THE ROLE OF ADAPTOR PROTEINS CRK AND CRKL IN LENS DEVELOPMENT

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DEDICATION

This work is dedicated to all my family and friends who supported me and encouraged me to keep going when I wanted to give up. This work is especially dedicated to my mother who worked hard to make sure I had the skills and drive to succeed and conquer every obstacle that comes my way. Lastly, I want to dedicate this work to GOD without him none of my achievements in life would be possible.

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Cell shape changes and signaling pathways are essential for the development and function of the lens. During lens development proliferating epithelial cells will migrate down to the equator of the lens, differentiate into lens fiber cells, and begin to elongate along the lens capsule. The Fibroblast Growth Factor (FGF) signaling pathway has been extensively studied for its role in lens fiber cell differentiation and elongation. However, the main mediators of FGF stimulated lens fiber cell elongation have not been identified. Adaptor proteins Crk and CrkL are SH2- and SH3-containing proteins that transduce signals from upstream tyrosine phosphorylated proteins to downstream effectors, including Ras, Rac1 and Rap1, which are important for cell proliferation, adhesion and migration. Underlying their diverse function, these two adaptor proteins have been implicated in receptor tyrosine kinase signaling, focal adhesion assembly, and cell shape. To explore the role of Crk and CrkL in FGF signaling-dependent lens development and fiber elongation, we employed Cre/LoxP system to generate a lens specific knockout of Crk/CrkL. This led to extracellular matrix defects, disorganization of the lens fiber cells, and a defect in lens fiber cell elongation. Deletion of Crk and CrkL in the lens also mitigated the gain-of-function phenotype caused by overexpression of FGF3, indicating an epistatic relationship between Crk/CrkL and FGF signaling during lens fiber cell elongation. Further studies, revealed that the activity of Crk and CrkL in FGF signaling is controlled by the phosphatase Shp2 and the defect observed in lens fiber cell elongation can be rescued by constitutive activation of the GTPases Ras and Rac1 in the Crk and CrkL mutant lens. Interestingly, the deletion of the GTPases Rap1 in the lens showed no obvious phenotype pertaining to

lens fiber cell elongation. These findings suggest that Crk and CrkL play an important role in integrating FGF signaling and mediating lens fiber cell elongation during lens development.

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ABBREVIATIONS

Abl	Abelson murine leukemia
Akt	Protein Kinase B
Brdu	5-Bromo-2'-deoxyuridine
Bcr	Breakpoint Cluster Region
C3G	CRK SH3 domain-binding guanine nucleotide-releasing factor
C-Maf	Musculoaponeurotic Fibrosarcoma
CML	Chronic myelogenous leukemia
Crk	Chicken tumor virus No. 10 [CT-10] regulator of kinase
DAPI	4',6-Diamidino-2-Phenylindole, Dihydrochloride
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribonucleic acid
Dock 180	Dedicator of cytokinesis
E	Embyronic day
ELMO	Engulfment and Cell Motility
Erk	Extracellular signal-regulated kinases
FAK	Focal Adhesion Kinase
FGF	Fibroblast Growth Factor
FBS	Fetal Bovine Serum
GDP	Guanosine diphosphate
GTP	Guanosine triphosphate
H	Hour
Ig	Immunoglobulin
kDa	Kilodaltons
LADD	Lacrimo-auriculo-dento-digital
LSL	Lox-Stop-Lox
MEK	Mitogen-activated protein kinase kinase
mm	Milimeter
mM	milli-Molar
mRNA	messenger ribonucleic acid
NaCl	Sodium Chloride
Na_3VO_4	Sodium orthovanadate
NaF	Sodium Flouride
OFT	Outflow tract
PAE	Primary Aortic Endothelial
PBS	Phosphate Buffer Solution
PAK	p21-activated kinases
PI3K	Phosphoinositide 3-kinase
PH	Pleckstrin homology
$p27^{kip1}$	Cyclin-dependent kinase inhibitor 1B
$p57^{Kip2}$	Cyclin-dependent kinase inhibitor 1C
p130Cas	Crk associated substrate
Prox1	Prospero Homeobox 1
Rap1	Ras-related protein 1

Ras	Rat Sarcoma
RIPA	Radio-Immunoprecipitation assay
Shp2	Src homology region 2 domain-containing phosphatase 2
SH2	Src Homology domain 2
SH3	Src Homology domain 3
Shb	Src Homology 2 Domain Containing Adaptor Protein B
Tiam	T-Cell Lymphoma Invasion and Metastasis
Tris-HCl	Tris-Hydrochloride
UO126	Monoethanolate
ug	Microgram
ul	Microliters
80KH	protein kinase C substrate

