve then transmits these signals to the visual cortex, the part of the brain where visual perception is created (Figure I.1).

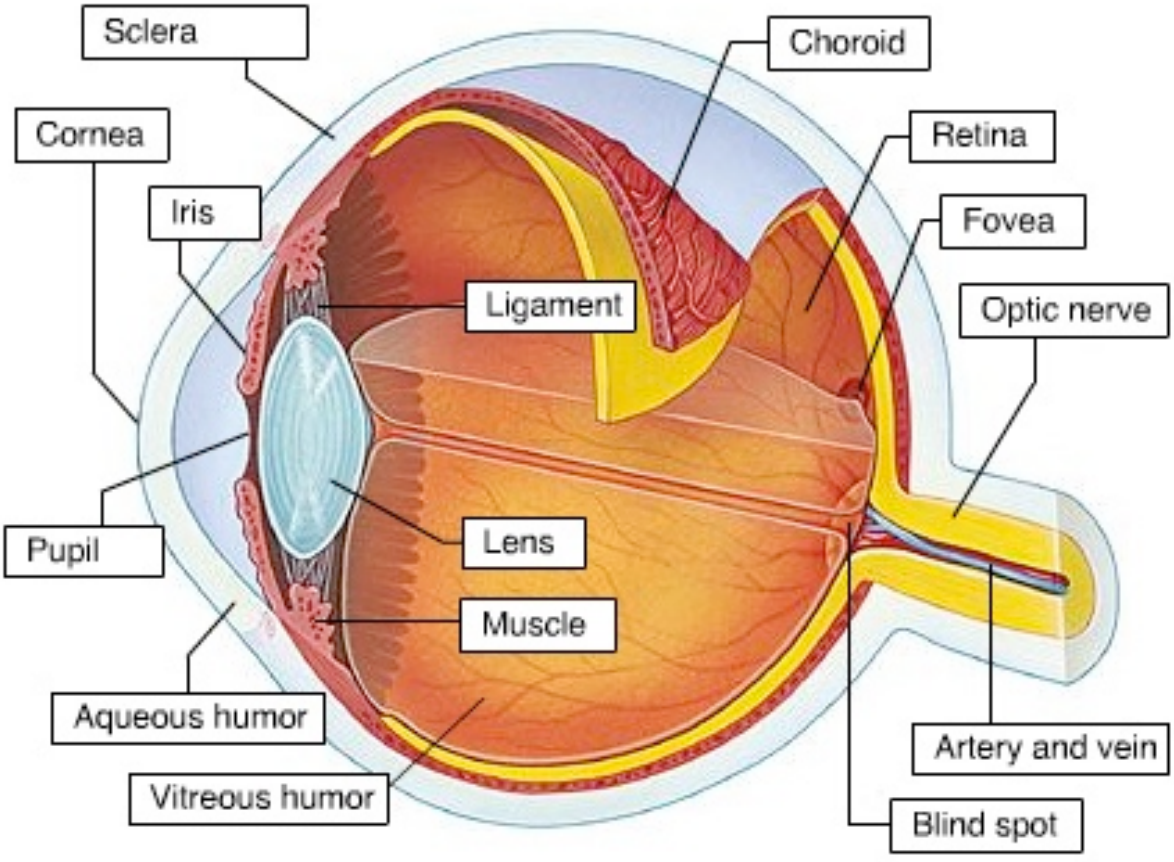
Cataract is defined as any opacity of the lens. Cataractous lenses are unable to properly focus light onto the retina, due to light scattering, thus creating blurred vision and eventually blindness. The World Health Organization (WHO) estimates that around 19.34 million people are bilaterally blind worldwide, representing 43% of all blindness, and this number is expected to increase further in the future due to the increased longevity of the population. Cataract surgery is the most common form of cataract treatment and is usually successful. However, in approximately 20% of cases, posterior capsular opacification develops within weeks to years after surgery (Wormstone, 2002). Cataract surgery costs Medicare insurances about $3.5 billion per year in United States (www.nei.nih.gov). Although surgery is considered a cost-effective treatment for cataract in United States, in many developing countries, cataract surgery is not

readily available or cost prohibitive thereby leaving many people blind (www.nei.nih.gov). Thus, cataract remains the leading cause of blindness and an important cause of visual impairment across the world.

The major risk factors of cataract are aging, hypertension, diabetes, ultraviolet light exposure, significant alcohol consumption, and smoking (Hennis et al., 2003; Hiratsuka et al., 2009; Leske et al., 1999; Lois et al., 2008; Wegener et al., 2002). Children are occasionally born with cataracts, mainly due to genetic mutations in genes that encode proteins important for the structure and clarity of the lens, such as crystallins, connexins, or transcription factors important for lens differentiation such as Pax6, Pitx3, c-Maf and Foxe3, or enzymes affecting galactose metabolism (Churchill and Graw, 2011). Women are at higher risk than men for getting cataracts. The possible risk factors for women that warrant further research may be those associated with pregnancy and childbearing (Reidy et al., 2002). At the cellular and molecular levels, cataracts can be caused by defects in cell-cell adhesion, polarity, proliferation, or differentiation, often involving aberrations in various signaling pathways.

In addition to the clinical importance of lens research, the lens is an excellent developmental model. The lens is a relatively simple tissue, being composed of two cell types, the epithelial cells and the fiber cells, which are derived from the epithelial cells. The lens has been very well characterized at the histological level. The cells are separated into spatially distinct regions of quiescent, cycling, and differentiating cells. Also there are many genetic and molecular tools available for studying the lens, making this distinct organ more accessible for research into elucidating the mechanisms of development.

Figure I.1 The anatomy of the eye. (Image from *Biology* 6th edition). The front of the eye is the cornea. The cornea is the clear part of the eye's protective covering. It allows light to enter the eye. The iris is the colored part of the eye that contracts and expands so the pupil can proper amount of light into the eye. The light is directed by the pupil to the lens. The lens focuses the light onto the retina. Nerve fibers in the retina carry images to the brain through the optic nerve.


Sclera
Choroid
Cornea
Retina
Iris
Fovea
Ligament
Optic nerve
Pupil
Lens
Muscle
Aqueous humor
Artery and vein
Vitreous humor
Blind spot

The Griep lab recently has shown that the **P**SD95/**D**lg/**Z**O-1 domain-containing proteins (PDZ domain proteins), Discs Large-1 (Dlg-1) and Scribble (Scrib), are required to maintain cell-cell adhesion, apical polarity, fiber differentiation, and proliferation in the mouse ocular lens (Rivera et al., 2013; Rivera et al., 2009; Yamben et al., 2013). Additionally, the lab has determined that *Dlg-1* is involved in Planar Cell Polarity (PCP), the polarization of cells in an epithelial sheet perpendicular to the direction of apico-basal polarity (Rivera et al., 2009). In my thesis work, I sought to understand the mechanism through which *Dlg-1* regulates fiber cell differentiation. In doing so, I have tested three main hypotheses. First, I addressed the hypothesis that *Dlg-1* regulates fiber cell differentiation by regulating the levels of Fibroblast growth factor receptor (Fgfr) signaling, a pathway known to be required for fiber cell differentiation (Liu et al., 1996; Richardson et al., 1993; Wang et al., 2010). Second, I tested the hypothesis that Dlg-1 interacts with and regulates the activity of the ephrin receptor, EphA2, and this interaction may link Dlg-1 to Fgfr signaling. Third, I tested the hypothesis that the interactions between *Dlg-1* and other PCP genes that control the hexagonal shape of the fiber cells affects Fgfr signaling.

The PDZ protein, Dlg-1, is a key regulator of cell adhesion, apical-basal cell polarity and cell proliferation. In *Drosophila*, *dlg* mutants are characterized by disruptions in adherens junctions and apico-basal polarity and exhibit a disorganized multilayered epithelium (Bilder et al., 2000; Bilder and Perrimon, 2000). Dlg is often associated with cell membrane complexes where it is thought to function as a scaffolding molecule (Humbert et al., 2003). Previous studies in the Griep lab have demonstrated that *Dlg-1,* the mouse homolog of the *Drosophila* gene *dlg*, is expressed in the lens and required for cell adhesion, polarity and differentiation in this tissue (Nguyen et al., 2003; Nguyen et al., 2005; Rivera et al., 2009). In the course of these studies, I observed that the level of activated Erk, a downstream signaling intermediate of the Fibroblast

growth factor receptor (Fgfr) signaling pathway, was reduced, suggesting that loss of *Dlg-1* function may impair Fgfr signaling. Fgf has been shown to be only growth factor capable of inducing lens fiber cell differentiation (Liu et al., 1996; Richardson et al., 1993; Wang et al., 2010). In *in vivo* experiments, simultaneous deletion of *Fgfr* 1,2, and 3 genes in the lens revealed complete arrested lens fiber differentiation, which demonstrated that Fgfr signaling is required for lens development (Chow et al., 1995; McAvoy and Chamberlain, 1989; Robinson, 2006; Stolen and Griep, 2000; Zhao et al., 2008). These data led me to hypothesize that Fgfr signaling in lens fiber cells is dependent on *Dlg-1*. Therefore, I 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. These data are described in Chapter III.

Although Fgf is the only growth factor known to be capable of inducing fiber cell differentiation, other growth factors are known to affect fiber cell structure and cell adhesion. Among various growth factors which expressed in the lens, Eph receptors form the largest subfamily of receptor tyrosine kinases (RTK). Although numerous Eph receptors are expressed in the lens, thus far only *Epha2* null lenses have been shown to exhibit a phenotype (Cooper et al., 2008; Shi et al., 2012). Activated EphA2 increases the interaction between the adherens junction protein, N-cadherin, and β-catenin, promoting cell-cell adhesion and linkage to the actin cytoskeleton (Cooper et al., 2008). In addition, mutations in *EPHA2* is associated with age-related and congenital lens cataract in humans (Jun et al., 2009; Shiels et al., 2008). A number of studies have shown that the Fgfr and Eph signaling pathways interact. Dlg-1 may regulate fiber cell differentiation through its interaction with other proteins via the interaction of its PDZ domains with the PDZ binding motifs on its target proteins. There is no known PDZ binding motif in Fgf receptors, but there is in EphA2. For this reason, I addressed the hypothesis that

Dlg-1's regulation of Fgfr signaling is mediated through Dlg-1's interaction with EphA2. In Chapter IV, I assessed the impact of loss of *Dlg-1* on Fgfr1, Fgfr2, and EphA2 activity and the interactions between these proteins. Based on my data, I have identified a new role for Dlg-1 in regulating EphA2 activity and propose that Dlg-1 regulates Fgfr signaling via its interaction with EphA2.

In *Drosophila*, *van gogh* has been identified as a core planar cell polarity (PCP) protein. Mutations in *van gogh* exhibit considerable disorganization of the epithelial structures such as leg bristles, wing cell hairs, and eye ommatidia, which are classic structures governed by PCP (Taylor et al., 1998). The Griep lab previously has shown that *Dlg-1* and *Scrib*, the mouse homologs of *Drosophila* tumor suppressors, *dlg* and *scrib*, are required for mouse lens development. In addition, the lab has demonstrated that these genes interact with *Van Gogh-Like2* (*Vangl2)*, the mammalian homolog of *van gogh,* to regulate lens fiber shape and the architecture of the lens (S. Shatadal and A.E. Griep, manuscript in preparation). I hypothesized that the genetic interactions between *Dlg-1*, *Scrib*, and *Vangl2* not only affect fiber cell shape but also similarly affect Fgfr signaling. Therefore, I assessed the effect of single and compound mutations in these genes on the levels of Fgfr2 and Fgfr signaling intermediates. My data show that these mutations similarly affect Fgfr signaling, thus linking PCP genes and fiber cell shape to the regulation of Fgfr signaling, which is required for fiber cell differentiation. These results are described in Chapter V.

In the following sections I will cover additional background that provide the rationale for my studies and the interpretation of my results.

Early lens development

By embryonic day 8.5 (E8.5), the lens starts to be derived from the ocular surface ectoderm. The inductional signals from optic vesicle initiate lens development in the overlying ocular surface ectoderm, which undergoes localized thickening to form the lens placode (E10). At E10.5, the lens placode and the optic vesicle simultaneously invaginate into optic cup forming the lens pit (Piatigorsky, 1981). In this process, bone morphogenetic protein 7 (BMP7) (Wawersik et al., 1999) and fibroblast growth factor (FGF) receptor signalings (Faber et al., 2001) are required as well as transcription factors such as Prox1 and Sox2 (Lang, 2004). These signaling pathways and transcription factors cooperate to promote Pax6 expression. Pax6 is required throughout all stages of lens development and it is considered as master regulator of lens development (Lang, 2004). The lens pit subsequently detaches from the overlying ectoderm, creating a hollow lens vesicle. The overlying surface ectoderm gives rise to the corneal epithelium. The optic cup will eventually form the retina and the retinal pigmented epithelium. At E12.5, cells in the posterior of the lens vesicle withdraw from the cell cycle and elongate towards the anterior of the lens vesicle until they make contact with the epithelium, thereby filling the hollow vesicle. These are referred to as the primary fiber cells. These fiber cell synthesize β and γ crystallins and finally lose all membrane-bound organelles. Fiber cell differentiation continues as cells in the periphery of the epithelium in the region referred to as the transition zone, withdraw from the cell cycle, elongate and move toward the center of the lens and eventually lose their membrane bound organelles. These are called secondary fiber cells. Secondary fiber cell differentiation goes on for the life of the animal, albeit at progressively slower rates as postnatal age increases. The mature lens is comprised of the lens capsule, the lens epithelium, and differentiated lens fiber cells (McAvoy et al., 1999).

Figure I.2. Schematic diagram of early mouse lens and cornea development. At E8.5 the lens morphogenesis begins in the layer of ectodermal cells that overlie the optic vesicle. Inductional signals from the optic vesicle cause the ectodermal cells to thicken, forming the lens placode by E10. By E10.5, the lens placode starts to invaginate into the optic vesicle forming the lens pit. The optic vesicle forms the double layered optic cup. The optic cup will eventually form the retina. By E11.5, the lens pit has detached from the ectoderm, forming the lens vesicle. By E12.5 cells in the posterior of the lens vesicle differentiate into fiber cells and elongate to fill the vesicle. Eventually, a mature lens is formed containing a monolayer of epithelial cells overlying a mass of differentiated fiber cells.

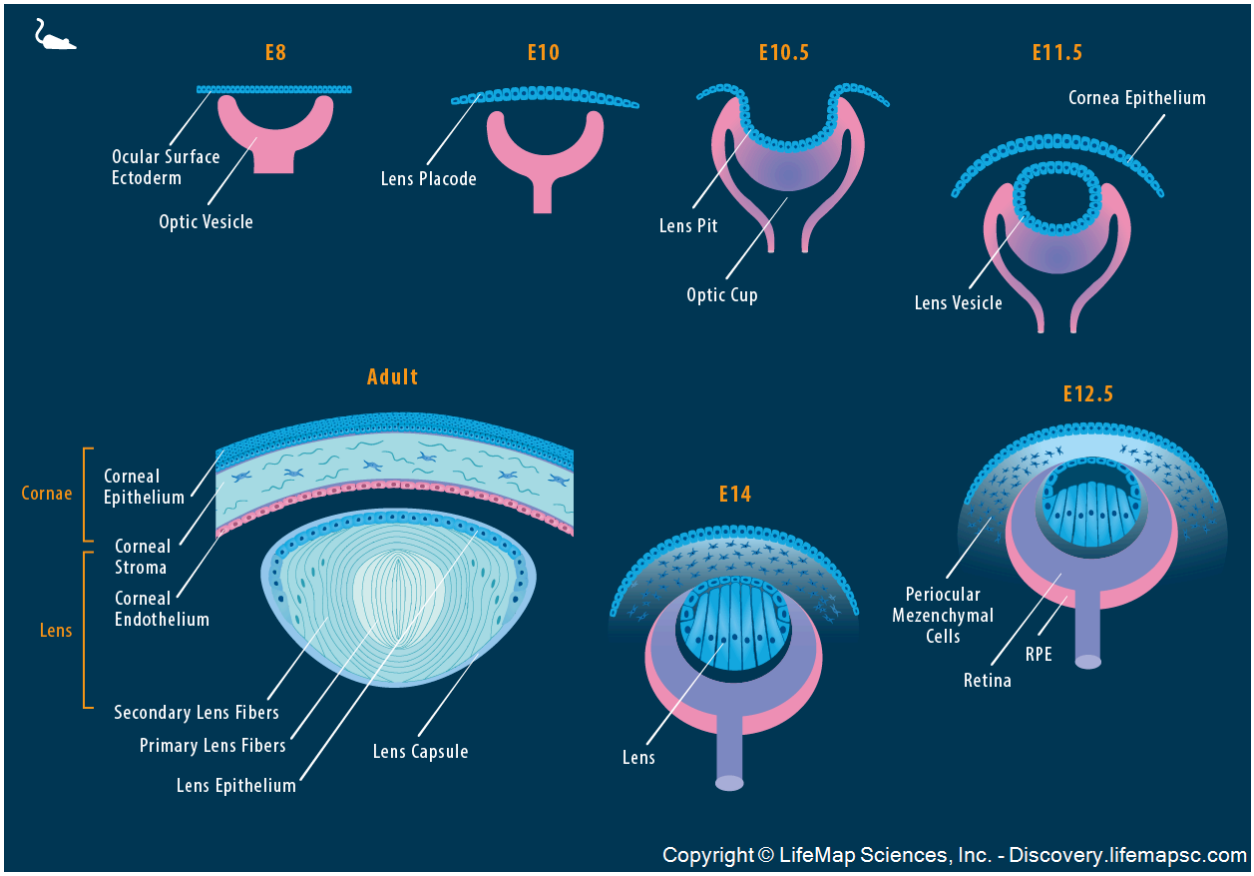


Figure I.2 (Image from http://discovery.lifemapsc.com)

General background on PDZ domain-containing proteins

Protein-protein interactions are key for building functional protein complexes. PDZ domains are modular protein-protein interaction domains that have a role in protein targeting and protein complex assembly. The name PDZ comes from the first three proteins in which these domains were found, PSD-95 (Postsynaptic density 95, PSD-95), Dlg (*Drosophila* discs large protein, Dlg), and ZO-1 (Zonula Occludens 1, ZO-1). PDZ domains consist of approximately 90 amino acid residues and identified as a region of sequence homology among a diverse signaling proteins. The PDZ domain also consists of two α-helices and six β-strands in a compactly arranged in a globular structure (Fan and Zhang, 2002; Hung and Sheng, 2002). PDZ domains have been identified in various proteins from organisms such as bacteria, plants, yeast, *Drosophila*, and mammals (Ponting, 1997; Ponting et al., 1997).

PDZ domain-containing proteins are often associated with cell membrane complexes where they function as scaffolding molecules. They play roles in directing the specificity of receptor tyrosine kinase-mediated signaling, in establishing cell polarity, and in directing protein trafficking (Dimitratos et al., 1999). PDZ proteins interact with their targets through the binding of the PDZ domain to the C-terminal PDZ binding motif of their target protein in a sequence-specific fashion. The specificity of PDZ domains is determined by the interaction of the first residue of helix αB and the side chain of the −2 residue of the C-terminal ligand (Hung and Sheng, 2002).

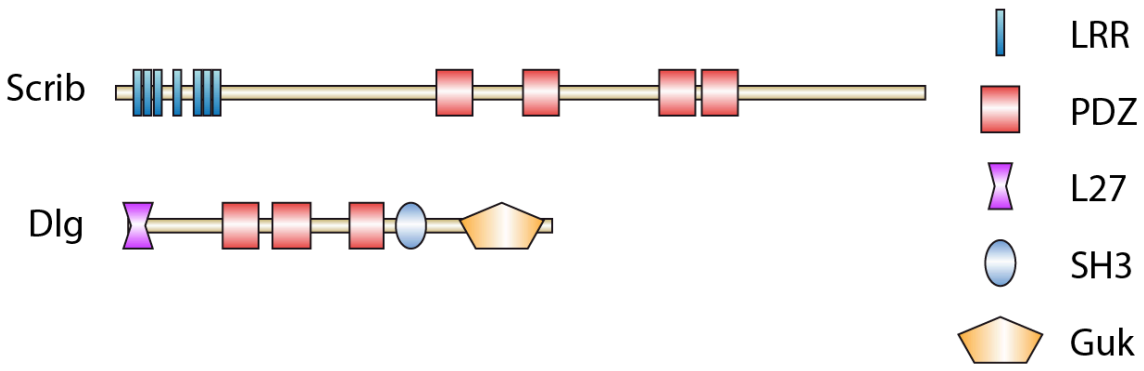
PDZ proteins frequently contain numerous PDZ domains often organized in tandem repeats. PDZ domains are found in a wide variety of proteins that are involved in protein-protein interactions such as the MAGUK superfamily and proteins that contain protein interaction

modules such as WW and LIM domains, sterile α-motif (*SAM*), ankyrin (*Ank*) and leucine-rich (*LRR*) repeats (Hung and Sheng, 2002).

PDZ domain-containing proteins, Dlg-1 and Scrib

The two PDZ domain-containing proteins studied in the Griep lab, Dlg-1 and Scrib, belong to two different families of PDZ proteins based on the other protein-protein interaction domains they contain. Dlg-1 is the founding member of a family of proteins known as membrane-associated guanylate kinases or MAGUKs. Members of this family are defined by three different protein interaction modules; the PDZ domains, an Src homology3 (SH3) domain, and an enzymatically inert guanylate kinase (GK) domain (Figure I.3). Scrib belongs to the LAP (Leucine rich and PDZ) family of PDZ proteins and contains 16 leucine rich-repeats (LRR) and four PDZ domains (Figure I.3). Dlg and Scrib associate in a complex in Drosophila that is referred to as the Scrib polarity complex. Genetic analyses of *dlg* and *scrib* in *Drosophila* have demonstrated that these genes play roles in biological processes including the regulation of proliferation, cell adhesion, apical-basal polarity and differentiation (Bilder, 2001, 2004; Bilder et al., 2000; Bilder and Perrimon, 2000; Harris and Lim, 2001; Johnson and Wodarz, 2003; Rivera et al., 2009). In vertebrates, *Dlg-1* and *Scrib* play a role in PCP, in addition to playing roles in cell proliferation, adhesion and apico-basal polarity (Montcouquiol et al. 2003 and Rivera et al 2013). Below I discuss roles for Dlg-1 and Scrib in cell adhesion, polarity, and interactions with a core PCP protein, Vangl2.

Figure I.3. Schematic diagram of the protein-protein interaction domains in Scrib and Dlg. Scrib belongs to LAP family of PDZ proteins. Scrib contains 16 leucine rich-repeats (LRR) and four PDZ domains. Dlg-1 belongs to the MAGUK family of PDZ proteins and contains three PDZ domains, an Src homology3 (SH3) domain, and the enzymatically inert guanylate kinase (GK) domain. (Bilder, 2001).



(Figure I.3. Figure modified from Kim and Sheng, 2004)

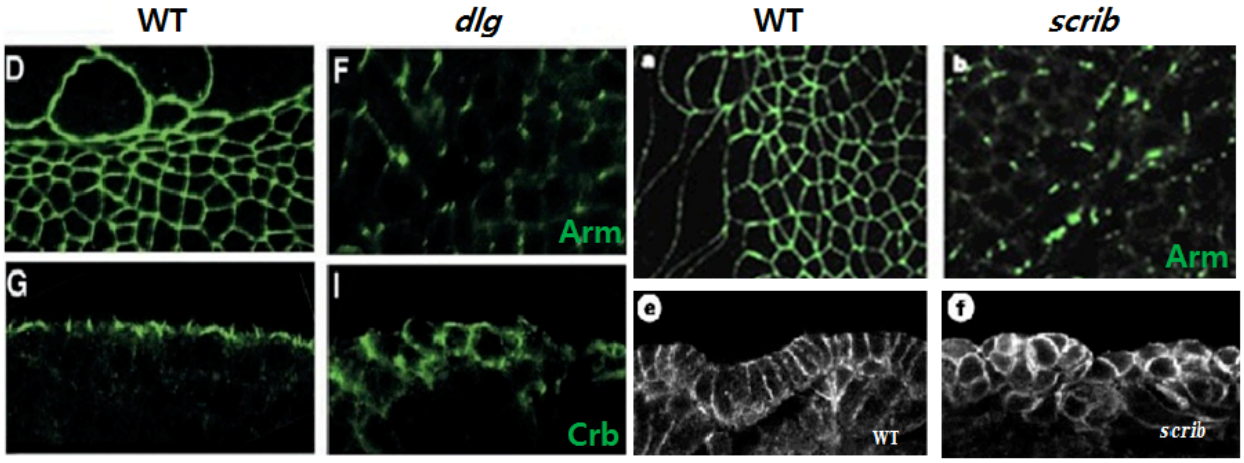
Role of PDZ domain-containing proteins in the cell-cell adhesion and polarity

Cell-cell adhesion is the mechanical force that drives cells into stable configurations during the assembly of tissues. Cell adhesion is linked to the cytoskeleton and subject to regulation by cell signaling. The adherens junction is an element of the cell–cell junction in which cadherin receptors bridge the neighboring plasma membranes via their interactions. Cadherins associate with cytoplasmic proteins, called catenins, which bind to cytoskeletal components, including actin filaments and microtubules (Gumbiner, 1996). The core components of adherens junctions are the transmembrane proteins, E-cadherin and N-cadherin, α-catenin and β-catenin (Armadillo in Drosophila, referred as Arm in Figure I.4.) (Humbert et al., 2003; Knust and Bossinger, 2002). In *Drosophila* and *C. elegans*, Dlg and Scrib have been demonstrated to play roles in maintaining and positioning the components of adherens junctions and apical determinants (Bilder et al., 2000; Bilder and Perrimon, 2000; Bossinger et al., 2001; Firestein and Rongo, 2001; Koppen et al., 2001; McMahon et al., 2001; Segbert et al., 2004; Woods et al., 1996). For example, in *Drosophila*, *dlg* deficiency results in acute disruption of epithelial structure with disorganization of intercellular junction formation and a loss of apico-basal cell polarity (Figure I.4). In *C. elegans*, DLG-1 is also required for proper adherens junction organization (Bilder et al., 2000; Bossinger et al., 2001; Firestein and Rongo, 2001). In *Drosophila, scrib* mutants show disruption of embryonic epidermis from a monolayered columnar epithelium to a multilayered pile of irregularly shaped cells and the apically localized transmembrane protein, Crb shows unrestricted localization in both apical and basolateral regions (Figure I.4) (Bilder et al., 2000; Bilder and Perrimon, 2000). Scrib and Dlg colocalize with another tumor suppressor, lethal giant larvae (Lgl) to form what is referred to as the Scrib polarity complex. Co-activity of these three factors is required for cortical localization of Lgl and

junctional localization of Scrib and Dlg. These three tumor suppressors, *scrib, dlg,* and *lgl,* show strong genetic interactions, in regulating cell polarity and growth control (Humbert et al., 2003; Humbert et al., 2008).

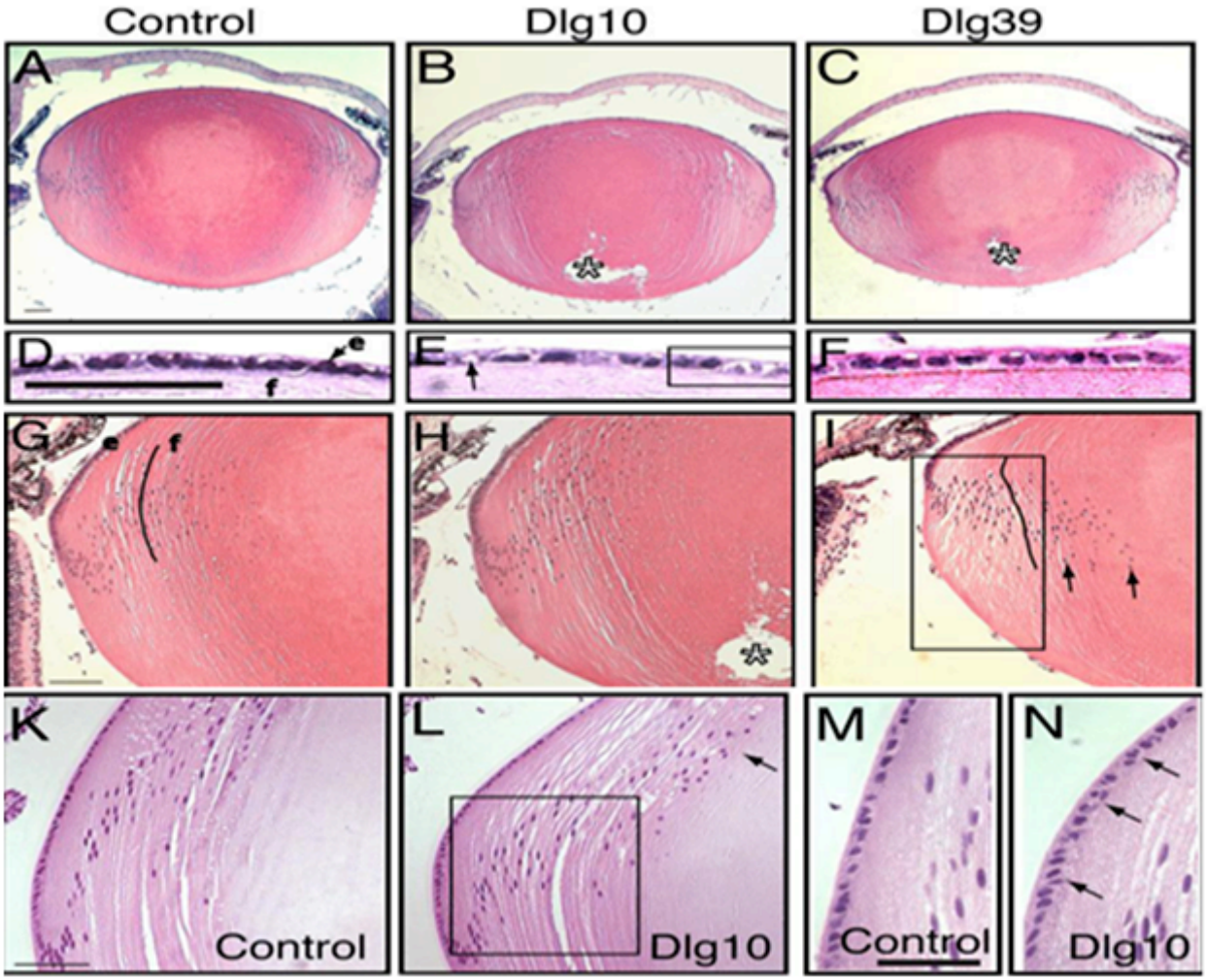
The roles for Dlg and Scrib in cell adhesion and polarity have been well characterized in *Drosophila* and *C. elegans*. In mammalian cell, the adherens junctions are highly conserved to *Drosophila,* and roles of *Dlg-1* and *Scrib* for localization of E-cadherin have been described (Qin et al., 2005; Reuver and Garner, 1998). Although mammalian *Dlg-1* and *Scrib* are known to be expressed in a variety of cell types, the functional roles for these proteins in adhesion and polarity is only recently been addressed. In mammalian studies have shown that *Dlg-1* is required for normal craniofacial and palatal morphogenesis (Caruana and Bernstein, 2001) and *Scrib* is also required for normal lung epithelial organization and lumen morphogenesis and by maintaining cell-cell contacts (Yates et al., 2013). The Griep lab has asked if the roles of *Dlg-1* and *Scrib* are conserved between *Drosophila* and mammalian development *in vivo* in the mouse. They have shown that *Dlg-1* and *Scrib* are required for multiple aspects of lens development. Deletion of *Dlg-1* in the lens disrupted cell adhesion, apico-basal polarity and altered the structure of both epithelial and fiber cells. (Figure I.5) (Rivera et al., 2009). *Scrib* deficiency results in shorter epithelial cell height, nuclei in the fiber cell compartment do not organize into the normal bow pattern but rather are scattered broadly, and lenses are small compared to the control (Figure I.6). Also *Scrib* has a role in maintaining N-cadherin at fiber cell membranes where it can participate in linkages with actin (I.F. Yamben Ph.D Thesis, 2010). Future studies will be needed to gain a deeper understanding of the role of these two PDZ proteins in the lens and other organs in mammalian species such as the mouse.

Figure I.4. *dlg* and *scrib* functions in *Drosophila*. (D, F, a, b) Confocal image for wild-type and *dlg* and *scrib* mutants stained for anti-arm and crb. Arm is the adherens junction marker Armadillo, which is the *Drosophila* homolog of mammalian β-catenin. Anti-Arm staining on wild type cellular blastoderms shows the normal `honeycomb' pattern, but anti-Arm staining on *dlg* and *scrib* mutants shows a severe disruption of the pattern. (G, I) In the wild-type, staining for the apical protein Crumbs (crb), is restricted in apical membrane, whereas in the *dlg* mutant, Crb staining is observed on all membranes of the cell, (e, f) Spectrin staining of cell membranes facilitates seeing the cell structure. In the wild type, the cells are columnar and form a monolayered epithelium. In the *scrib* mutant, cells have lost their rectangular structure and the epithelium has become multilayered.



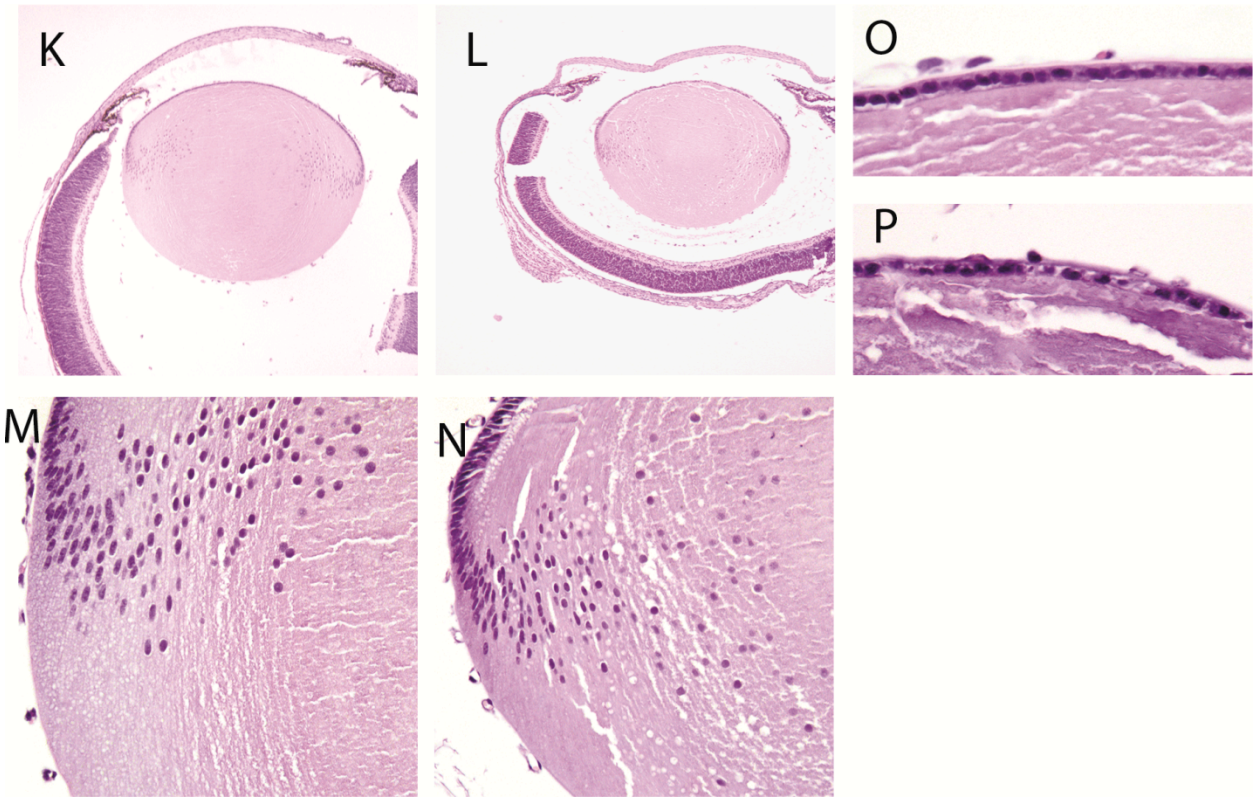
(Figure I.4. Figure modified from Bilder, 2000)

Figure I.5. Morphological defects in postnatal *Dlg-1* mutant lenses. A-C: Low magnification view of lenses from control (A), *Dlg10$^{f/f}$Cre* (B) and *Dlg39$^{f/f}$Cre* (C) P2 hematoxylin and eosin stained eye sections. The posterior suture defects shows in *Dlg10$^{f/f}$Cre* and *Dlg39$^{f/f}$Cre* lenses (asterisks). **D-F:** Higher magnification view of the epithelium from control (D), *Dlg10$^{f/f}$Cre* (E) and *Dlg39$^{f/f}$Cre* (F) P2 mice. The epithelium from the lenses of the *Dlg10$^{f/f}$Cre* mice show irregularities in the organization and appearance of the nuclei, vacuoles (arrow) and regions of loosely packed cells lacking nuclei (box). **G-I**: Higher magnification view of the transition zone in lenses of the control, *Dlg10$^{f/f}$Cre* and *Dlg39$^{f/f}$Cre* P2 mice. Nuclei did not form bow shape, but instead extended into the posterior region of the lens. Additionally, fibers did not maintain their normal "C" shaped curvature (black lines in G and I). Nuclei near the transition zone in the *Dlg$^{f/f}$10Cre* lenses fail to migrate interiorly. **K-L:** View of the transition zone from control (K) and *Dlg10$^{f/f}$Cre* (L) P24 mice. Compared to the tight bow region of the control lenses, the bow region in the lenses of the *Dlg10$^{f/f}$Cre* mice was broader and nuclei had failed to migrate anteriorly (box). Denucleation appeared delayed, as the cortical fiber region was expanded and rounded nuclei were observed encroaching on the center of the lens (arrow). **M-N:** High magnification of the epithelium in the bow region of control (M) and *Dlg10$^{f/f}$Cre* (N) mice. Compared to controls, the cells in the epithelium of the *Dlg10$^{f/f}$Cre* lenses were disorganized and irregularly shaped and the epithelium sometimes appeared to be multilayered (arrows). Scale bar=100 μm in A-C, G-I, K-L and 50 μm in D-F, M-N.



(Figure I.5. Figure modified from Rivera et al., 2009)

Figure I.6. Morphological defects in postnatal *Scrib10$^{f/f}$Cre* mutant lenses. K-L: Low magnification view of lenses from control (K) and *Scrib10$^{f/f}$Cre* (B) P2 hematoxylin and eosin stained eye sections. The *Scrib10$^{f/f}$Cre* lens is noticeably smaller than the control lens. **M-N:** Higher magnification of the transition zone region from control (M) and *Scrib10$^{f/f}$Cre* (N) lenses. The nuclei in the transition zone of the *Scrib10$^{f/f}$Cre* lens are scattered and disorganized as compared to the control and nuclei are found encroaching on the organelle free zone in the center of the lens. The curvature of the fiber cells in the *Scrib10$^{f/f}$Cre* lens was not the normal "C" shape resulting in distortion of the overall structure of the lens. **O-P:** High magnification view of the epithelium from control (O) and *Scrib10$^{f/f}$Cre* (P) lenses. The epithelium in the *Scrib10$^{f/f}$Cre* lens appeared flatter than in the control and some nuclei were missing, suggesting that structure of the epithelium was disorganized and/or cell death was occurring.



(Figure I.6. Figure modified from S. Shatadal and A.E. Griep, manuscript in preparation)

Interactions between Dlg-1 and Scrib with a core planar cell polarity (PCP) protein, Vangl2

Planar cell polarity (PCP), the polarization of cells in an epithelial sheet perpendicular to the orientation of apical-basal polarity, has been thought to be critically required for regulating changes in cell shape and the coordinated movement of cells that form the overall shape of an organ (Singh and Mlodzik, 2012; Wallingford, 2012). The framework of PCP was originally built in *Drosophila* where it was shown to be governed by a group of core genes that belong to the Wnt/PCP pathway. Genetic screening in *Drosophila* have identified several core PCP genes, including *Frizzled* (*Fz*), *Flamingo* (*Fmi*), *Van Gogh* (*Vang*), *Dishevelled* (*Dsh*), *Prickle* (*Pk*), and *Diego* (*Dgo*) (Feiguin et al., 2001; Singh and Mlodzik, 2012; Taylor et al., 1998; Wallingford and Habas, 2005). These proteins are asymmetrically distributed on the membranes of the cells and establish a polarizing signal across the epithelial sheet. Mammalian developmental processes such as orientation of sensory hair cells in the cochlea of the inner ear and neural tube closure also require PCP genes (Rida and Chen, 2009; Wang and Nathans, 2007). Hair cell orientation in the cochlea and neural tube closure are regarded as sensitive readouts for PCP in mammalian development.

Vangl is a component of the non-canonical Wnt/PCP pathway, which is implicated in diverse cell polarity functions, and is involved in early morphogenesis and patterning of cells. Vangl proteins are well conserved with relatives found in flies *(Vangl/Stbm)*, frogs *(Xstbm)*, and fish *(trilobite/Vangl2)* (Darken et al., 2002; Jessen et al., 2002; Taylor et al., 1998; Wolff and Rubin, 1998). Studies on Vangl in these organisms have led the way to studies on the function of mammalian Vangl during *in vivo* morphogenesis.

In *Drosophila*, mutations in *van gogh (vang)/strabismus (stbm)* cause notable disruption of epithelial structures, including the hairs (trichome) on the wing cells, the bristles on the legs, and the eye units, called ommatidia (Taylor et al., 1998). Plasma membrane formation is dependent on the interactions between Vang/Stbm and Dlg through the binding of Vang's C-terminal PDZ-binding motif to the PDZ domains of Dlg. Vang/Stbm localized to a specific membrane compartment efficiently recruits Dlg, whereas a mutant *Vang/Stbm* in which the C-terminal PDZ binding motif is deleted eliminates recruitment of Dlg (Lee et al., 2003). In addition, Scrib is also required for epithelial polarity and has been demonstrated to interact with Vangl2, where Vangl2 through its C-terminal PDZ binding motif binds to specific PDZ domains of Scrib (Kallay et al., 2006; Yoshioka et al., 2013). In zebrafish and Xenopus, cilia-mediated nodal flow results in altered left-right asymmetry in *Vangl2* mutants (Antic et al., 2010; Borovina et al., 2010; May-Simera et al., 2010).

In mouse embryogenesis, *Vangl2* plays a role in regulating the orientation of stereociliary bundles in the cochlea, a distinctive example of PCP in the mouse. In addition, *Vangl2* mutations cause defects in eyelid and neural tube closure, defects in lung and kidney development, and also a reduced size of brain size caused by premature differentiation of neuronal progenitors (Wang et al., 2005; Yates et al., 2010a; Yates et al., 2010b). These studies have demonstrated that *Vangl2* plays a role in establishing planar cell polarity in epithelial cells and regulating convergent extension movements during mammalian embryogenesis. Interestingly, in the mouse *Scrib* genetically interacts with the *Vangl2* to regulate of the orientation of the sterociliary bundles in the cochlea (Montcouquiol et al 2003), indicating that in the mouse *Scrib* is also a PCP gene, unlike in *Drosophila* where is has not been identified as a PCP gene. The Griep lab has shown that loss of *Dlg-1* resulted in defects in eyelid closure and neural tube closure, and the

disorganization of the stereociliary bundles of the cochlear hair cells (Rivera et al., 2013), thus identifying *Dlg-1* as a PCP gene in the mouse. Interestingly, the lenses of mice carrying a *Vangl2* gene have a flattened shape and defects in fiber cell curvature and suture formation, phenotypes also exhibited in the *Dlg-1* deficient lens (Rivera et al., 2009; Sugiyama et al., 2009; Sugiyama et al., 2011). In Chapter V, I address the possibility that the genetic interactions between *Dlg-1, Scrib*, and *Vangl2,* modulate the levels of Fgfr signaling and downstream intermediates in accordance with their modulation of fiber cell structure.

Growth factors that regulate lens development

The lens has a distinctive polarity and is located within the surrounding ocular media. The aqueous humor bathes the lens epithelial cells and the vitreous humor bathes the lens fiber cells. The vitreous humor contains a myriad of growth factors including, insulin-like growth factor (IGF), epidermal growth factor (EGF), fibroblast growth factor (FGF), bone morphogenetic proteins (BMPs), vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), and many other growth factors from vitreous humor or aqueous humor are required for lens development (Cheng and Gong, 2011; Lovicu et al., 2011; Wang et al., 2010) .