CHAPTER ONE

Introduction

1.1 Eye development

1.1.1 Overview of murine eye development

The formation of the eye begins during gastrulation, a very early stage of embryonic development. Specifically, eye development commences during the development of the forebrain with the specification of a single eye field (1). Gastrulation is the formation of a three layered structure called the gastrula, consisting of the ectoderm, mesoderm, and endoderm (2). Interestingly, only the ectoderm and mesoderm give rise to the important structures of the eye. The ectoderm is responsible for the formation of the lens, the cornea epithelium, and the eyelid. Also the neuroepithelium, a derivative of the ectoderm, gives rise to the retina, the ciliary body, the iris, and the optic nerve. The mesoderm and the neural crest subsequently form the extraocular mesenchyme (3).

During the formation of the midline structures of the embryo, the single eye field is separated into two distinct sections called the optic vesicles. While the optic vesicle is forming, the surface ectoderm begins to thicken and form the lens placode. During embryonic day 10, the lens placode comes in contact with the optic vesicle and begins to invaginate, forming the lens pit. The optic vesicle also invaginates and becomes the optic cup. At approximately embryonic day 11.5, the lens pit closes up to form the lens vesicle and the overlying surface ectoderm detaches from the lens vesicle and eventually forms the corneal epithelium. Between the stages of embryonic day 12 and 14, the murine lens develops into a well-defined structure that consists of a group of mostly quiescent epithelial cells at the anterior epithelium layer and a small group of proliferating epithelial cells at

the equatorial region that differentiate into lens fiber cells throughout the life of a mouse (4).

The optic cup continues to develop with the inner layer of the optic cup becoming the neural retina and the outer layer becoming the retinal pigment epithelium (RPE). The differentiating cells in the inner layer of the optic cup give rise to multiple cell types such as retinal ganglion cells, amacrine cells, bipolar cells, and photoreceptor cells (5). These cells are essential for the vision of the mouse and any aberration in their development will lead to retinal disorders. The RPE is an extremely important component of the vertebrate eye. It is a monolayer of polarized epithelial cells that serve multiple functions such as recycling the outer segments of the photoreceptors and maintaining water flow and adhesion in the retina (6). During development, the presumptive RPE is surrounded by the extraocular mesenchyme constituted by a group of mesodermal and neural crest cells that is responsible for regulating differentiation and patterning of the RPE (7). In addition, one of the interesting properties of the RPE is its ability to trans-differentiate into the neural retina during embryonic development under appropriate stimuli (6,8,8).

In addition to the neural retina and the RPE, the tip of the optic cup gives rise to the iris and the ciliary body. The iris, named after the Greek goddess of the rainbow, is one of the most colorful organs in the vertebrate body. The iris is positioned between the corneal epithelium and the lens and is important for regulating the amount of light that passes through the eye. The development of the iris begins mid-gestation and is solely dependent on the proper development of the neuroectoderm, the periocular mesenchyme, and

signaling from the developing lens. Made up of multiple cell types, the iris contains three major components, the iris pigment epithelium, the iridial muscle, and the iris stroma (9). Although the ciliary body and iris are derived from the same embryonic origin, the ciliary body is completely different in terms of structure and function. The ciliary body begins as the ciliary epithelium and differentiates into two layers, the outer pigmented layer and the inner non-pigmented layer. During development of the eye, the ciliary epithelium will fold to become the ciliary process and the mesenchyme cells from the neural crest will form the connective tissue and ciliary muscle of the ciliary body. In addition, the non-pigment layer secretes fibrillins to aid the production of the ciliary zonules. Some of the most important roles of the ciliary body is to maintain intraocular pressure, secrete glycoproteins and aqueous humor into the vitreous body, and maintain lens shape and structure (10) (11).

After light travels through the components of the eye that were previously discussed, its information is sent to the brain as an electrical signal by one of the most important components of the eye, the optic nerve. The development of the optic nerve begins at embryonic day 11.5 in the mouse and at this stage it is called the optic stalk. During eye development, the axons from the ganglion cells travel down toward the optic stalk and begin to grow into it to form the optic nerve (5). Once the optic nerve is formed it relays visual information to the brain, where it is further processed and perceived.