The lens epithelial explant system has been used extensively to identify growth factor signaling pathways that regulate lens cell proliferation and differentiation. For example, in rat explants FGF has been shown to be mitogenic for lens epithelial cells and to induce all aspect of lens fiber differentiation (Liu et al., 1996; Wang et al., 2010). IGF has also been shown to act in a synergistic fashion with EGF to be promote proliferation in rabbit lens explants (Reddan and Wilson-Dziedzic, 1983). Studies in chick lens explants have demonstrated roles for IGF (Beebe

et al., 1987), TGF-β (Chen et al., 2001; Ireland and Mrock, 2004), and BMP (Belecky-Adams et al., 2002) in promoting the fiber cell differentiation. Human lens epithelial cells have enhanced proliferation rate and increased Erk and Jnk kinase activities after treatment with HGF (Choi et al., 2004). PDGF-A (Reneker and Overbeek, 1996) or TGFβ-1 (Lovicu et al., 2004) overexpression in transgenic mice resulted in the expression of the fiber specific β crystallin expression in the disrupted lens epithelial cells. However, *in vitro* studies have shown that neither of these growth factors can directly induce lens fiber differentiation (Kok et al., 2002; Lovicu et al., 2004).

Although a number of growth factors have been reported to influence or support lens fiber differentiation, transgenic studies overexpressing many of different growth factors demonstrated that only Fibroblast growth factor (FGF) can induce differentiation (Lovicu and Overbeek, 1998). The results of all of these studies highlight the importance of the Fgfr signaling pathway in regulating fiber cell differentiation. This led me to focus on the hypothesis that *Dlg-1* affects fiber cell differentiation by modulating Fgfr signaling for my thesis project. I discuss details about roles of Fgf/Fgfr signaling in development in the following section.

Fibroblast growth factor receptor signaling

The Fgfr signaling pathway is known to have various biological functions such as regulating cellular proliferation, survival, migration and differentiation. Fgf receptors contain an extracellular ligand-binding domain, a transmembrane region and a highly conserved intracellular tyrosine kinase signaling domain. Signaling downstream of the receptor proceeds through two main pathways (1) via the intracellular receptor substrates Fgfr substrate 2 (Frs2)

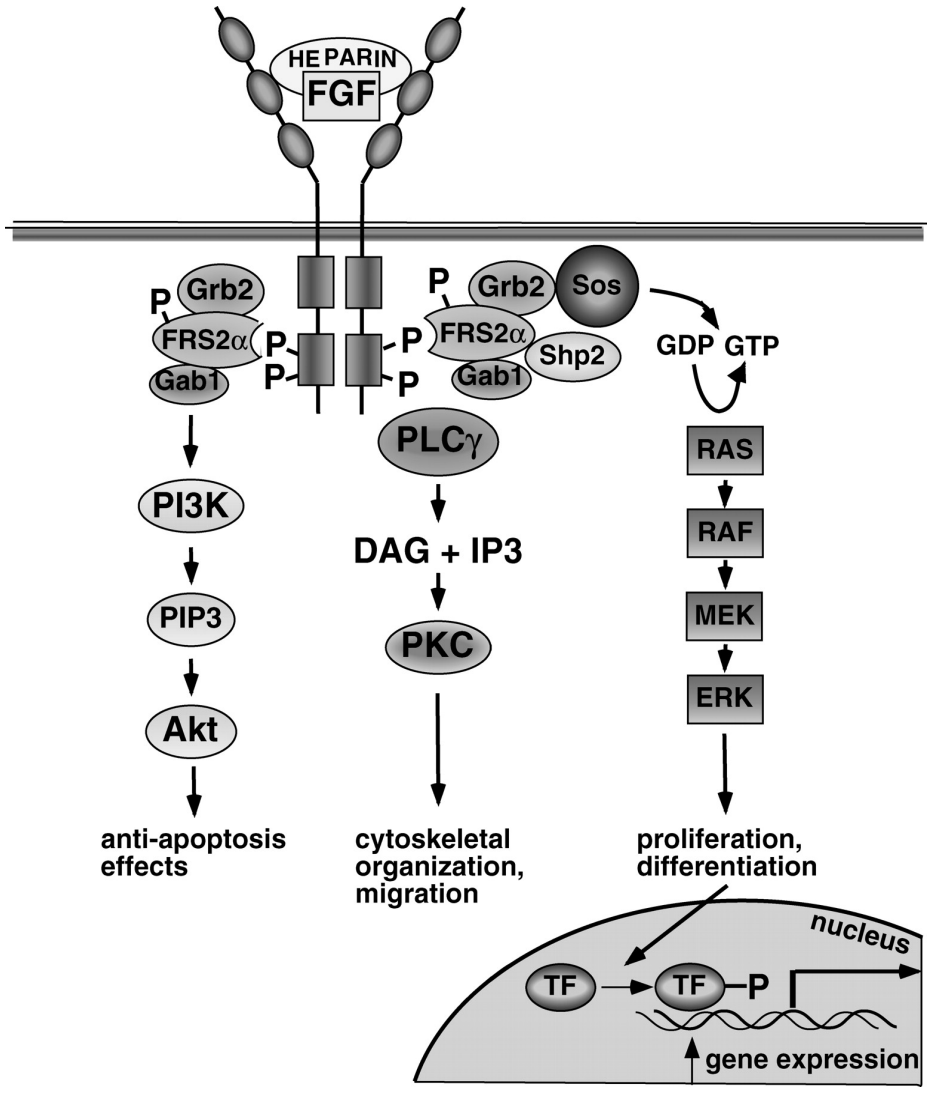
and (2) via phospholipase Cγ (PLCγ). Frs2 is then able to trigger two pathways, the Ras-dependent mitogen-activated protein kinase (MAPK) and Ras-independent phosphoinositide 3-kinase (PI3K)–Akt signaling pathways. Other pathways can also be activated by Fgfrs, including the signal transducers and activator of transcription (STAT) pathway (Figure I.7) (Eswarakumar et al., 2005; van der Noll et al., 2013).

Studies of Fgf/Fgfrs have shown Fgfrs are important in the development of many organs. For example, Fgfr2 and Fgfr3 can negatively regulate proliferation and positively induce differentiation in the development of membranous and endochondral bone (Schmahl et al., 2004; Yu et al., 2003). Gain of function germline mutations in the Fgfr genes shows skeletal dysplasias, with *Fgfr1* mutation causes Pfeiffer syndrome, *Fgfr2* mutation causes craniosynostosis syndromes, and *Fgfr3* mutation results in chondrodysplasia syndromes (Muenke et al., 1994; Ornitz and Marie, 2002). In the kidney, loss of *Fgfr1* interrupts nephron formation (Gerber et al., 2009) and *Fgfr2* deletion in the metanephric mesenchyme results in kidney and urinary tract anomalies, including vesicoureteral reflux (Hains et al., 2010). Gain of function mutations in *Fgfr3* leads to thanatophoric dysplasia, which is associated with cystic dysplasia or renal hypoplasia (Prontera et al., 2006).

Based on previous studies, at least four Fgf receptors are expressed in the mouse embryonic lens from four genes: *Fgfr1*, *Fgfr2, Fgfr3*, and *Fgfr4* (de Iongh et al., 1996; Garcia et al., 2005; Matsuo, 1993; Robinson, 2006). In seminal studies using the rat explant system, John McAvoy's group demonstrated that a low dose of Fgf can initiate cell proliferation, a medium dose can result in migration, and higher dose of Fgf can induce fiber differentiation (Figure I.8) (McAvoy and Chamberlain, 1989). These data led to the hypothesis that lens cell behavior is regulated by a gradient of Fgfr stimulation. Several experiments have shown that Fgf/Fgfr

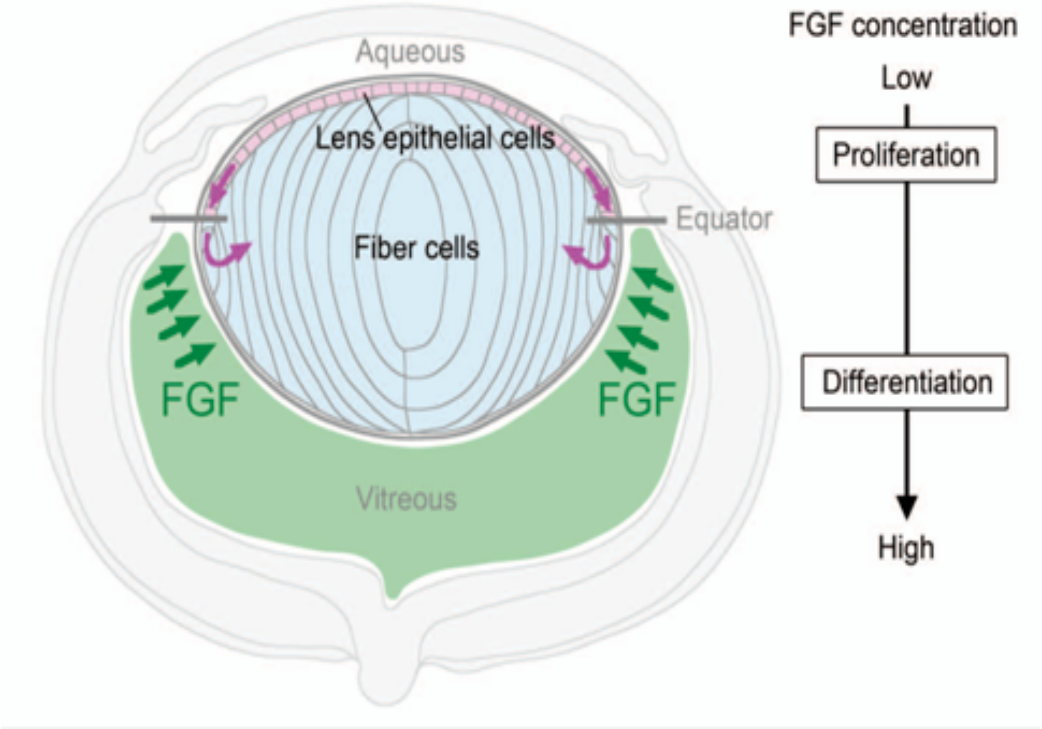
signaling is required for lens growth and differentiation. Blocking Fgf induced of fiber cell differentiation with pharmacological inhibitors of Fgf signaling in the epithelial explant system blocked the Fgf induced activation of Erk ½ (Lovicu and McAvoy, 2001). *In vivo,* experiments using transgenic mice overexpressing dominant-negative inhibitors of Fgf receptors have shown that these receptors are required for lens fiber differentiation (Chow et al., 1995; Robinson et al., 1995; Stolen and Griep, 2000). The clearest evidence for the requirement for Fgfrs in fiber cell differentiation is that conditional and simultaneous gene ablation of three *Fgfrs* (*Fgfr1-3*) resulted in lenses where fiber cell differentiation is completely arrested. These arrested lens vesicles showed an absence of fiber elongation, abnormal proliferation, and aberrant or reduced expression of crystallins, the transcription factors Prox1, Pax6, c-Maf, and the cyclin dependent kinase inhibitors, p27 and p57 (Zhao et al., 2008). In addition, *Fgfr2* is required in the early stages of lens development, at the lens placode stage, to allow lens cells to exit from the cell cycle, for lens epithelial cell survival (Garcia et al., 2005) and *Fgfrs 1* and *2* are required in the placode for expression of Fgfr target genes Erm and ER81 (Garcia et al., 2011). Together, all of these experiments demonstrate that Fgfrs are key regulators of cell fate and play pivotal roles in the lens by influencing lens induction, growth, and differentiation.

Figure I.7. Fgf/Fgfr signaling cascades. Fgfs induce dimerization, kinase activation and transphosphorylation of tyrosine residues of Fgfrs, leading to activation of downstream signaling pathways. Multiple pathways are stimulated by phosphorylated Fgfrs, including Ras-MAP kinase, PI-3 kinase/Akt and PLC-γ pathways. Fgf/Fgfr signaling plays a role in the regulation of differentiation, proliferation, and apoptosis via these downstream signaling pathways.



(Figure I.7. Figure modified from Zhang et al., 2003)

Figure I.8. Lens proliferation and differentiation are regulated by a gradient of Fgfr stimulation. Based on *in vitro* studies in the rat explant system, McAvoy proposed that a low dose of Fgf can stimulate cell proliferation of lens epithelial cells, a medium dose can result in migration, and higher dose of Fgf can induce fiber cell differentiation.



(Figure I.8. From Sugiyama et al., 2011)

Eph/Ephrin signaling

Eph receptors form the largest subfamily of receptor tyrosine kinases (RTK). The Eph family of receptor tyrosine kinases can be divided into two subclasses and includes 16 different members, EphA (1-10) and EphB (1-6) kinases. EphA receptors usually bind glycosyl-phosphatidylinositol (GPI)- linked ephrin-A ligands while EphB receptors bind transmembrane-bound ephrin-B ligands based on their binding affinity. Each receptor binds with multiple ligands and vice versa. (Himanen, 2012; Himanen et al., 2004). The cytoplasmic domain of Eph receptors is composed of a juxtamembrane region with two conserved regulatory tyrosine residues that control kinase activity, a single tyrosine kinase domain, a sterile alpha motif (SAM), and a PDZ-binding motif (Figure I.9) (Himanen, 2012; Kullander and Klein, 2002). Binding of an ephrin ligand to the extracellular globular domain of an Eph receptor leads to Eph phosphorylation and the activation or suppression of downstream signaling cascades. (McClelland et al., 2010)

Eph/ephrin signaling has emerged as one of the key regulators of cell-cell adhesion and coordinator of not only embryonic developmental processes but homeostasis of mature organs (Davy et al., 1999; Holland et al., 1996). Ephs/ephrins can lead to cell migration and positioning by initiating assembly of cell-cell contacts. During embryonic development, Ephs/ephrins are expressed in developing tissues and organs, where they have roles in gastrulation, organogenesis and tissue patterning in systems such as the skeletal, vascular, and nervous systems (Lackmann and Boyd, 2008; Merlos-Suarez and Batlle, 2008; Pasquale, 2008). Their various biological functions are achieved by modulating the adhesion or segregation between Eph expressing cells

and cells expressing their membrane-bound ephrins. In addition, Eph activation affects cell proliferation and apoptosis. (Genander and Frisen, 2010; Pasquale, 2010).

In the lens, *EphA2* mutations lead to age-dependent cortical cataracts in humans and mice (Jun et al., 2009; Kaul et al., 2010; Shiels et al., 2008; Tan et al., 2011). *EphA2*, which functions in the Eph-ephrin bidirectional signaling pathway of mammalian cells, plays a role in maintaining lens transparency. EphA2 is expressed in the cortical lens fiber cells (Cooper et al., 2008; Jun et al., 2009; Shi et al., 2012) and has been shown to be required for recruiting β-catenin to the adherens junction protein, N-cadherin, thereby promoting linkage to the cytoskeleton and cell-cell adhesion (Cooper et al., 2008). Cataract formation in *ephrin-A5* or *Epha2* mutant lenses is associated with the alteration of adherens junctions and the disruption of fiber cell organization (Cooper et al., 2008).

RTK signaling pathways are highly interconnected and they share prominent cytoplasmic signaling cascades, including the MAPK or AKT pathways (Kholodenko et al., 2010). A number of studies have shown interaction between Eph/ephrin and Fgfrs. For example, EphA4 and FGFR have been shown to trans-phosphorylate each other and potentiate downstream signaling, MAPK activation, although this does not require direct binding of Fgfr and Eph receptor (Fukai et al., 2008; Park et al., 2004; Yokote et al., 2005). In addition, interplay between these two RTK signaling pathways is involved in regulating cell fate determination and asymmetric cell division in *Ciona* embryos (Picco et al., 2007). In this report, activated Eph receptor inhibits Erk activation whereas Fgfr signaling promotes Erk activation. Thus, the authors propose that Fgfr signaling and Eph/ephrin signaling antagonistically regulate developmental processes (Picco et al., 2007). In addition, FGFR1 also seems to inhibit ephrin-dependent EphB2 by blocking a positive feedback loop via downregulation of the Ras-Erk pathway while enhancing the

phosphorylation of EphB2 (Poliakov et al., 2008). Further studies of the crosstalk between Ephs and Fgfrs will be needed for a better understanding of role that the interaction between these pathways plays in development *in vivo*.

Figure I.9. Structure of ephrin and Eph receptors. Eph receptors have an extracellular region, that contains an ephrin-binding domain and two fibronectin type III repeats. Eph receptors have an intracellular region that contains a tyrosin kinase domain, a SAM domain, and a PDZ binding domain. EphrinA ligands bind to the extracellular cell membrane with GPI anchor. EphrinB ligands are transmembrnae proteins with cytoplasmic tail and PDZ binding domain.

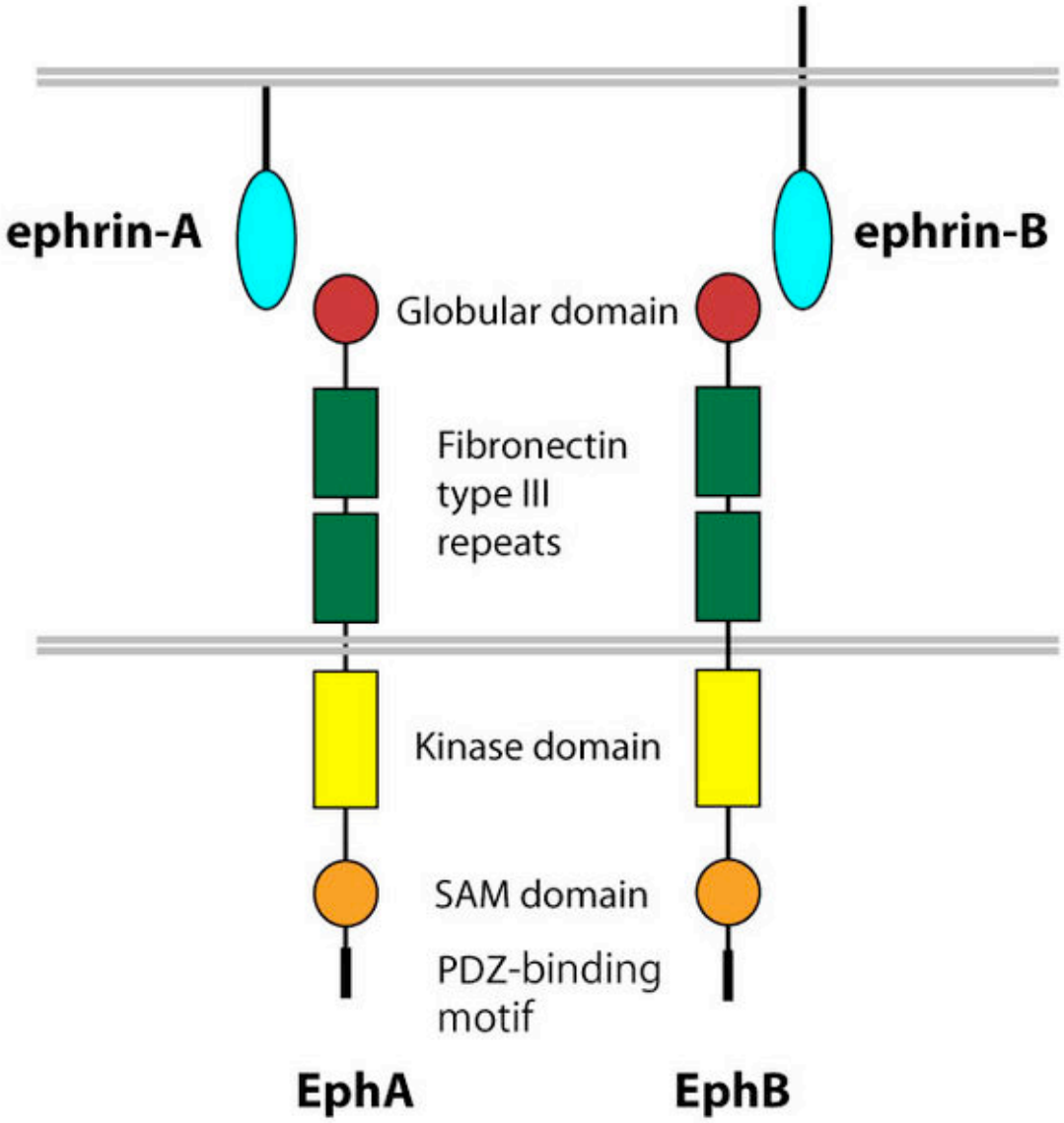


Figure I.9 (Image from http://bsd.neuroinf.jp)

Summary

Overall, this thesis represents my studies in which I address the hypotheses that *Dlg-1* regulates Fgfr signaling cascades via EphA2, and that *Dlg-1* and *Scrib* through their interaction with *Vangl2* maintains lens cell structure which is necessary for normal Fgfr signaling. Chapter II contains the Materials and Methods used in these experiments. Chapter III presents work demonstrating that *Dlg-1* is a modulator of Fgfr signaling pathway at the level of the receptors and suggests that Dlg-1 regulates fiber cell differentiation through its role in PCP. In Chapter IV, I present work demonstrating that *Dlg-1* regulates EphA2 function based on which I suggest that the interaction between with Dlg-1 and EphA2 mediates the effect of *Dlg-1* on Fgfr signaling and adherens junction formation. In Chapter V, I show that *Dlg-1* and *Scrib* may act in opposing ways with respect to *Vangl2* in modulating Fgfr signaling in ocular lens. Finally, in Chapter VI, I summarize my findings and discuss their significance. In addition, I suggest future experiments that may support my findings and further elucidate the roles of *Dlg-1, Scrib*, and *Vangl2* together with signaling pathways, including Fgf receptor signaling and Eph/ephirin signaling, in lens development.

CHAPTER II: Materials and Methods

To avoid reiteration the materials and methods used in the following chapters, these have been compiled into this chapter.

Generation of *Dlg$^{f/f}$10Cre* and *Dlg$^{f/f}$ 39Cre* mutant mice

Mice carrying a conditional allele of *Dlg-1* were generated initially in collaboration with Sara J. Simonson and Paul F. Lambert (University of Wisconsin, Madison) and the Transgenic Animal Facility (University of Wisconsin Biotechnology Center) (Lee and Griep, 2014; Rivera et al., 2009). The Cre driver transgenic mice, *MLR10Cre* and *MLR39Cre* (Zhao et al., 2004), were generated in the laboratory of Dr. Michael Robinson (Miami University, Oxford, OH) and have been described previously. *Dlg-1$^{f/f}$* mice were crossed to either *MLR10Cre* or *MLR39Cre* mice to generate *Dlg$^{f/}$;MLR10Cre* or *Dlg$^{f/+;}$MLR39Cre* mice and these mice were then mated to *Dlg-1$^{f/+}$* mice to generate *Dlg$^{f/f}$;MLR10Cre* mice (here after referred to as *Dlg$^{f/f}$10Cre*) or *Dlg$^{f/f}$;MLR39Cre* mice (here after referred to as *Dlg$^{f/f}$39Cre* mice) such that all progeny positive for *MLR10Cre* or *MLR39Cre* had received only a single copy of the *MLR10Cre* or the *MLR39CRE* transgene. To confirm Cre mediated deletion, genotyping was carried out on DNA isolated from tail using primer Dlg-1 3′, Dlg-1 5′ and Anti-cre primers (Table II. I) Details about lens specific Cre mediated deletion were described previously (Rivera et al., 2009; Zhao et al., 2004). Loss of Dlg-1 protein following cre-mediated excision of *Dlg-1* sequences was confirmed by immunofluorescent staining for Dlg-1 using a mouse anti-rat SAP97 (Novus Cat# NBP1-48054) at 1:500 dilution.

Generation of *Dlg$^{f/f}$;Fgfr1$^{f/+}$10Cre* and *Dlg$^{f/f}$;Fgfr2$^{f/+}$10Cre* $^{+}$ mutant mice

The generation of mice carrying conditional alleles of *Dlg-1$^{f/f}$* mice have been described above. Mice carrying conditional null alleles of *Fgfr1* and *Fgfr2* (referred to as *Fgfr1^{flox}* and *Fgfr2^{flox}*) were obtained from Dr. Xin Sun (University of Wisconsin-Madison). *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 on tail DNA using primers Fgfr1 flox Sun3, Fgfr1 flox Sun4 for *Fgfr1*^{flox} and Fgfr2 flox Fr439 and Fgfr2 flox Fr639 for *Fgfr1*^{flox} or *Fgfr2*^{flox}, respectively, to confirm specific cre mediated deletion (Table II.I).

Generation of *Dlg*$^{f/+}$*10Cre;Vangl2*$^{Lp/+}$ and *Scrib*$^{f/+}$*10Cre;Vangl2*$^{Lp/+}$ mutant mice

Dlg-1 or *Scrib* conditional null mice were crossed with *MLR10Cre* mice and the progeny crossed with *Vangl2*$^{Lp/+}$ mice to generate *Dlg*$^{f/+}$*10Cre;Vangl2*$^{Lp/+}$ and *Scrib*$^{f/+}$*10Cre;Vangl2*$^{Lp/+}$ day E16.5 embryos. Embryos containing the floxed *Dlg-1* allele and the *MLR10Cre* transgene were identified as described above. Mice carrying a point mutation in *Vangl2*, referred to as *Vangl2*$^{Lp/+}$ mice were obtained from Dr. Rivka A. Rachel (National Institutes of Health). To identify *Scrib* floxed containing progeny, primers ScribF5'and Scrib1-X2-Rev were used and primer Lp-F1 and Lp-R1 were used for identifying *Vangl2* (Table II. I).

Table II.1 Oligonucleotide PCR primers

Name	Sequence and description
Pr4	5'GCA TTC CAG CTG CTG ACG GT 3' 5' primer for the mouse *αACry* promoter for MLR10Cre and MLR39Cre (Zhao et al., 2004)
Anti-Cre	5'CAG CCC GGA CCG ACG ATG AAG 3' 3' primer for *Cre recombinase* (Zhao et al., 2004)
Dlg-1 3'	5' GGA AGG AAA CTC ACG GAT GGT CC 3' 3' primer for intron 9 of mouse *Dlg-1*
Dlg-1 5'	5' CAT CAT GGT TGA AGT GCT CTG GGC 3' 5' primer for exon 7 of mouse *Dlg-1*
Dlg-1 I8R	5' AAA TGT GGC CTG AGG ATC TAC CTC CG 3' 3' primer for intron 8 of mouse *Dlg-1*
Fgfr1 flox Sun3	5' CTA GTA TCC TGT GCC TAT C 3' 5' primer for $Fgfr1^{flox}$ (Xu *et al*, 2004)
Fgfr1 flox Sun4	5' CAA TCT GAT CCC AAG ACC AC 3' 3' primer for $Fgfr1^{flox}$ (Xu *et al*, 2004)
Fgfr2 flox Fr439	5' TTC CTG TTC GAC TAT AGG AGC AAC AGG CGG 3' 5' primer for $Fgfr1^{flox}$ (Yu *et al*, 2003)
Fgfr2 flox Fr639	5' GAG AGC AGG GTG CAA GAG GCG ACC AGT CAG 3' 3' primer for $Fgfr1^{flox}$ (Yu *et al*, 2003)
ScribF5'	5' GCA CAC TGG GTA TCA TGG CTA 3' 5' primer upstream of exon 1 of mouse *Scrib* (Yamben et al., 2013)
Scrib1-X2-Rev	5' CAC CAG CTG CAT GAA GTT 3' 3' primer for intron 8 of mouse *Scrib* (Yamben et al., 2013)
Lp-F1	5' CCTTCCTGGAGCGATATTTG 3' 5' primer for mouse $Vangl2^{Lp}$ (R. Rachel, personal communication)
Lp-R1	5' CTGCAGCCGCATGACGAACT3' 3' primer for mouse $Vangl2^{Lp}$ (R. Rachel, personal communication)

Tissue Preparation

Heads from embryonic day 12.5 (E12.5), E13.5, E15.5 and E17.5 control (*Dlg-1*$^{f/f}$), *Dlg*$^{f/f}$*10Cre*, and *Dl*$^{f/f}$*39Cre* embryos were fixed in 4% paraformaldehyde overnight at 4ºC, washed in 1X phosphate buffered saline (PBS), dehydrated in increasing concentrations of ethanol, and embedded in paraffin oriented for longitudinal sectioning. Eyes of P2 eyes control, *Fgfr1*$^{f/+}$*10Cre, Fgfr2*$^{f/+}$*10Cre, Dlg*$^{f/f}$*;Fgfr1*$^{f/+}$*10Cre* and *Dlg*$^{f/f}$*;Fgfr2*$^{f/+}$*10Cre* mice were similarly fixed and embedded in paraffin. Heads from day E16.5 *Dlg*$^{f/+}$*10Cre*, *Scrib*$^{f/+}$*10Cre*, *Vangl2*$^{Lp/+}$, *Dlg*$^{f/+}$*10Cre;Vangl2*$^{Lp/+}$ and *Scrib*$^{f/+}$*10Cre;Vangl2*$^{Lp/+}$ embryos were similarly fixed and embedded in paraffin. Sections (5μm) 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 4^0C, incubated in 10% and 20% sucrose for 1.5 hour each at 4^0C and then in 30% sucrose overnight at 4^0C and embedded in TissueTek oriented for transverse sectioning. Cryogenic sections (10μm) were cut and used for immunofluorescence.

Western Blot Analysis

Fiber cells were dissected from postnatal day 2 (P2) control, *Dlg*$^{f/f}$*10Cre, Dlg*$^{f/f}$*39Cre* mice and protein lysates were prepared by extraction in RIPA buffer with protease inhibitors. A total of 50-100μg 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, rabbit anti-human ERM (Santa Cruz Cat# SC-22807) at 1:1000 dilution, rabbit anti-human-β-catenin (Sigma Cat# C2206) at 1:100 dilution, rabbit anti human- active β-catenin (Millipore Cat# 05-665) at 1:100 dilution, rabbit anti-human-Fgfr1 (Santa Cruz Biotechnology Cat# SC-7945) at 1:100 dilution, rabbit anti-human-Fgfr2 (Santa Cruz Biotechnology Cat# SC-122) at 1:100 dilution, and goat anti-mouse EphA2 (BD Transduction Laboratories Cat# AF639) antibodies at 1:100 dilution were incubated overnight at 4°C. Membranes were washed in 1X PBST or 1X TBST and were then 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 *Dlg$^{f/f}$10Cre* lenses were lysed in Triton X-100 buffer (1% Triton X-100, 10mM imidazole, 100mM NaCl, 1mM $MgCl_2$, 5mM EDTA) and centrifuged at 13,200 rpm for 15 minutes at 4^0C to generate the detergent soluble supernatant (cytoplasmic) and detergent insoluble pellet (cytoskeletal associated) fractions. The pellets were resuspended in Urea buffer (7M urea, 0.1M Tris, pH 7.9, 5mM EDTA) and centrifuged to generate the cytoskeletal associated, urea soluble fraction. A total of 50μg of each soluble and 100μg 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# 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 Lifesciences) 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 $\pm$ standard deviation across 3-4 pools. For statistical analysis, the two-sided One Sample t-test was carried out 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 (Benjamini and Hochberg, 1995) rather than the unadjusted per comparison P-values from the statistical tests. A false discovery rate (FDR) $\leq$ 0.05 was considered to be statistically significant.

Immunofluorescence

Heads from day E12.5, E13.5, E15.5, and E17.5 control, *Dlg$^{f/f}$10Cre* or *Dlg$^{f/f}$39Cre* embryos were fixed, embedded in paraffin, and sectioned as described above. For immunofluorescence detection, sections were deparaffinized in xylenes and rehydrated using graded ethanols. 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 0.3M glycine in 1XPBS 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 Dlg-1 immunofluorescent staining, antigen retrieval was accomplished by boiling the sections in 0.1M 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 1XPBSfor 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. Sections were counterstained with To-Pro3 (Invitrogen Cat# T3605) to visualize nuclei. Stained sections viewed by confocal microscopy. Fluorescence intensities in the regions of the lenses were quantified by drawing a box around the area (epithelium, transition zone, anterior region of fiber cells) and measuring the intensity using ImageJ. Fluorescence intensities in the corresponding retinae were also measured and used as internal controls. At least 3 sections over two slides for at least 3 eyes per time point were analyzed. The relative Fgfr2 or pErk levels were calculated by setting the lens/retina value for the controls at 1. The data for each eye was combined to give a single value for the eye. The data reported are the mean $\pm$ standard deviation across the samples for each time point. For statistical analysis, the two-sided One Sample t-test was conducted using MSTAT software. A FDR ≤ 0.05 was considered to be statistically significant.

To verify the specificity of the FGFR2 antibody, lens sections from control and *Fgfr2*$^{f/f}$*;LeCre* E17.5 embryos, which cre expression starts at E9.5 in the lens placode and gives rise to the lens and other ectodermally derived cells (Ashery-Padan et al., 2000), were subjected to immunofluorescence analysis and the signal intensities quantified (samples provided by Dr. Michael L. Robinson, Miami University, Oxford, OH). Stained *Fgfr2* conditional null lens

sections showed a greater than 90% reduction in signal intensity compared to control lens sections (Figure III.S1).

For immunofluorescent staining of P30 cryogenic sections, sections were trypsinized for 45 minutes at room temperature, washed twice in PBS, blocked with 10% horse serum in 0.5% Triton X-100 for 1 hour at RT, incubated with mouse anti-rat SAP97 diluted at 1:500 in 10% horse serum and anti-human FGFR2 diluted at 1:200 in 10% horse serum overnight at 4^0C.Sections were washed in 1X PBS and were incubated with the AlexaFluor 568 conjugated goat anti-rabbit (Molecular Probes) and FITC-conjugated horse anti-mouse (Vector Labs) for 1 hour at RT. For immunofluorescent staining of P30 cryogenic sections, sections were trypsinized for 25 minutes at room temperature, washed twice in PBS, blocked with 10% horse serum in 0.5% Triton X-100 for 1 hour at RT, incubated with goat anti-mouse EphA2 (BD Transduction Laboratories Cat# AF639) diluted at 1:500 in 10% horse serum and mouse anti-human N-cadherin (BD Transduction Laboratories Cat# 610921) diluted at 1:500 in 10% horse serum overnight at 4^0C.Sections were washed in 1X PBS and were incubated with the FITC-conjugated horse anti-goat (Vector Labs) and FITC-conjugated horse anti-mouse (Vector Labs) for 1 hour at RT.

Sections from P2 eyes of control, *Fgfr1*$^{f/+}$*10Cre, Fgfr2*$^{f/+}$*10Cre, Dlg*$^{f/f}$*;Fgfr1*$^{f/+}$*10Cre* and *Dlg*$^{f/f}$*;Fgfr2*$^{f/+}$*10Cre* mice were immunostained for Fgfr2, pAkt and pErk as described above for *Dlg*$^{f/f}$*10Cre* and *Dlg*$^{f/f}$*39Cre* embryos. Immunostaining for Fgfr1 was carried out using a rabbit anti-human FGFR1 antibody (Abcam Cat# ab10646) at a 1:500 dilution. Sections from E16.5 control, *Dlg*$^{f/+}$*10Cre*, *Scrib*$^{f/+}$*10Cre*, *Vangl2*$^{Lp/+}$, *Dlg*$^{f/+}$*10Cre;Vangl2*$^{Lp/+}$, and *Scrib*$^{f/+}$*10Cre;Vangl2*$^{Lp/+}$ embryos were immunostained for Fgfr2, pAkt and pErk as described above for *Dlg*$^{f/f}$*10Cre* and *Dlg*$^{f/f}$*39Cre* embryos.

PCR Array profiling

Mouse Tyrosine Kinases RT² Profiler™ PCR Arrays (Qiagen Cat# PAMM-161Z) containing 84 receptor tyrosine kinases genes, including panel of five housekeeping genes to normalize PCR array data, genomic DNA control primers, reverse transcription controls and positive PCR controls. RNA was prepared from ten lenses from control and *Dlg$^{f/f}$10Cre* E17.5 embryos using a Qiagen RNeasy Mini Kit (Qiagen). RNA integrity and concentration was determined using a NanoDrop spectrophotometer. 500ng of RNA from each pool were used for each cDNA synthesis. Four control and four *Dlg$^{f/f}$10Cre* cDNA pools were synthesized using RT² First Strand kit (Qiagen). The integrity of each cDNA pool was determined by real time-PCR with Gapdh and Actin primers. The resultant cDNA pools were amplified by real-time PCR using SYBR Green PCR master mix (Qiagen) as follows: (1) denaturation for 10 min at 95^{0}C, (2) 40 cycles of 15s denaturation at 95^{0}C and (3) annealing for 60s at 60^{0}C using a BioRad CFX real-time PCR machine on independent array. The $\Delta\Delta C_T$ method provided from Qiagen was used for averaging Ct values for each genes analysis. Relative gene expression values of control and *Dlg$^{f/f}$10Cre* samples were determined using the web-based analysis software. (http://pcrdataanalysis.sabiosciences.com/pcr/arrayanalysis.php). The fold change of gene expression was calculated as $2^{-\Delta\Delta C_T}$.

Co-immunoprecipitation

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 fraction was resuspended in NP-40 buffer (1% NP-40, 150mM NaCl, 2mM EDTA, 50mM Tris-HCl, pH8.0). A total of 175µg of protein were pre-cleared with Protein A Sepharose (Invitrogen) or Protein G Sepharose

(Invitrogen) for 1 hour at 4°C and then incubated with 10μg of anti-β-catenin, anti-Dlg-1, anti-Fgfr1, anti-Fgfr2, and anti-EphA2 antibody or 10μg of corresponding IgGs (Thermo Scientific) as controls, overnight at 4°C, followed by incubation with Protein A Sepharose or Protein G Sepharose beads for 2 hours in 4°C. Protein A Sepharose or Protein G Sepharose pellets were washed three times with NP-40 buffer, resuspended in 5 μl loading dye/20 μl urea buffer and then denatured 7 minutes at 95^0C. The released proteins were fractionated by SDS/PAGE (7.5% polyacrylamide gels), and then transferred to PVDF membranes and immunoblotted for β-catenin, Dlg-1, Fgfr1, Fgfr2, 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 co-immunoprecipitation experiments were repetitively carried out to verify the consistency.

CHAPTER III: Loss of Dlg-1 in the Mouse Lens Impairs Fibroblast Growth Factor Receptor Signaling

This chapter is composed of a paper published by Sungkyoung Lee and Griep to *PLOS One*. The materials and methods section has been moved to Chapter II. All data were collected by Sungkyoung Lee.

SUMMARY

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. Our lab has 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, I 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 (Lovicu et al., 2011; Robinson, 2006). 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 (Gao et al., 2011; Singh and Mlodzik, 2012; Wallingford, 2012). In this study, I 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 (Piatigorsky, 1981). 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 (Piatigorsky, 1981).

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) (Lovicu et al., 2011; Wang et al., 2010). Among these various growth factors, FGF has been demonstrated to be the only growth factor capable of initiating lens fiber differentiation (Liu et al., 1996; Richardson et al., 1993; Wang et al., 2010). 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* (de Iongh et al., 1996; Garcia et al., 2005; Matsuo, 1993; Robinson, 2006). *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 (Chow et al., 1995; Robinson et al., 1995; Stolen and Griep, 2000). 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 (Zhao et al., 2008), 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 (Sugiyama et al., 2011; Sugiyama et al., 2010). Furthermore, transgenic expression of an inhibitor of Wnt signaling, secreted frizzled related protein 2 (Sfrp2) inhibited fiber cell differentiation (Sugiyama et al., 2010). Finally, it has been shown that FGF-induced fiber cell differentiation in rat explant cultures is dependent on Wnt signaling (Dawes et al., 2013). All these studies point toward a requirement for Wnt/PCP signaling in the transmission of Fgfr signaling to induce fiber cell differentiation.

Recently, the Griep lab discovered that *Discs large-1* (*Dlg-1)*, the mouse homolog of the *Drosophila* gene *discs-large* (*dlg),* is expressed in the lens (Nguyen et al., 2003; Nguyen et al., 2005) 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 (Rivera et al., 2009), consistent with the role documented for *dlg* in *Drosophila* (Bilder and Perrimon, 2000). However, in addition, the phenotype of the *Dlg-1* deficient lens resembled that described for the $Vangl2^{Lp/Lp}$ 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 $Vangl2^{Lp/Lp}$ 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 (Rivera et al., 2013), thereby identifying *Dlg-1* as a new PCP gene.

In the course of the Griep lab's analysis of the phenotype of the *Dlg-1* deficient lens, I observed that levels of activated Erk, a signaling intermediate of numerous growth factor signaling pathways, were reduced in the*Dlg-1* deficient fiber cells (Rivera et al., 2009). Erk activation downstream of FGF stimulation is necessary for lens proliferation (Iyengar et al., 2007), and differentiation (Chen et al., 2008). 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, I tested this hypothesis. Specifically, I 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. I 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.

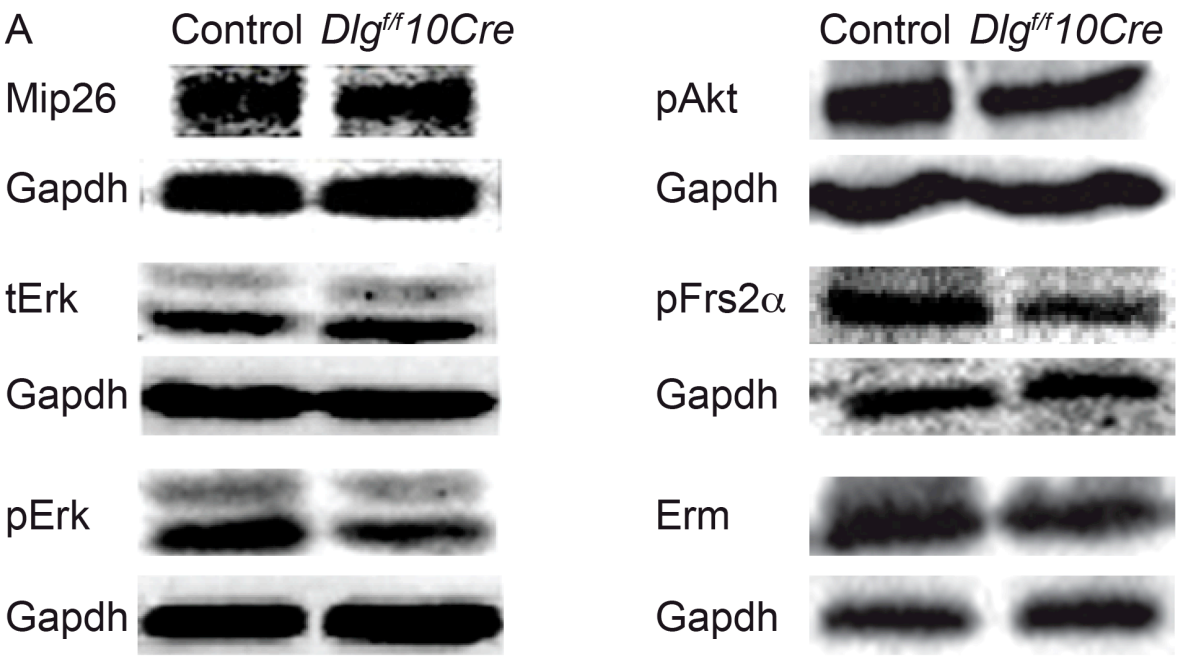
RESULTS

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

The Griep lab previously has 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 (Lovicu et al., 2011; Robinson, 2006) appeared to be reduced in *Dlg-1* deficient fiber cells (Rivera et al., 2009). Because Fgfr signaling has been shown to be essential for fiber cell differentiation(Robinson, 2006; Stolen and Griep, 2000), I hypothesized that Fgfr signaling is compromised in *Dlg-1* deficient lenses. To test this hypothesis, *Dlg-1*$^{f/f}$ mice, which carry a conditional null allele of *Dlg-1*, were crossed to *MLR10CRE* mice (Rivera et al., 2009) to generate *Dlg-1*$^{f/f}$ mice (controls) and *Dlg*$^{f/f}$*10Cre* 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 *Dlg*$^{f/f}$*10Cre* 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 *Dlg*$^{f/f}$*10Cre* lenses as compared to controls and levels of pErk were reduced by 30%, despite an increase in levels of total Erk (tErk; Figure III.1). Fgfr signaling is also known to activate another signaling intermediate, pAkt, via the PI3K pathway (Lovicu et al., 2011). Levels of pAkt, were reduced by 25% in *Dlg*$^{f/f}$*10Cre* fiber cell extracts as compared to controls (Figure III.1).

Fgfr signaling stimulates the activation of signaling cascades starting with the phosphorylation of the docking protein, Frs2α (Gotoh, 2008; van der Noll et al., 2013), which binds directly to the receptor. Although Frs2αcan be activated by receptors in addition to Fgfrs, these other receptors are not expressed in the lens (Madakashira et al., 2012). Thus, activation of Frs2αin 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 $Dlg^{f/f}$*10Cre* lenses using anti-pFrs2α antibodies. The level of pFrs2α was decreased by 30% in extracts from $Dlg^{f/f}$*10Cre* lenses as compared to controls. Finally, western blot analysis showed that the protein level of Erm, an Fgfr target, was decreased by 30% in extracts from $Dlg^{f/f}$*10Cre* 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 $Dlg^{f/f}$*10Cre* mice, indicating that *Dlg-1* is a modulator of Fgfr signaling and fiber cell differentiation in the lens.

Figure III.1. Components of the Fgfr signaling pathway are reduced in *Dlg$^{f/f}$10Cre* fiber cells. (A) RIPA lysates from P2 control and *Dlg$^{f/f}$10Cre* fiber cells were immunoblotted for the indicated proteins and the blots reprobed for Gapdh as a loading control. Representative blots are shown. (B) Quantification of protein levels. Shown are the levels of the indicated proteins in extracts from *Dlg$^{f/f}$10Cre* fiber cells relative to levels in the controls (control levels set a 1.0). Signal intensities were quantified by phosphorimager analysis, as described in Materials and Methods, and the data subjected to statistical analysis using the two-sided One Sample t-test. At least 3 protein pools were blotted in triplicate over 1-3 blots. The relative levels of Fgfr signaling intermediates and target as well as the fiber cell specific protein, Mip26, were all reduced in *Dlg$^{f/f}$10Cre* lens fibers as compared to control. Error bars=standard deviation. *=FDR<0.05, **=FDR<0.01.


A
Control
Dlg f/f 10Cre
Mip26
Gapdh
tErk
Gapdh
pErk
Gapdh
Control
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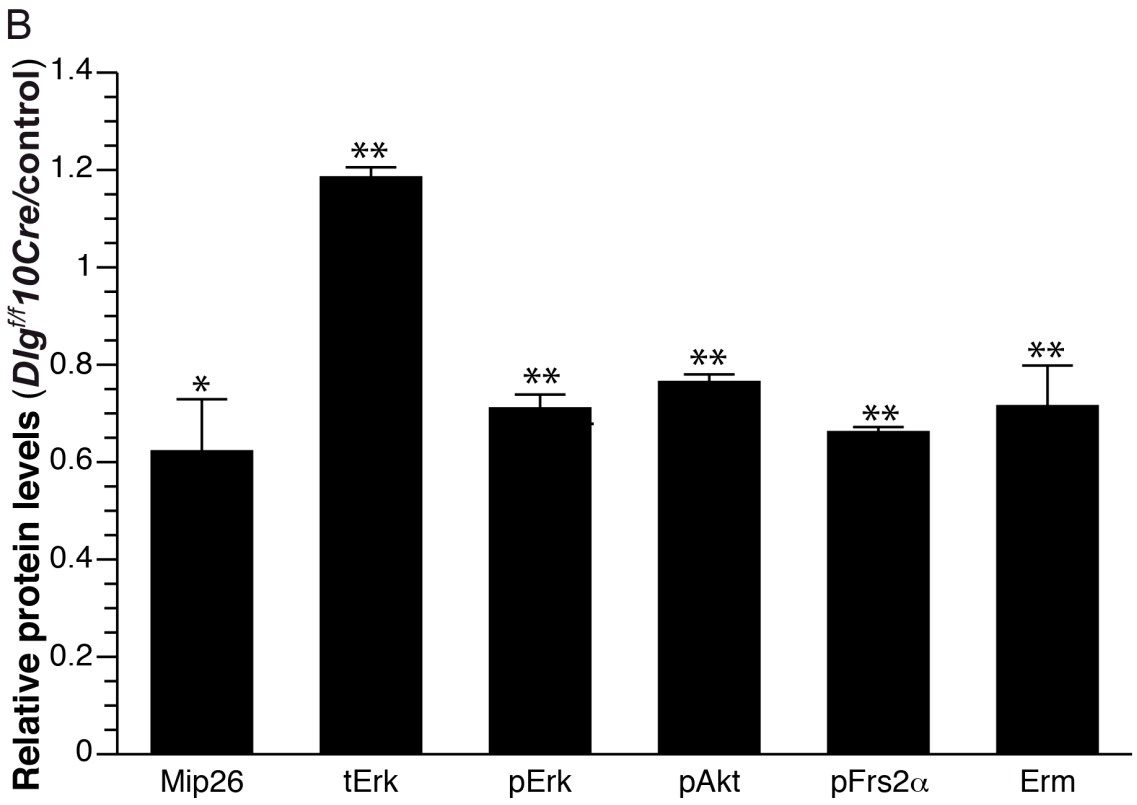
Gapdh
Erm
Gapdh


B
Relative protein levels (Dlg f/f 10Cre/control)
1.4
1.2
1
0.8
0.6
0.4
0.2
0
*
**
**
**
**
**
Mip26
tErk
pErk
pAkt
pFrs2α
Erm

In *Dlg$^{f/f}$10Cre* mice, *Dlg-1* is ablated in both epithelial and fiber cells. Previously, by comparing the phenotype of the fiber cells in these mice to mice in which *Dlg-1* is ablated only in the fiber cells (referred to as *Dlg$^{f/f}$39Cre* mice), we showed that the epithelial and fiber cell defects were independent of each other (Rivera et al., 2009). To verify that the effects of *Dlg-1* deficiency on Fgfr signaling in the fiber cells is a fiber cell autonomous effect, I quantified the levels of pErk and pFrs2α in the fibers of *Dlg$^{f/f}$39Cre* mice as compared to *Dlg$^{f/f}$10Cre* mice and controls by western blot analyses. As shown in Figure III.3, levels of pErk and pFrs2α were significantly reduced in the fiber cells from *Dlg$^{f/f}$39Cre* mice as compared to controls (20% reduction in each protein), although to a lesser extent than in the fiber cells from *Dlg$^{f/f}$10Cre* mice (30% reduction in pErk and 35% reduction in pFrs2α). That the reduction in pErk and pFrs2α in *Dlg$^{f/f}$39Cre* fibers is less than in *Dlg$^{f/f}$10Cre* 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 (Zhao et al., 2004). These data indicate that the effect of *Dlg-1* deficiency on Fgfr signaling is fiber cell autonomous.

Figure III.2. Documentation of the specificity of the anti-FGFR2 antibody. Paraffin embedded sections of day E17.5 control and *Fgfr2$^{f/f}$;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 *Fgfr2$^{f/f}$;LeCre* embryo was nearly absent while immunoreactivity in the retina was similar between control and *Fgfr2$^{f/f}$;LeCre* embryo. The signal intensity for Fgfr2 staining in the *Fgfr2$^{f/f}$;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 = 50μm

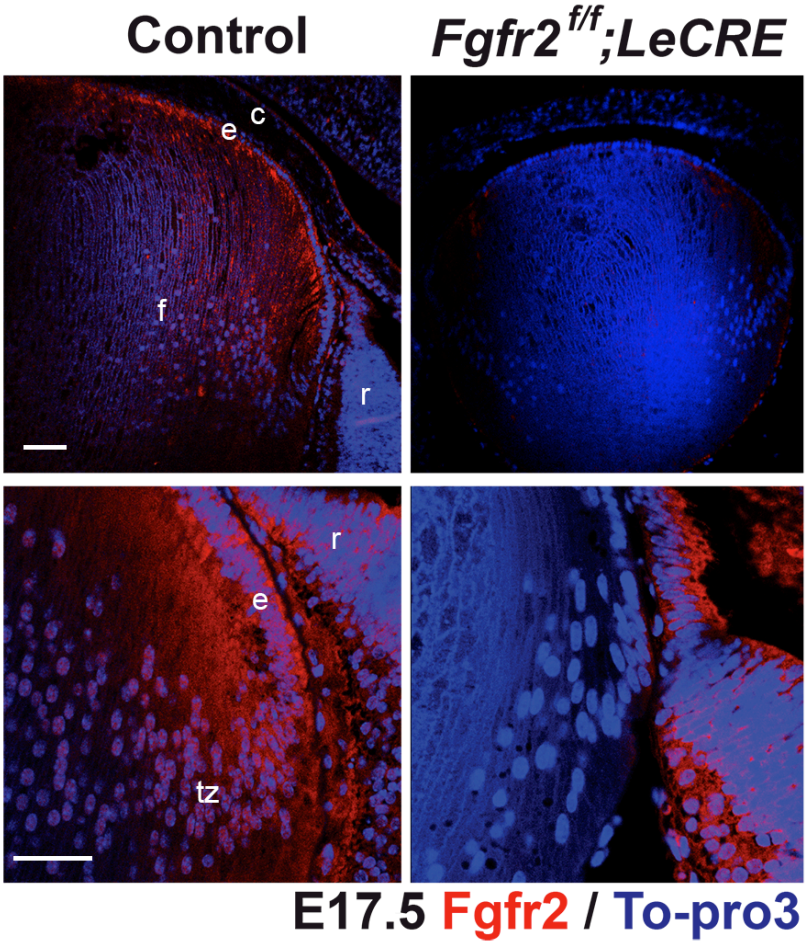
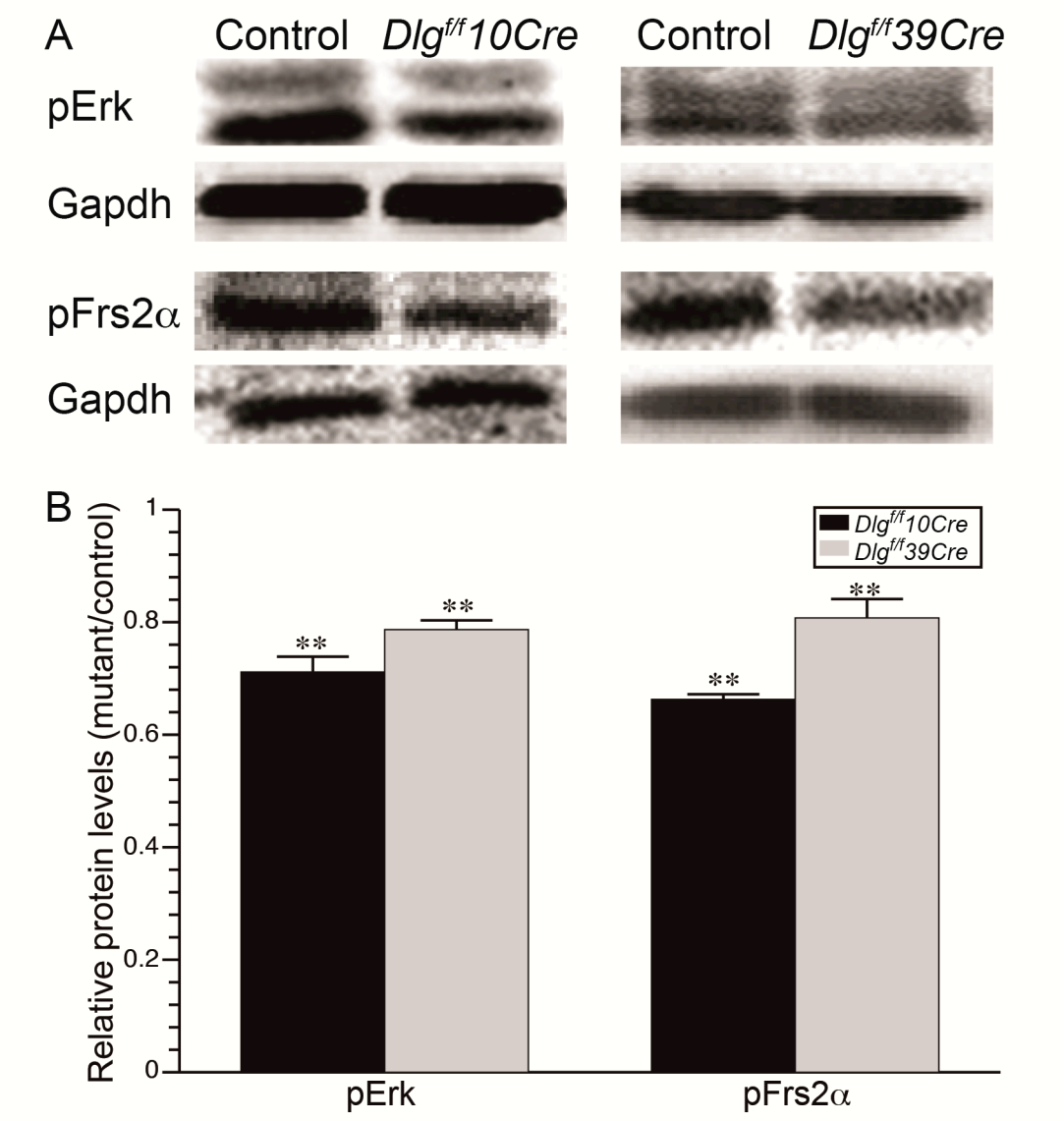

Control
Fgfr2 f/f;LeCRE
c
e
f
r
r
e
tz
E17.5 Fgfr2 / To-pro3

Figure III.3. Levels of Fgfr signaling intermediates are reduced in both *Dlg$^{f/f}$10Cre* and *Dlg$^{f/f}$39Cre* fiber cells. (A) RIPA lysates from P2 control, *Dlg$^{f/f}$10Cre*, and *Dlg$^{f/f}$39Cre* fiber cells were immunoblotted for the pErk and pFrs2α and the blots reprobed for Gapdh as a loading control. Representative blots are shown. (B) Quantification of protein levels. Shown are the levels of the indicated proteins in extracts from *Dlg$^{f/f}$10Cre* fiber cells relative to levels in the controls (control levels set at 1.0). Signal intensities were quantified by phosphorimager analysis, as described in Materials and Methods, and the data subjected to statistical analysis using the two-sided One Sample t-test. At least 3 protein pools were blotted in triplicate over 1-3 blots. The relative levels of Fgfr signaling intermediates were reduced in the fiber cells of *Dlg$^{f/f}$39Cre* mice as well as *Dlg$^{f/f}$10Cre* mice compared to controls, indicating that the effect was fiber cell autonomous. Error bars=standard deviations.**=FDR<0.01.


A
Control
Dlg^f/f10Cre
Control
Dlg^f/f39Cre
pErk
Gapdh
pFrs2α
Gapdh
B
Relative protein levels (mutant/control)
1
0.8
0.6
0.4
0.2
0
Dlg^f/f10Cre
Dlg^f/f39Cre
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pErk
pFrs2α

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 *Dlg$^{f/f}$10Cre* mice. To address this possibility, whole cell extracts from P2 fiber cells of *Dlg$^{f/f}$10Cre* and control mice were subjected to western blot analyses using antibodies specific to Fgfr1, Fgfr2 or Fgfr3. Levels of Fgfr2 were significantly reduced by 40% in *Dlg$^{f/f}$10Cre* lenses compared to controls; however, levels of Fgfr1 and Fgfr3 trended toward being increased in the *Dlg-1* deficient lenses (Figure III. 4 and 5). To understand if the subcellular distribution of Fgfrs were affected, lenses from P2 control and *Dlg$^{f/f}$10Cre* mice were extracted in Triton X-100 to generate Triton X-100 soluble and insoluble fractions. The triton soluble fraction contains mainly cytosolic proteins while the triton insoluble fraction contains cytoskeletal proteins, membrane proteins, and cytosolic proteins that are tightly associated with the membrane. Immunoblotting the triton soluble and insoluble fractions for Mip26, an intrinsic membrane protein, confirmed that the fractionation was successful as the amount of Mip26 in the soluble fraction was less than 2% of that in the insoluble fraction (data not shown). The levels of Fgfrs were altered in the insoluble fraction from the *Dlg$^{f/f}$10Cre* lenses, relative to control lenses whereas the levels of soluble fractions of *Dlg$^{f/f}$10Cre* were similar to the levels in controls. The Fgfr2 levels were reduced by 60% in the cytoskeletal-associated fraction of *Dlg$^{f/f}$10Cre* lenses. Fgfr1 was significantly increased by 75% in *Dlg$^{f/f}$10Cre* insoluble fractions and levels of Fgfr3 were increased, although the increase was not statistically significant (Figure III.5). These results demonstrate that *Dlg-1* is required to maintain the normal levels of Fgfrs in the lens and thereby regulate Fgfr signaling.

Figure III.4. Levels of Fgfr2 are reduced in *Dlg$^{f/f}$10Cre* lenses. RIPA lysates of lenses from control and *Dlg$^{f/f}$10Cre* P2 mice were subjected to western blot analysis for Fgfr2 and the blots reprobed for Gapdh as a loading control. Shown is a representative full western blot of lysates from control and *Dlg$^{f/f}$10Cre* lenses for Fgfr2. Two independently prepared pools of lysates from control and two independently prepared pools of lysates from *Dlg$^{f/f}$10Cre* lenses are included on this blot. Lanes: (1) control extract #1, (2) *Dlg$^{f/f}$10Cre* extract #1, (3) control extract #2, (4) *Dlg$^{f/f}$10Cre* extract #2, (5) molecular mass markers with sizes (in kD) indicated.

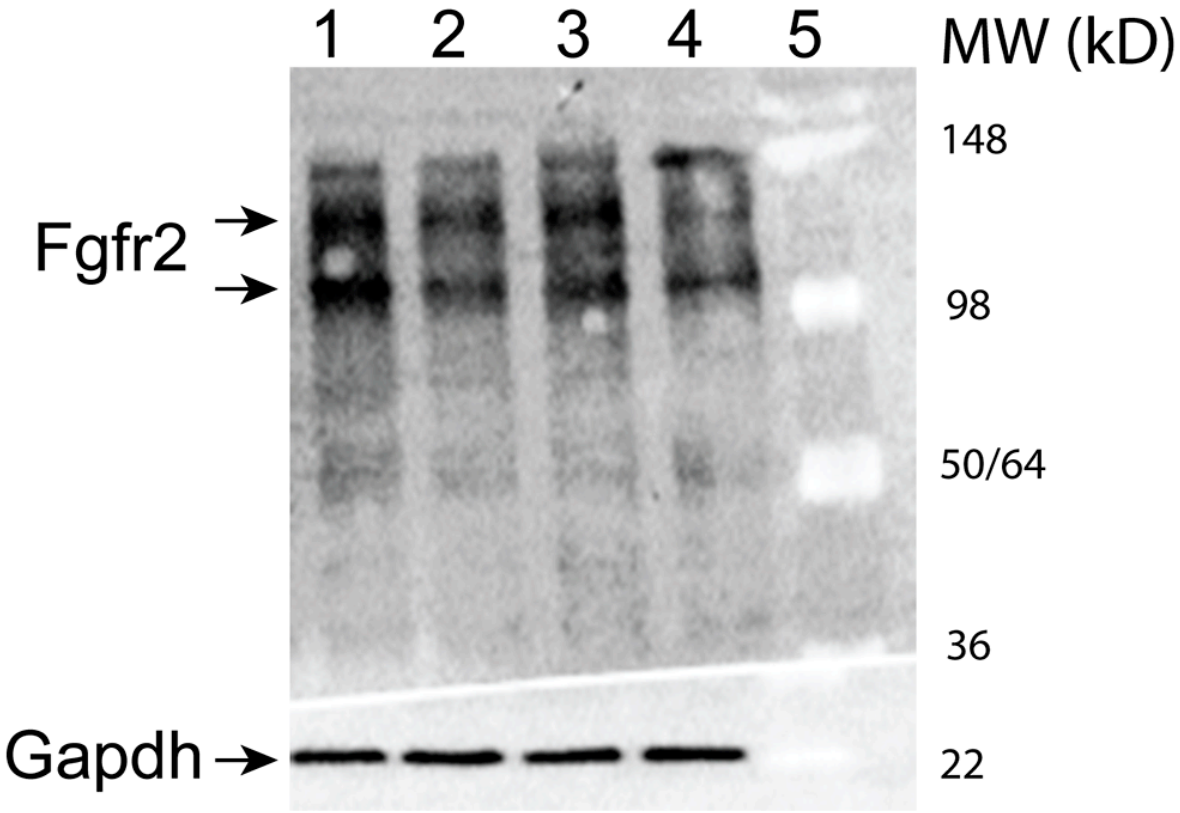
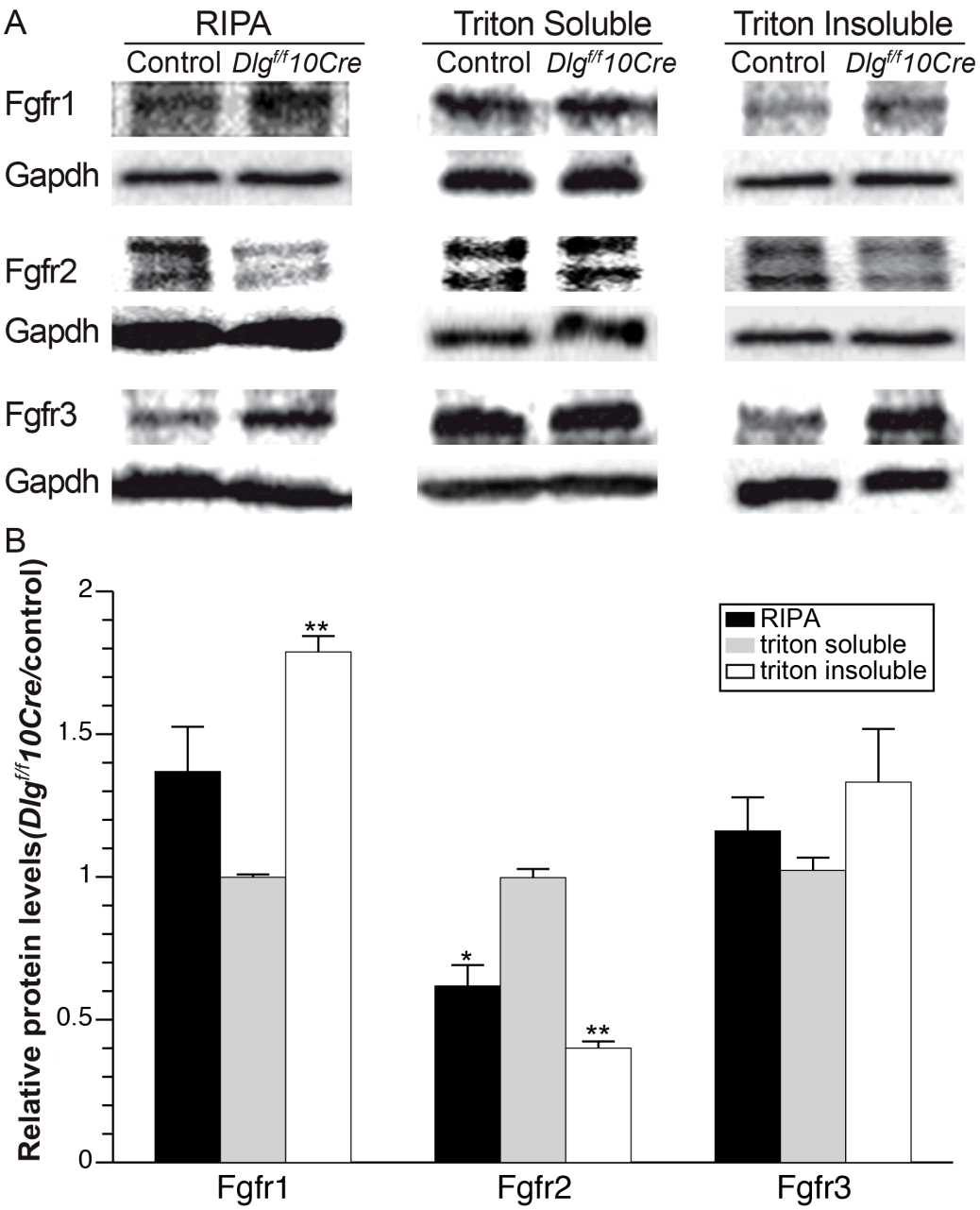

1
2
3
4
5
MW (kD)
148
98
50/64
36
22
Fgfr2
Gapdh

Figure III.5. Levels of Fgfrs are altered in the lenses of *Dlg$^{f/f}$10Cre* mice. (A) RIPA, triton soluble (cytosolic) and triton insoluble (cytoskeletal-associated) extracts from P2 control and *Dlg$^{f/f}$10Cre* lenses were subjected to western blot analysis for Fgfr1, Fgfr2, and Fgfr3 and the blots reprobed for Gapdh as a loading control. Representative blots are shown. (B) Quantification of protein levels. Shown are the levels of each Fgfr in extracts from *Dlg$^{f/f}$10Cre* 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.


A
RIPA
Triton Soluble
Triton Insoluble
Control
Dlg f/f 10Cre
Fgfr1
Gapdh
Fgfr2
Gapdh
Fgfr3
Gapdh
B
Relative protein levels(Dlg f/f 10Cre/control)
RIPA
triton soluble
triton insoluble
2
1.5
1
0.5
0
**
*
**
Fgfr1
Fgfr2
Fgfr3

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 $Dlg^{f/f}$*10Cre* and $Dlg^{f/f}$*39Cre* lenses, immunofluorescence experiments were carried out on paraffin embedded sections of E12.5 and E13.5 $Dlg^{f/f}$*10Cre* and E13.5 and E14.5 $Dlg^{f/f}$*39Cre* embryos. In keeping with previous reports (Cain et al., 2008; Zhao et al., 2004), immunoreactivity for Dlg-1 in $Dlg^{f/f}$*10Cre* lenses was reduced at E12.5 and absent at E13.5 (Figure III.6). Immunoreactivity for Dlg-1 in the fiber cell compartment of $Dlg^{f/f}$*39Cre* lenses was reduced at E13.5 and absent at E14.5 (Figure III. 6).

To determine if Fgfr signaling is compromised at the time Dlg-1 protein is lost from the $Dlg^{f/f}$*10Cre* lens, paraffin embedded sections from day E13.5 control and $Dlg^{f/f}$*10Cre* embryos were immunostained with anti-pERK antibodies. The intensities of staining in transition zone of control and $Dlg^{f/f}$*10Cre* 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 (Figure III.7). In contrast, the intensity of pErk staining was reduced in $Dlg^{f/f}$*10Cre* lenses by 64% (Figure III.7). 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.

Figure III.6. Loss of Dlg-1 protein following cre-mediated excision of *Dlg-1* sequences. Paraffin embedded sections of eyes from control, *Dlg$^{f/f}$10Cre* and *Dlg$^{f/f}$39Cre* day E12.5, E13.5, and E14.5 embryos were subjected to immunoflourescent staining for Dlg-1 (green). For *Dlg$^{f/f}$10Cre* lenses, the intensity of staining throughout the lens was greatly reduced at E12.5 and staining was undetectable at E13.5. For *Dlg$^{f/f}$39Cre* 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 = 50μm.

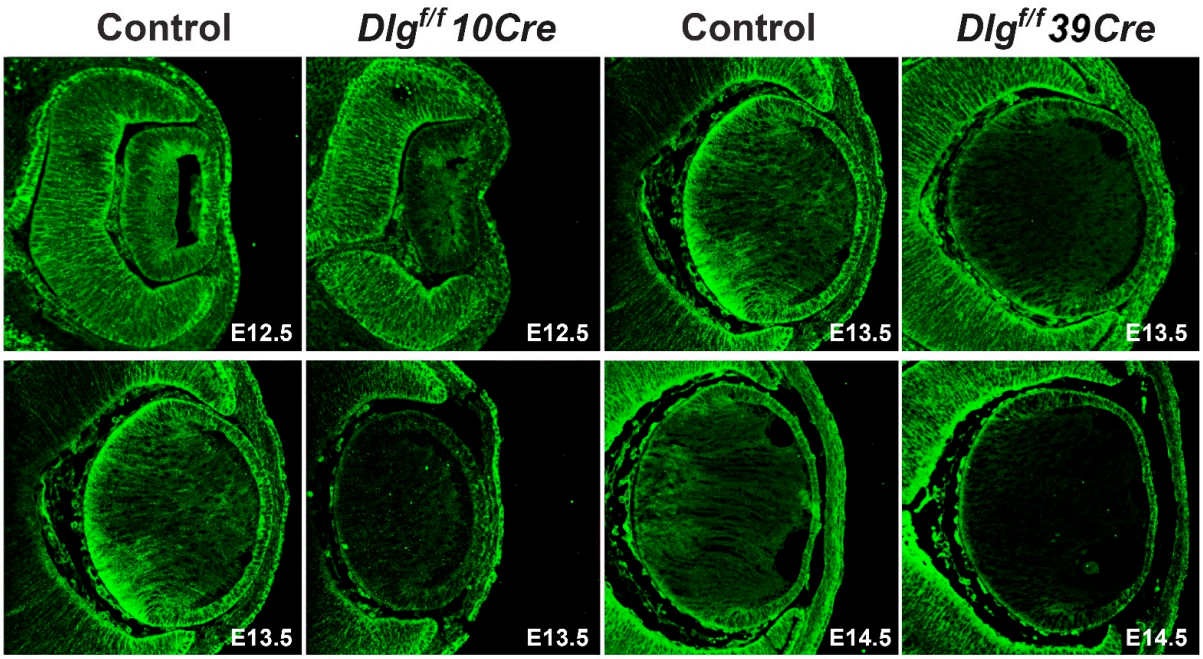
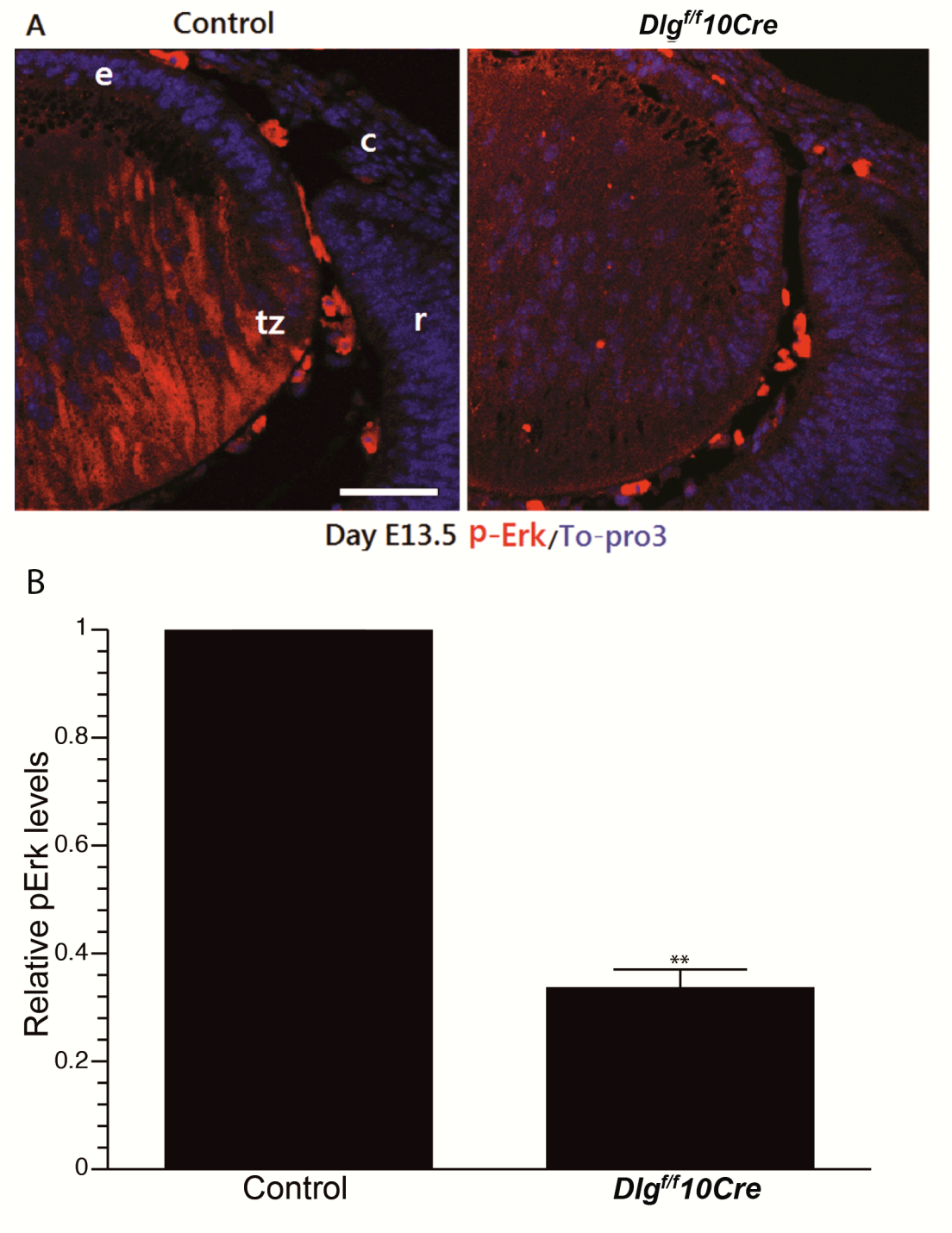

Control
Dlg^f/f 10Cre
Control
Dlg^f/f 39Cre
E12.5
E12.5
E13.5
E13.5
E13.5
E13.5
E14.5
E14.5

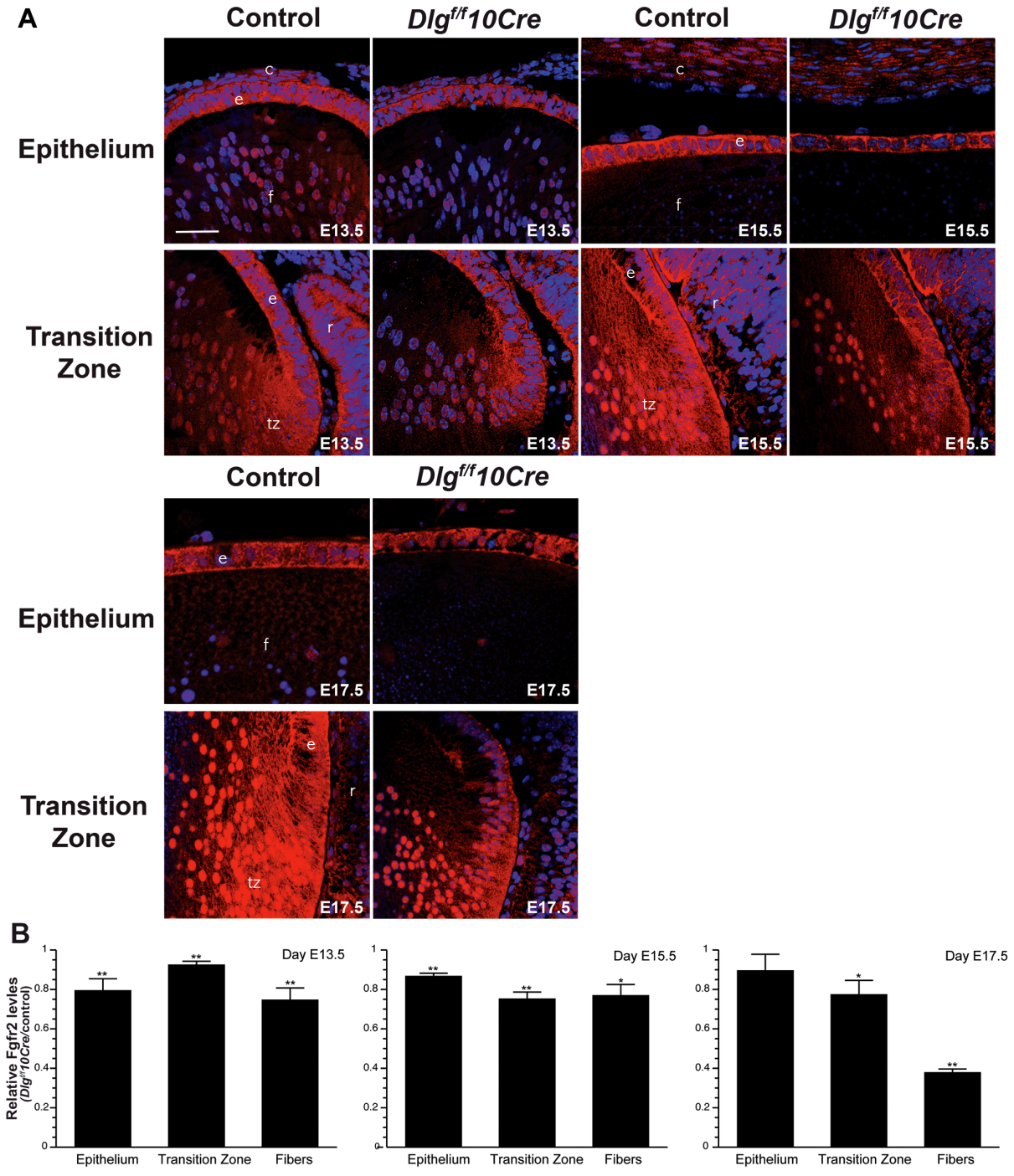
Figure III.7. Levels of the Fgfr signaling intermediate, pErk, are reduced in embryonic *Dlg$^{f/f}$10Cre* lenses. (A) Paraffin embedded sections of eyes from E13.5 control and *Dlg$^{f/f}$10Cre* embryos were subjected to immunofluorescence analysis using an anti-pErk antibody (red) and the nuclei counterstained with To-Pro3 (blue). Representative images of the transition zone are shown. c, cornea; e, lens epithelium; r, retina; tz, transition zone. Bar = 50μm. (B) Quantification of pErk levels. Shown are the relative levels of pErk in the transition zone of *Dlg$^{f/f}$10Cre* and control lenses (control levels set at 1.0) Quantification of signal intensities in the transition zone was carried out using ImageJ, as described in Materials and Methods, and the data subjected to statistical analysis using the two-sided One Sample t-test. At least 3 different sections from at least 3 different lenses were evaluated. The relative levels of pErk in the transition zone of the *Dlg$^{f/f}$10Cre* lens were reduced as compared to levels in the corresponding region of the control lenses. Error bars=standard deviations. *=FDR<0.01.


A
Control
Dlg^f/f10Cre
e
c
tz
r
Day E13.5 p-Erk/To-pro3
B
Relative pErk levels
1
0.8
0.6
0.4
0.2
0
**
Control
Dlg^f/f10Cre

Effect of Loss of *Dlg-1* on Fgfr2 Levels During Embryogenesis

Given that levels of pErk were reduced in the lenses of E13.5 *Dlg$^{f/f}$10Cre* embryos, I asked if levels of Fgfr2 were similarly reduced in *Dlg$^{f/f}$10Cre* lenses at earlier time points. To do so, immunofluorescence experiments were carried out on paraffin embedded sections from control and *Dlg$^{f/f}$10Cre* E13.5, E15.5, and E17.5 embryos using an anti-FGFR2 antibody. Representative images are shown in Figure III.8A. The intensities of staining in epithelium, transition zone and fiber cells in control and *Dlg$^{f/f}$10Cre* lenses were measured by ImageJ (see Materials and Methods). In control lenses, staining for Fgfr2 was seen in the epithelium, the transition zone, and the fiber cells (Figure III.8B). Also, Fgfr2 was detected in fiber cell nuclei. The intensity of staining for Fgfr2 in *Dlg$^{f/f}$10Cre* lenses was significantly reduced at E13.5 in all three areas of the lens (Figure III.8B). Levels of Fgfr2 were similarly reduced at E15.5 and were reduced in the transition zone and fibers at E17.5. The reduced intensity of staining for Fgfr2 in the transition zone and fiber cells appeared to be in both the cell membranes and the nuclei of the *Dlg$^{f/f}$10Cre* 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.

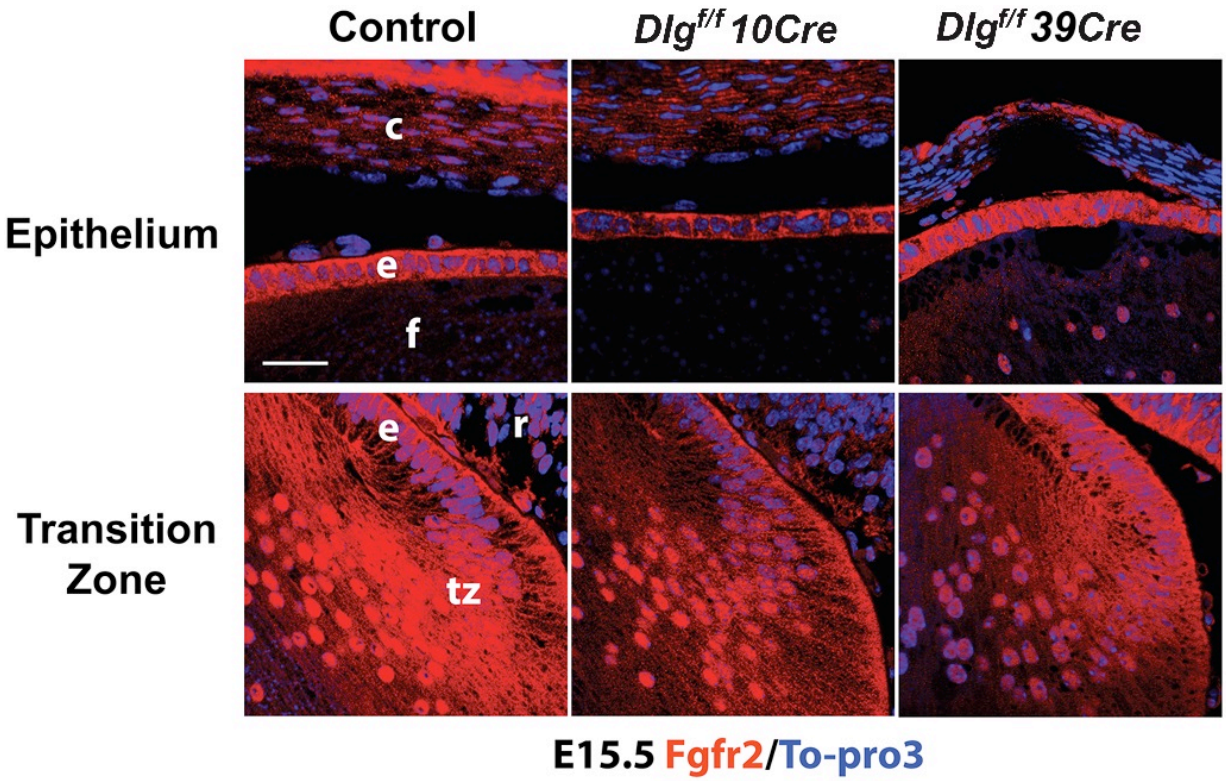
Figure III.8. Levels of Fgfr2 are reduced in embryonic *Dlg$^{f/f}$10Cre* lenses. (A) Paraffin sections of eyes from day E13.5, E15.5 and E17.5 control and *Dlg$^{f/f}$10Cre* embryos were subjected to immunofluorescence analysis using an anti-FGFR2 antibody (red) and the nuclei counterstained with To-Pro3 (blue). Representative images of the epithelium and transition zone/fiber cell compartment from control and *Dlg$^{f/f}$10Cre* eyes are shown for each time point. c, cornea; e, lens epithelium; f, lens fibers; r, retina; tz, transition zone. Bar = 50μm. (B) Quantification of Fgfr2 levels. Shown are the relative levels of FGFR2 in regions of the *Dlg$^{f/f}$10Cre* lens as compared to levels in the corresponding regions of the control lenses (control levels set at 1.0). Quantification of signal intensities in epithelium, transition zone and fibers was carried out using ImageJ, as described in Materials and Methods, and the data subjected to statistical analysis using the two-sided One Sample t-test. At least 3 different sections from at least 3 different lenses were evaluated for each time point. The Fgfr2 levels were reduced in transition zone and fiber cells of *Dlg$^{f/f}$10Cre* lenses compared to controls at all time points and in the epithelium of the *Dlg$^{f/f}$10Cre* lenses compared to controls at E13.5 and E15.5. Error bars=standard deviations. *=FDR<0.05, **=FDR<0.01.