1.1.2 Overview of murine lens development

Lens development is considered one of the most essential processes in eye development. The lens also serves as a great model for molecular and genetics studies and has been used

by prominent scientist over the years to explain many developmental processes. One of the earliest known studies using the lens was done by Hans Spemann in 1901 in which he established the principle of induction. He destroyed the optic vesicle of the frog and found that the lens did not form, indicating that the optic vesicle is necessary for lens formation. This study paved the way for studies that use the lens as a model to understand development.

The development of the mouse lens begins at embryonic day 9 when the head ectoderm comes in close contact with an extension of the forebrain, called the optic vesicle (Figure 1A). This is followed by the thickening of this region of the head ectoderm to form the lens placode (Figure 1B). It is now known that development of the lens placode is controlled by a plethora of transcription factors such as Pax6, Six3 and Sox2. In addition, the induction and inhibition of signaling pathways such as the Bone Morphogenetic Protein (BMP) signaling pathway and the Wnt signaling pathway, respectively, play a role in the formation of the lens placode (12) (13).

Another important step in lens morphogenesis is the invagination of the lens placode to form the lens pit (Figure 1C). The invagination of the lens placode has been extensively studied and is regulated by shroom3-mediated apical constriction and a balance between Rho GTPases Rac1 and RhoA that leads to cell shape changes and proper invagination (14,15). The lens pit will continue to deepen and the connection between the overlying head ectoderm will narrow to form the lens stalk (Figure 1D). The lens vesicle will eventually detach from the overlying surface ectoderm and the anterior cells of the lens

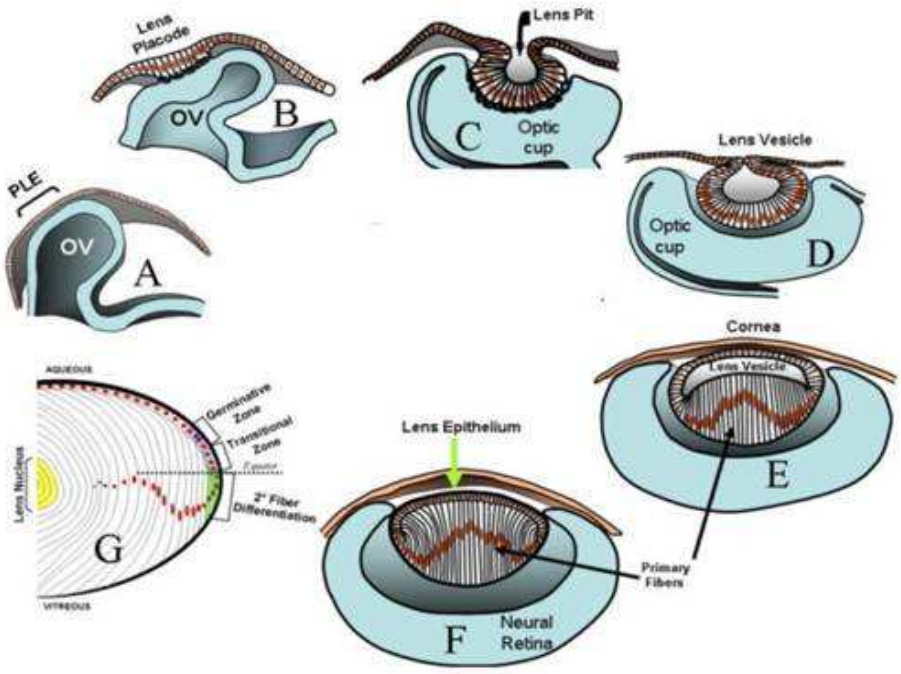

Figure 1. Lens Development. At mouse embryonic day 9.5 (E9.5), the optic vesicle (OV) extends toward the presumptive lens ectoderm (PLE) (Fig. 1A), inducing the latter to thicken into a cuboidal layer called a lens placode (Fig. 1B). At E10.5, the lens placode invaginates to form a lens pit (Fig. 1C), which eventually closes at the anterior surface to form a lens vesicle (Fig. 1D). Subsequently, the anterior epithelial cells migrate posteriorly to the equatorial regions of the lens, where they move inside the lens to elongate into lens fiber cells (Fig. 1E and F). In the mature lens, the lens fiber cells are organized into an exquisitely concave pattern to maintain structural integrity and transparency (Fig. 1G) **(Adapted from Robinson ML)(4).**

vesicle will become the lens epithelium. The posterior cells of the lens vesicle will differentiate into the primary lens fiber cells and fill the lumen of the lens vesicle (Figure 1F)(4). The primary lens fiber cells will remain in the lens throughout life and are some of the oldest cells in the body. As the development of the lens continues, a group of proliferating epithelial cells will migrate to the equator of the lens and differentiate into the secondary lens fiber cells (Figure 1E). The secondary lens fiber cells will elongate along the anterior and posterior axis of the lens. It is important to note that the lens fiber cells make up 99 percent of the lens and upon terminal differentiation lens fiber cells undergo cytoplasmic organelle degradation. Also, the synthesis and packing of crystallin protein is one of the most important processes of lens development because it creates the transparency and refractive medium needed for light to pass through to the retina (16).

1.2 Fibroblast Growth Factor (FGF) signaling pathway

1.2.1 The Fibroblast Growth Factors

Fibroblast Growth Factors (FGFs) are a group of glycoproteins that control multiple developmental processes, during vertebrate and invertebrate development, by binding to FGF receptors. FGFs consists of 120-130 amino acids that are organized into 12 antiparallel β-strands, flanked by a N and C terminal (17). Interestingly, FGF was first identified in a bovine pituitary extract, as a protein with fibroblast growth promoting activity (18). It was eventually purified in 1983 and was then named basic FGF. Since its discovery, 22 members of the FGF family have been identified and can be divided into seven subfamilies (19). FGFs can also be characterized based on their signaling mechanism, specifically paracrine, intracrine, and endocrine signaling (Figure 2).

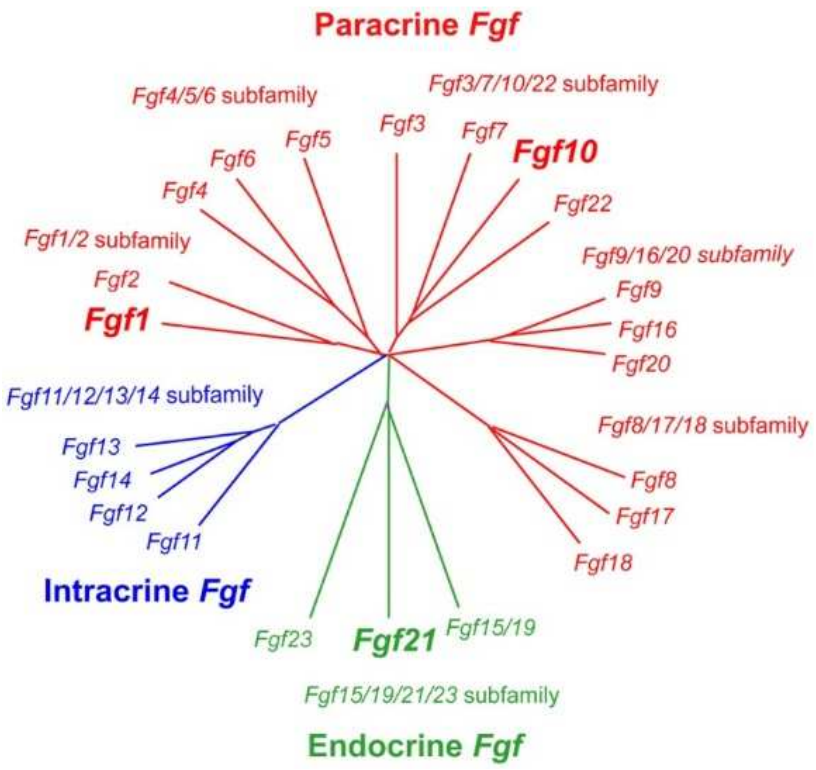

Figure 2. Evolutionary relationships within the *Fgf* gene family by phylogenetic analysis. Phylogenetic analysis shows that 22 *Fgf* genes can be arranged into seven subfamilies. **(Adapted from H.Otha and N.Itoh Human Disorders and the FGF Signaling Pathway)**

Paracrine FGFs bind to FGF receptors and their cofactor heparan sulfate to elicit biological responses. Intracrine FGFs differ from paracrine FGFs in that they are not secreted but are important for generating action potentials in neurons.