A
Control
Dlg^f/f10Cre
Control
Dlg^f/f10Cre
Epithelium
Transition Zone
c
e
f
r
tz
E13.5
E15.5
Control
Dlg^f/f10Cre
Epithelium
Transition Zone
E17.5
B
Relative Fgfr2 levles (Dlg^f/f10Cre/control)
Day E13.5
Day E15.5
Day E17.5
Epithelium
Transition Zone
Fibers

Effect of Loss of *Dlg-1* on Fgfr2 Levels in *Dlg$^{f/f}$39Cre* Embryonic Lenses

In the *Dlg$^{f/f}$10Cre* lenses, reduced levels of Fgfr2 were found in the epithelium as well as in the fiber cells (Figure III.8). To determine if the effect of *Dlg-1* deficiency on Fgfr2 levels in the fiber cells was independent of the effect in the epithelium, sections from day E15.5 control, *Dlg$^{f/f}$10Cre* and *Dlg$^{f/f}$39Cre* embryos were subjected to immunofluorescence experiments using an anti-Fgfr2 specific antibody. As shown in Figure III.9, the intensity of immunofluorescent staining for Fgfr2 in the transition zone of *Dlg$^{f/f}$39Cre* 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.

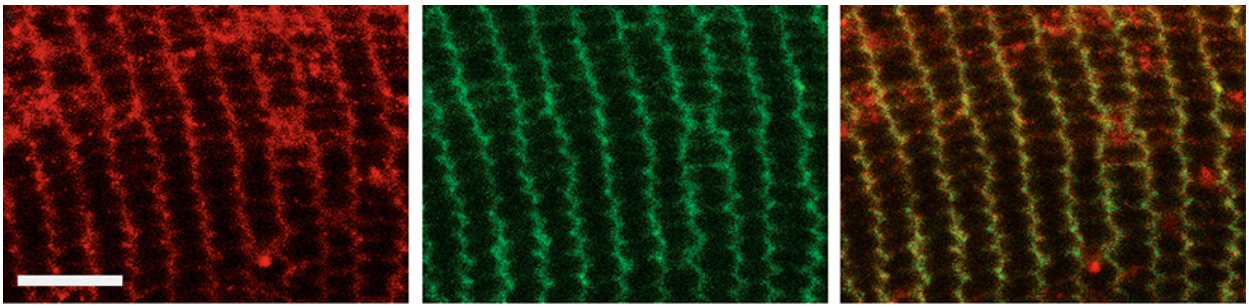
Figure III.9. Fgfr2 levels are reduced in the transition zone and fiber cells of *Dlg$^{f/f}$39Cre* embryos. Paraffin embedded sections from day E15.5 control, *Dlg$^{f/f}$10Cre* and *Dlg$^{f/f}$39Cre* embryos were subjected to immunofluorescence analysis using an anti-FGFR2 antibody (red) and the nuclei counterstained with To-Pro3 (blue). Representative images of the epithelium and transition zone of each genotype are shown. The intensity of staining in the epithelium from the *Dlg$^{f/f}$39Cre* embryo is similar to that in control whereas intensity in the epithelium of *Dlg$^{f/f}$10Cre* embryos is less than in the control. The intensity of Fgfr2 staining was reduced in the transition zone of both *Dlg$^{f/f}$10Cre* and *Dlg$^{f/f}$39Cre* 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 = 50μm.


Control
Dlg f/f 10Cre
Dlg f/f 39Cre
Epithelium
Transition Zone
c
e
f
e
r
tz
E15.5 Fgfr2/To-pro3

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 (Harris and Lim, 2001). 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 P30 control mice. Immunostaining for Fgfr2 was predominantly localized to the short sides of the fiber cells in the outer cortex, as was immunostaining for Dlg-1 (Figure III.10). In merged images, overlap in staining was observed (Figure III.10), suggesting that Dlg-1 and Fgfr2 colocalize on the short sides of the fiber cells.

Figure III.10. Dlg-1 and Fgfr2 co-localize on the short sides of the fiber cells in the outer cortex. Cryogenic sections from control P30 lenses were subjected to immunofluorescent staining using anti-Fgfr2 anti-Dlg-1 antibodies. Staining for Fgfr2 (red) was predominantly localized on the short sides of the fiber cells as was staining for Dlg-1 (green). Overlap (yellow) can be seen in the merged image. Bar = 50μm.


FGFR2
Dlg-1
Merged

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, I examined the relationship between Fgfr signaling, which is known to be required for lens fiber cell differentiation (Zhao et al., 2008), and the regulation of cell structure and tissue architecture by *Dlg-1*, a gene involved in both apical-basal polarity and planar cell polarity (Rivera et al., 2013), which has also been shown to be required for fiber cell differentiation (Rivera et al., 2009). I 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.

Dlg-1 is a modulator of Fgfr levels and Fgfr signaling in fiber cell differentiation

In this study, I asked if *Dlg-1* deficient lenses would exhibit 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 $Dlg^{f/f}$*10Cre* 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 (Iyengar et al., 2007; Rivera et al., 2009). Diminished levels of pAkt may be responsible for the apoptosis observed in the $Dlg^{f/f}$*10Cre* lenses (Rivera et

al., 2009), as it is known that pAkt is necessary for cell survival (Pap and Cooper, 1998). I also found reduced levels of pFrs2α, which is directly activated by the Fgfrs (Gotoh, 2008; van der Noll et al., 2013), 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 *Dlg$^{f/f}$10Cre* 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, I 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 (Tarkkonen et al., 2012), providing a precedent for our observations.

I found that the subcellular distribution of Fgfr2 differed between epithelium and fiber cells. In the epithelium, the staining was only membrane associated. However, in the fiber cells, staining was associated with both the membrane and the nucleus (Figure III.8). In the *Dlg$^{f/f}$10Cre* fiber cells, levels of Fgfr2 appeared to be reduced in both membrane and nuclear compartments, suggesting multiple functions of Fgfr2 are affected by loss of *Dlg-1*. Shifts in the subcellular distribution of Fgfrs have been observed previously. For instance, in certain cancers, shift in localization of Fgfrs 1 and 2 has been correlated with the stage of the cancer (Behrens et al., 2008; Martin et al., 2011). Perhaps more relevant to lens fiber differentiation, nuclear staining for Fgfr2has been observed in the terminal end buds of the mammary gland (Lu et al., 2008) and also in Sertoli cell precursors (Schmahl et al., 2004). The role of nuclear Fgfr2 is unknown at the present time. However, given the circumstances under which nuclear Fgfr2 has been found, it is

possible that nuclear Fgfr2 might be important for regulating gene expression tied to entering or maintaining the differentiated state.

Reduced Fgfr signaling in *Dlg-1* deficient lenses is not sufficient to account for the differentiation defects in the *Dlg$^{f/f}$10Cre* lens.

Previously, our lab showed that *Dlg-1* deficiency leads to inhibition of fiber cell differentiation, which was characterized by numerous changes in gene expression and defects in fiber cell structure (Rivera et al., 2009). Herein, I 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 (Lovicu et al., 2011; Robinson, 2006; Zhao et al., 2008), 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 (Garcia et al., 2005). This phenotype bears some similarity to the *Dlg$^{f/f}$10Cre* phenotype as *Dlg$^{f/f}$10Cre* lenses display small changes in rates of proliferation in the epithelium (unpublished data) and apoptosis in both the epithelium and fiber cell compartment of postnatal mice (Rivera et al., 2009), suggesting that these aspects of the *Dlg$^{f/f}$10Cre* 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 has formed. 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 (Zhao et al., 2008), suggesting that in the lens the Fgfrs function redundantly or can compensate

for each other. Ablating Fgfrs 1, 2, and 3 with *MLR10CRE* results in a near complete failure of fiber cell differentiation (Zhao et al., 2008), a pleiotropic phenotype that differs from that of the *Dlg$^{f/f}$10Cre* fiber cells. Taken together these data suggest that it is likely that loss of *Dlg-1* disrupts signaling pathways in addition to Fgfr 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 (Boswell et al., 2008; Faber et al., 2002), TGFβ superfamily (Beebe et al., 2004; de Iongh et al., 2001), Notch (Rowan et al., 2008; Saravanamuthu et al., 2009)and Eph/Ephrin (Cheng and Gong, 2011). Additionally, signaling pathways via cell adhesion proteins such as N-cadherin (Pontoriero et al., 2009) and integrins (Hayes et al., 2012; Simirskii et al., 2007; Walker et al., 2002; Wederell and de Iongh, 2006) are known to play roles in lens development. Impairment of any of these signaling pathways could cooperate with defects in Fgfr 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*.

Dlg-1 modulates Fgfr signaling and fiber cell differentiation through PCP

Recent studies in rat lens explants have shown that Fgf-induced differentiation is dependent on Wnt signaling (Dawes et al., 2013). This appears to be due to Wnt/PCP signaling for several reasons. First, studies using Wnt/β-catenin reporter mice have shown an absence of activity in the differentiating fibers (Dawes et al., 2013; Liu et al., 2006). Second, blocking Wnt signaling *in vivo* in transgenic mice by overexpressing Sfrp2 inhibits fiber differentiation and results in a phenotype similar to the lens phenotype of mouse mutants in core PCP genes, *Vangl2*

and *Celsr1* (Sugiyama et al., 2011; Sugiyama et al., 2010). Third, in the outer cortical fiber cells, the core PCP proteins are asymmetrically localized and that these cells have a cilium that is oriented toward the anterior pole of the lens, showing that these cells exhibit PCP (Sugiyama et al., 2011; Sugiyama et al., 2010). The lenses of the *Vangl2* and *Celsr1* mutant mice, *Vangl2*$^{Lp/Lp}$ and *Crsh*, respectively, and the Sfrp2 transgenic mice are characterized by a flattened and elongated shape, defects in fiber cell curvature, and defects in suture formation (Sugiyama et al., 2011; Sugiyama et al., 2010). These same defects are observed in *Dlg-1* mutant lenses (Rivera et al., 2009). Given that *Dlg-1* plays a role in PCP (Rivera et al., 2013) and the phenotype of the *Dlg-1* mutant lens resembles that of the *Vangl2*$^{Lp/Lp}$ and *Crsh* mutant lenses, it is likely that the mechanism through which *Dlg-1* affects Fgfr signaling and fiber differentiation is through its role in PCP. Thus, *Dlg-1* is the first PCP gene that has been shown to modulate Fgfr signaling and lens fiber cell differentiation. A connection between Dlg-1 and Fgf signaling has been suggested previously. During intestinal tube formation in *C. elegans*, DLG-1 interacts with VANG-1 and is phosphorylated by the FGF-like receptor EGL-15 (Hoffmann et al., 2010). In the mouse, *Dlg-1* and *Cask* cooperate to regulate kidney development through a mechanism that involves regulation of Fgf8 expression (Ahn et al., 2013). Together, these and our findings support the concept that there is interaction between Dlg-1 and Fgfr pathways and this interaction has an impact on developmental processes.

Interestingly, I observed that Fgfr2 and Dlg-1 overlap on the short sides of the cortical fiber cells, suggesting that Dlg-1 and Fgfr2 may interact in a complex. This interaction is not likely to be direct as there is no known PDZ binding motif in Fgfr2. Dlg-1 also has been shown to be involved in vesicle trafficking(Lee et al., 2002), which could play a role in Dlg-1's modulation of Fgfr2 and PCP proteins. Cell adhesion and cytoskeletal organization are also

compromised in the *Dlg$^{f/f}$10Cre* lenses (Rivera et al., 2009), which could impair PCP and, consequently, Fgfr signaling and fiber cell differentiation. Of note, I have observed that the levels of the cell adhesion protein, N-cadherin, (Lee and Griep, unpublished data) and α-catenin (Rivera et al., 2009) are reduced in *Dlg$^{f/f}$10Cre* lenses. Future studies will be needed to determine the detailed mechanism through which PCP and Dlg-1 modulate Fgfr signaling and fiber cell differentiation.

Conclusion

In this chapter, I have shown that loss of Dlg-1 in the lens impairs Fgfrs signaling and leads to altered levels of Fgfrs. Dlg-1 deficient lenses showed that levels of the Fgf receptor downstream intermediates pErk, pAkt, and pFrs2α, the Fgfr target, Erm, and the fiber cell specific protein, Mip26, were downregulated. The levels of Fgfr2 were decreased in Dlg-1 deficient lenses compared to controls, while levels of Fgfr1 in Dlg-1 deficient lenses were increased compared to controls. Additionally, levels of Fgfr2 and pErk were reduced in *Dlg$^{f/f}$10Cre* 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. *Dlg-1* is required for the proper levels of Fgfrs and Fgfr signaling, and these data suggest that *Dlg-1* acts as modulator of fiber differentiation in the lens through its role in PCP.

CHAPTER IV: Fibroblast Growth Factor Receptor Signaling and Adherens Junction Proteins are Regulated by *Dlg-1* via Interactions with EphA2 in Mouse Lens

This chapter is composed of a paper in the process of being submitted by S. Lee and AE. Griep. The materials and methods have been moved to Chapter II. The generation of *Dlg*$^{f/f}$;*Fgfr1*$^{f/+}$*10Cre* and *Dlg*$^{f/f}$;*Fgfr2*$^{f/+}$*10Cre* mice described in Chapter II.

SUMMARY

Growth factors, through activation of growth factor receptor kinases (RTK) and their downstream signaling intermediates, are crucial regulators of cell proliferation and differentiation, essential processes for both normal development and the long-term maintenance of organ structure and function. We have previously shown that *Dlg-1 (Discs large-1)* is a regulator of lens fiber cell structure and differentiation and a modulator of the Fgfr signaling pathway, a pathway known to be required for fiber cell differentiation. Herein, I investigated the mechanism through which Dlg-1, a PDZ domain containing scaffolding protein, regulates Fgfr signaling. Immunofluorescent staining for Fgfr1 and Fgfr2 in lenses that are deficient for *Dlg-1* and haplodeficient for one of these *Fgfrs* showed that downregulation of one Fgfr leads to upregulation of the other Fgfr. The effects of these mutations on the levels of pErk and pAkt, downstream signaling intermediates in the Fgfr pathway, correlated with the level of Fgfr2. Western blot analysis of control and *Dlg-1* deficient lens extracts showed that loss of *Dlg-1* leads to redistribution of N-cadherin from the cytoskeletal-associated protein fraction to the cytosolic fraction. Co-immunoprecipitation analysis demonstrated that Dlg-1 complexes with β-catenin, EphA2, and, weakly, with Fgfrs 1 and 2. In addition, EphA2 complexed strongly with N-cadherin, β-catenin, and Fgfrs 1 and 2 and the levels of these interactions were altered in the absence of *Dlg-1*. Finally, loss of *Dlg-1* not only led to changes in the levels of Fgfr1, Fgfr2, and EphA2 but also led to even greater changes in the levels of activation of these receptors. Based on these results and the knowledge that EphA2 contains a PDZ binding motif whereas Fgfrs do not, I propose that Dlg-1 modulates Fgfr signaling through regulation of EphA2.

INTRODUCTION

Growth factors, through activation of growth factor receptor tyrosine kinases (RTK) and their downstream signaling intermediates, are crucial regulators of cell proliferation and differentiation, essential process for both normal development and the long-term maintenance of organ structure and function (Lovicu et al., 2011; Robinson, 2006; Wang et al., 2010). The formation and maintenance of the architecture of specialized organs is also dependent on signaling pathways that regulate cell-cell adhesion, cytoskeletal structure, apical-basal polarity and planar cell polarity. Identifying factors that coordinate the signaling pathways known to regulate these diverse biological processes is crucial to our understanding of normal development and disease that can occur when these pathways are disrupted (Lovicu et al., 2011; Robinson, 2006; Wang et al., 2010). *Dlg-1 (Discs large-1)*, the mouse homolog of *Drosophila dlg*, is known to be required for developmental processes in multiple organs (Cavatorta et al., 2004; Gardiol et al., 2006; Naim et al., 2005) through Dlg-1's regulation of cell-cell adhesion, cell shape, apical-basal and planar cell polarity (Nguyen et al., 2005; Rivera et al., 2013; Rivera et al., 2009). Previously, we showed that *Dlg-1* is required for lens fiber cell differentiation and maintenance of the architecture of the lens (Lee and Griep, 2014) and also is a modulator of the Fgfr signaling pathway in the mouse lens (Lee and Griep, 2014). In this study, I address the possibility that *Dlg-1* is a factor that regulates the interaction of the ephrin/Eph (Cheng and Gong, 2011; Cooper et al., 2008) and Fgf/Fgfr (Gu et al., 1996; Huang et al., 2003; Lai et al., 2011) signaling pathways, two signaling pathway that are crucial for fiber cell structure and differentiation.

The lens has been widely used as a system for studying mechanisms of mammalian development because it consists of one cell type, epithelial cells, which are organized into spatially separated regions of quiescent, cycling and differentiating cells. It is composed of a monolayer of epithelial cells that overlies a mass of derivative, differentiated, highly elongated cells, the fiber cells. The lens derives from a layer of ectodermal cells, the lens placode, that overlie the optic vesicle. Signals from the optic vesicle induce the ectodermal cells to thicken, invaginate, and pinch off from the overlying ectoderm, forming the lens vesicle. Next, cells in the anterior form the lens epithelial cells while cells in the posterior undergo a terminal differentiation process in which they withdraw from the cell cycle, elongate to occlude the hollow vesicle, and upregulate express differentiation specific proteins, forming the primary fiber cells. Subsequently, secondary fiber cells differentiate from the periphery of the lens as epithelial cells withdraw from the cell cycle, elongate and migrate along the capsule and epithelial cells until they meet their counterpart forming the lens sutures (McAvoy et al., 1999; Piatigorsky, 1981).

Growth factor signaling pathways play crucial roles in lens development. Among the various growth factors that are expressed in the lens, Fgfs have been demonstrated to be the only growth factors capable of inducing lens fiber cell differentiation (Liu et al., 1996; Richardson et al., 1993; Wang et al., 2010). 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 (Zhao et al., 2008). Eph receptors form the largest subfamily of receptor tyrosine kinases (RTK). EphA2, an Eph receptor which is expressed in the cortical lens fiber cells (Cooper et al., 2008; Jun et al., 2009; Shi et al., 2012), has been shown to be required for linking the adherens junction protein, N-cadherin, to β-catenin, thereby promoting linkage to the

cytoskeleton and cell-cell adhesion (Cooper et al., 2008). In addition, mutations in *EPHA2* are associated with age-related lens cataract in humans (Jun et al., 2009). The interplay between the Eph/ephrin and Fgfr signaling pathways has been shown to be involved in regulating cell fate determination and asymmetric cell division in *Ciona* embryos (Picco et al., 2007) where it has been shown that activated Eph receptor inhibited Erk activation whereas Fgfr signaling promoted Erk activation. These findings led to the proposal that Fgfr signaling and Eph/ephrin signaling antagonistically regulate developmental processes (Picco et al., 2007). Other studies have reported an agonistic interaction between Fgfr and Eph/ephrin signaling pathways. In mammalian cells, Fgfr and EphA4 phosphorylated each other and activated downstream signaling pathways. Furthermore, co-stimulation of both receptors resulted in the potentiation of MAPK signaling; although, it does not require direct binding of Fgfr and Eph receptor (Yokote et al., 2005).

Recently, the Griep lab discovered that *Discs large-1* (*Dlg-1)*, the mouse homolog of the *Drosophila* gene *discs-large* (*dlg),* is expressed in the lens (Nguyen et al., 2003; Nguyen et al., 2005) and we demonstrated that *Dlg-1* is required for cell adhesion, apical-basal polarity and fiber cell differentiation in the lens using lens specific deletion of *Dlg-1*. I also showed that loss of *Dlg-1* in the lens disrupted Fgfr signaling and 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 Fgfr signaling pathway (Lee and Griep, 2014). Dlg-1 belongs to a class of proteins referred to as PDZ domain containing proteins which are thought to act as scaffolding proteins capable of assembling large macromolecular complexes at the cell membrane (Harris and Lim, 2001). PDZ proteins interact directly with proteins that contain a PDZ binding motif, usually positioned at the C-terminus of the proteins (Harris and Lim, 2001). Because Fgfrs are

not known to contain PDZ binding motifs, it is likely that the regulation of Fgfr signaling by Dlg-1 is indirect, mediated by other proteins. Interestingly, EphA2 does contain a PDZ binding motif (Kalo and Pasquale, 1999) and both *Dlg-1* (Rivera et al., 2009) and *Epha2* (Cooper et al., 2008) mutant lenses show disruption in the hexagonal architecture of the fiber cell and cell adhesion defects, suggesting that these proteins may interact in the lens.

In this study, I first addressed the finding that Fgfr levels are oppositely affected in the *Dlg-1* deficient state and the impact of this imbalance on Fgfr signaling. I found that levels of Fgfr1 negatively correlated with levels of Fgfr2 and that the levels of activated Fgfr downstream signaling intermediates positively correlated with Fgfr2 levels. Second, I addressed the hypothesis that Dlg-1's regulation of Fgfr signaling is mediated through Dlg-1's regulation of EphA2 signaling. I found that ablation of *Dlg-1* led to reduced EphA2 levels and to disruption of the association of N-cadherin and β-catenin in a manner that phenocopies ablation of *EphA2*. I found that Dlg-1 complexes with EphA2, that EphA2 complexes with Fgfr1 and Fgfr2 and that Dlg-1 complexes with Fgfr1 and Fgfr2, albeit more weakly than does EphA2. Finally, I 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 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.

RESULTS

Cross regulation of Fgfrs in the absence of Dlg-1

I previously have shown that ablation of *Dlg-1* in the mouse lens led to decreased levels of Fgfr2; however, the levels of Fgfr1 were increased (Lee and Griep, 2014). Therefore, I hypothesized that in the absence of Dlg-1, downregulation of one Fgfr results in upregulation of the other Fgfr. To test this hypothesis, I generated $Dlg^{f/f}$*10Cre* micethat also carried one mutant allele of *Fgfr1* or *Fgfr2*. I 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 the levels of Dlg-1 were 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 (Fig. 2). 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. The intensities of Fgfr1 or Fgfr2 staining in transition zone in control and mutant lenses were quantified by ImageJ (see Materials and Methods). Levels of Fgfr1 in $Fgfr1^{f/+}$*10Cre*lenses and Fgfr2 in the $Fgfr2^{f/+}$*10Cre* lenses were reduced approximately 50%, in keeping with the genetic disruption of one allele (Fig. 3). 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 (Fig. 3). 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 quantified.

Interestingly, Fgfr1 levels were increased by 45% in *Dlg$^{f/f}$;Fgfr2$^{f/+}$10Cre* lenses as compared to 25% in *Dlg$^{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/+}$10Cre* mice were immunostained with anti-Fgfr2 antibodies and the staining intensities quantified. Fgfr2 levels were decreased by only 19% in *Dlg$^{f/f}$;Fgfr1$^{f/+}$10Cre*as compared to 53% in *Dlg$^{f/f}$10Cre* lenses (Fig. 1).Thus, deficiency of Fgfr1 in the absence of Dlg-1 reverses the effect on Fgfr2 levels. These results demonstrate 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, downregulation of one Fgfr leads to an upregulation of another Fgfr, perhaps as an attempt at compensation.

Figure IV.1. Relative Fgfr levels in *Dlg-1*, *Fgfr* compound mutant lenses. (A) Paraffin sections of eyes from P2 control, *Fgfr2*$^{f/+}$*1Ccre*, *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*$^{f/+}$*1Ccre*, *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. Bar = 50μm. (B) Quantification of Fgfr1 and Fgfr2 levels. Shown are the relative levels of Fgfr1 and Fgfr2 in the transition zone of the mutant lenses as compared to levels in the corresponding regions of the control lenses (control levels set at 1.0). Quantification of signal intensities 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 Fgfr1 levels in *Fgfr2*$^{f/+}$*10Cre* lenses were the same as in controls. The Fgfr1 levels in *Dlg*$^{f/f}$*10Cre* lenses were increased as compared to 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 as compared to 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=standard deviations. *=FDR<0.05, **=FDR<0.01.

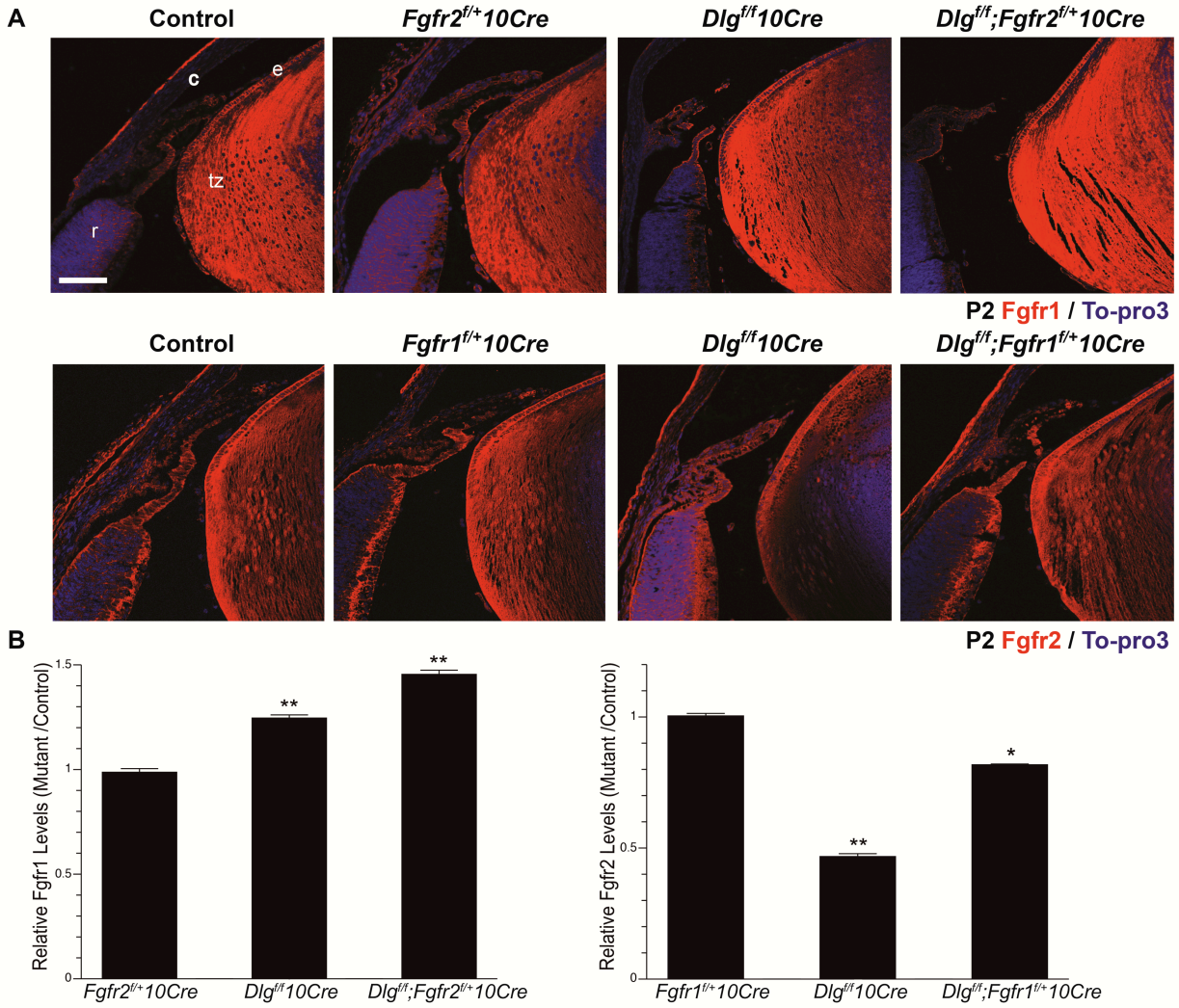

A
Control
Fgfr2f/+10Cre
Dlgf/f10Cre
Dlgf/f;Fgfr2f/+10Cre
c
e
tz
r
P2 Fgfr1 / To-pro3
Control
Fgfr1f/+10Cre
Dlgf/f10Cre
Dlgf/f;Fgfr1f/+10Cre
B
P2 Fgfr2 / To-pro3
Relative Fgfr1 Levels (Mutant /Control)
1.5
1
0.5
0
**
**
Fgfr2f/+10Cre
Dlgf/f10Cre
Dlgf/f;Fgfr2f/+10Cre
Relative Fgfr2 Levels (Mutant /Control)
1
0.5
0
**
*
Fgfr1f/+10Cre
Dlgf/f10Cre
Dlgf/f;Fgfr1f/+10Cre

Figure IV.2. Loss of Dlg-1 protein following cre-mediated excision of *Dlg-1* sequences. Paraffin embedded sections of eyes from P2 control, *Dlg$^{f/f}$10Cre*, *Dlg$^{f/f}$;Fgfr1$^{f/+}$10Cre*, and *Dlg$^{f/f}$;Fgfr2$^{f/+}$10Cre* were subjected to immunoflourescent staining for Dlg-1 (green). For each genotype, the intensity of staining throughout the lens was virtually undetectable. c, cornea; e, lens epithelium; f, lens fiber cells; r, retina; tz, transition zone. Bar = 50μm.

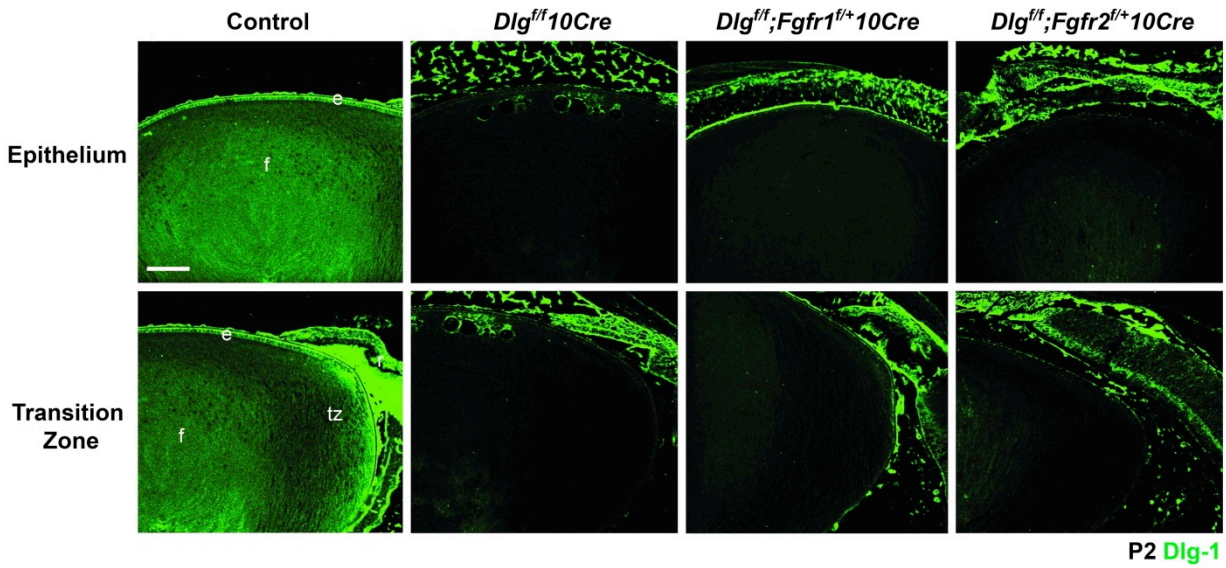
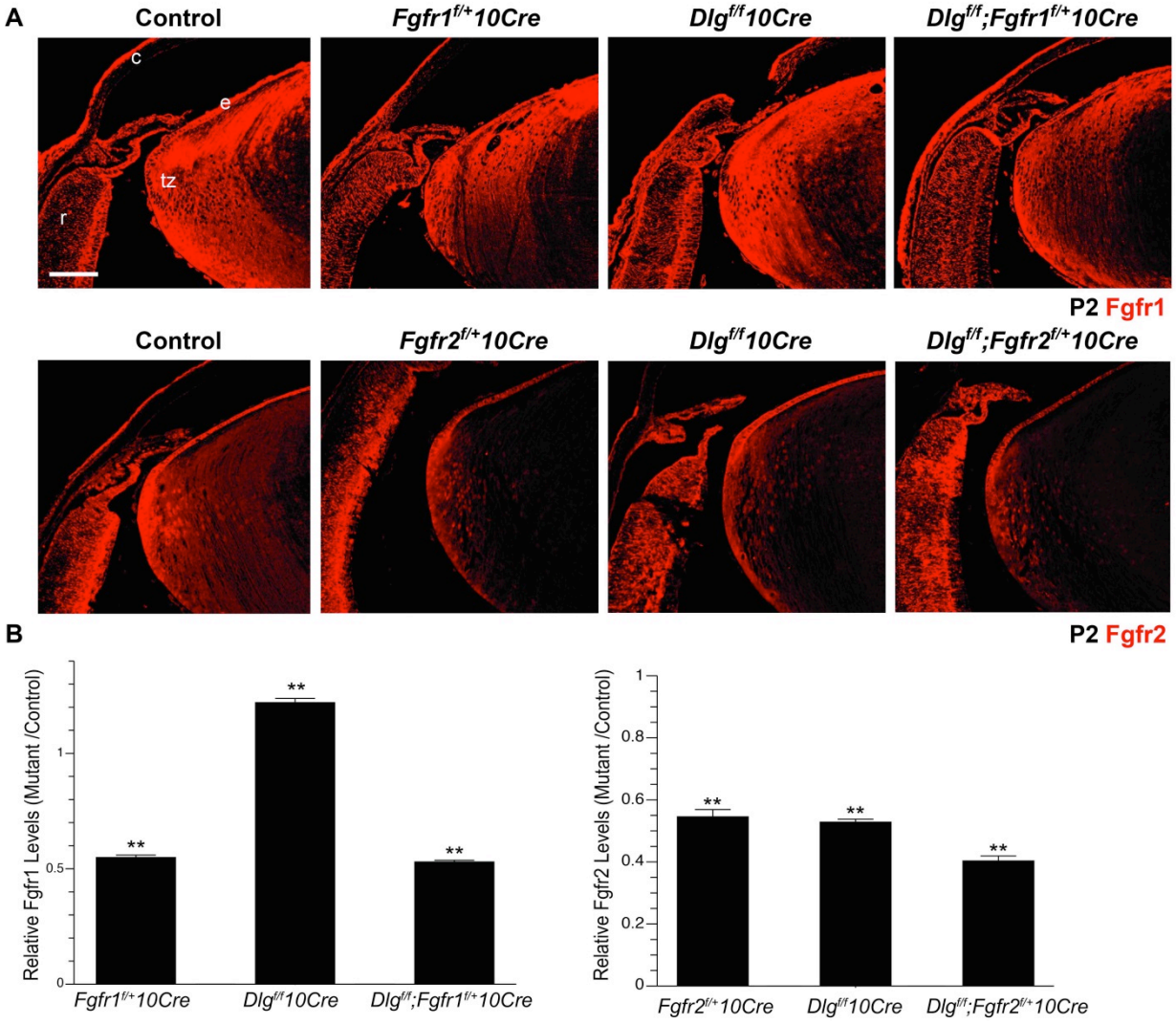

Control
Dlg^f/f10Cre
Dlg^f/f;Fgfr1^f/+10Cre
Dlg^f/f;Fgfr2^f/+10Cre
Epithelium
Transition Zone
e
f
tz
P2 Dlg-1

Figure IV.3. Loss of Fgfr1 and Fgfr2 protein following cre-mediated excision of one allele of*Fgfr1* and *Fgfr2*. (A) Paraffin sections of eyes from P2 control, *Fgfr1$^{f/+}$10Cre*, *Dlg$^{f/f}$10Cre*, and *Dlg$^{f/f}$;Fgfr1$^{f/+}$10Cre* were subjected to immunofluorescent staining using an anti-Fgfr1 antibody (red) and eyes from *Fgfr2$^{f/+}$10Cre*, *Dlg$^{f/f}$10Cre*, *Dlg$^{f/f}$;Fgfr2$^{f/+}$10Cre* mice were subjected to immunofluorescent staining using an anti-Fgfr2 antibody(red). Representative images of the transition zone are shown for each lens. c, cornea; e, lens epithelium; r, retina; tz, transition zone. Bar = 50μm. (B) Quantification of Fgfr1 and Fgfr2 levels. Shown are the relative levels of Fgfr1 and Fgfr2 in the transition zone of the mutant lenses as compared to levels in the control lenses (control levels set at 1.0). Quantification of signal intensities was carried out using ImageJ, as described in Materials and Methods, and the data subjected to statistical analysis using the two-sided One Sample t-test. At least 3 different sections from at least 3 different lenses were evaluated. Fgfr1 levels in *Fgfr1$^{f/+}$10Cre* and *Dlg$^{f/f}$;Fgfr1$^{f/+}$10Cre* lenses were reduced by 50% as compared to control lenses. Fgfr2 levels in *Fgfr2$^{f/+}$10Cre* and *Dlg$^{f/f}$10Cre* lenses were reduced 45% as compared to control lenses. Fgfr2 levels in *Dlg$^{f/f}$;Fgfr2$^{f/+}$10Cre* lenses were reduced by 60% as compared to controls.


A
Control
Fgfr1f/+10Cre
Dlgf/f10Cre
Dlgf/f;Fgfr1f/+10Cre
c
e
tz
r
P2 Fgfr1
Control
Fgfr2f/+10Cre
Dlgf/f10Cre
Dlgf/f;Fgfr2f/+10Cre
B
P2 Fgfr2
Relative Fgfr1 Levels (Mutant /Control)
0
0.5
1
**
**
**
Fgfr1f/+10Cre
Dlgf/f10Cre
Dlgf/f;Fgfr1f/+10Cre
Relative Fgfr2 Levels (Mutant /Control)
0
0.2
0.4
0.6
0.8
1
**
**
**
Fgfr2f/+10Cre
Dlgf/f10Cre
Dlgf/f;Fgfr2f/+10Cre

Effect of Fgfr1 and Fgfr2 on activation of downstream signaling intermediates

The Griep lab have previously shown 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 (Lee and Griep, 2014). Although it is not well specified each Fgf receptors effects on the downstream signaling pathways and seems to compensate each other in the lens(Zhao et al., 2008), from prior studies, it has been suggested that Fgfr2 is the primary Fgf receptor driving this pathway in the lens (Garcia et al., 2011). If this is the case, reducing the level of Fgfr1 in *Dlg-1* null lenses should result in increases levels of activated Fgfr signaling intermediates because Fgfr2 levels increase while reducing further the level of Fgfr2 should result in further decreases in levels of these activated intermediates. To address these predictions, paraffin embedded 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 quantified. In control lenses, pErk staining was observed throughout the lens fiber cell compartment and was notably more concentrated in transition zone, a region of the lens that occurs cell differentiation, and toward the posterior tips of the fiber cells. Levels of pErk in*Fgfr1*$^{f/+}$*10Cre* and*Fgfr2*$^{f/+}$*10Cre* lenses were the same as in control lenses (Fig. 4).The intensity of pErk staining 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 only 22%, as compared to 33% in *Dlg*$^{f/f}$*10Cre*lenses (Fig 4). This pattern was similar to the pattern of another Fgfr downstream intermediate, pAkt. 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 while the intensity of pAkt staining in *Dlg*$^{f/f}$;*Fgfr1*$^{f/+}$*10Cre* was reduced by 22% as compared to 39% in *Dlg*$^{f/f}$*10Cre*lenses (Fig. 5).

Thus, the levels of activated Fgfr signaling intermediates correlate positively with the levels of Fgfr2.

Figure IV.4. 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*, *Fgfr1*$^{f/+}$*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. Bar = 50μm. (B) Quantification of pErk levels. Shown are the relative levels of pErk in the transition zone of lenses (control levels set at 1.0). The relative levels of pErk in the transition zone of the *Fgfr1*$^{f/+}$*10Cre* and *Fgfr2*$^{f/+}$*10Cre* lenses were the same as controls. The relative p-Erk levels in *Dlg*$^{f/f}$*10Cre* lenses were reduced as compared to levels in the control lenses. The relative p-Erk levels in the *Dlg*$^{f/f}$;*Fgfr1*$^{f/+}$*1Cre* lenses were higher than in the *Dlg*$^{f/f}$*10Cre* lenses, whereas the p-Erk levels in the *Dlg*$^{f/f}$;*Fgfr2*$^{f/+}$*10Cre* lenses were lower than in the *Dlg*$^{f/f}$*10Cre* lenses. Error bars=standard deviations. *=FDR<0.05, **=FDR<0.01.

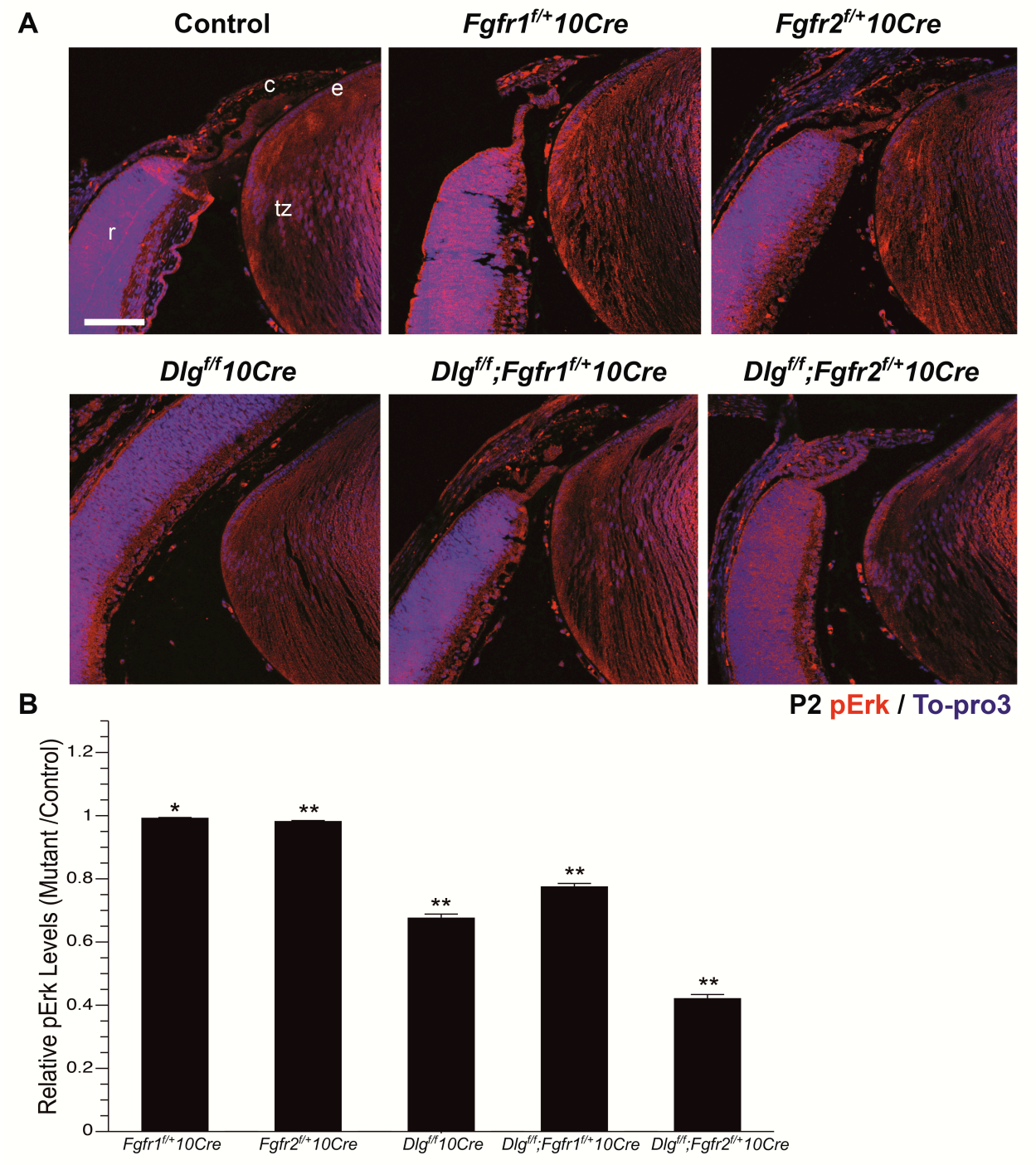
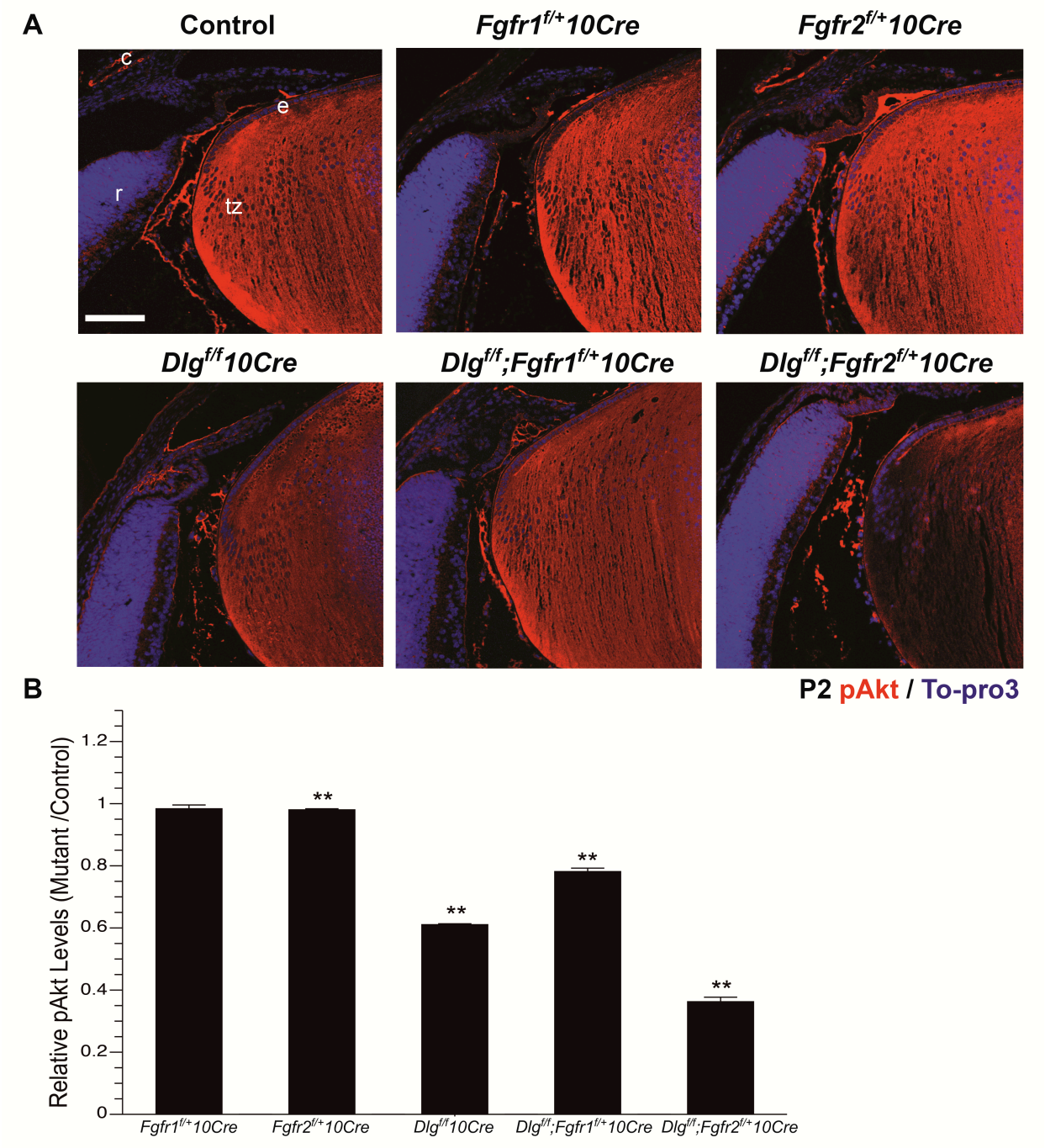

A
Control
Fgfr1f/+10Cre
Fgfr2f/+10Cre
c
e
tz
r
Dlgf/f10Cre
Dlgf/f;Fgfr1f/+10Cre
Dlgf/f;Fgfr2f/+10Cre
B
P2 pErk / To-pro3
Relative pErk Levels (Mutant /Control)
0
0.2
0.4
0.6
0.8
1
1.2
*
**
**
**
**
Fgfr1f/+10Cre
Fgfr2f/+10Cre
Dlgf/f10Cre
Dlgf/f;Fgfr1f/+10Cre
Dlgf/f;Fgfr2f/+10Cre

Figure IV.5. 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*, *Fgfr1*$^{f/+}$*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. Bar = 50μm. (B) Quantification of pAkt levels. Shown are the relative levels of pAkt in the transition zone of lenses (control levels set at 1.0). The relative levels of pAkt in the transition zone of the *Fgfr1*$^{f/+}$*10Cre* and *Fgfr1*$^{f/+}$*10Cre* lens were the same as control. The relative pAkt levels in *Dlg*$^{f/f}$*10Cre* lenses were reduced as compared to the levels in the control lenses. The relative pAkt levels 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=standard deviations. *=FDR<0.05, **=FDR<0.01.


A
Control
Fgfr1f/+10Cre
Fgfr2f/+10Cre
c
e
r
tz
Dlgf/f10Cre
Dlgf/f;Fgfr1f/+10Cre
Dlgf/f;Fgfr2f/+10Cre
B
P2 pAkt / To-pro3
Relative pAkt Levels (Mutant /Control)
1.2
1
0.8
0.6
0.4
0.2
0
**
**
**
**
Fgfr1f/+10Cre
Fgfr2f/+10Cre
Dlgf/f10Cre
Dlgf/f;Fgfr1f/+10Cre
Dlgf/f;Fgfr2f/+10Cre

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

The results of our experiments show that loss of *Dlg-1* affects the protein levels of Fgf receptors, specifically in the cytoskeletal associated fraction, suggesting that the regulation of Fgfr levels by Dlg-1 is at the protein level rather than at the RNA level. To determine if RNA levels of Fgf receptors were altered in $Dlg^{f/f}$*10Cre* lenses, I 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 1). Thus, the effect of *Dlg-1*deficiency on Fgf receptor levels is not at RNA level, supporting the concept that *Dlg-1*'s regulation of Fgfrs is at the protein level.

Table IV.1. QPCR Array on E17.5 mRNA from *Dlg$^{f/f}$10Cre*and Control lenses

Symbol	$2^{\Delta C_T}$		Ratio	Fold change	*t*-Test
	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

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

Previous studies in our lab have shown that Dlg-1 and Fgfr2 colocalize on the short sides of the fiber cells (Lee and Griep, 2014). However, any interaction between these proteins may be indirect as there is no known PDZ binding motif in Fgfr2. Interestingly, N-cadherin, which has been suggested to be a non-canonical ligand for Fgfr (Murakami et al., 2008), and β-catenin, which has PDZ binding motif at its C terminus, also localize to the short sides of the fiber cells. Our lab have shown that Dlg-1 colocalizes with N-cadherin and β-catenin (Rivera et al., 2009) and in the absence of *Dlg-1,* cell-cell contact between fiber cells is loosened and the tight association of N-cadherin with the membrane appeared to be reduced (Rivera et al., 2009). Thus, it is possible that the interaction of Dlg-1 with N-cadherin and β-catenin mediate the effects of Dlg-1 on Fgfr2. To begin to address this possibility, I asked if loss of *Dlg-1* affected the levels and subcellular distribution of N-cadherin and β-catenin. To determine if less N-cadherin is associated with the cytoskeleton, lenses from P2 control and $Dlg^{f/f}10Cre$ mice were extracted with Triton-X 100. Extracts were subjected to western blot analysis with anti-N-cadherin antibodies. The levels of N-cadherin were increased more than two-fold in the triton soluble fraction from the$Dlg10^{f/f}Cre$ lenses relative to control lenses whereas the levels of N-cadherin in the triton insoluble fractions of $Dlg^{f/f}10Cre$ were decreased by 30% compared to controls (Fig. 6). 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 was altered in

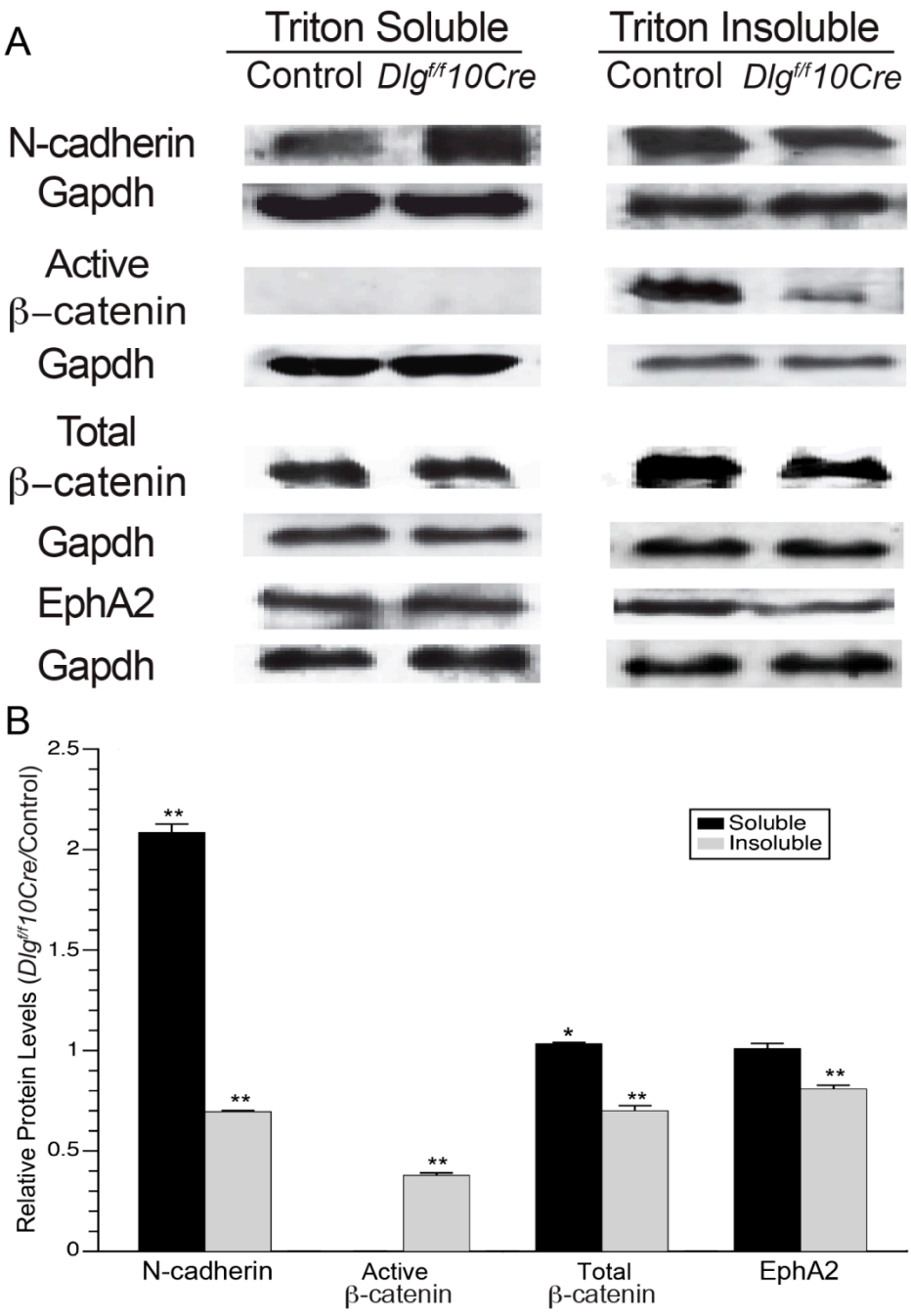
the absence of *Dlg-1*. While the levels of membrane-associated β-catenin were altered, the distribution of active β-catenin was not.

The ephrin receptor, EphA2, which also has a PDZ binding motif at its C terminus, is required for localizing N-cadherin to the membrane and promoting the linkage of N-cadherin to β-catenin (Cooper et al., 2008). In the absence of EphA2, N-cadherin was found redistributed to the cytoplasm, although β-catenin localization was not affected (Cooper et al., 2008). The similarity of the effect of loss of Dlg-1 on N-cadherin and β-catenin localization to that of loss of EphA2 raises the possibility that EphA2 is affected by the loss of Dlg-1. Therefore, extracts from control and *Dlg$^{f/f}$10Cre* lenses were also subjected to western blot analysis with anti-EphA2 antibodies. While EphA2 levels in the triton soluble fraction from *Dlg$^{f/f}$10*Cre lenses were not different from that of controls, levels in the triton insoluble fraction were decreased by 20% in *Dlg$^{f/f}$10Cre* lenses (Fig. 6). 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 1). Thus, loss of *Dlg-1* correlates with reduced levels of EphA2 and reduced levels of cytoskeletal associated N-cadherin, which is dependent on EphA2 function.

It is known that N-cadherin and EphA2 colocalize on the short sides of the fiber cells (Cooper et al., 2008). As the subcellular distribution of N-cadherin was altered in *Dlg$^{f/f}$10Cre* lenses, I asked if colocalization of N-cadherin and EphA2 localization was lost in the *Dlg$^{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}$10Cre*mice using anti-N-cadherin and anti-EphA2 antibodies. As shown previously (Cooper et al., 2008), immunostaining for EphA2 and N-cadherin was predominantly localized to the short sides of the fiber cells in the outer cortex (Fig. 7) and co-localization was observed in control lenses. In *Dlg$^{f/f}$10Cre*lenses,the

intensity of EphA2 and N-cadherin staining was reduced, cytoplasmic N-cadherin was observed, and, therefore, co-localization of EphA2 and N-cadherin was reduced (Fig. 7). Collectively, these data demonstrate that *Dlg-1* is required to maintain the normal levels and distribution of β-catenin, N-cadherin, and EphA2.

Figure IV.6. 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}$10Cre* lenses were subjected to western blot analysis for the indicated proteins and the blots reprobed for Gapdh as a loading control. Representative blots are shown. (B) Quantification of protein levels. Shown are the levels of the indicated proteins in extracts from *Dlg$^{f/f}$10Cre*lenses relative to levels in the controls (control levels set a 1.0). Signal intensities were quantified by phosphorimager analysis, as described in Materials and Methods, and the data subjected to statistical analysis using the two-sided One Sample t-test. At least 3 protein pools were blotted in triplicate over 1-3 blots. The relative levels of N-cadherin in the cytosolic fraction from *Dlg$^{f/f}$10Cre* lenses were increased as compared to controls whereas the levels of cytoskeletal associated N-cadherin were reduced. The levels of active β-catenin, total β -catenin and EphA2in the cytoskeletal associated fraction from *Dlg$^{f/f}$10Cre* lenses were reduced as compared to controls. Error bars=standard deviations. *=FDR<0.05, **=FDR<0.01.


A
Triton Soluble
Control Dlg^f/f10Cre
Triton Insoluble
Control Dlg^f/f10Cre
N-cadherin
Gapdh
Active β−catenin
Gapdh
Total β−catenin
Gapdh
EphA2
Gapdh
B
Relative Protein Levels (Dlg^f/f10Cre/Control)
2.5
2
1.5
1
0.5
0
Soluble
Insoluble
**
**
**
*
**
**
N-cadherin
Active β-catenin
Total β-catenin
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 contains PDZ binding motifs at their C-termini, suggesting that these three proteins may complex with each other. Fgf receptors, on the other hand, have no known PDZ binding motif. However, a direct interaction between another Eph receptor, EphA4and Fgfrs 1 and 2 has been documented (Fukai et al., 2008; Yokote et al., 2005). 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 (Cooper et al., 2008) and this could be the mechanism through which Dlg-1 status affects Fgfr signaling. As levels of N-cadherin, β-catenin, EphA2 and Fgfr2 are reduced (Figs. 1 and 6), and co-localization between N-cadherin and EphA2 is lost (Fig. 7) in the *Dlg-1* null lenses, I hypothesized that these proteins would be complexed in the lens.

To test this hypothesis, co-immunoprecipitation 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 triton insoluble 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 while the Dlg-1 immunoprecipitates were blotted with antibodies against β-catenin or EphA2 (Fig. 8). As expected, in control extracts N-cadherin and EphA2 co-immunoprecipitated with β-catenin and N-cadherin co-immunoprecipitated with EphA2. The levels of N-cadherin and EphA2 co-immunoprecipitating

with β-catenin were reduced in extracts from *Dlg$^{f/f}$10Cre* lenses as were the levels of N-cadherin co-immunoprecipating with EphA2. Dlg-1 co-immunoprecipitated with β-catenin and EphA2 in control extracts but not from *Dlg$^{f/f}$10Cre* extracts. Next, I asked if EphA2 and Dlg-1 co-immunoprecipitate with N-cadherin, Fgfr1 and/or Fgfr2. Extracts from control and *Dlg$^{f/f}$10Cre* lenses were subjected to immunoprecipiation with antibodies against Fgfr2 and EphA2 and the immunoprecipitates blotted with antibodies for N-cadherin, EphA2 and Fgfr1, respectively. Extracts were also subjected to immunoprecipitation with anti-Dlg-1 antibody and the immunoprecipitates blotted with antibodies for Fgfr2 or Fgfr1. In control extracts EphA2 immunoprecipitated with both Fgfr2 and Fgfr1. The levels of N-cadherin co-immunoprecipitating with EphA2 were reduced in extracts from *Dlg$^{f/f}$10Cre* lenses compared to control lenses. Interestingly, in extracts from *Dlg$^{f/f}$10Cre* lenses, the amount of Fgfr2 co-immunoprecipitating with EphA2 was reduced as compared to controls while the amount of Fgfr1 co-immunoprecipitating with EphA2 was increased. In control extracts Fgfr1 and Fgfr2 also co-immunoprecipitated with Dlg-1. However, the amount of Fgfr co-immunoprecipitating with Dlg-1 was less than the amount that co-immunoprecipitated with EphA2, suggesting that the interaction between EphA2 and the Fgf receptors may be more direct than the interaction between Dlg-1 and the Fgf receptors. 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 involved 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.

Figure IV.7. EphA2 and N-cadherin co-localization is disrupted and 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 diffuseand co-localization was lost. Bar = 50μm.

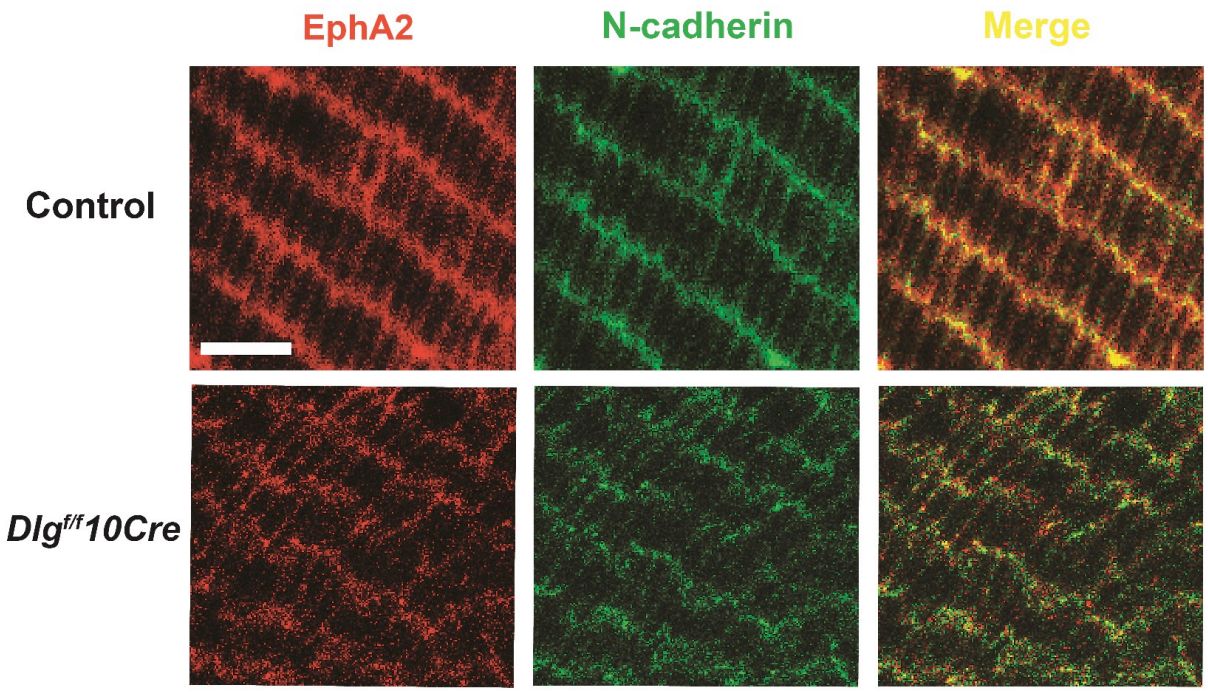
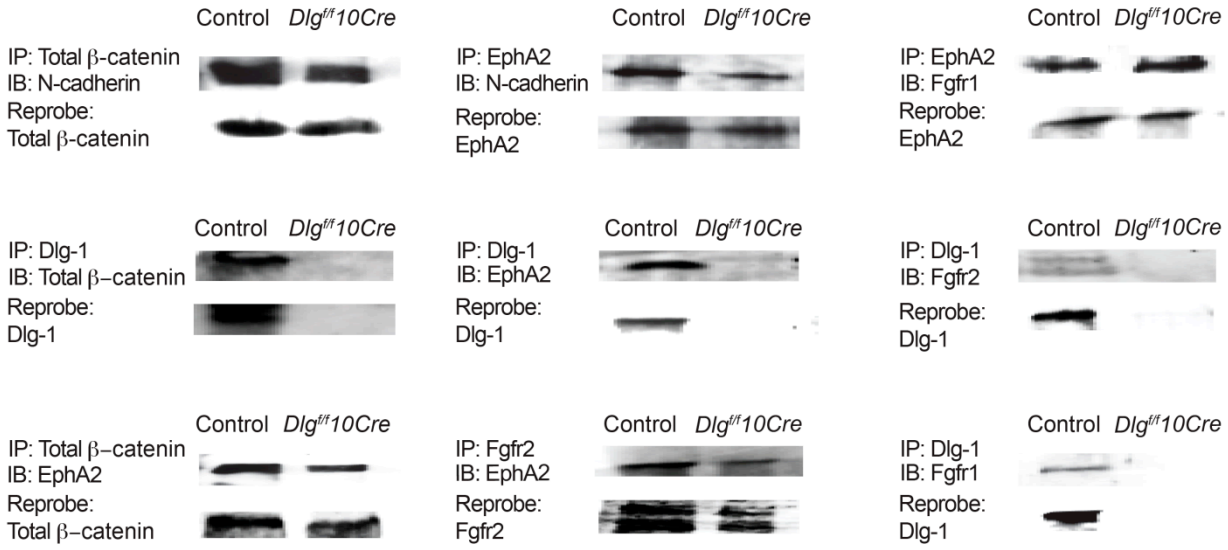

EphA2
N-cadherin
Merge
Control
Dlg^f/f10Cre

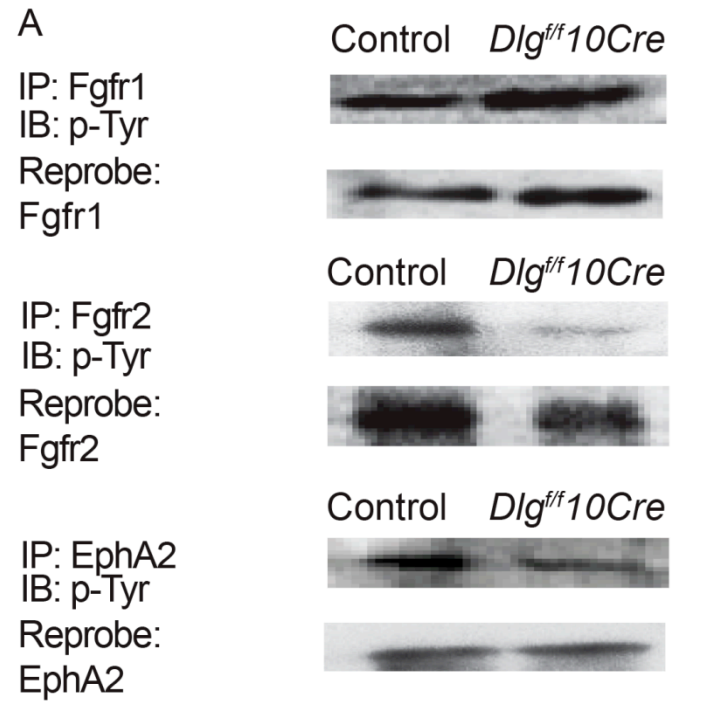
Figure IV.8. Levels of protein-protein interactions are altered in *Dlg$^{f/f}$10Cre* lenses. Lenses from P10 control and *Dlg10Cre* mice were extracted with Triton X-100 and the pellets resuspended in NP-40 buffer. Extracts were subjected to IP and IB using antibodies against the indicated proteins. As a loading control, the blots were reprobed for the respective immunoprecipitated proteins. In control lenses, β-catenin interacts with N-cadherin, Dlg-1 interacts with β-catenin, EphA2, Fgfr1 and Fgfr2, and EphA2 interacts with β-catenin, Fgfr1 and Fgfr2. In *Dlg$^{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 as compared to controls whereas the interaction between EphA2 and Fgfr1 was increased as compared to controls.


Control
Dlg^f/f 10Cre
IP: Total β-catenin
IB: N-cadherin
Reprobe:
Total β-catenin
IP: EphA2
IB: N-cadherin
Reprobe:
EphA2
IP: EphA2
IB: Fgfr1
Reprobe:
EphA2
IP: Dlg-1
IB: Total β–catenin
Reprobe:
Dlg-1
IP: Dlg-1
IB: EphA2
Reprobe:
Dlg-1
IP: Dlg-1
IB: Fgfr2
Reprobe:
Dlg-1
IP: Total β–catenin
IB: EphA2
Reprobe:
Total β–catenin
IP: Fgfr2
IB: EphA2
Reprobe:
Fgfr2
IP: Dlg-1
IB: Fgfr1
Reprobe:
Dlg-1

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

Based on the finding that Dlg-1 is found in a complex with Fgfrs 1, 2 and EphA2 and that *Dlg-1* status affects the 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 levels 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 Fgf receptors and EphA2, lenses from P10 control and $Dlg^{f/f}$*10Cre*mice were extracted with triton-X 100 buffer and the insoluble fraction resuspended in NP-40 buffer. The extracts subjected to immunoprecipitation with antibodies against Fgfr1, Fgfr2 and EphA2 and the immunoprecipitates subjected to western blotting using an anti-phosphotyrosine antibody (Fig. 9).In extracts from control lenses, all three receptors were tyrosine phosphorylated. In extracts from $Dlg^{f/f}$*10Cre*lenses, p-Tyr levels were140% higher for Fgfr1 as compared to the levels in control extracts. The level of tyrosine phosphorylation of Fgfr2was reduced by 89% and the level of tyrosine phosphorylated EphA2 was reduced by 45%. Thus, in the absence of Dlg-1, the activation levels of these receptors were altered. These results demonstrate that, Dlg-1 is required not only for maintaining normal levels of Fgfr1, Fgfr2, and EphA2, but also for maintaining proper levels of receptor activation.

Figure IV.9. **Levels of activated Fgfrs and activatedEphA2 are altered in *Dlg$^{f/f}$10Cre* lenses**. (A) Lenses from P10 control and *Dlg$^{f/f}$10Cre* lenses were extracted with Triton X-100 and the pellets resuspended in NP-40 buffer. The extracts were subjected to immunoprecipitation with antibodies against the indicated proteins followed by western blotting with antibody against phosphtyrosine (IB: p-Tyr). As a loading control, blots were reprobed for their respective immunoprecipitated receptor proteins. (B) Quantification of protein levels. Shown are the levels of the indicated proteins in extracts from *Dlg$^{f/f}$10Cre* lenses relative to levels in the controls (control levels set a 1.0). Signal intensities were quantified by phosphorimager analysis, 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 immunoprecipitated and immunoblotted in triplicate over 1-3 blots. Error bars=standard deviations. *=FDR<0.05, **=FDR<0.01.


A
Control
Dlg^f/f 10Cre
IP: Fgfr1
IB: p-Tyr
Reprobe:
Fgfr1
Control
Dlg^f/f 10Cre
IP: Fgfr2
IB: p-Tyr
Reprobe:
Fgfr2
Control
Dlg^f/f 10Cre
IP: EphA2
IB: p-Tyr
Reprobe:
EphA2

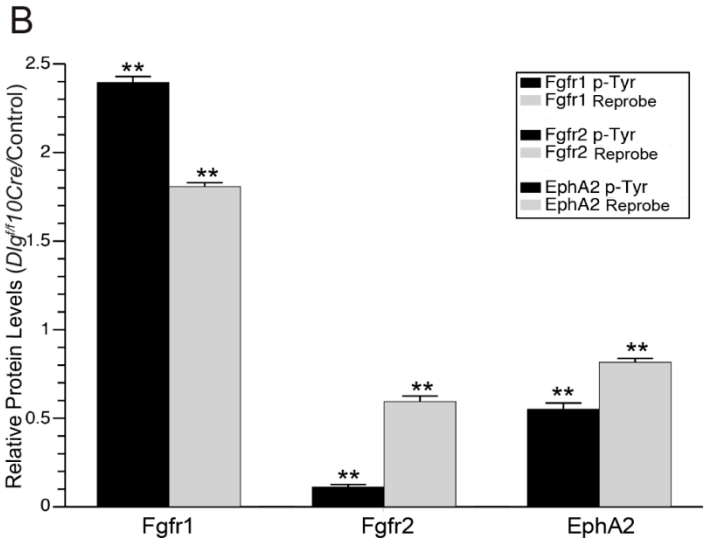
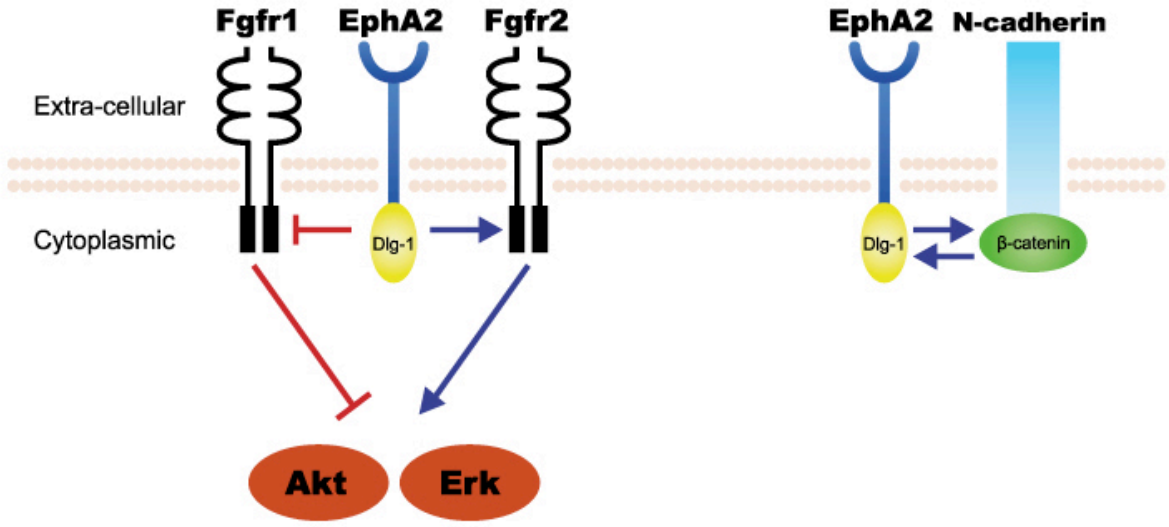

B
Relative Protein Levels (Dlg^f/f 10Cre/Control)
2.5
2
1.5
1
0.5
0
**
Fgfr1 p-Tyr
Fgfr1 Reprobe
Fgfr2 p-Tyr
Fgfr2 Reprobe
EphA2 p-Tyr
EphA2 Reprobe
Fgfr1
Fgfr2
EphA2

Figure IV.10. Proposed model of Dlg-1 function in the lens. EphA2 is known to associate with Fgfr1 and Fgfr2 to activate downstream Akt and Erk. The result obtained in this study support a model in which Dlg-1 inhibits Fgfr1 signaling and enhances Fgfr2 signaling through regulation of EphA2 activity. In addition, Dlg-1, by regulating EphA2 activity, promotes EphA2's interaction between N-cadherin and β-catenin, thus stabilizing membrane anchored adherens junction complexes.


Fgfr1
EphA2
Fgfr2
EphA2
N-cadherin
Extra-cellular
Cytoplasmic
Dlg-1
Dlg-1
β-catenin
Akt
Erk

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, I examined the relationship between Dlg-1, a scaffolding protein involved in cell-cell adhesion, apical-basal polarity and planar cell polarity (Rivera et al., 2013), EphA2, which plays a role in lens fiber cell packing and adherens junction formation (Cooper et al., 2008),and Fgfr signaling, which is required for lens fiber cell differentiation (Zhao et al., 2008). I found that in the *Dlg-1* deficient state, the effects on pErk and pAkt correlated with levels of Fgfr2. Loss of *Dlg-1* also led to redistribution of N-cadherin to the cytosol. I found that Dlg-1 complexes with β-catenin, EphA2 and Fgfrs 1 and 2. I found that EphA2 complexes with N-cadherin, β-catenin and Fgfrs 1 and 2 and the level of these interactions are altered in the absence of Dlg-1. I also found that loss of *Dlg-1* not only led to changes in the levels of Fgfr1, Fgfr2 and EphA2 but also led to altered levels of their activation. Based on our results, I propose that *Dlg-1* modulates Fgfr signaling and lens fiber cell packing through EphA2 (see Fig. 7). Thus, I suggest that the mechanism through which *Dlg-1* regulates fiber cell differentiation is, at least in part, through regulation of EphA2.

Dlg-1-dependent Regulation of Fgfr1 and Fgfr2 Signaling

I previously showed that Fgfr1 is increased and Fgfr2 is decreased in the *Dlg-1* deficient lenses (Lee and Griep, 2014). In this study, I 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). I also showed that the level of activation of Fgfr1 was increased while the level of activation of Fgfr2 was decreased (Fig. 9). These results suggest that Dlg-1 is an activator of

Fgfr2 and a suppressor of Fgfr1. The mechanism through which changes in Fgfr levels occur is not known. However, these changes in receptor levels are occurring at the protein level rather than at the RNA level (Table 1). It is possible that the lens attempts to compensate for loss of Fgfr2 activity by increasing activity of other Fgfrs. This compensation does not occur in the Dlg-1 sufficient state as deletion of one allele of Fgfr1 or Fgfr2 did not result in increased levels of the other or significantly alter the levels of pErk and pAkt (Zhao et al., 2008). It is possible that the changes in cell shape that occur as a consequence of ablation of *Dlg-1* result in changes in the stability of the protein complexes at the membrane (see below), leading to altered levels of Fgfrs. No changes in fiber cell shape have been reported to occur in single Fgfr deficient lenses.

I further showed that the levels of activated signaling intermediates, pErk and pAkt, correlated with Fgfr2 levels (Figs. 4 and 5), consistent with the previous suggestion that this is the major Fgfr driving the MAPK and Pi3Kinase pathways (Lovicu et al., 2011). However, the reduction in Fgfr2 levels and activity was greater than the reduction in pErk and pAkt levels. I previously showed that Fgfr3 levels were increased in the Dlg-1 deficient lenses (Lee and Griep, 2014). Thus, if Fgfr3 is a positive regulator of Erk and Akt activation, it is possible that increased Fgfr3 activity might account for the differences between the reduction in Fgfr2 activation and reduction in pErk and pAkt levels.

Dlg-1 is a Regulator of EphA2 Activity

Previous studies have shown that EphA2 colocalizes with N-cadherin and β-catenin on the short sides of the lens fiber cells and EphA2 can be complexed with N-cadherin and β-catenin(Cooper et al., 2008). This interaction is required for linking N-cadherin to β-catenin at the cell membrane, an important part of regulating cell shape and cell-cell adhesion (Cooper et

al., 2008). In this study, I demonstrated that loss of *Dlg-1* results in a redistribution of N-cadherin from the cytoskeletal associated fraction to the cytosolic fraction (Fig. 6), disrupts the colocalization of N-cadherin and EphA2 (Fig. 7), and disrupts the interaction of N-cadherin and membrane associated β-catenin (Fig. 8). I furthermore demonstrated loss of Dlg-1 results in reduced levels of activated EphA2 (Fig. 9), indicating that Dlg-1 is required for stimulating the appropriate level of EphA2 activation and function. Thus, I have identified Dlg-1 as an upstream regulator of EphA2. These results suggest that the mechanism through which Dlg-1 carries out its well-known role in cell-cell adhesion is through its regulation of EphA2. However, the reduction in activation of EphA2 when *Dlg-1* is ablated was only partial (Fig. 9), suggesting that there may be factors in addition to Dlg-1 that play a role in regulating EphA2 activity. It is possible that *Dlg-1* and other well known PCP factors cowork for maintaining cell-cell adhesion. In the lens, core PCP genes, *Vangl2* and *Celsr* mutants exhibited similar structural defects (Sugiyama et al., 2009; Sugiyama et al., 2011). Interestingly, this lens phenotype resembled that of Dlg-1 and EphA2 mutants suggesting that this subset of PDZ domain proteins are playing a role in PCP, it is likely that the mechanism through Dlg-1 affect theses core PCP proteins and growth factor signalings.

Certain receptor tyrosine kinases have been shown to interact with PDZ proteins (Hock et al., 1998). For example, in neurons some members of the Eph receptor family have been shown to interact with PDZ proteins via their C-terminal PDZ binding motif (Torres et al., 1998). However, neither EphA2 nor Dlg-1 was among the proteins identified in that screen. Given the similarities in the phenotypes of lenses deficient for these proteins and the effect of loss of Dlg-1 on EphA2 function, I hypothesized that they might interact. Indeed, I showed by co-immunoprecipitation that Dlg-1 and EphA2 are found in the same cytoskeletal-associated

complex in the lens (Fig. 8). These results suggest that Dlg-1through its role as a scaffolding protein may assemble 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 co-immunoprecipitation 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 as well and the association of Dlg-1 with β-catenin was strong (Subbaiah et al., 2012) (Fig. 8). 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, I have shown that Dlg-1 affects the activation levels of Fgfrs 1 and 2 (Fig. 9), indicating that not only is Dlg-1 a regulator of Fgfr levels but also a regulator of the activation of these receptors. The regulation of Fgfrs by Dlg-1 is not likely to occur through direct binding, as Fgfrs do not have a known PDZ binding motif. Based on our results and the precedent that EphA4 binds to Fgfr1 and Fgfr2, (Picco et al., 2007), I hypothesized that in the lens EphA2 interacts with Fgfrs 1 and 2 and that Dlg-1 influences this interaction through its regulation of EphA2. Consistent with this hypothesis, I found that Dlg-1, EphA2, Fgfr1 and Fgfr2 co-immunoprecipitate with each other (Fig. 8), suggesting that they complex with each other. Because the interaction between Dlg-1 and the Fgfrs appeared to be weaker than the interaction between EphA2 and the Fgfrs, I suggest that EphA2 is a bridge between Dlg-1 and the Fgfrs. Thus, I put forth a model in which Dlg-1 regulates EphA2 activity, which in turn regulates Fgfr activity. Based on our results, I suggest that EphA2 is a positive regulator of Fgfr2 activity whereas it is a negative regulator of Fgfr1 activity. Cross-talk between Ephrin/Eph and

Fgf/Fgfr signaling on cell development and downstream regulation has been shown previously (Chong et al., 2000; Moody, 2004; Picco et al., 2007; Yokote et al., 2005). In *Ciona* embryo study, antagonistic regulation of Eph receptor and Fgfr signaling in Erk activation have proposed (Picco et al., 2007) whereas agonistic interaction of Fgfr and EphA4 in MAPK signaling activation have reported in mammalian cells study (Fukai et al., 2008; Yokote et al., 2005). Despite the efforts made towards the understanding of interplay between Eph receptor and Fgfr signaling, it remains unclear how they regulate downstream signaling in the lens cell development and how these signaling events are coupled to the lens.

Taken together, our study provides new insight into the role of *Dlg-1*in vertebrate development. Our study provides evidence to 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.

Conclusion

Herein, I have proposed Dlg-1 modulates Fgfr signaling through regulation of EphA2. I found that Dlg-1 complexes with EphA2, that EphA2 complexes with Fgfr1 and Fgfr2 and Dlg-1 complexes with Fgfr1 and Fgfr2, albeit more weakly than does EphA2. I found that loss of Dlg-1 leads to reduced complexing of EphA2 with N-cadherin, β-catenin and Fgfr2 but increased complexing of EphA2 with Fgfr1 and these changes correlate with the altered levels of activated EphA2, Fgfr1 and Fgfr2. I have 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. I also showed that the level of activation of Fgfr1 was increased while the level of activation of Fgfr2

was decreased. The data proposed the idea that Dlg-1 inhibit Fgfr1 signaling and enhance Fgfr2 signaling indirectly, but through EphA2 activation. Based on these results and the knowledge that EphA2 contains a PDZ binding motif whereas Fgfrs do not, I propose that Dlg-1 modulates Fgfr signaling through regulation of EphA2 and also Dlg-1 is required to proper Fgfr signaling and adherens junction formation mediated by EphA2.

CHAPTER V: Genetic Interaction between *Dlg-1* and Planar Cell Polarity Genes Modulates Fgfr Signaling in the Mouse Lens

This chapter is composed of unpublished data obtained by Sungkyoung Lee. The Materials and Methods have been moved to Chapter II. The generation of *Dlg*$^{f/+}$*10Cre;Vangl2*$^{Lp/+}$and *Scrib*$^{f/+}$*10Cre;Vangl2*$^{Lp/+}$ compound heterozygote mice is also described in Chapter II.

SUMMARY

Planar Cell Polarity (PCP) is thought to be a key determinant in development and in the maintenance of tissue architecture during embryogenesis and in the adult organism. Growth factor receptor signaling pathways are also regulators of many developmental processes. The Griep lab has previously shown that the PCP genes, *Dlg-1*, *Scrib*, and *Vangl2* are required for epithelial cell structure and polarity and fiber cell structure and differentiation. Fibroblast growth factor receptors (Fgfr) signaling are involved in the regulation of many developmental processes and are known to be required for fiber cell differentiation. In Chapter III, I showed that *Dlg-1* is a regulator of Fgfr2 signaling, a pathway required for fiber cell differentiation. In this chapter, I asked if the interactions between PCP genes *Dlg-1*, *Scrib*, and *Vangl2,* that control fiber cell structure also modulate the Fgfr signaling pathway, which is known to be required for fiber cell differentiation. Results indicate that *Dlg-1* haplodeficiency enhances $Vangl2^{Lp/+}$ phenotype of reduced Fgfr signaling whereas *Scrib* haplodeficiency rescues the $Vangl2^{Lp+}$ reduction of Fgfr2 signaling. Thus, *Dlg-1* and *Scrib* may act in opposing ways with respect to *Vangl2* in modulating PCP and Fgfr signaling pathways in ocular lens.

INTRODUCTION

Fiber cell differentiation in the mouse ocular lens is a multi-step process involving changes in cell's proliferative capacities and the differentiation of the progenitor epithelial cells into the terminally differentiated fiber cells. It is thought that certain growth factor signaling pathways are critical for fiber cell differentiation (Lovicu et al., 2011; Robinson, 2006). In addition, extensive changes in cell shape that result in transformation of the cuboidal epithelial cell into the elongated, hexagonal shape of the fiber cell and the orderly packing of these fibers and their coordinated movement into the center of the lens are necessary to form the overall structure of this tissue (Kuszak et al., 2004; Singh and Mlodzik, 2012). Planar cell polarity (PCP), the polarization of cells in an epithelial sheet perpendicular to the orientation of apical-basal polarity, has been identified to be critically required for regulating changes in cell shape and the coordinated movement of cells that form the overall shape of an organ (Singh and Mlodzik, 2012; Wallingford, 2012). An open question is whether PCP and their regulation of cell shape modulates growth factor signaling pathways that are required to form the differentiated structure of cells in tissues. In this chapter, I ask whether fiber cell shape, which we have recently shown is regulated by the interactions between the PCP genes *Dlg-1*, *Scrib* and *Vangl2*, modulates the activation of the Fgfr2 signaling pathway, which is known to be required for fiber cell differentiation.