Lastly, Endocrine FGFs are also unique because they are hormone-like, do not require heparan sulfates, and utilize a cofactor which belongs to the Klotho family of molecules (20). Secreted FGFs (intracrine and paracrine) regulate cellular processes such as proliferation, differentiation, migration. Importantly, these FGFs are essential to early developmental processes such as differentiation of the inner cell mass, as well as late developmental stages such as organogenesis. Endocrine FGFs specifically plays a role in regulating various processes in adult organisms such as lipid and carbohydrate metabolism (21).

1.2.2 FGF Receptors

As previously mentioned, FGFs carry out their functions by binding to FGF receptors. FGF receptors are a group of tyrosine kinase receptors that are essential for the activation of downstream signaling pathways involved in cell proliferation, differentiation, and cell growth (22). There are four known FGF receptors (FGFR1-FGFR4); each contains an immunoglobulin domain, a transmembrane domain, and a cytoplasmic tyrosine kinase domain. Specifically, the immunoglobulin domain consists of approximately 2 to 3 Ig loops that occur through alternate splicing and is essential for ligand binding and specificity. The first Ig loop is thought to be involved in FGF receptor auto-inhibition and the second and

third loops play an important role in FGF binding and the array of ligand binding affinities based of the subtypes and isoforms of FGF receptors (23). In addition, there is a stretch of amino acids between each Ig I and II loop and a heparin binding domain that is essential for increasing the half-life of the FGF and FGF receptor complex (24) . Lastly, the crystal structure of the cytoplasmic tyrosine kinase domain of the FGF receptor reveals an activation-loop that contains two tyrosine residues that remains in the inactive or low activity do to a kinase invariant proline rich residue at the C-terminal end of the activation loop that interferes with substrate binding. Upon activation of the receptor and auto-phosphorylation of the tyrosine residues signaling proteins are directly recruited to or linked to the receptor via docking proteins to induce downstream signaling (19).

1.2.3 FGF Signaling

FGF signaling begins by the release of FGF from the extracellular matrix and its binding to heparan sulfate proteogylcans (HSPGs), which stabilize the FGF-FGF receptor binding. This leads to ligand-dependent dimerization of FGF receptors, which causes a conformational change and activation of the tyrosine kinase domain. Subsequent trans-phosphorylation of tyrosine residues on the FGF receptor creates docking sites for multiple adaptor protein such as FGFR substrate 2 (FRS2) and PLCγ1. Extensively studied for its role in FGF signaling, FRS2 is known as the main mediator of FGF signaling. FRS2 can be phosphorylated by the FGF receptor on multiple sites, which can then interact with multiple proteins, leading to important downstream signaling (25).

One of the most well established signaling pathways mediated by FRS2 is the MAPK signaling pathway. In MAPK signaling, phosphorylated FRS2 recruits son of sevenless (SOS) and Growth-factor receptor bound protein 2 (GRB2). SOS is a guanine nucleotide exchange factor (GEF) that activates the Ras GTPase. Ras will then stimulate the Raf-to-MEK-to-ERK pathway. The phosphorylation of ERK leads to activation of transcription factors that induce multiple cellular processes such as proliferation and differentiation (26). Although the MAPK signaling pathway is one of the main pathways activated by FGF, other pathways such as the PI3K-AKT pathway, Phospholipase C pathway, and the STAT3 pathway can also be activated. In addition, the FGF signaling pathway can be regulated by multiple negative regulators such as SEF, FGFR like 1, and Sprouty (25).

1.3 Human Disorders and the FGF Signaling Pathway

The FGF signaling pathway is one of the crucial signaling mechanisms during embryonic development (27). Many developmental diseases are associated with the abnormal function of the FGF signaling pathways. In particular, FGF signaling plays a major role in skeletal diseases such as dwarfing chondrodysplasias and Craniosynostosis. Dwarfing chondrodysplasia is a skeletal disorder that causes abnormal formation of bone. There are multiple types of chondrodysplasia such as achondrodysplasia, hypochondrodysplasia, and thanatophoric dysplasia that are caused by mutations in the tyrosine kinase domain of FGF receptors. Specifically, most of these mutations are caused by mutations in FGF receptor 3, including the G380R mutation underlying achondroplasia and dwarfism. FGF18 and FGF2 are also possible candidates for some of the causes of chondrodysplasias (28).