PCP was originally described in *Drosophila* where it is governed by a group of core genes that belong to the Wnt/PCP pathway. In *Drosophila*, *Strabismus/Van Gogh* is identified as a core Wnt/PCP gene where it is known to be required in epithelial structures such as leg bristles, wing cell hairs, and eye ommatidia (Taylor et al., 1998). The vertebrate homolog, *Vangl2*, is a

PCP gene in vertebrates as it is required for formation of the polarized structure of the sterociliary bundles in the hair cells of the cochlea and neural tube closure, two developmental processes that require multiple Wnt/PCP genes (Dabdoub et al., 2003; Dabdoub and Kelley, 2005). However, in vertebrates the group of Wnt/PCP genes is much larger, as it includes some genes that are not known as PCP genes in *Drosophila*. For example, *Scrib*, the vertebrate homologs of the *Drosophila* gene, *scrib,* is a prerequisite for the proper localization of PCP proteins, such as Vangl2 (Montcouquiol 2003). We identified *Dlg-1*, the vertebrate homolog of the *Drosophila* gene, *dlg*, as another new PCP gene in the mouse (Rivera et al., 2009). Dlg-1 and Scrib are PDZ domain (PSD-95, Dlg-1, ZO-1) containing proteins, which are thought to function as scaffolding molecules to organize and maintain large and complicated protein complexes at the membrane (Bilder et al., 2000; Bilder and Perrimon, 2000; Humbert et al., 2003). The PDZ domain is a protein-protein interaction module that typically binds to a PDZ binding motif at the C-terminus of other proteins. In both *Drosophila* and human cells Strabismus/Van Gogh*,* a core member of Wnt/PCP pathway, has been shown to directly bind to the PDZ domain of proteins such as Scrib and Dlg via its C-terminal PDZ binding motif (Harris and Lim, 2001; Wang and Nathans, 2007; Yoshioka et al., 2013).

Growth factor signaling pathways play essential roles in regulating lens cell differentiation. The ocular vitreous humor, 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), and vascular endothelial growth factor (VEGF) (Lovicu et al., 2011; Wang et al., 2010). Among the various growth factors that are expressed in the lens, fibroblast growth factor (Fgf) has been demonstrated to be only growth factor capable of lens fiber cell differentiation (Lovicu

and Overbeek, 1998). Binding of a ligand to an Fgfr results in receptor activation by phosphorylation on tyrosines, which then stimulates the activation of a cascade, starting with the activation of the docking protein, Frs2α. Frs2α is then able to trigger two pathways, Erk pathway and Akt pathway (Van der Noll et al., 2013). In *in vivo* experiments, simultaneous deletion of *Fgfr* 1,2, and 3 genes revealed the complete arrest of lens fiber differentiation, which demonstrated that Fgfr signaling is required for lens development (Zhao et al., 2008). From previous studies, it has been suggested that Fgfr2 is the primary Fgf receptor driving MAPK pathway in the lens (Garcia et al., 2011).

The Griep lab previously showed that mouse *Dlg-1* and *Scrib* are required for mouse lens development (Nguyen et al., 2003; Rivera et al., 2009; Yamben et al., 2013). In addition, the laboratory has shown that these genes interact at the genetic level with *Vangl2* to regulate fiber cell shape and hexagonal packing (S. Shatadal and A.E. Griep, manuscript in preparation). In Chapter III, I demonstrated that ablation of *Dlg-1* resulted in reduced Fgfr2 signaling. In this chapter, I address the possibility that the genetic interactions between the mammalian PCP genes, *Dlg-1, Scrib*, and *Vangl2,* modulate the levels of Fgfr signaling in accordance with their modulation of fiber cell structure which ultimately regulates fiber cell differentiation.

RESULTS

Generation of $Dlg^{f/+}1Cre;Vangl2^{Lp/+}$ and $Scrib^{f/+}10Cre;Vangl2^{Lp/+}$ embryos

Dlg-1$^{f/f}$ or *Scrib*$^{f/f}$ mice were crossed with *MLR10Cre* mice and the progeny crossed with *Vangl2*$^{Lp/+}$ mice to generate $Dlg^{f/+}10Cre;Vangl2^{Lp/+}$ and $Scrib^{f/+}10Cre;Vangl2^{Lp/+}$ mice along with the control, and single mutant $Dlg^{f/+}10Cre$, $Scrib^{f/+}10Cre$, and *Vangl2*$^{Lp/+}$ mice. Progeny

containing the floxed *Dlg-1* allele and *MLR10Cre* recombinase gene were identified by PCR genotyping as described in Chapter. II. *Vangl2*$^{Lp/+}$ mice were obtained from Dr. Rivka Rachel (National Institutes of Health). To validate that haplodeficiency *for Dlg-1* or *Scrib* did not alter the levels of Vangl2, we carried out immunofluorescence experiments on paraffin embedded sections from E16.5 embryos using an anti-Vangl2 antibody. As shown in Figure 1, the intensity of staining for Vangl2 was similar in control, *Dlg*$^{f/+}$*10Cre,* and *Scrib*$^{f/+}$*10Cre* lenses. Staining for Vangl2 in the *Vangl2*$^{Lp/+}$ lens appeared to be less membrane associated, which would be expected due to the fact that the *Loop tail* mutation disrupts Vangl2's ability to be membrane associated. Some membrane staining was also evident, as would be expected from the protein arising from the wild type Vangl2 allele. Staining for Vangl2 in the *Dlg*$^{f/+}$*10Cre;Vangl2*$^{Lp/+}$ and *Scrib*$^{f/+}$*10Cre;Vangl2*$^{Lp/+}$ lenses showed reduced membrane associated protein in the cortical fiber cells. Therefore, haplodeficiency for *Dlg-1* or *Scrib* did not appear to greatly alter Vangl2 levels or localization. The *Vangl2*^{Lp} allele disrupted the amount of membrane associated Vangl2, which was not altered by *Dlg-1* or *Scrib* haplodeficiency.

Figure.V.1. Loss of Cre-mediated excision of one allele of Vangl2 protein. Paraffin embedded sections of eyes from E16.5 Control, *Vangl2*$^{Lp/+}$,*Dlg*$^{f/+}$*10Cre*, *Scrib*$^{f/+}$*10Cre*, *Dlg*$^{f/+}$*10Cre;Vangl2*$^{Lp/+}$ and *Scrib*$^{f/+}$*10Cre;Vangl2*$^{Lp/+}$ were subjected to immunoflourescent staining for Vangl2 using an anti-Vangl2 antibody (red). Compared to control, *Dlg*$^{f/+}$*10Cre*, and *Scrib*$^{f/+}$*10Cre* lenses showed similar intensity of staining for Vangl2. Compared to control, *Vangl2*$^{Lp/+}$, *Dlg*$^{f/+}$*10Cre;Vangl2*$^{Lp/+}$ and *Scrib*$^{f/+}$*10Cre; Vangl2*$^{Lp/}$. Representative images of the transition zone are shown. c, cornea; e, lens epithelium; r, retina; tz, transition zone. Scale Bar = 50μm.

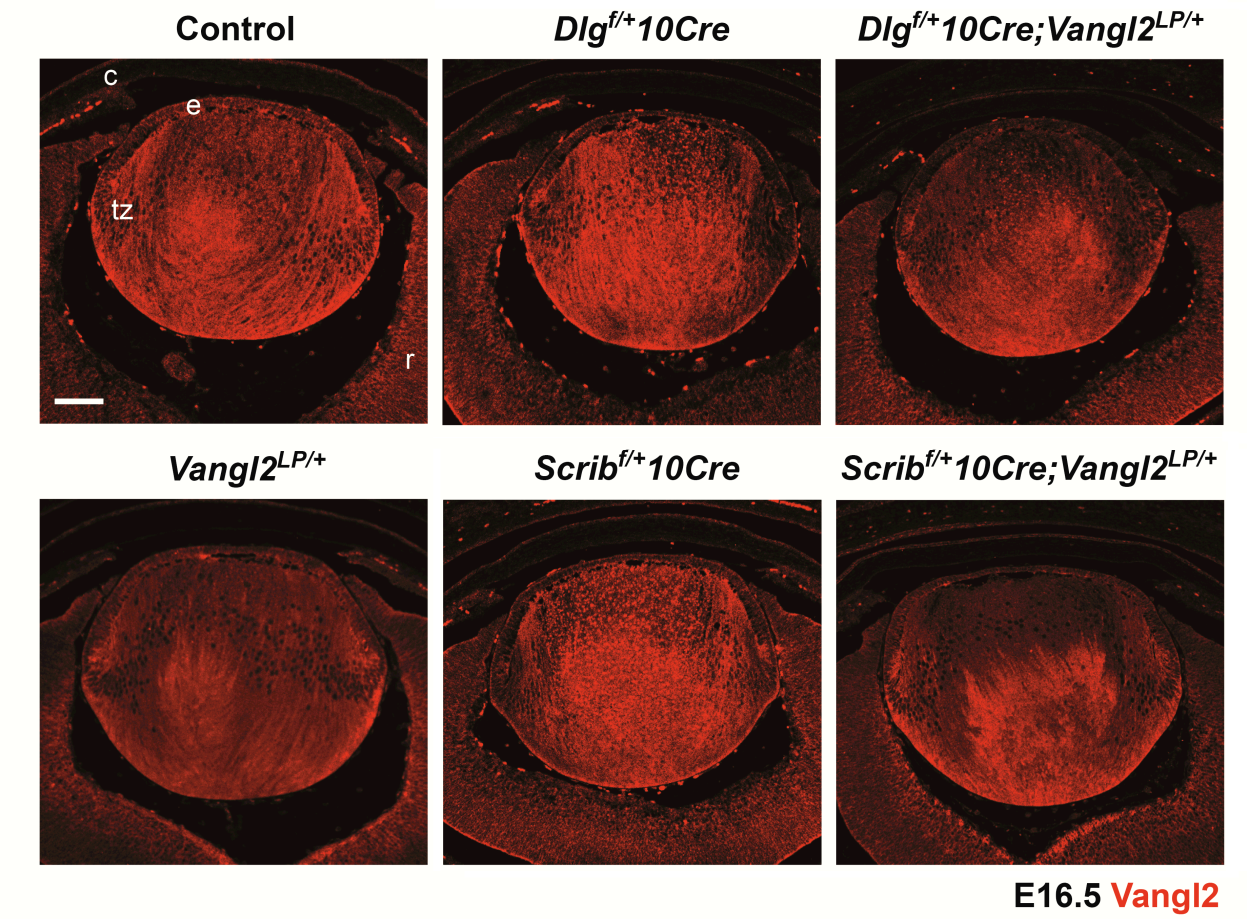
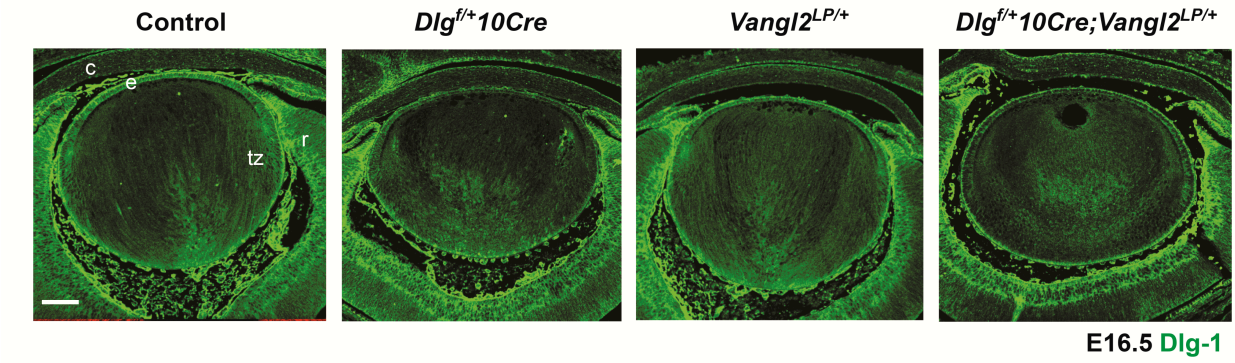
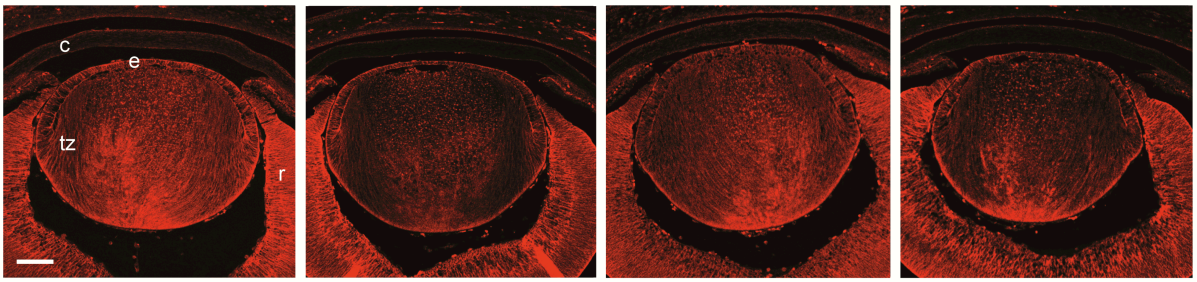

Control
Dlg^f/+10Cre
Dlg^f/+10Cre;Vangl2^LP/+
c
e
tz
r
Vangl2^LP/+
Scrib^f/+10Cre
Scrib^f/+10Cre;Vangl2^LP/+
E16.5 Vangl2

Figure V.2. Loss of each protein following mutation of one allele of *Dlg-1* and *Scrib*. Paraffin embedded sections of eyes from E16.5 control, *Vangl2*$^{Lp/+}$, *Dlg*$^{f/+}$*10Cre,* and *Dlg*$^{f/+}$*10Cre;Vangl2*$^{Lp/+}$ embryos were subjected to immunoflourescence analysis for Dlg-1 using an anti-Dlg-1 antibody (green). Eyes from E16.5 control, *Vangl2*$^{Lp/+}$, *Scrib*$^{f/+}$*10Cre,* and *Scrib*$^{f/+}$*10Cre;Vangl2*$^{Lp/+}$ embryos were subjected to immunofluorescence analysis for Scrib using an anti-Scrib antibody (red). Dlg-1 levels in *Dlg*$^{f/+}$*10Cre*, and *Dlg*$^{f/+}$*10Cre;Vangl2*$^{Lp/+}$ lenses were reduced as compared to control and *Vangl2*$^{Lp/+}$ lenses. Scrib levels in *Scrib*$^{f/+}$*10Cre*, and *Scrib*$^{f/+}$*10Cre;Vangl2*$^{Lp/+}$ lenses were reduced as compared to control and *Vangl2*$^{Lp/+}$ lenses. Representative images of the transition zone are shown. c, cornea; e, lens epithelium; r, retina; tz, transition zone. Scale Bar = 50μm.



E16.5 Dlg-1



E16.5 Scrib

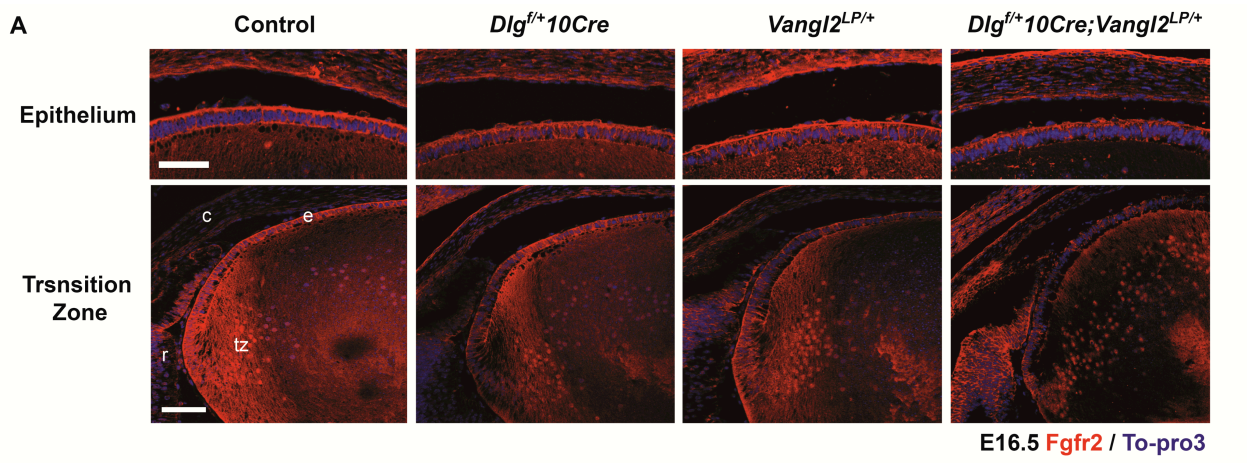
Effect of the *Dlg-1*, *Scrib*, *Vangl2*^{Lp} single and compound heterozygote mutations on Fgfr2 levels

The Griep lab has recently determined that *Dlg-1* is a PCP gene in the mouse (Rivera et al., 2013), and that *Dlg-1* interacts genetically with *Vangl2* and *Scrib*, two other PCP genes in the mouse, to control fiber cell shape and hexagonal packing. Haplodeficiency for *Dlg-1* and mutation in *Vangl2* individually disrupted fiber cell structure and fiber cell shape and the combination of these two mutations led to further disruption in fiber cell structure, (Shatadal and Griep, in preparation), I showed previously that ablation of *Dlg-1* in the lens altered levels of Fgfr2 (Lee and Griep, 2014). I hypothesized that the genetic interaction between these PCP genes that controls fiber cell shape also would regulate the level of Fgfr2 in a manner consistent with the effect of the mutations in these genes on cell shape. PCP is also thought to be a regulator of cell shape and organization in the central epithelium. I also hypothesized that genetic interactions between these PCP genes would affect Fgfr2 levels in the epithelium. To address these hypothesis, I first generated paraffin embedded eye sections from E16.5 control, *Dlg*$^{f/+}$*10Cre*, *Vangl2*$^{Lp/+}$and, *Dlg*$^{f/+}$*10Cre;Vangl2*$^{Lp/+}$ and these sections were subjected to immunofluorescent staining for Fgfr2 using an anti-Fgfr2 antibody (Figure. V.3). The intensities of staining for Fgfr2 were quantified by ImageJ, as described in Chapter II. In the epithelium, Fgfr2 levels were decreased by 22% in *Dlg*$^{f/+}$*10Cre*, and 31% in *Vangl2*$^{Lp/+}$ compared to control. In higher magnification images it is apparent that in *Vangl2*$^{Lp/+}$ heterozygotes the epithelium is taller and Fgfr2 expression was reduced especially on apical membrane. The defects in the apical membrane of *Dlg*$^{f/+}$*10Cre;Vangl2*$^{Lp/+}$ compound heterozygote lenses were more severe as staining for Fgfr2 is nearly lost. In the transition zone, the intensity for Fgfr2 staining was reduced by 29% in *Dlg*$^{f/+}$*10Cre*, and 38% in *Vangl2*$^{Lp/+.}$ The intensity for Fgfr2 staining in the

Dlg$^{f/+}$10Cre;Vangl2$^{Lp/+}$ lenses was reduced by 66% compared to control. Thus, both Dlg-1 deficiency and the *Lp* mutant *Vangl2* allele led to reduced levels of Fgfr2 in both the epithelium and fiber cells and the combination of these two mutant alleles resulted in further reduced intensities. These data indicate that haplodeficiency for *Dlg-1 or Vangl2* each lead to a reduction in Fgfr2 levels and the combination leads to more severe reduction in Fgfr2 levels. Thus, deficiency for *Dlg-1* enhanced the effect of the V*angl2$^{L/+P}$* allele on Fgfr2 levels, indicating that these two genes act similarly on the pathway that links fiber cell shape to Fgfr2 levels.

Similarly, to determine if *Scrib* deficiency alone and/or in combination with *Vangl2^{Lp}* allele affected Fgfr2 levels, paraffin embedded eye sections from E16.5 embryos were subjected to immunofluorescence analysis for Fgfr2. In epithelium region, intensity of staining for Fgfr2 was reduced by 18% in *Scrib$^{f/+}$10Cre*, and 32% in *Vangl2$^{Lp/+}$* compared to control. The long sides of the epithelial cell, especially in the central region of the epithelium appeared to be shorter in the *Vangl2$^{Lp/+}$* and *Scrib$^{f/+}$10Cre* lenses. However, fiber cell length in the *Vangl2$^{Lp/+}$;Scrib$^{f/+}$10Cre* lenses was similar to controls. In the transition zone, the intensity of staining for Fgfr2 was reduced by 31% in *Scrib$^{f/+}$10Cre*, and 35% in *Vangl2$^{Lp/+}$*. However, staining intensities for Fgfr2 in *Scrib$^{f/+}$10Cre;Vanlg2$^{Lp/+}$* compound heterozygote lenses were closer to that in controls, (Figure.V.4), indicating that the haplodeficiency for *Scrib* at least partially rescued the defect due to the *Vangl2^{Lp}* allele. This finding suggests that *Scrib* and *Vangl2* act in opposing was on the same pathway that regulates cell shape and, ultimately, Fgfr2 levels.

Figure V.3. Fgfr2 Levels are lower in *Dlg$^{f/+}$10Cre;Vangl2$^{Lp/+}$* compound heterozygotes than in the single heterozygotes. (A) Paraffin embedded sections of eyes from E16.5 control, in *Dlg$^{f/+}$10Cre, Vangl2$^{Lp/+}$*, and *Dlg$^{f/+}$10Cre;Vangl2$^{Lp/+}$* mice 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 compartment from control and mutant lenses are shown. Representative images of the transition zone are shown. c, cornea; e, lens epithelium; r, retina; tz, transition zone. Scale Bar = 50μm. (B) Quantification of Fgfr2 levels. Shown are the relative levels of Fgfr2 in regions of the each mutant lenses as compared to levels in the corresponding regions of the control lenses (control levels set at 1.0). Quantification of signal intensities in epithelium and transition zone was carried out using ImageJ, as described in Materials and Methods (Chapter. II), 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. Error bars=standard deviations. *=FDR<0.05, **=FDR<0.01


A
Control
Dlg^f/+10Cre
Vangl2^LP/+
Dlg^f/+10Cre;Vangl2^LP/+
Epithelium
Trsnsition Zone
c
e
r
tz
E16.5 Fgfr2 / To-pro3

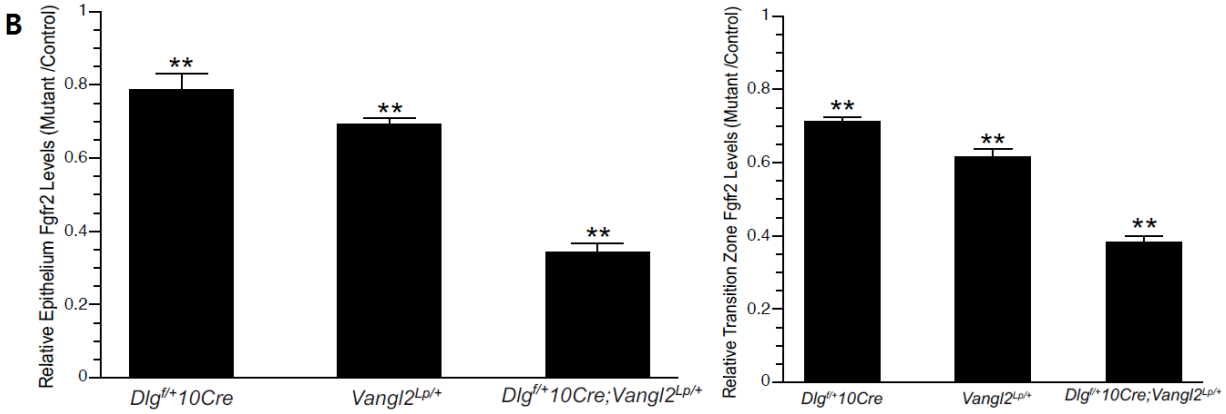
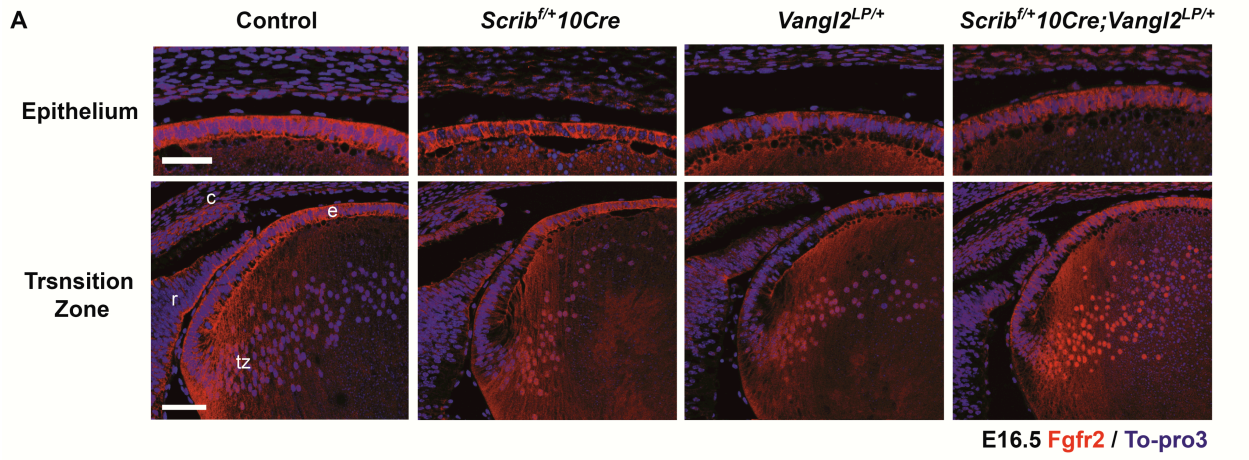

B
Relative Epithelium Fgfr2 Levels (Mutant /Control)
Relative Transition Zone Fgfr2 Levels (Mutant /Control)
0
0.2
0.4
0.6
0.8
1
**
Dlg^f/+10Cre
Vangl2^Lp/+
Dlg^f/+10Cre;Vangl2^Lp/+

Figure V.4. The reduced level of Fgfr2 levels in the *Vangl2*$^{Lp/+}$ lens is partially rescued by *Scrib* haplodeficiency. (A) Paraffin embedded sections from day E16.5 control, *Vangl2*$^{Lp/+}$, *Scrib*$^{f/+}$*10Cre*, and *Scrib*$^{f/+}$*10Cre;Vangl2*$^{Lp/+}$ 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 and transition zone from the *Vangl2*$^{Lp/+}$ lens was lower than in the *Scrib*$^{f/+}$*10Cre* lens and both were lower than in control. The intensity of Fgfr2 staining in the *Scrib*$^{f/+}$*10Cre;Vangl2*$^{Lp/+}$ lenses was indistinguishable from that in the *Scrib*$^{f/+}$*10Cre* lenses, indicating that the effect of the *Vangl2*$^{Lp/+}$ allele on Fgfr2 levels was rescued by *Scrib* haplodeficiency. At least 3 different sections from at least 3 different lenses were evaluated Representative images of the transition zone are shown. c, cornea; e, lens epithelium; r, retina; tz, transition zone. Scale Bar = 50μm. (B) Quantification of Fgfr2 levels. Shown are the relative levels of Fgfr2 in regions of the each mutant lenses as compared to levels in the corresponding regions of the control lenses (control levels set at 1.0). Quantification of signal intensities in epithelium and transition zone was carried out using ImageJ, as described in Materials and Methods (Chapter. II), 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. Error bars=standard deviations. *=FDR<0.05, **=FDR<0.01


A
Control
Scrib f/+ 10Cre
Vangl2 LP/+
Scrib f/+ 10Cre;Vangl2 LP/+
Epithelium
Trsnsition Zone
c
e
r
tz
E16.5 Fgfr2 / To-pro3

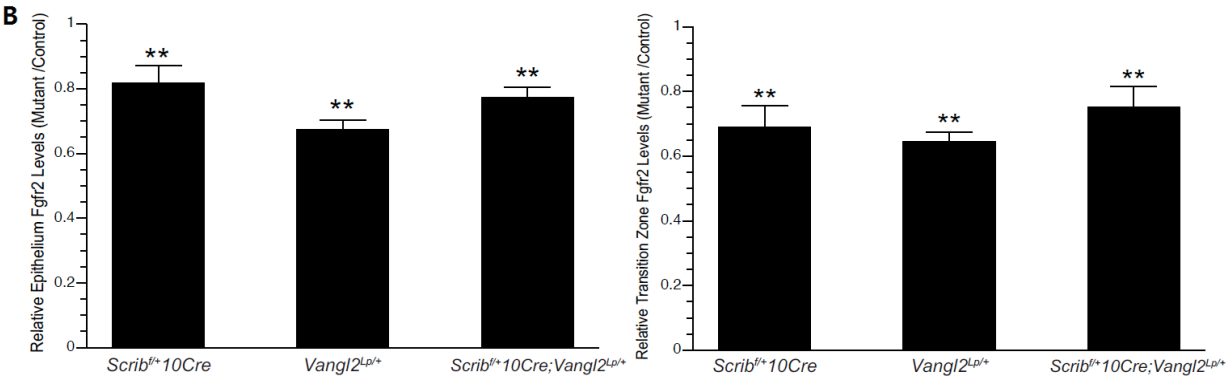

B
Relative Epithelium Fgfr2 Levels (Mutant /Control)
Relative Transition Zone Fgfr2 Levels (Mutant /Control)
0
0.2
0.4
0.6
0.8
1
**
Scrib f/+ 10Cre
Vangl2 Lp/+
Scrib f/+ 10Cre;Vangl2 Lp/+

Figure V.5. Fgfr2 expression in lens epithelium of *Dlg$^{f/+}$10Cre;Vangl2$^{Lp/+}$* compound heterozygotes as compared to *Dlg$^{f/+}$10Cre or Vangl2$^{Lp/+}$* single heterozygotes. Paraffin embedded sections of eyes from E16.5 control, in *Dlg$^{f/+}$10Cre*, *Vangl2$^{Lp/+}$*,and *Dlg$^{f/+}$10Cre;Vangl2$^{Lp/+}$* mice were subjected to immunofluorescence analysis using an anti-Fgfr2 antibody (red) and the nuclei counterstained with To-Pro3 (blue). *Dlg$^{f/+}$10Cre and Vangl2$^{Lp/+}$* were reduced levels of Fgfr2 in epithelium and transition zone near epithelium. Fgfr2 levels in the *Dlg$^{f/+}$10Cre;Vangl2$^{Lp/+}$* lenses were lower than in either of the single heterozygotes. Bar = 50μm.

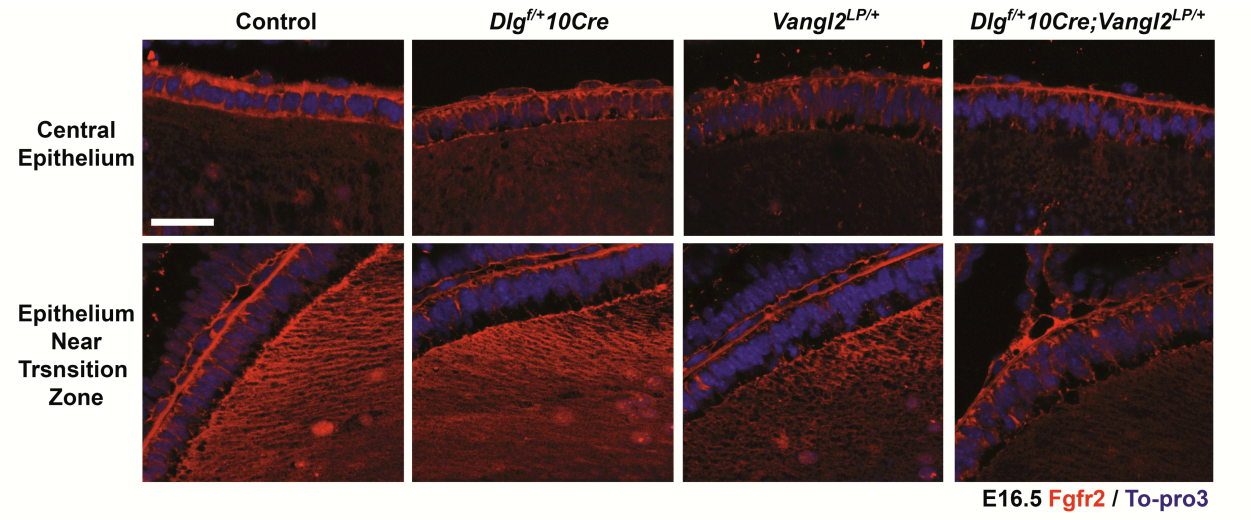
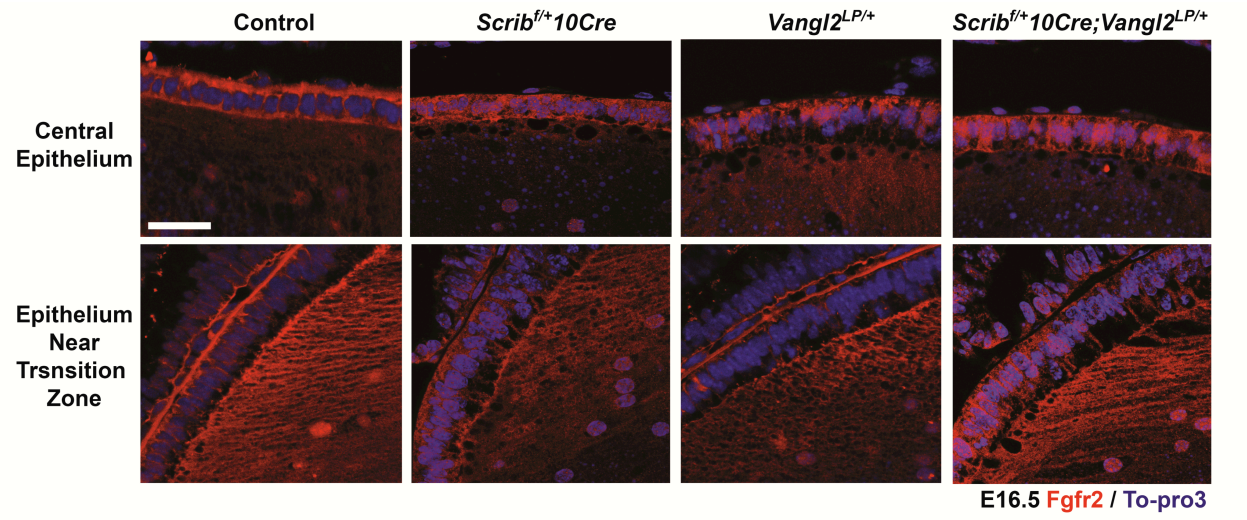

Control
Dlg^f/+10Cre
Vangl2^LP/+
Dlg^f/+10Cre;Vangl2^LP/+
Central Epithelium
Epithelium Near Trsnsition Zone
E16.5 Fgfr2 / To-pro3

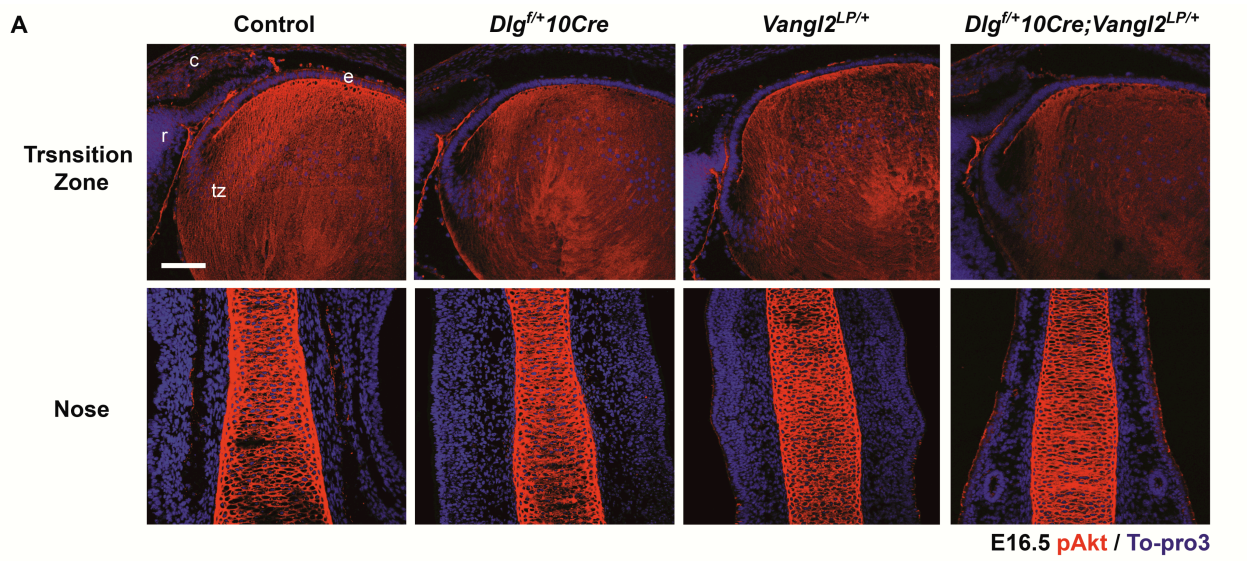
Figure V.6. Fgfr2 expression in lens epithelium of *Scrib$^{f/+}$10Cre;Vangl2$^{Lp/+}$* compound heterozygotes as compared to *Scrib$^{f/+}$10Cre or Vangl2$^{Lp/+}$* single heterozygotes. Paraffin embedded sections from day E16.5 Control, *Vangl2$^{Lp/+}$*, *Scrib$^{f/+}$10Cre,* and *Scrib$^{f/+}$10Cre;Vangl2$^{Lp/+}$* embryos were subjected to immunofluorescence analysis using an anti-Fgfr2 antibody (red) and the nuclei counterstained with To-Pro3 (blue). The expression of Fgfr2 staining in the epithelium and transition zone near epithelium from the *Vangl2$^{Lp/+}$* and *Scrib$^{f/+}$10Cre* were reduced compared to controls. However, Fgfr2 staining patterns in *Scrib$^{f/+}$10Cre;Vangl2$^{Lp/+}$* compound heterozygotes in both epithelium and transition zone near the epithelium were more similar to controls. Bar = 50μm.


Control
Scrib f/+ 10Cre
Vangl2 LP/+
Scrib f/+ 10Cre;Vangl2 LP/+
Central Epithelium
Epithelium Near Trsnsition Zone
E16.5 Fgfr2 / To-pro3

Effect of the *Dlg-1*, *Scrib*, *Vangl2*^{Lp} single and compound heterozygote alleles on pAkt levels

In Chapter III, I showed that the levels of the activated Fgfr signaling intermediates, pErk and pAkt, are reduced in lenses of mice deficient for *Dlg-1*. Given these results on the effects of single and double heterozygous mutations in *Dlg-1*, *Scrib,* and *Vangl2*, I hypothesized that activation of Fgfr2 downstream signaling effectors would be compromised. To determine if deficiency in *Dlg-1* and/or the *Vangl2*^{Lp} mutant protein would reduce pAkt levels, paraffin embedded sections of eyes from E16.5 embryos were subjected to immunofluorescent staining for pAkt using an anti-pAkt antibody. The relative levels of pAkt in transition zone regions of each mutant lenses were compared to levels in the nasal tube regions as quantification controls (control levels set at 1.0). As shown in Figure.V.5 and V.6., the intensity of pAkt were reduced in the transition zone 44% from *Dlg*$^{f+}$*10Cre,* 30% from *Scrib*$^{f/+}$*10Cre,* and 41% from *Vanlg2*$^{Lp/+}$ as compared to control, respectively. Whereas each single heterozygotes showed some reduced levels of pAkt, the intensity of staining for pAkt in the *Dlg*$^{f/+}$*10Cre;Vangl2*$^{Lp/+}$ compound heterozygote lenses was further reduced by 62% as compared to control (Figure.V.7). Conversely, the intensity of staining for pAkt was partially rescued in *Scrib*$^{f/+}$*10Cre;Vangl2*$^{Lp/+}$ compound heterozygote lenses (Figure.V.8). These data indicate that PCP genes, *Dlg*-1 and *Vangl2* act similarly to regulate pAkt levels whereas *Scrib* and *Vangl2* act in opposing manners to regulate pAkt levels.