Craniosynostosis is also a disorder of skeletal development and was discovered in 1830 as premature fusion of the cranial sutures. It can further be divided into the fusion of one suture or the fusion of multiple sutures and characterized as primary or secondary craniosynostosis. Primary being that the disorder was caused by a biological event and secondary meaning there was some type of external force that caused premature fusion. Craniosynostosis occurs in 1 of 2500 live births. Although most craniosynostosis cases are sporadic, there are some syndromic cases, which are due to genetics or specifically autosomal dominant inheritance caused by a gain of function mutation in the FGF receptor. Most of the time, the gain of function mutation is in the region of the FGF receptor that regulates the binding affinity of the FGF ligand to the FGF receptor. This gain of function mutation can increase the receptors binding affinity leading to enhanced sensitivity to FGF signal, perturbing developmental processes like cell differentiation (29).

There are multiple syndromes associated with craniosynostosis such as Pfeiffer syndrome and Crouzon syndrome. Pfeiffer Syndrome is one of the most common syndromes that are associated with craniosynostosis, partial syndactyl and midface hypoplasia. Pfeiffer syndrome is caused by mutations in both FGF receptors 1 and 2. The mutations in FGF receptor 1 are in the linker region between the second and third domain. In contrast, FGF receptor 2 mutations are in the Ig III and tyrosine kinase domains (30). Crouzon syndrome unlike Pfeiffer syndrome mainly has mutations on FGF receptor 2. The chromosome mapping of Crouzon syndrome revealed that it is located in chromosome 10q25-q26. In a search for possible genes that can cause Crouzon syndrome, FGF receptor 2 was a possible candidate because it was also mapped to chromosome 10q25-q26 (31). This mutation in

FGF receptor 2 causes a ligand-independent activation and disrupts ligand binding (32). Unlike patients with Pfeiffer syndrome, patients with Crouzon syndrome have normal intelligence and normal extremities but exhibit abnormal protrusions of the eye ball and jaw (29).

In addition to extensive studies done on FGF signaling and its role in development and developmental disease, there is a plethora of studies addressing FGF signaling in cancer. The pathogenic outcome of FGF signaling can be caused by distinct alterations in either the FGF ligand or the FGF receptor. One of the most common alterations in FGF signaling is in the FGF receptor, leading to ligand-independent signaling. It is characterized by activation mutations, chromosomal translocation, and receptor gene amplification. Bladder cancer has been most frequently linked to FGF receptor mutations. Specifically, approximately 50 percent of bladders cancers are caused by mutations in FGF receptor 3 and most mutations are seen in the transmembrane domain. Hematological malignancies are another example of how FGF signaling is linked to cancer. They can be caused by chromosomal translocation of the FGF receptor, which creates a fusion protein that contains an N terminus of a transcription factor fused with a FGF receptor kinase domain. This leads to constant FGF receptor dimerization and a constitutively active FGF receptor. In addition to chromosomal translocation, gene amplification has been shown to play a significant role in cancer. Gene amplification in FGF receptor 1 and 2 has been implicated in certain cancers like gastric cancer and breast cancer. In breast cancer, amplification of the FGF receptor 1 chromosomal region 8p11-12 is a very common focal amplification, especially in Estrogen Receptor positive breast cancers (25).

Although mutations in the FGF receptor are the most common mutations seen in cancers, there are some mutations found in the FGF ligand that can also lead to cancer. Somatic mutations in FGF9 has been known to cause colorectal and endometrial cancer (33). In addition, overproduction of autocrine FGF has been seen in many types of tumors. The upregulation of multiple FGFs such as FGF 2, FGF 8, and FGF 18 is known to be involved in cell survival and neovascularization. Similar to autocrine FGFs, paracrine FGFs has also been reported in many cancers. Other examples included the overexpression of serum FGF2 which is associated with small-cell lung cancer and poor prognosis (34).

1.4 FGF Signaling and Eye Development

1.4.1 FGF Signaling and Lens Development

As previously discussed, FGF signaling plays an extensive role in embryonic development, particularly in lens development. It has been reported that in the presence of a FGF receptor inhibitor, there was a reduction of Pax6, a prominent marker of lens induction. Similarly, after expression of a truncated FGF receptor, lens development was perturbed as early as the lens induction stage (35). In addition examination of embryos, containing lens Cre conditional knockout of FGF receptor 1 and 2 ,displayed a thin lens placode and an increase in the TUNEL labeling index at embryonic day 9 compared to wild type litter mates (36). Taken together these data reveal how essential FGF signaling is for lens induction.

It has also been established that lens epithelial cell proliferation is regulated by growth factors, such as FGFs, in the aqueous humour of the eye. *In vitro* and *in vivo* studies have

also revealed that there is a gradient of FGF present in the lens that establish polarity and induces different cellular response, such as cell proliferation. This gradient was first observed by McAvoy and Chamberlin in 1989 using rat lens explants. They found that as you increase the concentration of FGF, cell proliferation occurs first but as you continue to increase the concentration cell elongation and differentiation follows (37). Using a thymidine incorporation assay and a cell labeling method on lens epithelial cells, it was shown that as the concentration of basic FGF increased the first cellular response was cell proliferation (38). More recent studies have found that in the presence of a low dose of FGF, lens explants displayed increased Erk activity compared to wild type and an increase in the amount of Brdu incorporated cells compared to explants treated with the MEK inhibitor UO126. The discovery that there is a gradient of FGF present in the lens has been a landmark in understanding the role of FGF signaling (Figure 3) (39).

From this discovery, the role of FGF signaling in lens cell differentiation was further characterized by using a dominant negative form of FGF receptor 1. Transgenic mice with the dominant negative receptor 1 exhibited a reduction in the cell differentiation process and an increase in cellular apoptosis (40). It was also found that the lens epithelial cells in FGF receptor 2 knock out mice fail to exit the cell cycle indicated by a decrease in cell cycle inhibitors like $p27^{kip1}$ (41). In addition, FGF receptor 1, 2, and 3 can compensate for each other during lens fiber cell differentiation, indicated by the more severe phenotypes when all three receptors are deleted (42).

Studies displaying ectopic and overexpression of FGFs also revealed important roles of FGF signaling during lens development. Transgenic expression of a human FGF-1, using a Aα-crystallin promoter for expression in the ocular lens, induced lens fiber cell elongation and expressed β-crystallin (43).To further understand the role of FGFs in lens development, transgenic expression of a secreted from of FGF3 in the ocular lens using the same Aα-crystallin promoter caused differentiation of the entire lens epithelium and subsequently lead to degeneration of the lens (44). These studies further confirmed one of the crucial roles of FGF signaling in lens development is lens fiber cell elongation and differentiation.

Lastly, recent studies have delved into the co-factors and downstream proteins that are essential for FGF-mediated lens fiber cell differentiation, which include heparan sulfates, the adaptor protein Frs2, and the phosphatase Shp2. Disruption of heparan sulfates prevents lens fiber cell differentiation shown by a decrease in differentiation markers and an increase in the lens epithelial cell marker E-cadherin (46). Similarly, conditional knockout of Frs2 in the mouse lens leads to decrease in lens differentiation markers, Prox1 and β- crystallin (47). The genetic interaction of Frs2 and Shp2 has been shown to be essential for FGF-mediated lens fiber cell differentiation in that it completely abolishes lens development by as early as embryonic day 12.5 (48).