Figure V.7. The reduced level of pAkt in the *Vangl2*$^{Lp/+}$ lens is enhanced reduction by *Dlg-1* haplodeficiency. (A) Paraffin embedded sections of eyes from E16.5 control, in *Dlg*$^{f/+}$*10Cre*, *Vanlg2*$^{Lp/+}$, and *Dlg*$^{f/+}$*10Cre;Vanlg2*$^{Lp/+}$ mice were subjected to immunofluorescence analysis using an anti-pAkt antibody (red) and the nuclei counterstained with To-Pro3 (blue). Shown are representative images. c, cornea; e, lens epithelium; r, retina; tz, transition zone. (B) Quantification of pAkt levels. Shown are the intensities of staining for pAkt in transition zone of the lenses of each mutant as compared controls. The intensity of staining in the cartilage in the snout was used as a staining control (control levels set at 1.0). Scale Bar = 50μm. Error bars=standard deviations. *=FDR<0.05, **=FDR<0.01


A
Control
Dlg f/+ 10Cre
Vangl2 LP/+
Dlg f/+ 10Cre;Vangl2 LP/+
Trsnsition Zone
Nose
c
e
r
tz
E16.5 pAkt / To-pro3

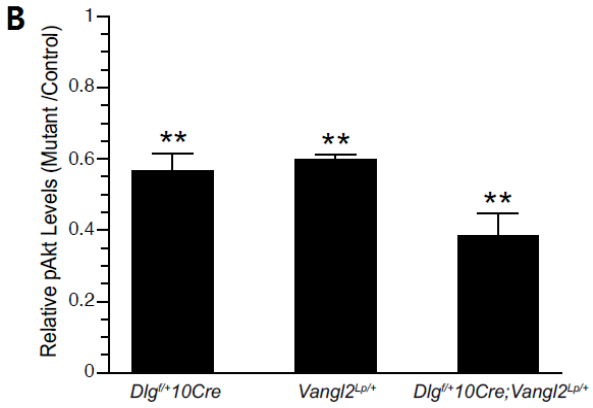
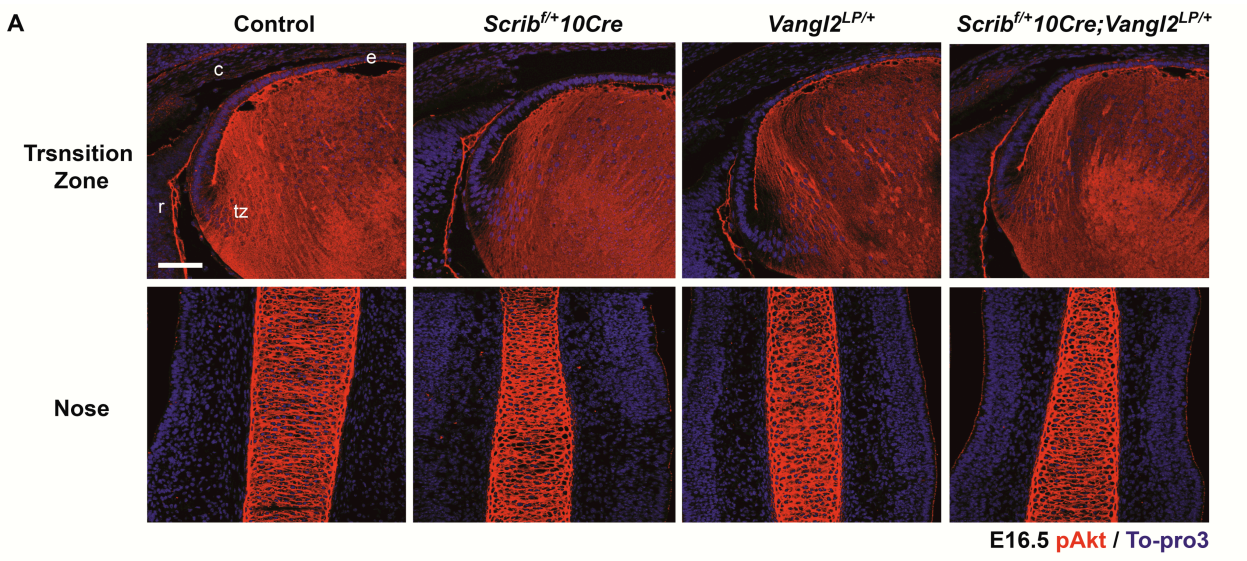
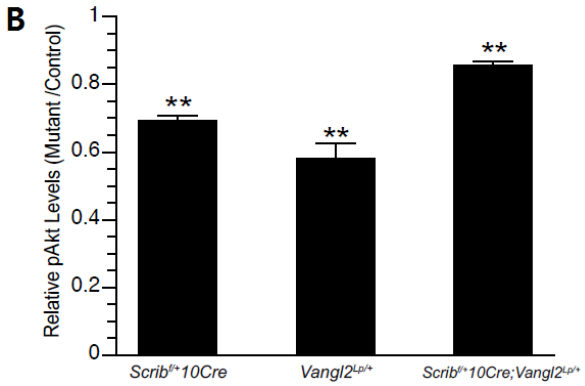

B
Relative pAkt Levels (Mutant /Control)
1
0.8
0.6
0.4
0.2
0
**
**
**
Dlg f/+ 10Cre
Vangl2 Lp/+
Dlg f/+ 10Cre;Vangl2 Lp/+

Figure V.8. The reduced level of pAkt in the *Vangl2*$^{Lp/+}$ lens is partially rescued by *Scrib* haplodeficiency. Paraffin embedded sections of eyes from E16.5 control, *Vangl2*$^{Lp/+}$, *Scrib*$^{f/+}$*10Cre*, and *Scrib*$^{f/+}$*10Cre;Vangl2*$^{Lp/+}$ mice were subjected to immunofluorescence analysis using an anti-pAkt antibody (red) and the nuclei counterstained with To-Pro3 (blue). c, cornea; e, lens epithelium; r, retina; tz, transition zone. (B) Quantification of pAkt levels. Shown are the relative levels of pAkt in transition zone regions and each mutant lenses as compared to levels in the nose regions as quantification controls (control levels set at 1.0). Quantification of signal intensities in transition zone was carried out using ImageJ, as described in above, 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. Scale Bar = 50μm. Error bars=standard deviations. *=FDR<0.05, **=FDR<0.01

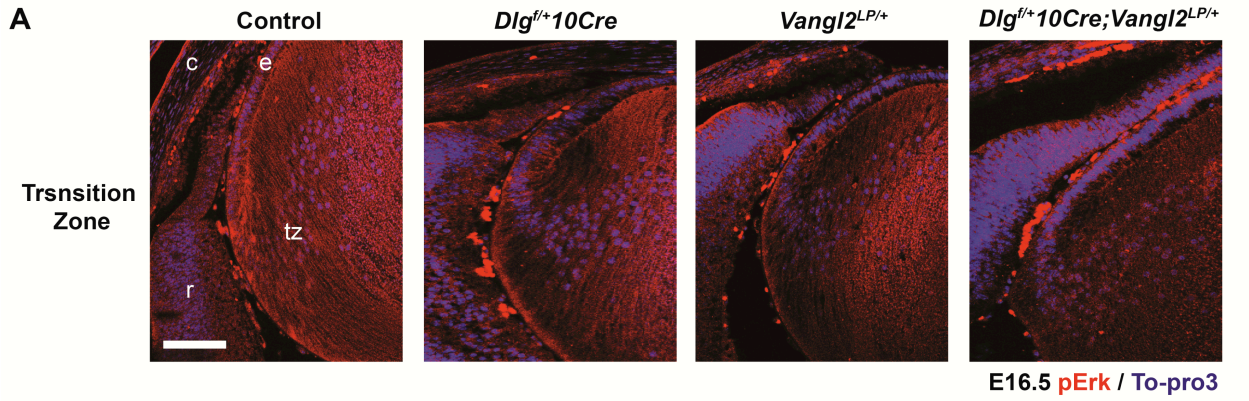

A
Control
Scrib f/+ 10Cre
Vangl2 LP/+
Scrib f/+ 10Cre;Vangl2 LP/+
Trsnsition Zone
Nose
c
e
r
tz
E16.5 pAkt / To-pro3


B
Relative pAkt Levels (Mutant /Control)
1
0.8
0.6
0.4
0.2
0
**
**
**
Scrib f/+ 10Cre
Vangl2 Lp/+
Scrib f/+ 10Cre;Vangl2 Lp/+

Effect of the *Dlg-1*, *Scrib*, *Vangl2*^{Lp} single and compound heterozygote mutants on pErk levels

I have previously shown that the level of the activated Fgfr signaling intermediate, pErk, is reduced in lenses of mice deficient for *Dlg-1* (Chapter III). Given the results above on the effects of single and double heterozygous mutations in *Dlg-1*, *Scrib,* and *Vangl2* on Fgfr2 and pAkt levels, I hypothesized that activation of Fgfr2 downstream signaling effectors would be compromised. To determine if deficiency in *Dlg-1* and/or the *Vangl2*^{Lp} mutant protein would reduce pErk levels, paraffin embedded sections of eyes from E16.5 embryos were subjected to immunofluorescent staining for pErk using an anti-pErk antibody. To determine if deficiency in *Dlg-1, Scrib* and/or the *Vangl2*^{Lp} mutant protein would reduce pErk levels, paraffin embedded sections of eyes from E16.5 embryos were subjected to immunofluorescent staining for pErk using an anti-pErk antibody. Eyes sections from E16.5 control, *Dlg*$^{f/+}$*10Cre, Scrib*$^{f/+}$*10Cre*, *Vangl2*$^{Lp/+}$,*Dlg*$^{f/+}$*10Cre; Vangl2*$^{Lp/+}$, and *Scrib*$^{f/+}$*10Cre;Vangl2*$^{Lp/+}$ lenses were subjected into immunofluorescence experiments using anti-pErk antibody and measure the intensity of signal in the transition zone region.. The reduced intensity of staining for pErk in the transition zone in the *Dlg*$^{f/+}$*10Cre, Scrib*$^{f/+}$*10Cre*,and *Vangl2*$^{Lp/+}$ lenses was 28%, 28% and 31%, respectively (Figure.V.9 and V.10). Similar with pAkt results, the intensity of pErk levels of *Dlg*$^{f/+}$*10Cre;Vangl2*$^{Lp/+}$ showed further reduction by 51% compared to control (Figure.V.9), whereas *Scrib*$^{f/+}$*10Cre;Vangl2*$^{Lp/+}$ compound heterozygotes lenses were partially rescued by 17% as compared to control (Figure.V.10). , These data indicate that PCP genes, *Dlg*-1 and *Vangl2* act similarly to regulate pErk levels whereas *Scrib* and *Vangl2* act in opposing manners to regulate pErk levels.

Figure V.9. The reduced level of pErk in the *Vangl2*$^{Lp/+}$ lens is further reduced by *Dlg-1* haplodeficiency. (A) Paraffin embedded sections of eyes from E16.5 control, in *Dlg*$^{f/+}$*10Cre, Vangl2*$^{Lp/+}$, and *Dlg*$^{f/+}$*10Cre;Vangl2*$^{Lp/+}$ mice were subjected to immunofluorescence analysis using an anti-pErk antibody (red) and the nuclei counterstained with To-Pro3 (blue). c, cornea; e, lens epithelium; r, retina; tz, transition zone. (B) Quantification of pErk levels. Shown are the relative levels of pErk in transition zone regions of the each mutant lenses as compared to control levels (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. Error bars=standard deviations.*=FDR<0.05, **=FDR<0.01


A
Control
Dlg^f/+10Cre
Vangl2^LP/+
Dlg^f/+10Cre;Vangl2^LP/+
Trsnsition Zone
c
e
tz
r
E16.5 pErk / To-pro3

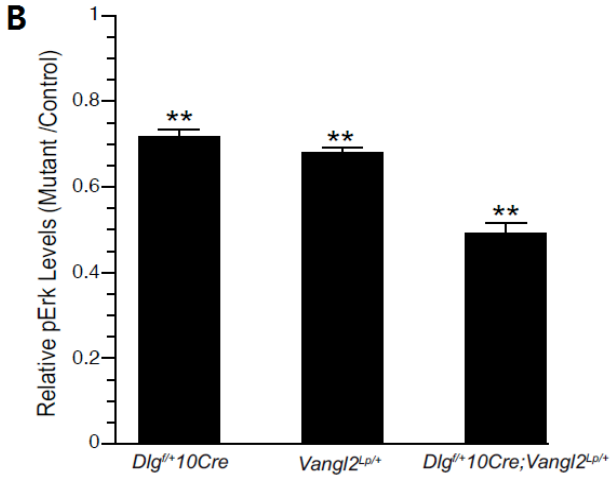
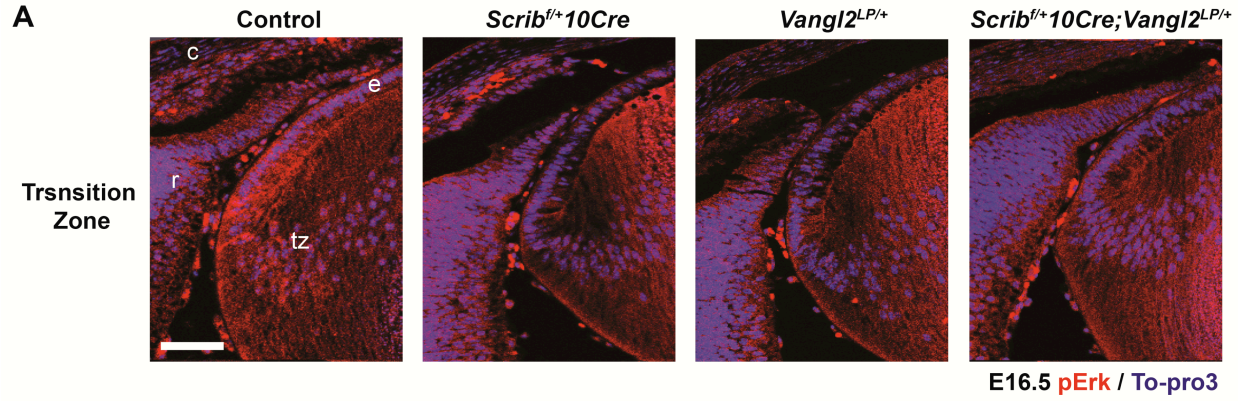
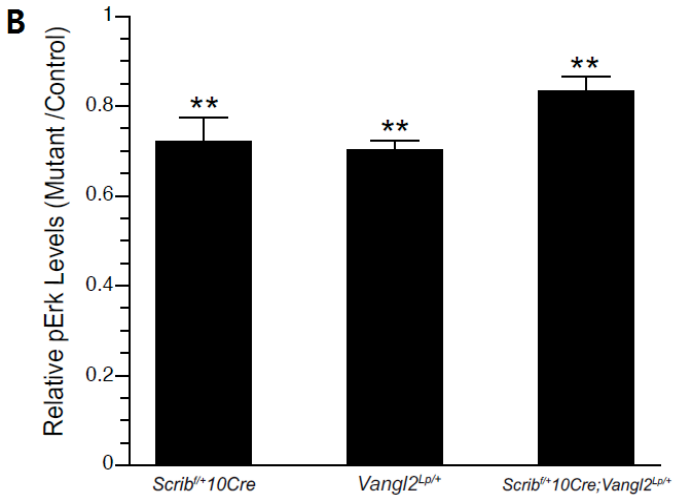

B
Relative pErk Levels (Mutant /Control)
1
0.8
0.6
0.4
0.2
0
**
**
**
Dlg^f/+10Cre
Vangl2^Lp/+
Dlg^f/+10Cre;Vangl2^Lp/+

Figure V.10. The reduced level of pErk in the *Vangl2*$^{Lp/+}$ lens is partially rescued by *Scrib* haplodeficiency. (A) Paraffin embedded sections of eyes from E16.5 control, *Vangl2*$^{Lp/+}$, *Scrib*$^{f/+}$*10Cre*, and *Scrib*$^{f/+}$*10Cre;Vangl2*$^{Lp/+}$ mice were subjected to immunofluorescent staining using an anti-pAkt antibody (red) and the nuclei counterstained with To-Pro3 (blue). (A) Shown are representative images from the transition zone of the lenses of each genotype. (B) Quantification of pErk levels. Shown are the relative levels of pErk in transition zone regions of the each mutant lenses as compared to control levels (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. Error bars=standard deviations.*=FDR<0.05, **=FDR<0.01


A
Control
Scrib f/+ 10Cre
Vangl2 LP/+
Scrib f/+ 10Cre;Vangl2 LP/+
Trsnsition Zone
c
e
r
tz
E16.5 pErk / To-pro3


B
Relative pErk Levels (Mutant /Control)
1
0.8
0.6
0.4
0.2
0
**
**
**
Scrib f/+ 10Cre
Vangl2 Lp/+
Scrib f/+ 10Cre;Vangl2 Lp/+

DISCUSSION

Cell growth and differentiation in the lens are dependent on numerous growth factor signaling pathways. Of key importance is the Fgfr signaling pathway, which has been shown to be essential for fiber cell differentiation (Lovicu et al., 2011; Wang et al., 2010). In Chapter III, I showed that the PCP gene, *Dlg-1,* is required for attaining normal levels of Fgfr signaling and lens fiber differentiation. In this Chapter, I asked if the interaction between the PCP genes *Dlg-1, Scrib,* and *Vangl2* affects Fgfr2 levels and the activation of downstream signaling intermediates of Fgfr2, I found that haplodeficiency of *Dlg-1,* and *Scrib,* and also a $Vangl2^{Lp}$ mutant allele led to reduced levels of Fgfr2 and its downstream signaling intermediates, pAkt and pErk. I further showed that levels of Fgfr2 and signaling intermediates were further reduced in lenses of $Dlg^{f/+}10Cre;Vangl2^{Lp/+}$ compound heterozygotes, indicating that haplodeficiency of *Dlg-1* enhanced the Fgfr2 signaling defects observed in the $Vangl2^{Lp/+}$ lenses. Conversely, the Fgfr2 signaling defects observed in the $Vangl2^{Lp/+}$ lens were rescued in the $Scrib^{f/+}10Cre;Vangl2^{Lp/+}$ lenses. The Griep lab has shown that fiber cell shape and size are disrupted in *Dlg-1, Scrib,* or $Vangl2^{Lp/+}$ lenses. The defects in fiber cell shape are more severe in $Dlg^{f/+}10Cre;Vangl2^{Lp/+}$ lenses whereas the defects in fiber cell shape and size are rescued in the $Scrib^{f/+}10Cre;Vangl2^{Lp/+}$ lenses (Shatadal and Griep, in preparation). Together, my data correlate the dependence of fiber cell structure by *Dlg-1*, *Scrib,* and *Vangl2* with the regulation of the Fgfr signaling pathway is essential for fiber cell differentiation.

PCP genes are required for proper cell differentiation and levels of Fgfr signaling intermediates in ocular lens.

PDZ proteins Scrib and Dlg-1 have been shown to localize to sites of cell-cell contacts in mammalian cells (Dow et al., 2003; Laprise et al., 2004; Reuver and Garner, 1998) and their roles in cell adhesion and differentiation in *Drosophila* are well known (Bilder, 2001; Bilder et al., 2000; Bilder and Perrimon, 2000). Vangl2 is a PCP protein that regulates the induction of polarized cellular and tissue morphology during animal development. It has been shown that core PCP proteins interact with other PDZ proteins in vertebrate systems. Scrib interact with Vangl2 to regulate PCP and it is known that Vangl2 selectively bind to PDZ domain in Scrib (Kallay et al., 2006; Yoshioka et al., 2013). In previous studies, it has been suggested that Dlg-1 has a role in proper localization of Vangl2 (Lee et al., 2003; Yoshioka et al., 2013). The epithelial cells in *Scrib* haplodeficient lenses are shorter in height compared to those in the control lens and in *Dlg-1* deficient lenses the epithelium is disorganized and sometimes multilayered (Figure.V.5 and 6). I found that the lens epithelial organization in $Dlg^{f/+}$*10Cre;*$Vanlg2^{Lp/+}$ was more disrupted as compared to that in the single heterozygotes. Also, the shorter length of the cells in the epithelium of *Scrib* mutant lens and wider size of epithelial cells in the *Vangl2* mutant lens phenotype were rescued in $Scrib^{f/+}$*10Cre;*$Vangl2^{Lp/+}$ compound heterozygotes. These data are consistent with our lab unpublished data (Griep and Shatadal, In preparation) about how the PCP factors, *Dlg-1*, *Scrib* and *Vangl2* affect lens fiber cell structure. Interestingly, Fgfr2 expression was nearly absent in the apical membrane of $Dlg^{f/+}$*10Cre;*$Vanlg2^{Lp/+}$ (Figure.V.5), whereas levels of Fgfr2 and its downstream intermediate pAkt and pErk were partially rescued in $Scrib^{f/+}$*10Cre;*$Vanlg2^{Lp/+}$ compound heterozygotes both transition zone and epithelium region as compared to that in lenses of the single heterozygotes (Figure.V.6, 8, and 10). In Chapter III, I

suggested that *Dlg-1* is required for proper levels of Fgfr signaling and modulation of fiber cell differentiation through its roles in PCP. In transgenic mouse lenses, the overexpression of Sfrp2 (Secreted frizzled-related protein2) results in disorganized lens fiber formation and downregulation of PCP signaling components (Chen et al., 2008). Together, the results of myself and others indicate that PCP genes, *Dlg-1, Scrib*, and *Vangl2* are required for normal fiber and epithelial cell structure and fiber cell structure may have an impact of the levels of Fgfr signaling which in turn affects the level of Fgfr signaling, which is necessary for fiber cell differentiation.

Taken together, these data indicate that PCP genes, *Dlg-1, Scrib* and *Vangl2* are modulators of cell shape in the lens and cell shape is integrated with Fgfr signaling to promote lens fiber cell differentiation.

Dlg-1 and *Scrib* modulate Fgfr2 signaling through interactions with *Vangl2*

As a result of my studies in Chapter III, I suggested that *Dlg-1* is required for attaining the normal levels of Fgfr signaling. Other studies in the Griep have shown that *Scrib* is also required for normal levels of Fgfr signaling lab (Yamben, PhD thesis, 2010). In this chapter, to look at the consequences of PCP regulation of fiber cell structure, Fgfr2, pAkt, and pErk levels were examined in lenses of control, single and compound *Dlg-1, Scrib*, and *Vangl2* heterozygotes by immunofluorescence. Whereas each single heterozygote lens showed some reduced levels of Fgfr2 and their downstream intermediates pAkt and pErk, the intensity levels in lenses of $Dlg^{f/+}10Cre;Vangl2^{Lp/+}$ compound heterozygotes were further reduced as compared to that in lenses of the single heterozygotes (Figure.V.7 and 9). Furthermore, Fgfr2 expression was nearly undetectable in the apical membrane of $Dlg^{f/+}10Cre;Vanlg2^{Lp/+}$ (Figure.V.5). Conversely, levels of Fgfr2, pAkt and pErk were partially rescued in $Scrib^{f/+}10Cre;Vanlg2^{Lp/+}$

compound heterozygotes as compared to single heterozygotes (Figure.V.6, 8, and 10). The precise mechanism of PCP genes' regulation on Fgfr signaling is unclear. It is possible that structural defects of mutant lenses led to mislocalization of Fgfrs and impairs Fgfr signaling. Reduced levels of Fgfr2 and its downstream signaling intermediates in *Dlg$^{f/+}$10Cre* and *Vangl2$^{Lp/+}$* lenses and rescued levels in *Scrib$^{f/+}$10Cre* in combination with either of these two mutant alleles suggest that Fgfr signaling may be impaired dependent on interactions between Dlg-1 or Scrib with *Vangl2* In addition, *Scrib* required for proper formation of epithelial polarity, has been shown to interact with *Vangl2*, where Vangl2 binds to specific PDZ domains of Scrib (Kallay et al., 2006). Further study need to aimed to understand how Dlg-1 and Scrib is mechanistically interact to regulate Fgfr signaling and role in mammalian cell shape formation.

Wnt/PCP signaling is an important part of an Fgf-induced lens fiber differentiation. In lens explants *in vitro*, Fgf2 treatment of explants activates Wnt-Frizzled signaling (Dawes et al., 2013), which in turn increases Fgfr signaling which supports fiber cell differentiation. *Vangl2$^{Lp/Lp}$* mutant lenses show defects in lens fiber cell differentiation that are similarly observed in lenses of other PCP mutants such as *Crsh* (Sugiyama et al., 2010). The interaction between *Dlg-1* and *Scrib* toward *Vangl2* may be one factor in these Wnt/PCP and Fgfr signaling pathways that are essential for lens fiber differentiation.

Taken together, these data indicate that PCP genes are modulators of lens epithelium morphology and Fgfr signaling. As the *Dlg-1* heterozygotous state enhances *Vanlg2$^{Lp/+}$* phenotype whereas *Scrib* heterozygotous state suppresses the *Vanlg2$^{Lp/+}$* phenotype. *Dlg-1* and *Scrib* may act in opposing ways with respect to *Vangl2* in modulating PCP and Fgfr signaling in ocular lens. Further studies will be required to elucidate the role of PCP genes in the regulation

of fiber cell shape and the mechanism through which the Dlg-1 interaction regulates Fgfr signaling and, ultimately, fiber cell differentiation.

Conclusion

Herein, I asked if the genetic interactions between *Dlg-1, Scrib*, and *Vangl2,* modulate the levels of Fgfr signaling and downstream intermediates in accordance with their modulation of fiber cell structure, which ultimately regulates fiber cell differentiation. *Dlg-1* haplodeficiency enhances $Vangl2^{Lp/+}$ phenotype of reduced Fgfr signaling whereas *Scrib* haplodeficiency rescues the $Vangl2^{Lp+}$ reduction of Fgfr2 signaling. Thus, *Dlg-1* and *Scrib* may act in opposing ways with respect to *Vangl2* in modulating PCP and Fgfr signaling pathways in ocular lens.

CHAPTER VI: THESIS SUMMARY

This chapter summarizes and discusses in terms of their significance the studies presented in the previous chapters (III-V). Also future directions are proposed to extend these studies.

Certain PDZ domain containing proteins, Dlg-1 and Scrib, have been shown to modulate cell adhesion, apical-basal cell polarity and differentiation They are thought to function as scaffolding molecules that can modulate cell fate and the signaling pathways that dictate cell fate through their ability to participate in multiple protein-protein interactions. Growth factor

signaling also plays roles in diverse biological responses in different organs and organisms activating or inhibiting downstream signaling pathways that regulate gene expression patterns which ultimately lead to proper development and maintenance of the structure and function of tissues and organs that make up the body. Previous studies in the Griep lab have shown that PDZ proteins, Dlg-1 and Scrib, regulate various aspects of lens development *in vivo*. Results from these studies suggests that Dlg-1 and Scrib may play a role in cell differentiation through the modulation of cell adhesion-cytoskeletal proteins and certain growth factor signaling pathways that are essential for lens cell growth and differentiation.

In my thesis work, I have explored the potential association of Dlg-1 with Fgfr and Eph receptor signaling pathways, the consequences of these interactions on lens fiber cell differentiation and the mechanisms through which these PDZ proteins affect Fgfr and Eph signaling. I have addressed three major hypotheses. First, *Dlg-1* is a modulator of the Fgfr signaling pathway and fiber cell differentiation. Second, Dlg-1 regulates Fgfr signaling and adherens junction formation via interaction with the Eph receptor, EphA2. Third, *Dlg-1* and *Scrib* regulate Fgfr signaling through their interaction with the core PCP gene, *Vangl2*.

My studies have shown that loss of Dlg-1 in the lens impairs Fgfr signaling and leads to altered levels of Fgfrs, implicating *Dlg-1* as a modulator of Fgfr signaling pathway at the level of the receptors (Chapter III). Also, my studies have shown that Dlg-1 interacts with and regulates the activation of EphA2 (Chapter IV). As EphA2 interacts with N-cadherin to link this cell adhesion molecule to β-catenin and, thereby, the underlying actin cytoskeleton (cooper et al. 2008). I propose that Dlg-1 affects cell adhesion through its regulation of EphA2. Similarly, my studies have shown that EphA2 interacts with Fgfrs 1 and 2 more robustly than Dlg-1 interacts with Fgfrs 1 and 2. Therefore, I propose that EphA2 mediates the effect of Dlg-1 on Fgfr

activation and signaling. Finally, I have shown that the genetic interactions between *Dlg-1, Scrib*, and *Vangl2* are required for proper levels of Fgfr signaling and downstream intermediates, pErk and pAkt, which is important for lens differentiation and cell survival (Chapter V). Below, I discuss the significance of the findings of my thesis work and suggest future directions to extend these studies.

Dlg-1 **is a modulator of Fgfr signaling in lens fiber cell differentiation**

Dlg-1 is required for cell adhesion, apico-basal polarity and fiber differentiation (Rivera et al., 2009) and PCP (Rivera et al., 2013). In Chapter III, I asked if *Dlg-1* is a regulator of the Fibroblast growth factor receptor (Fgfr) signaling pathway, which is required for lens fiber cell differentiation. Our laboratory first became to suggest that PCP gene, *Dlg-1* regulate Fgfr signaling. I found that loss of *Dlg-1* results in altered levels of Fgfrs, Fgfr signaling intermediates and Fgfr downstream targets. Loss of *Dlg-1* also led to reduced levels of Fgfr2 and pErk, an Fgfr signaling intermediate, in a site of the lens that exhibits PCP. In the lens, Fgfr signaling activates signaling cascades starting with only the phosphorylation of the docking protein, Frs2α (Gotoh, 2008; van der Noll et al., 2013), which binds directly to the receptor. Activation of Frs2α in the lens is the anchor for Fgfr signaling (Madakashira et al., 2012). To determine if loss of *Dlg-1* affected the levels of Frs2α activation, I analyzed protein levels of *Dlg-1* mutant lenses with control. I found reduced levels of pFrs2α and reduced levels of Fgfr2 were similar, suggesting that the alteration of Fgfr signaling occurs at the level of the receptor. Also levels of Fgfr2 and downstream, pErk were reduced in *Dlg-1* deficient lenses at least from embryonic day13.5, indicating that loss of *Dlg-1* on Fgfr signaling occurred early in the course of secondary fiber cell differentiation. Prior studies have demonstrated that in the lens Fgfrs and Fgfr downstream

signaling intermediates are initiators of fiber cell differentiation (Chow et al., 1995; Lovicu and McAvoy, 2001). In addition, Fgfr2 is required in the early stages of lens development to allow lens cells to exit from the cell cycle, for lens epithelial cell survival (Garcia et al., 2005). I showed that Fgfr2 and Dlg-1 overlap on the short side of the cortical fiber cells and, using co-immunoprecipitation experiment, confirmed they have protein interactions. However, there is no known PDZ binding motif in Dlg-1, so this interaction is thought to be indirect. Dlg-1 has been known to involved in vesicle transport, which could regulate Fgfr2 and PCP proteins (Lee et al., 2002). Adherens junction proteins also defected in Dlg-1 deficient status. Dlg-1 may be important for targeting the complex to the membrane either by stabilizing cytoskeletal attachment or mediating the interaction between the complex and the vesicle transport system.

To evaluate the Fgfr2 turnover, I would examine the colocalization of Dlg-1 with early endosome, late endosome, and recycling endosome with double immunofluorescence. This experiment may allow us to understand if Dlg-1 segregation regulates signal transduction and Fgf receptor turnover during endocytosis. Further studies is required to understand the mechanisms through which PCP and Dlg-1 regulate Fgfr signaling and fiber differentiation.

Despite of reduced Fgfr2 levels, levels of Fgfr1 and Fgfr3 were increased in *Dlg-1* deficient lenses. Furthermore, in Chapter IV, I showed that in the *Dlg-1* deficient state, further reduction in Fgfr1 or Fgfr2 through genetic manipulation resulted in increased levels of Fgfr2 or Fgfr1, respectively and the levels of activated signaling intermediates correlated positively with the levels of Fgfr2. It is possible that the lens attempts to compensate for loss of Fgfr2 activity by increasing the activity of other Fgfrs in the lens. These changes in Fgfr levels are dependent on *Dlg-1* status as there has not been observed any changes in the levels of one Fgfr when another is deleted on an otherwise wild type background. It is also possible that the changes in cell shape

that occur as a consequence of ablation of *Dlg-1* results in changes in the organization of protein complexes or stability of the protein complexes at the membrane that are important for receptor activation or turnover. Ablation of an individual Fgfr has not been shown to alter fiber cell shape (Zhao et al., 2008). I began to address the potential role of fiber cell shape and Dlg-1's influence on this in Chapter V (see below for discussion).

Another question that remains to be answered is how *Dlg-1* status affects Fgfr3 activity. As I showed in Chapter III, Fgfr3 levels were increased in *Dlg-1* deficient lenses. Therefore, if Fgfr3 is a positive regulator of Erk and Akt activation, it is possible that increased Fgfr3 activity might account for the differences between the reduction in Fgfr2 activation and reduction in pErk and pAkt levels. To address this question, I would generate mice that are deficient for *Dlg-1* in the lens and also haplodeficient for *Fgfr3* (*Dlg$^{f/f}$;Fgfr3$^{f/+}$10Cre* mice) just as I did for Fgfr1 and 2. I would measure the levels of pErk and pAkt in the (*Dlg$^{f/f}$;Fgfr3$^{f/+}$10Cre* lenses and compare with control and other *Dlg-1* deficient, *Fgfr* haplodeficient lenses. Additionally, I would perform co-immunoprecipitation experiments to determine if Fgfr3 complexes with Dlg-1 and assess the intensity of Fgf receptors interactions and other downstream components interactions with Fgfrs in *Dlg-1* deficient state. Thus, these mice will give further insights into the relationship between Dlg-1 and Fgfr signaling which is essential for fiber cell differentiation. Furthermore, these studies will provide further insight into how the different Fgfrs direct downstream signaling in the pathway in the lens that was not elucidated through studies on lens specific knockouts of *Fgfrs*.

Dlg-1 regulates Fgfr signaling and adherens junction proteins via EphA2

Ephrin/Eph signaling is emerging as an important pathway in regulating lens development. Mutations in *EPHA2* are associated with age-related lens cataract (Jun et al., 2009) and congenital cataracts in humans (Dave et al., 2013; Kaul et al., 2010). Loss of ephrin-A5 leads to reduced EphA2 activity, disrupts lens fiber cell shape and leads to cataract (Cooper et al., 2008). Various Receptor tyrosine kinases (RTK) share prominent downstream signaling cascades, including the MAPK or AKT pathways (Jun et al., 2009; Kholodenko et al., 2010; Shiels et al., 2008). Such is the case for Eph/ephrin and Fgfr as both have been shown to activate Frs2 and the MAPK pathway (Fukai et al., 2008; Park et al., 2004; Yokote et al., 2005). I hypothesized that EphA2 interacts with Fgfrs 1 and 2 and that Dlg-1 participates in this interactions as a regulator in the lens, because there is no known PDZ domain in Fgfrs. During various biological effects in tissues, Ephrin/Eph signaling involves several highly conserved tyrosine phosphorylation sites in the cytoplasmic domain, a C-terminal PDZ motif, and the binding to PDZ domain proteins (Kalo and Pasquale, 1999). My data show that as protein levels of N-cadherin, β-catenin, EphA2 and Fgfr2 are reduced, and co-localization between N-cadherin and EphA2 is decreased and disrupted in the *Dlg-1* null lenses, I hypothesized that these proteins may interact in a complex in the lens. Interestingly, the intensity of co-immunoprecipitation from $Dlg^{f/f}$*10Cre* lenses also reduced in adherens junction proteins N-cadherin, β-catenin, and EphA2 with Fgfr2 as compared to controls while the amount of Fgfr1 co-immunoprecipitating with EphA2 was increased. However, the amount of Fgfr co-immunoprecipitating with Dlg-1 was less than the amount that co-immunoprecipitated with EphA2, suggesting that the interaction between EphA2 and the Fgf receptors may be more direct than the interaction between Dlg-1 and the Fgf receptors. Therefore, based on my data, I propose that EphA2 is a bridge between Dlg-1 and the Fgfrs.

To address my hypothesis, I would examine direct binding of Dlg-1 with EphA2 using GST (glutathione-S-transferase) pull-down assay. GST pull-down assay is an effective way to examine the direct binding of two proteins *in vitro*. Also it would be worthy to examine whether if Dlg-1 directly bind with Fgfrs or not to support my former data. I have confirmed interactions among these proteins through co-immunoprecipitation, however this experiment could not allow me to verify whether if Dlg-1 is directly binding with those receptors. Some researches insist that Eph and Fgfr potentiate downstream signaling in complex form (Fukai et al., 2008). However, there have been no researches about theses complexes in the lens. Through GST pull-down assay, I also would examine Eph/Fgfr complex form in the lens. These future studies will provide greater details into the mechanistic interactions of Dlg-1, Fgfrs, and EphA2 in the lens in regulating growth factor signaling pathways.

To understand the Ephrin/Eph signaling pathway and mechanisms through which Dlg-1 regulate Fgfr signaling through interactions with EphA2, it should be worthwhile to investigate *EphA2* null mice. I would examine the Fgfrs and dowmstream intermediate expression by immunofluorescence and western blotting to determine how EphA2 affects to Fgfr signaling pathways. I would cross *EphA2* deficient mice to *Dlg-1* or *Scrib* null mice to generate to explore genetic interactions between PDZ proteins with EphA2. I would then examine the histology of the mutant lenses and would examine the pattern and levels of Fgfr signaling components expression in control and mutant mouse lenses by immunofluorescence and western blotting to determine how *Dlg-1* or *Scrib* regulate EphA2 in the lens. This experiment will give us deeper understanding of genetic interactions and support the hypothesis that Dlg-1, through its PDZ interaction, regulate Fgfr signaling through EphA2 signaling.

To further gain insight into the signaling pathways which Dlg-1 may regulate, it would be worth expanding the search to other receptors which have PDZ-binding motifs. For example, approximately 20% of G protein coupled receptor proteins (including ionic channels, ionotropic receptors, and single transmembrane proteins) have PDZ-binding motif (Fredriksson et al., 2003; Lee and Zheng, 2010) PDZ domain containing proteins regulate GPCR trafficking and various siganling pathways (Dunn and Ferguson, 2015). This expanded experiment may further allow us to understand global roles of PDZ domain protein such as Dlg-1 in receptor tyrosine kinases and signaling pathways. Further resources and studies will be required to address these questions.

Dlg-1, Scrib and the regulation of cell adhesion and apical-basal polarity in the lens

In *Drosophila*, mutations in discs large (*dlg*), scribble (*scrib*) and lethal giant larvae (*lgl*) have revealed close connection in the regulation of cell polarity and cell proliferation (Wodarz, 2000). In *dlg, scrib,* or *lgl* homozygous mutant larvae, there was disruption of apical–basal polarity and the structure of the epithelial cells in the epidermis (Wodarz, 2000). These phenotypes suggested that *dlg*, *scrib* or *lgl* have roles in cell polarity. In addition, in *dlg*, *scrib* or *lgl* homozygous mutant larvae, brain cells and imaginal disc continue to proliferate, forming large amorphous masses (Wodarz, 2000). Furthermore, *dlg* or *lgl* mutants enhance the *scrib* mutant phenotype, indicating that they function in genetic interactions (Bilder et al., 2000; Bilder and Perrimon, 2000; Humbert et al., 2003).

In the mouse, the height of the epithelial cells in *Scrib* deficient lenses are shorter than in controls (Shatadal and Griep, in preparation) and in *Dlg-1* deficient lenses the cuboidal epithelial cell shape is frequently lost and the monolayered epithelium is sometimes multilayered (Rivera et al., 2009). There are two *Lgl* genes in the mouse, *Lgl-1* and *Lgl-2* (Froldi et al., 2008; Mason

et al., 1988; Musch et al., 2002). To date, neither gene has been studied in the lens. I propose that Lgl also interacts with Dlg-1 and Scrib in the lens and these interactions are essential for apical-basal polarity. First I would examine the pattern and levels of Lgl-1 and Lgl-2 expression in control mouse lenses by immunofluorescence and western blotting to determine which *Lgl*(s) is(are) expressed in the lens. Based on those results, I would examine the pattern and level of expression of the Lgl(s) expressed in the lens in *Dlg-1* or *Scrib* mutant lenses using quantitative immunofluoresence, I would next generate mice carrying a conditional allele of the relevant *Lgl(s)*, and cross them to *MLR10Cre* mice to generate a lens specific deletion of *Lgl*. I would then examine the histology of the mutant lenses and the patterns and levels of Dlg-1 and Scrib expression in the *Lgl* mutant lenses. I also would immunostain these lenses with anti-ZO-1 antibodies, as ZO-1 is specifically located on the apical membrane of cells in the epithelium and the apical restriction of ZO-1 is lost in the *Dlg-1* mutant lens (Rivera et al. 2009). In addition, to understand if Lgl interacts genetically with Dlg-1 and/or Scrib, to regulate apico-basal polarity and lens architecture, I propose to further generate compound heterozygotes, $Dlg^{f/+};Lgl^{f/+}10Cre$ or $Scrib^{f/+};Lgl^{f/+}10Cre$, compare the histology of these mutant lenses to that of the individual heterozygous and null lenses. To determine if apico-basal polarity has been disrupted, I would immunostain for ZO-1. In neuroblasts, Dlg is required for localization of Scrib and Lgl, but localization of Dlg is normal in *scrib* and *lgl* mutants. Dlg is required for the cortical recruitment of both Scrib and Lgl (Albertson and Doe, 2003), suggesting that Dlg is an upstream of Scrib and Lgl. The analysis of our *Dlg-1* and *Scrib* mutant lenses showed these genes are required for proper lens development. Determining Lgl expression in control and Dlg-1 and Scrib mutant lenses and characterizing *Lgl* mutant lenses may allow us to understand if *Lgl* play a roles in these complexes and how *Lgl* interacts with *Dlg-1* and *Scrib* in regulating cell polarity in the lens.

***Dlg-1* and *Scrib* modulate Fgfr2 signaling and lens cell structure via interactions with *Vangl2*.**

In chapter V, I presented data showing that the levels of Fgfr2 and its downstream signaling intermediates, pAkt, and pErk, were reduced in each of the single heterozygotes, *Dlg-1*$^{f/+}$, *Scrib*$^{f/+}$, and *Vangl2*$^{Lp/+}$. *Dlg*$^{f/+}$*10Cre;Vangl2*$^{Lp/+}$ compound heterozygous lenses showed significant reduction in the levels of Fgfr2, pAkt and pErk both in the transition zone and in the epithelium as compared to control and single heterozygotes. On the other hand, expression levels of these proteins were partially rescued in *Scrib*$^{f/+}$*10Cre;Vangl2*$^{Lp/+}$ compound heterozygotes., My data also showed that the organization of the epithelium was more disrupted in the lenses of the *Dlg*$^{f/+}$*10Cre;Vangl2*$^{Lp/+}$ compound heterozygotes than in the single heterozygotes and that Fgfr2 levels were lower in the compound heterozygous lenses as compared to single heterozygous lenses. Furthermore, the shorter size of the epithelial cells in the *Scrib* mutant lens and taller epithelium of *Vangl2* mutant lens were partially rescued in *Scrib*$^{f/+}$*10Cre;Vangl2*$^{Lp/+}$ compound heterozygotes, as the epithelium appeared more similar in size to that in control lenses. These data suggest the possibility that *Vangl2* supports or enhances *Dlg-1* and suppresses or limits *Scrib* effects on the regulation of Fgfr2 signaling and on the structure of the epithelium. In Chapters III and IV, I showed that *Dlg-1* status not only affected Fgfr2 levels and activity but also affected Fgfr1 and Fgfr3 in opposite ways than Fgfr2. To understand if the interaction between *Vangl2* and *Dlg-1* affects Fgfrs in addition to Fgfr2, I will determine the Fgfr1 and Fgfr3 expression levels in lenses of *Dlg*$^{f/+}$*10Cre;Vangl2*$^{Lp/+}$ and *Scrib*$^{f/+}$*10Cre;Vangl2*$^{Lp/+}$ compound heterozygotes. It is possible that the effect on Fgfr1 and Fgfr3 will be opposite from the effect on Fgfr2, as I have already observed in *Dlg-1* mutant lenses. This experiment may

further help to understand if Dlg-1 and Scrib play a role in regulating Fgfr signaling via interaction with Vangl2.

Another question that still remains to be answered is whether Vangl2 is also complex with Fgfrs as Dlg-1 and Scrib. Complexity of Vangl2 and Fgfrs can be assessed by co-immunoprecipitation and co-immunofluorescence. If Vangl2 is in complexes with Fgfrs, I propose to address the possibility that *Vangl2* genetically interacts with *Fgfrs*, in a way similar to the way *Dlg-1* interacts with *Fgfrs*. I would mate mice conditionally deleted for *Fgfr1* or *Fgfr2* with *Vangl2* mutant mice to generate $Fgfr1^{f/+}10Cre;Vangl2^{Lp/+}$ and $Fgfr2^{f/+}10Cre;Vangl2^{Lp/+}$ mice. I would then examine the histology of the mutant lenses and the patterns and levels of Dlg-1 and Scrib expression in the mutant lenses. I also would immunostain these lenses with adheren juction proteins and Fgfr downstream intermediates antibodies and measure expression levels and patterns. This approach will further inform me of the extent to which *Vangl2* and *Dlg-1* similarly affect Fgfr signaling and adherens junction formation.

In *Drosophila dlg, scrib,* and *lgl* mutants, apical membrane determinants are disrupted, while basal proteins appear to be unaffected, suggesting that other proteins are required for the localization of basal membrane (Woods et al., 1996; Woods et al., 1997). Interestingly, in $Dlg^{f/+}10Cre;Vangl2^{Lp/+}$ compound heterozygote lenses Fgfr2 expression was almost absent at apical membrane of the epithelial cells while this phenotype was partially rescued in lenses of the $Scrib^{f/+}10Cre;Vangl2^{Lp/+}$ compound heterozygotes (In Chapter V). These observations support the hypothesis that Vangl2 regulates Fgfr2 expression and the localization of this protein to the apical membrane through interaction with Dlg-1 and Scrib. As mentioned above, it will be useful to generate compound heterozygote mice by mating mice containing conditional *Lgl* deletion allele to $Dlg^{f/+}10Cre;Vangl2^{Lp/+}$ compound heterozygotes and $Scrib^{f/+}10Cre;Vangl2^{Lp/+}$

compound heterozygotes. The lenses of these triple mutant animals can be scored for defects in epithelial cell polarity, Fgfr localization and Fgfr signaling. Taken together, these future studies will provide greater details into the *in vivo* mechanistic interactions of *Dlg-1, Scrib* and *Vangl2* in the lens in regulating growth factor signaling pathways that are important for lens development.

Dlg-1 may directly regulate β-catenin in lens development

β-catenin is required for the proliferation of lens progenitor cells and their proper differentiation into fiber cells (Cain et al., 2008). In Chapter III and IV, I showed that *Dlg-1* mutant lenses have defects in fiber cell differentiation and levels of cytoskeletal associated activated β-catenin. The loss of *Ctnnb1* (the gene name for β-catenin) leads to defects that are similar to those observed in *Dlg-1* mutant lenses (Cain et al., 2008). The similarity in the phenotype of these two mutants suggests that *Dlg-1* and *Ctnnb1* may lie in the same pathway in lens development. It is known that β-catenin has a PDZ binding motif, suggesting that β-catenin could directly interact with Dlg-1 (Subbaiah et al., 2012). In Chapter IV, I showed that Dlg-1 regulates the levels of EphA2 activation. EphA2 is known to play a role in lens fiber cell packing and adherens junction formation through its recruitment of N-cadherin to β-catenin at the cell membrane (Cooper et al., 2008). In ephrinA5 mutant lenses, there is no difference in the levels of cytoskeletal associated β-catenin, which differs from my observations on the *Dlg-1* mutant lenses. Thus, the question arises as to whether the impact of Dlg-1 on cytoskeletal associated β-catenin is only due to Dlg-1's regulation of EphA2 or whether Dlg-1 also regulates the levels of cytoskeletal associated β-catenin via other mechanisms.

To address this question, I will quantitate the levels of activated β-catenin in the cytoskeletal associated protein fraction in *Dlg-1* and *Epha2* mutant lenses by western blot analyses. If I find that *Dlg-1* deficiency has a greater effect on the levels of activated b-catenin, I cross *Ctnnb1flox* mice with *MLR10Cre* mice and determine if Fgfr signaling in *Ctnnb1*$^{f/f}$*10* lenses is compromised and the extent to which any changes are similar to the changes in *Dlg-1* deficient lenses. I would compare the phenotype of *Ctnnb1*$^{f/f}$*10* lenses (the gene name for β-catenin) to *Dlg*$^{f/f}$*10* lenses by hematoxylin and eosin staining, and quantitate the levels of N-cadherin, activated Fgfr and activated Fgfr signaling intermediates by western blot analyses as I have done in Chapters III-V. I would also cross *Ctnnb1*$^{f/+}$ mice to *Dlg*$^{f//+}$*10* mice to determine if there is a genetic interaction between *Dlg-1* and *Ctnnb1*. I would assess the expression of adherens junction proteins in the lenses of *Dlg-1* and *Ctnnb1* compound heterozygotes as compared to controls by quantitative immunofluorescence and western blot analyses. I would perform immunofluorescence and western blot analysis on activated Fgfr signaling components to determine if the interactions between Dlg-1 and β-catenin are responsible for Dlg-1's effect on Fgfr signaling. From these experiments, I would learn if Dlg-1 affects the levels of cytoskeletal associated β-catenin solely by its interaction with EphA2 or if Dlg-1 affects β-catenin through multiple mechanisms. The latter finding would prompt additional experiments to test for direct interaction between Dlg-1 and β-catenin (through the PDZ domains in Dlg-1 and the PDZ binding motif in β-catenin) in lens and the impact of that interaction on lens development.

Conclusion

Previously, the Griep lab has shown that the PDZ domain-containing proteins, Dlg-1 (Discs Large) and Scribble are required to maintain cell-cell adhesion, apical polarity, fiber differentiation, and proliferation in the mouse ocular lens and that *Dlg-1* is a PCP gene in the mouse. In my thesis studies, I have sought to determine if there is a linkage between PDZ domain proteins and Fgfr signaling pathways in lens fiber cell differentiation and if this linkage involved *Dlg-1*'s role in PCP. I showed that Dlg-1 inhibited Fgfr1 activation but promoted Fgfr2 activation and the activation of Fgfr downstream signaling intermediates. Also, I showed that Dlg-1 complexes with and regulates the activity of EphA2, which also associates with Fgfrs, providing a potential mechanism through which Dlg-1 affects Fgfr signaling. Finally, I showed that the genetic interactions between *Dlg-1*, *Scrib*, and *Vangl2* that the Griep lab has shown regulate cell shape, similarly affect Fgfr signaling, suggesting that cell shape is a determinant of regulating the signaling pathways necessary for fiber cell differentiation. Taken together, the results in my thesis uncovered new roles for Dlg-1 and PCP factors in the mechanisms of lens cell development. These results, along with future directions have proposed in this chapter, help deepen our understanding the molecular, genetic and signaling mechanisms that regulate lens development and may influence how we view mammalian development at large.

References

Ahn, S.Y., Kim, Y., Kim, S.T., Swat, W., Miner, J.H., 2013. Scaffolding proteins DLG1 and CASK cooperate to maintain the nephron progenitor population during kidney development. J Am Soc Nephrol 24, 1127-1138.

Albertson, R., Doe, C.Q., 2003. Dlg, Scrib and Lgl regulate neuroblast cell size and mitotic spindle asymmetry. Nat Cell Biol 5, 166-170.

Antic, D., Stubbs, J.L., Suyama, K., Kintner, C., Scott, M.P., Axelrod, J.D., 2010. Planar cell polarity enables posterior localization of nodal cilia and left-right axis determination during mouse and Xenopus embryogenesis. PloS one 5, e8999.

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

Beebe, D., Garcia, C., Wang, X., Rajagopal, R., Feldmeier, M., Kim, J.Y., Chytil, A., Moses, H., Ashery-Padan, R., Rauchman, M., 2004. Contributions by members of the TGFbeta superfamily to lens development. Int J Dev Biol 48, 845-856.

Beebe, D.C., Silver, M.H., Belcher, K.S., Van Wyk, J.J., Svoboda, M.E., Zelenka, P.S., 1987. Lentropin, a protein that controls lens fiber formation, is related functionally and immunologically to the insulin-like growth factors. Proceedings of the National Academy of Sciences of the United States of America 84, 2327-2330.

Behrens, C., Lin, H.Y., Lee, J.J., Raso, M.G., Hong, W.K., Wistuba, II, Lotan, R., 2008. Immunohistochemical expression of basic fibroblast growth factor and fibroblast growth factor receptors 1 and 2 in the pathogenesis of lung cancer. Clin Cancer Res 14, 6014-6022.

Belecky-Adams, T.L., Adler, R., Beebe, D.C., 2002. Bone morphogenetic protein signaling and the initiation of lens fiber cell differentiation. Development 129, 3795-3802.

Benjamini, Y., Hochberg, Y., 1995. CONTROLLING THE FALSE DISCOVERY RATE - A PRACTICAL AND POWERFUL APPROACH TO MULTIPLE TESTING. Journal of the Royal Statistical Society Series B-Methodological 57, 289-300.

Bilder, D., 2001. PDZ proteins and polarity: functions from the fly. Trends in genetics : TIG 17, 511-519.

Bilder, D., 2004. Epithelial polarity and proliferation control: links from the Drosophila neoplastic tumor suppressors. Genes & development 18, 1909-1925.

Bilder, D., Li, M., Perrimon, N., 2000. Cooperative regulation of cell polarity and growth by Drosophila tumor suppressors. Science 289, 113-116.

Bilder, D., Perrimon, N., 2000. Localization of apical epithelial determinants by the basolateral PDZ protein Scribble. Nature 403, 676-680.

Borovina, A., Superina, S., Voskas, D., Ciruna, B., 2010. Vangl2 directs the posterior tilting and asymmetric localization of motile primary cilia. Nature cell biology 12, 407-412.

Bossinger, O., Klebes, A., Segbert, C., Theres, C., Knust, E., 2001. Zonula adherens formation in Caenorhabditis elegans requires dlg-1, the homologue of the Drosophila gene discs large. Developmental biology 230, 29-42.

Boswell, B.A., Overbeek, P.A., Musil, L.S., 2008. Essential role of BMPs in FGF-induced secondary lens fiber differentiation. Dev Biol 324, 202-212.

Cain, S., Martinez, G., Kokkinos, M.I., Turner, K., Richardson, R.J., Abud, H.E., Huelsken, J., Robinson, M.L., de Iongh, R.U., 2008a. Differential requirement for beta-catenin in epithelial and fiber cells during lens development. Dev Biol 321, 420-433.

Cain, S., Martinez, G., Kokkinos, M.I., Turner, K., Richardson, R.J., Abud, H.E., Huelsken, J., Robinson, M.L., de Iongh, R.U., 2008b. Differential requirement for beta-catenin in epithelial and fiber cells during lens development. Dev Biol 321, 420-433.

Caruana, G., Bernstein, A., 2001. Craniofacial dysmorphogenesis including cleft palate in mice with an insertional mutation in the discs large gene. Molecular and cellular biology 21, 1475-1483.

Cavatorta, A.L., Fumero, G., Chouhy, D., Aguirre, R., Nocito, A.L., Giri, A.A., Banks, L., Gardiol, D., 2004. Differential expression of the human homologue of drosophila discs large oncosuppressor in histologic samples from human papillomavirus-associated lesions as a marker for progression to malignancy. International journal of cancer. Journal international du cancer 111, 373-380.

Chen, F., Mrock, L.K., Ireland, M.E., 2001. A role for endogenous TGFalpha and associated signaling pathways in the differentiation of lens fiber cells. Journal of cellular physiology 186, 288-297.

Chen, Y., Stump, R.J., Lovicu, F.J., Shimono, A., McAvoy, J.W., 2008. Wnt signaling is required for organization of the lens fiber cell cytoskeleton and development of lens three-dimensional architecture. Dev Biol 324, 161-176.

Cheng, C., Gong, X., 2011. Diverse roles of Eph/ephrin signaling in the mouse lens. PLoS One 6, e28147.

Choi, J., Park, S.Y., Joo, C.K., 2004. Hepatocyte growth factor induces proliferation of lens epithelial cells through activation of ERK1/2 and JNK/SAPK. Invest Ophthalmol Vis Sci 45, 2696-2704.

Chong, L.D., Park, E.K., Latimer, E., Friesel, R., Daar, I.O., 2000. Fibroblast growth factor receptor-mediated rescue of x-ephrin B1-induced cell dissociation in Xenopus embryos. Molecular and cellular biology 20, 724-734.

Chow, R.L., Roux, G.D., Roghani, M., Palmer, M.A., Rifkin, D.B., Moscatelli, D.A., Lang, R.A., 1995. FGF suppresses apoptosis and induces differentiation of fibre cells in the mouse lens. Development 121, 4383-4393.

Churchill, A., Graw, J., 2011. Clinical and experimental advances in congenital and paediatric cataracts. Philosophical transactions of the Royal Society of London. Series B, Biological sciences 366, 1234-1249.

Cooper, M.A., Son, A.I., Komlos, D., Sun, Y., Kleiman, N.J., Zhou, R., 2008. Loss of ephrin-A5 function disrupts lens fiber cell packing and leads to cataract. Proceedings of the National Academy of Sciences of the United States of America 105, 16620-16625.

Dabdoub, A., Donohue, M.J., Brennan, A., Wolf, V., Montcouquiol, M., Sassoon, D.A., Hseih, J.C., Rubin, J.S., Salinas, P.C., Kelley, M.W., 2003. Wnt signaling mediates reorientation of outer hair cell stereociliary bundles in the mammalian cochlea. Development 130, 2375-2384.

Dabdoub, A., Kelley, M.W., 2005. Planar cell polarity and a potential role for a Wnt morphogen gradient in stereociliary bundle orientation in the mammalian inner ear. Journal of neurobiology 64, 446-457.

Darken, R.S., Scola, A.M., Rakeman, A.S., Das, G., Mlodzik, M., Wilson, P.A., 2002. The planar polarity gene strabismus regulates convergent extension movements in Xenopus. The EMBO journal 21, 976-985.

Dave, A., Laurie, K., Staffieri, S.E., Taranath, D., Mackey, D.A., Mitchell, P., Wang, J.J., Craig, J.E., Burdon, K.P., Sharma, S., 2013. Mutations in the EPHA2 gene are a major contributor to inherited cataracts in South-Eastern Australia. PLoS One 8, e72518.

Davy, A., Gale, N.W., Murray, E.W., Klinghoffer, R.A., Soriano, P., Feuerstein, C., Robbins, S.M., 1999. Compartmentalized signaling by GPI-anchored ephrin-A5 requires the Fyn tyrosine kinase to regulate cellular adhesion. Genes & development 13, 3125-3135.

Dawes, L.J., Sugiyama, Y., Tanedo, A.S., Lovicu, F.J., McAvoy, J.W., 2013. Wnt-frizzled signaling is part of an FGF-induced cascade that promotes lens fiber differentiation. Investigative ophthalmology & visual science 54, 1582-1590.

de Iongh, R.U., Gordon-Thomson, C., Chamberlain, C.G., Hales, A.M., McAvoy, J.W., 2001. Tgfbeta receptor expression in lens: implications for differentiation and cataractogenesis. Exp Eye Res 72, 649-659.

de Iongh, R.U., Lovicu, F.J., Hanneken, A., Baird, A., McAvoy, J.W., 1996. FGF receptor-1 (flg) expression is correlated with fibre differentiation during rat lens morphogenesis and growth. Dev Dyn 206, 412-426.

Dimitratos, S.D., Woods, D.F., Stathakis, D.G., Bryant, P.J., 1999. Signaling pathways are focused at specialized regions of the plasma membrane by scaffolding proteins of the MAGUK family. Bioessays 21, 912-921.

Dow, L.E., Brumby, A.M., Muratore, R., Coombe, M.L., Sedelies, K.A., Trapani, J.A., Russell, S.M., Richardson, H.E., Humbert, P.O., 2003. hScrib is a functional homologue of the Drosophila tumour suppressor Scribble. Oncogene 22, 9225-9230.

Dunn, H.A., Ferguson, S.S., 2015. PDZ Protein Regulation of GPCR Trafficking and Signaling Pathways. Mol Pharmacol.

Eswarakumar, V.P., Lax, I., Schlessinger, J., 2005. Cellular signaling by fibroblast growth factor receptors. Cytokine & growth factor reviews 16, 139-149.

Faber, S.C., Dimanlig, P., Makarenkova, H.P., Shirke, S., Ko, K., Lang, R.A., 2001. Fgf receptor signaling plays a role in lens induction. Development 128, 4425-4438.

Faber, S.C., Robinson, M.L., Makarenkova, H.P., Lang, R.A., 2002. Bmp signaling is required for development of primary lens fiber cells. Development 129, 3727-3737.

Fan, J.S., Zhang, M., 2002. Signaling complex organization by PDZ domain proteins. Neuro-Signals 11, 315-321.

Feiguin, F., Hannus, M., Mlodzik, M., Eaton, S., 2001. The ankyrin repeat protein Diego mediates Frizzled-dependent planar polarization. Developmental cell 1, 93-101.

Firestein, B.L., Rongo, C., 2001. DLG-1 is a MAGUK similar to SAP97 and is required for adherens junction formation. Molecular biology of the cell 12, 3465-3475.

Fredriksson, R., Lagerstrom, M.C., Lundin, L.G., Schioth, H.B., 2003. The G-protein-coupled receptors in the human genome form five main families. Phylogenetic analysis, paralogon groups, and fingerprints. Mol Pharmacol 63, 1256-1272.

Froldi, F., Ziosi, M., Tomba, G., Parisi, F., Garoia, F., Pession, A., Grifoni, D., 2008. Drosophila lethal giant larvae neoplastic mutant as a genetic tool for cancer modeling. Curr Genomics 9, 147-154.

Fukai, J., Yokote, H., Yamanaka, R., Arao, T., Nishio, K., Itakura, T., 2008. EphA4 promotes cell proliferation and migration through a novel EphA4-FGFR1 signaling pathway in the human glioma U251 cell line. Mol Cancer Ther 7, 2768-2778.

Gao, B., Song, H., Bishop, K., Elliot, G., Garrett, L., English, M.A., Andre, P., Robinson, J., Sood, R., Minami, Y., Economides, A.N., Yang, Y., 2011. Wnt signaling gradients establish planar cell polarity by inducing Vangl2 phosphorylation through Ror2. Dev Cell 20, 163-176.

Garcia, C.M., Huang, J., Madakashira, B.P., Liu, Y., Rajagopal, R., Dattilo, L., Robinson, M.L., Beebe, D.C., 2011. The function of FGF signaling in the lens placode. Developmental biology 351, 176-185.

Garcia, C.M., Yu, K., Zhao, H., Ashery-Padan, R., Ornitz, D.M., Robinson, M.L., Beebe, D.C., 2005. Signaling through FGF receptor-2 is required for lens cell survival and for withdrawal from the cell cycle during lens fiber cell differentiation. Dev Dyn 233, 516-527.

Gardiol, D., Zacchi, A., Petrera, F., Stanta, G., Banks, L., 2006. Human discs large and scrib are localized at the same regions in colon mucosa and changes in their expression patterns are correlated with loss of tissue architecture during malignant progression. International journal of cancer. Journal international du cancer 119, 1285-1290.

Genander, M., Frisen, J., 2010. Ephrins and Eph receptors in stem cells and cancer. Current opinion in cell biology 22, 611-616.

Gerber, S.D., Steinberg, F., Beyeler, M., Villiger, P.M., Trueb, B., 2009. The murine Fgfrl1 receptor is essential for the development of the metanephric kidney. Developmental biology 335, 106-119.

Gotoh, N., 2008. Regulation of growth factor signaling by FRS2 family docking/scaffold adaptor proteins. Cancer Sci 99, 1319-1325.

Gu, X., Seong, G.J., Lee, Y.G., Kay, E.P., 1996. Fibroblast growth factor 2 uses distinct signaling pathways for cell proliferation and cell shape changes in corneal endothelial cells. Investigative ophthalmology & visual science 37, 2326-2334.

Gumbiner, B.M., 1996. Cell adhesion: the molecular basis of tissue architecture and morphogenesis. Cell 84, 345-357.

Hains, D.S., Sims-Lucas, S., Carpenter, A., Saha, M., Murawski, I., Kish, K., Gupta, I., McHugh, K., Bates, C.M., 2010. High incidence of vesicoureteral reflux in mice with Fgfr2 deletion in kidney mesenchyma. The Journal of urology 183, 2077-2084.

Harris, B.Z., Lim, W.A., 2001a. Mechanism and role of PDZ domains in signaling complex assembly. Journal of cell science 114, 3219-3231.

Harris, B.Z., Lim, W.A., 2001b. Mechanism and role of PDZ domains in signaling complex assembly. J Cell Sci 114, 3219-3231.

Hayes, J.M., Hartsock, A., Clark, B.S., Napier, H.R., Link, B.A., Gross, J.M., 2012. Integrin alpha5/fibronectin1 and focal adhesion kinase are required for lens fiber morphogenesis in zebrafish. Mol Biol Cell 23, 4725-4738.

Hennis, A., Wu, S.Y., Nemesure, B., Leske, M.C., Barbados Eye Studies, G., 2003. Hypertension, diabetes, and longitudinal changes in intraocular pressure. Ophthalmology 110, 908-914.

Himanen, J.P., 2012. Ectodomain structures of Eph receptors. Seminars in cell & developmental biology 23, 35-42.

Himanen, J.P., Chumley, M.J., Lackmann, M., Li, C., Barton, W.A., Jeffrey, P.D., Vearing, C., Geleick, D., Feldheim, D.A., Boyd, A.W., Henkemeyer, M., Nikolov, D.B., 2004. Repelling class discrimination: ephrin-A5 binds to and activates EphB2 receptor signaling. Nature neuroscience 7, 501-509.

Hiratsuka, Y., Ono, K., Murakami, A., 2009. Alcohol use and cataract. Current drug abuse reviews 2, 226-229.

Hock, B., Bohme, B., Karn, T., Yamamoto, T., Kaibuchi, K., Holtrich, U., Holland, S., Pawson, T., Rubsamen-Waigmann, H., Strebhardt, K., 1998. PDZ-domain-mediated interaction of the Eph-related receptor tyrosine kinase EphB3 and the ras-binding protein AF6 depends on the kinase activity of the receptor. Proceedings of the National Academy of Sciences of the United States of America 95, 9779-9784.

Hoffmann, M., Segbert, C., Helbig, G., Bossinger, O., 2010. Intestinal tube formation in Caenorhabditis elegans requires vang-1 and egl-15 signaling. Dev Biol 339, 268-279.

Holland, S.J., Gale, N.W., Mbamalu, G., Yancopoulos, G.D., Henkemeyer, M., Pawson, T., 1996. Bidirectional signalling through the EPH-family receptor Nuk and its transmembrane ligands. Nature 383, 722-725.

Huang, J.X., Feldmeier, M., Shui, Y.B., Beebe, D.C., 2003. Evaluation of fibroblast growth factor signaling during lens fiber cell differentiation. Investigative ophthalmology & visual science 44, 680-690.

Humbert, P., Russell, S., Richardson, H., 2003. Dlg, Scribble and Lgl in cell polarity, cell proliferation and cancer. BioEssays : news and reviews in molecular, cellular and developmental biology 25, 542-553.

Humbert, P.O., Grzeschik, N.A., Brumby, A.M., Galea, R., Elsum, I., Richardson, H.E., 2008. Control of tumourigenesis by the Scribble/Dlg/Lgl polarity module. Oncogene 27, 6888-6907.

Hung, A.Y., Sheng, M., 2002. PDZ domains: structural modules for protein complex assembly. J Biol Chem 277, 5699-5702.

Ireland, M.E., Mrock, L.K., 2004. Expression and activation of the epidermal growth factor receptor in differentiating cells of the developing and post-hatching chicken lens. Experimental eye research 79, 305-312.

Iyengar, L., Wang, Q., Rasko, J.E., McAvoy, J.W., Lovicu, F.J., 2007. Duration of ERK1/2 phosphorylation induced by FGF or ocular media determines lens cell fate. Differentiation 75, 662-668.

Jessen, J.R., Topczewski, J., Bingham, S., Sepich, D.S., Marlow, F., Chandrasekhar, A., Solnica-Krezel, L., 2002. Zebrafish trilobite identifies new roles for Strabismus in gastrulation and neuronal movements. Nature cell biology 4, 610-615.

Johnson, K., Wodarz, A., 2003. A genetic hierarchy controlling cell polarity. Nature cell biology 5, 12-14.

Jun, G., Guo, H., Klein, B.E., Klein, R., Wang, J.J., Mitchell, P., Miao, H., Lee, K.E., Joshi, T., Buck, M., Chugha, P., Bardenstein, D., Klein, A.P., Bailey-Wilson, J.E., Gong, X., Spector, T.D., Andrew, T., Hammond, C.J., Elston, R.C., Iyengar, S.K., Wang, B., 2009. EPHA2 is associated with age-related cortical cataract in mice and humans. PLoS genetics 5, e1000584.

Kallay, L.M., McNickle, A., Brennwald, P.J., Hubbard, A.L., Braiterman, L.T., 2006. Scribble associates with two polarity proteins, Lgl2 and Vangl2, via distinct molecular domains. Journal of cellular biochemistry 99, 647-664.

Kalo, M.S., Pasquale, E.B., 1999. Signal transfer by eph receptors. Cell and tissue research 298, 1-9.

Kaul, H., Riazuddin, S.A., Shahid, M., Kousar, S., Butt, N.H., Zafar, A.U., Khan, S.N., Husnain, T., Akram, J., Hejtmancik, J.F., Riazuddin, S., 2010. Autosomal recessive congenital cataract linked to EPHA2 in a consanguineous Pakistani family. Mol Vis 16, 511-517.

Kholodenko, B.N., Hancock, J.F., Kolch, W., 2010. Signalling ballet in space and time. Nature reviews. Molecular cell biology 11, 414-426.

Knust, E., Bossinger, O., 2002. Composition and formation of intercellular junctions in epithelial cells. Science 298, 1955-1959.

Kok, A., Lovicu, F.J., Chamberlain, C.G., McAvoy, J.W., 2002. Influence of platelet-derived growth factor on lens epithelial cell proliferation and differentiation. Growth Factors 20, 27-34.

Koppen, M., Simske, J.S., Sims, P.A., Firestein, B.L., Hall, D.H., Radice, A.D., Rongo, C., Hardin, J.D., 2001. Cooperative regulation of AJM-1 controls junctional integrity in Caenorhabditis elegans epithelia. Nat Cell Biol 3, 983-991.

Kullander, K., Klein, R., 2002. Mechanisms and functions of Eph and ephrin signalling. Nature reviews. Molecular cell biology 3, 475-486.

Kuszak, J.R., Zoltoski, R.K., Sivertson, C., 2004. Fibre cell organization in crystalline lenses. Experimental eye research 78, 673-687.

Lackmann, M., Boyd, A.W., 2008. Eph, a protein family coming of age: more confusion, insight, or complexity? Science signaling 1, re2.

Lai, W.T., Krishnappa, V., Phinney, D.G., 2011. Fibroblast growth factor 2 (Fgf2) inhibits differentiation of mesenchymal stem cells by inducing Twist2 and Spry4, blocking extracellular regulated kinase activation, and altering Fgf receptor expression levels. Stem Cells 29, 1102-1111.

Lang, R.A., 2004. Pathways regulating lens induction in the mouse. Int J Dev Biol 48, 783-791.

Laprise, P., Viel, A., Rivard, N., 2004. Human homolog of disc-large is required for adherens junction assembly and differentiation of human intestinal epithelial cells. J Biol Chem 279, 10157-10166.

Lee, H.J., Zheng, J.J., 2010. PDZ domains and their binding partners: structure, specificity, and modification. Cell Commun Signal 8, 8.

Lee, O.K., Frese, K.K., James, J.S., Chadda, D., Chen, Z.H., Javier, R.T., Cho, K.O., 2003. Discs-Large and Strabismus are functionally linked to plasma membrane formation. Nature cell biology 5, 987-993.

Lee, S., Fan, S., Makarova, O., Straight, S., Margolis, B., 2002. A novel and conserved protein-protein interaction domain of mammalian Lin-2/CASK binds and recruits SAP97 to the lateral surface of epithelia. Mol Cell Biol 22, 1778-1791.

Lee, S., Griep, A.E., 2014. Loss of Dlg-1 in the mouse lens impairs fibroblast growth factor receptor signaling. PloS one 9, e97470.

Leske, M.C., Wu, S.Y., Hennis, A., Connell, A.M., Hyman, L., Schachat, A., 1999. Diabetes, hypertension, and central obesity as cataract risk factors in a black population. The Barbados Eye Study. Ophthalmology 106, 35-41.

Liu, H., Thurig, S., Mohamed, O., Dufort, D., Wallace, V.A., 2006. Mapping canonical Wnt signaling in the developing and adult retina. Invest Ophthalmol Vis Sci 47, 5088-5097.

Liu, J., Chamberlain, C.G., McAvoy, J.W., 1996. IGF enhancement of FGF-induced fibre differentiation and DNA synthesis in lens explants. Exp Eye Res 63, 621-629.

Lois, N., Abdelkader, E., Reglitz, K., Garden, C., Ayres, J.G., 2008. Environmental tobacco smoke exposure and eye disease. The British journal of ophthalmology 92, 1304-1310.

Lovicu, F.J., McAvoy, J.W., 2001. FGF-induced lens cell proliferation and differentiation is dependent on MAPK (ERK1/2) signalling. Development 128, 5075-5084.

Lovicu, F.J., McAvoy, J.W., de Iongh, R.U., 2011. Understanding the role of growth factors in embryonic development: insights from the lens. Philosophical transactions of the Royal Society of London. Series B, Biological sciences 366, 1204-1218.

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

Lovicu, F.J., Steven, P., Saika, S., McAvoy, J.W., 2004. Aberrant lens fiber differentiation in anterior subcapsular cataract formation: a process dependent on reduced levels of Pax6. Investigative ophthalmology & visual science 45, 1946-1953.

Lu, P., Ewald, A.J., Martin, G.R., Werb, Z., 2008. Genetic mosaic analysis reveals FGF receptor 2 function in terminal end buds during mammary gland branching morphogenesis. Dev Biol 321, 77-87.

Madakashira, B.P., Kobrinski, D.A., Hancher, A.D., Arneman, E.C., Wagner, B.D., Wang, F., Shin, H., Lovicu, F.J., Reneker, L.W., Robinson, M.L., 2012. Frs2alpha enhances fibroblast growth factor-mediated survival and differentiation in lens development. Development 139, 4601-4612.

Martin, A.J., Grant, A., Ashfield, A.M., Palmer, C.N., Baker, L., Quinlan, P.R., Purdie, C.A., Thompson, A.M., Jordan, L.B., Berg, J.N., 2011. FGFR2 protein expression in breast cancer: nuclear localisation and correlation with patient genotype. BMC Res Notes 4, 72.

Mason, L., Giardina, S.L., Hecht, T., Ortaldo, J., Mathieson, B.J., 1988. LGL-1: a non-polymorphic antigen expressed on a major population of mouse natural killer cells. J Immunol 140, 4403-4412.

Matsuo, T., 1993. The genes involved in the morphogenesis of the eye. Jpn J Ophthalmol 37, 215-251.

May-Simera, H.L., Kai, M., Hernandez, V., Osborn, D.P., Tada, M., Beales, P.L., 2010. Bbs8, together with the planar cell polarity protein Vangl2, is required to establish left-right asymmetry in zebrafish. Developmental biology 345, 215-225.

McAvoy, J.W., Chamberlain, C.G., 1989. Fibroblast growth factor (FGF) induces different responses in lens epithelial cells depending on its concentration. Development 107, 221-228.

McAvoy, J.W., Chamberlain, C.G., de Iongh, R.U., Hales, A.M., Lovicu, F.J., 1999. Lens development. Eye 13 (Pt 3b), 425-437.

McClelland, A.C., Hruska, M., Coenen, A.J., Henkemeyer, M., Dalva, M.B., 2010. Trans-synaptic EphB2-ephrin-B3 interaction regulates excitatory synapse density by inhibition of

postsynaptic MAPK signaling. Proceedings of the National Academy of Sciences of the United States of America 107, 8830-8835.

McMahon, L., Legouis, R., Vonesch, J.L., Labouesse, M., 2001. Assembly of C. elegans apical junctions involves positioning and compaction by LET-413 and protein aggregation by the MAGUK protein DLG-1. J Cell Sci 114, 2265-2277.

Merlos-Suarez, A., Batlle, E., 2008. Eph-ephrin signalling in adult tissues and cancer. Current opinion in cell biology 20, 194-200.

Moody, S.A., 2004. To differentiate or not to differentiate: regulation of cell fate decisions by being in the right place at the right time. Cell Cycle 3, 564-566.

Muenke, M., Schell, U., Hehr, A., Robin, N.H., Losken, H.W., Schinzel, A., Pulleyn, L.J., Rutland, P., Reardon, W., Malcolm, S., et al., 1994. A common mutation in the fibroblast growth factor receptor 1 gene in Pfeiffer syndrome. Nature genetics 8, 269-274.

Murakami, M., Elfenbein, A., Simons, M., 2008. Non-canonical fibroblast growth factor signalling in angiogenesis. Cardiovascular research 78, 223-231.

Musch, A., Cohen, D., Yeaman, C., Nelson, W.J., Rodriguez-Boulan, E., Brennwald, P.J., 2002. Mammalian homolog of Drosophila tumor suppressor lethal (2) giant larvae interacts with basolateral exocytic machinery in Madin-Darby canine kidney cells. Mol Biol Cell 13, 158-168.

Naim, E., Bernstein, A., Bertram, J.F., Caruana, G., 2005. Mutagenesis of the epithelial polarity gene, discs large 1, perturbs nephrogenesis in the developing mouse kidney. Kidney international 68, 955-965.

Nguyen, M.M., Nguyen, M.L., Caruana, G., Bernstein, A., Lambert, P.F., Griep, A.E., 2003. Requirement of PDZ-containing proteins for cell cycle regulation and differentiation in the mouse lens epithelium. Molecular and cellular biology 23, 8970-8981.

Nguyen, M.M., Rivera, C., Griep, A.E., 2005. Localization of PDZ domain containing proteins Discs Large-1 and Scribble in the mouse eye. Mol Vis 11, 1183-1199.

Ornitz, D.M., Marie, P.J., 2002. FGF signaling pathways in endochondral and intramembranous bone development and human genetic disease. Genes & development 16, 1446-1465.

Pap, M., Cooper, G.M., 1998. Role of glycogen synthase kinase-3 in the phosphatidylinositol 3-Kinase/Akt cell survival pathway. J Biol Chem 273, 19929-19932.

Park, E.K., Warner, N., Bong, Y.S., Stapleton, D., Maeda, R., Pawson, T., Daar, I.O., 2004. Ectopic EphA4 receptor induces posterior protrusions via FGF signaling in Xenopus embryos. Molecular biology of the cell 15, 1647-1655.

Pasquale, E.B., 2008. Eph-ephrin bidirectional signaling in physiology and disease. Cell 133, 38-52.

Pasquale, E.B., 2010. Eph receptors and ephrins in cancer: bidirectional signalling and beyond. Nature reviews. Cancer 10, 165-180.

Piatigorsky, J., 1981. Lens differentiation in vertebrates. A review of cellular and molecular features. Differentiation 19, 134-153.

Picco, V., Hudson, C., Yasuo, H., 2007. Ephrin-Eph signalling drives the asymmetric division of notochord/neural precursors in Ciona embryos. Development 134, 1491-1497.

Poliakov, A., Cotrina, M.L., Pasini, A., Wilkinson, D.G., 2008. Regulation of EphB2 activation and cell repulsion by feedback control of the MAPK pathway. The Journal of cell biology 183, 933-947.

Ponting, C.P., 1997. Evidence for PDZ domains in bacteria, yeast, and plants. Protein science : a publication of the Protein Society 6, 464-468.

Ponting, C.P., Phillips, C., Davies, K.E., Blake, D.J., 1997. PDZ domains: targeting signalling molecules to sub-membranous sites. BioEssays : news and reviews in molecular, cellular and developmental biology 19, 469-479.

Pontoriero, G.F., Smith, A.N., Miller, L.A., Radice, G.L., West-Mays, J.A., Lang, R.A., 2009. Co-operative roles for E-cadherin and N-cadherin during lens vesicle separation and lens epithelial cell survival. Dev Biol 326, 403-417.

Prontera, P., Sensi, A., Pilu, G., Baldi, M., Baffico, M., Bonasoni, R., Calzolari, E., 2006. FGFR3 mutation in thanatophoric dysplasia type 1 with bilateral cystic renal dysplasia: coincidence or a new association? Genetic counseling 17, 407-412.

Qin, Y., Capaldo, C., Gumbiner, B.M., Macara, I.G., 2005. The mammalian Scribble polarity protein regulates epithelial cell adhesion and migration through E-cadherin. The Journal of cell biology 171, 1061-1071.

Reddan, J.R., Wilson-Dziedzic, D., 1983. Insulin growth factor and epidermal growth factor trigger mitosis in lenses cultured in a serum-free medium. Invest Ophthalmol Vis Sci 24, 409-416.

Reidy, A., Minassian, D.C., Desai, P., Vafidis, G., Joseph, J., Farrow, S., Connolly, A., 2002. Increased mortality in women with cataract: a population based follow up of the North London Eye Study. The British journal of ophthalmology 86, 424-428.

Reneker, L.W., Overbeek, P.A., 1996. Lens-specific expression of PDGF-A alters lens growth and development. Dev Biol 180, 554-565.

Reuver, S.M., Garner, C.C., 1998. E-cadherin mediated cell adhesion recruits SAP97 into the cortical cytoskeleton. Journal of cell science 111 (Pt 8), 1071-1080.

Richardson, N.A., Chamberlain, C.G., McAvoy, J.W., 1993. IGF-1 enhancement of FGF-induced lens fiber differentiation in rats of different ages. Investigative ophthalmology & visual science 34, 3303-3312.

Rida, P.C., Chen, P., 2009. Line up and listen: Planar cell polarity regulation in the mammalian inner ear. Seminars in cell & developmental biology 20, 978-985.

Rivera, C., Simonson, S.J., Yamben, I.F., Shatadal, S., Nguyen, M.M., Beurg, M., Lambert, P.F., Griep, A.E., 2013. Requirement for Dlgh-1 in planar cell polarity and skeletogenesis during vertebrate development. PloS one 8, e54410.

Rivera, C., Yamben, I.F., Shatadal, S., Waldof, M., Robinson, M.L., Griep, A.E., 2009. Cell-autonomous requirements for Dlg-1 for lens epithelial cell structure and fiber cell morphogenesis. Dev Dyn 238, 2292-2308.

Robinson, M.L., 2006. An essential role for FGF receptor signaling in lens development. Semin Cell Dev Biol 17, 726-740.

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

Rowan, S., Conley, K.W., Le, T.T., Donner, A.L., Maas, R.L., Brown, N.L., 2008. Notch signaling regulates growth and differentiation in the mammalian lens. Dev Biol 321, 111-122.

Saravanamuthu, S.S., Gao, C.Y., Zelenka, P.S., 2009. Notch signaling is required for lateral induction of Jagged1 during FGF-induced lens fiber differentiation. Dev Biol 332, 166-176.

Schmahl, J., Kim, Y., Colvin, J.S., Ornitz, D.M., Capel, B., 2004. Fgf9 induces proliferation and nuclear localization of FGFR2 in Sertoli precursors during male sex determination. Development 131, 3627-3636.

Segbert, C., Johnson, K., Theres, C., van Furden, D., Bossinger, O., 2004. Molecular and functional analysis of apical junction formation in the gut epithelium of Caenorhabditis elegans. Developmental biology 266, 17-26.

Shi, Y., De Maria, A., Bennett, T., Shiels, A., Bassnett, S., 2012. A role for epha2 in cell migration and refractive organization of the ocular lens. Investigative ophthalmology & visual science 53, 551-559.

Shiels, A., Bennett, T.M., Knopf, H.L., Maraini, G., Li, A., Jiao, X., Hejtmancik, J.F., 2008. The EPHA2 gene is associated with cataracts linked to chromosome 1p. Mol Vis 14, 2042-2055.

Simirskii, V.N., Wang, Y., Duncan, M.K., 2007. Conditional deletion of beta1-integrin from the developing lens leads to loss of the lens epithelial phenotype. Dev Biol 306, 658-668.

Singh, J., Mlodzik, M., 2012. Planar cell polarity signaling: coordination of cellular orientation across tissues. Wiley Interdiscip Rev Dev Biol 1, 479-499.

Stolen, C.M., Griep, A.E., 2000. Disruption of lens fiber cell differentiation and survival at multiple stages by region-specific expression of truncated FGF receptors. Dev Biol 217, 205-220.

Subbaiah, V.K., Narayan, N., Massimi, P., Banks, L., 2012. Regulation of the DLG tumor suppressor by beta-catenin. Int J Cancer 131, 2223-2233.

Sugiyama, Y., Akimoto, K., Robinson, M.L., Ohno, S., Quinlan, R.A., 2009. A cell polarity protein aPKClambda is required for eye lens formation and growth. Developmental biology 336, 246-256.

Sugiyama, Y., Lovicu, F.J., McAvoy, J.W., 2011. Planar cell polarity in the mammalian eye lens. Organogenesis 7, 191-201.

Sugiyama, Y., Stump, R.J., Nguyen, A., Wen, L., Chen, Y., Wang, Y., Murdoch, J.N., Lovicu, F.J., McAvoy, J.W., 2010. Secreted frizzled-related protein disrupts PCP in eye lens fiber cells that have polarised primary cilia. Developmental biology 338, 193-201.

Tan, W., Hou, S., Jiang, Z., Hu, Z., Yang, P., Ye, J., 2011. Association of EPHA2 polymorphisms and age-related cortical cataract in a Han Chinese population. Molecular vision 17, 1553-1558.

Tarkkonen, K.M., Nilsson, E.M., Kahkonen, T.E., Dey, J.H., Heikkila, J.E., Tuomela, J.M., Liu, Q., Hynes, N.E., Harkonen, P.L., 2012. Differential roles of fibroblast growth factor receptors (FGFR) 1, 2 and 3 in the regulation of S115 breast cancer cell growth. PLoS One 7, e49970.

Taylor, J., Abramova, N., Charlton, J., Adler, P.N., 1998. Van Gogh: a new Drosophila tissue polarity gene. Genetics 150, 199-210.

Torres, R., Firestein, B.L., Dong, H., Staudinger, J., Olson, E.N., Huganir, R.L., Bredt, D.S., Gale, N.W., Yancopoulos, G.D., 1998. PDZ proteins bind, cluster, and synaptically colocalize with Eph receptors and their ephrin ligands. Neuron 21, 1453-1463.

van der Noll, R., Leijen, S., Neuteboom, G.H., Beijnen, J.H., Schellens, J.H., 2013. Effect of inhibition of the FGFR-MAPK signaling pathway on the development of ocular toxicities. Cancer Treat Rev 39, 664-672.

Walker, J.L., Zhang, L., Zhou, J., Woolkalis, M.J., Menko, A.S., 2002. Role for alpha 6 integrin during lens development: Evidence for signaling through IGF-1R and ERK. Dev Dyn 223, 273-284.

Wallingford, J.B., 2012. Planar cell polarity and the developmental control of cell behavior in vertebrate embryos. Annu Rev Cell Dev Biol 28, 627-653.

Wallingford, J.B., Habas, R., 2005. The developmental biology of Dishevelled: an enigmatic protein governing cell fate and cell polarity. Development 132, 4421-4436.

Wang, J., Mark, S., Zhang, X., Qian, D., Yoo, S.J., Radde-Gallwitz, K., Zhang, Y., Lin, X., Collazo, A., Wynshaw-Boris, A., Chen, P., 2005. Regulation of polarized extension and planar cell polarity in the cochlea by the vertebrate PCP pathway. Nature genetics 37, 980-985.

Wang, Q., McAvoy, J.W., Lovicu, F.J., 2010. Growth factor signaling in vitreous humor-induced lens fiber differentiation. Investigative ophthalmology & visual science 51, 3599-3610.

Wang, Y., Nathans, J., 2007. Tissue/planar cell polarity in vertebrates: new insights and new questions. Development 134, 647-658.

Wawersik, S., Purcell, P., Rauchman, M., Dudley, A.T., Robertson, E.J., Maas, R., 1999. BMP7 acts in murine lens placode development. Developmental biology 207, 176-188.

Wederell, E.D., de Iongh, R.U., 2006. Extracellular matrix and integrin signaling in lens development and cataract. Semin Cell Dev Biol 17, 759-776.

Wegener, A., Heinitz, M., Dwinger, M., 2002. Experimental evidence for interactive effects of chronic UV irradiation and nutritional deficiencies in the lens. Developments in ophthalmology 35, 113-124.

Wodarz, A., 2000. Tumor suppressors: linking cell polarity and growth control. Curr Biol 10, R624-626.

Wolff, T., Rubin, G.M., 1998. Strabismus, a novel gene that regulates tissue polarity and cell fate decisions in Drosophila. Development 125, 1149-1159.

Woods, D.F., Hough, C., Peel, D., Callaini, G., Bryant, P.J., 1996. Dlg protein is required for junction structure, cell polarity, and proliferation control in Drosophila epithelia. J Cell Biol 134, 1469-1482.

Woods, D.F., Wu, J.W., Bryant, P.J., 1997. Localization of proteins to the apico-lateral junctions of Drosophila epithelia. Dev Genet 20, 111-118.

Wormstone, I.M., 2002. Posterior capsule opacification: a cell biological perspective. Experimental eye research 74, 337-347.

Yamben, I.F., Rachel, R.A., Shatadal, S., Copeland, N.G., Jenkins, N.A., Warming, S., Griep, A.E., 2013. Scrib is required for epithelial cell identity and prevents epithelial to mesenchymal transition in the mouse. Developmental biology 384, 41-52.

Yates, L.L., Papakrivopoulou, J., Long, D.A., Goggolidou, P., Connolly, J.O., Woolf, A.S., Dean, C.H., 2010a. The planar cell polarity gene Vangl2 is required for mammalian kidney-branching morphogenesis and glomerular maturation. Human molecular genetics 19, 4663-4676.

Yates, L.L., Schnatwinkel, C., Hazelwood, L., Chessum, L., Paudyal, A., Hilton, H., Romero, M.R., Wilde, J., Bogani, D., Sanderson, J., Formstone, C., Murdoch, J.N., Niswander, L.A., Greenfield, A., Dean, C.H., 2013. Scribble is required for normal epithelial cell-cell contacts and lumen morphogenesis in the mammalian lung. Developmental biology 373, 267-280.

Yates, L.L., Schnatwinkel, C., Murdoch, J.N., Bogani, D., Formstone, C.J., Townsend, S., Greenfield, A., Niswander, L.A., Dean, C.H., 2010b. The PCP genes Celsr1 and Vangl2 are required for normal lung branching morphogenesis. Human molecular genetics 19, 2251-2267.

Yokote, H., Fujita, K., Jing, X., Sawada, T., Liang, S., Yao, L., Yan, X., Zhang, Y., Schlessinger, J., Sakaguchi, K., 2005. Trans-activation of EphA4 and FGF receptors mediated by direct interactions between their cytoplasmic domains. Proceedings of the National Academy of Sciences of the United States of America 102, 18866-18871.

Yoshioka, T., Hagiwara, A., Hida, Y., Ohtsuka, T., 2013. Vangl2, the planar cell polarity protein, is complexed with postsynaptic density protein PSD-95 [corrected]. FEBS letters 587, 1453-1459.

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

Zhao, H., Yang, T., Madakashira, B.P., Thiels, C.A., Bechtle, C.A., Garcia, C.M., Zhang, H., Yu, K., Ornitz, D.M., Beebe, D.C., Robinson, M.L., 2008. Fibroblast growth factor receptor signaling is essential for lens fiber cell differentiation. Dev Biol 318, 276-288.

Zhao, H., Yang, Y., Rizo, C.M., Overbeek, P.A., Robinson, M.L., 2004. Insertion of a Pax6 consensus binding site into the alphaA-crystallin promoter acts as a lens epithelial cell enhancer in transgenic mice. Investigative ophthalmology & visual science 45, 1930-1939.