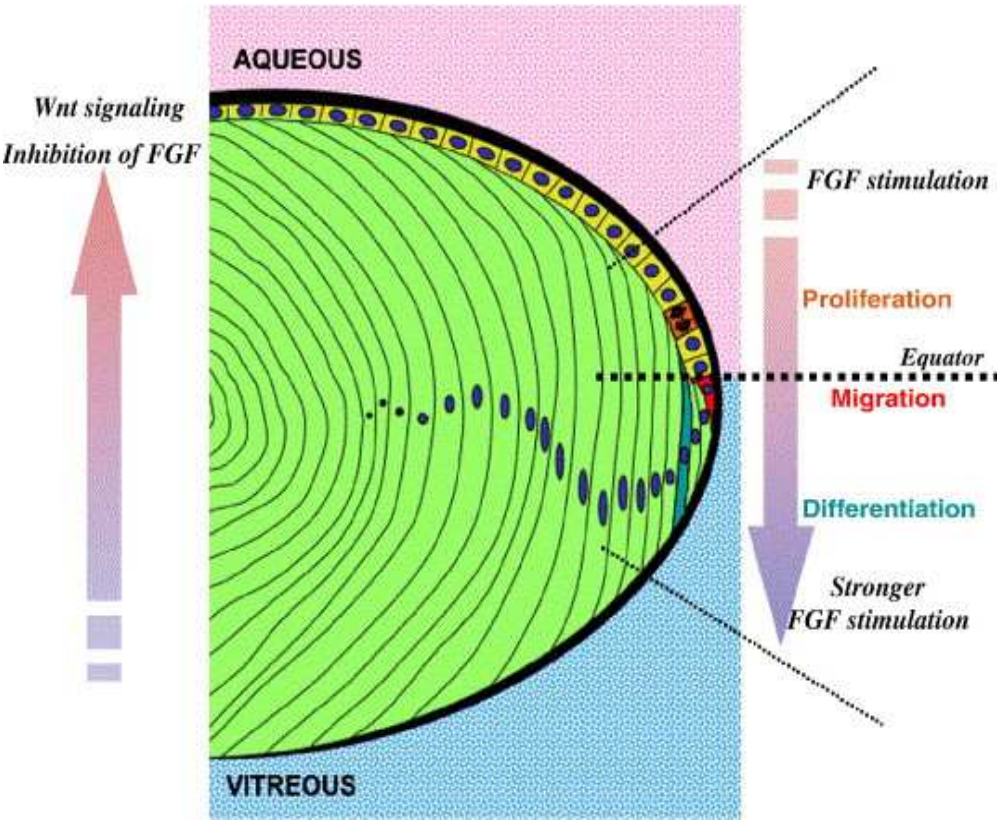

Figure 3. Gradient of FGF stimulation in lens cell behavior. The gradient of FGF establishes the cellular response needed for the development of the lens. As FGF signaling increases along the anterior to posterior axis of the lens, a transition from cell proliferation to differentiation is induced. (**Adapted from F.J. Lovicu and J.W. McAvoy** (45)**)** .

1.4.2 The FGF Signaling Pathways and Diseases of the Eye

FGF signaling has been implicated in some eye disorders such as ocular coloboma. Ocular coloboma is caused the failure of the optic vesicle fissure to close properly and can affect multiple components of the eye including the lens, retina, and the optic nerve. Ocular coloboma is known to be one of the most common eye disorder in children that causes visual impairment and blindness (49). Recent studies have found that conditional deletion of FGF receptors 1 and 2 in the optic vesicle of the mouse leads to ocular coloboma and optic nerve dysgenesis, indicating the importance of FGF signaling in eye development and disease. Another disease caused by mutations in FGF or its FGF receptor is the Lacrimo-auriculo-dento-digital (LADD) syndrome with lacrimal duct aplasia being is one of the main features. Mutations in the tyrosine kinase domain of the genes encoding FGF receptor 2 or 3 and in the ligand FGF 10 has been implicated in this disease (50). Lastly, loss of the FGF receptor and downstream effectors in the FGF signaling pathway during mouse eye development lead to microphthalmia and anophthalmia, indicating that loss of FGF signaling is crucial during mammalian eye development (51).

1.5 FGF Adaptor Proteins Crk and CrkL

1.5.1 Overview of FGF Adaptor Proteins Crk and CrkL

In the late 1980s, Bruce Mayer discovered a novel oncogene derived from a chicken tumor sample. Interestingly, Mayer and his colleagues also found that this novel oncogene did not contain a catalytic domain but was a fusion gene that consisted of a retroviral gene encoding the protein gag and a cellular gene that encoded for SH2 and SH3 domains. Characterized with no catalytic activity, this viral oncogene was surprisingly able to

dramatically increase tyrosine phosphorylation of proteins in transformed chicken embryo fibroblast cells. This unique feature led to the idea that this novel viral oncogene could mediate tyrosine kinase activity hence its name, *Crk* (Chicken tumor virus No. 10 [CT-10] regulator of kinase) (52) .

Since the discovery of viral (v)-Crk, due to alternative splicing at a single gene locus, two species CrkI and CrkII have been identified in mammalian cells (Figure 4). Although these two species are homologs of v-Crk, they differ in their oncogenic activity. CrkI has significantly more transforming activity than CrkII and is similar to the oncogenic tyrosine kinases such as Src. The decrease in transforming activity in CrkII is attributed to the phosphorylation of tyrosine residue at 221, a feature that is absent in CrkI (Figure 4). In particular, the phosphorylation of tyrosine 221 leads to its intramolecular binding to the SH2 domain, which sequesters the SH2 and SH3 domains of CrkII, preventing binding of target proteins (53).

Another gene was also cloned by *Hoeve et al.,* with the goal of identifying genes located near the *BCR* gene on chromosome 22. The exons at this cloned location encoded for a SH2 domain, which gives some insight on the role of this gene. Eventually, the complete cDNA sequencing was done and it revealed a novel Crk like (*CrkL)* gene. The CrkL protein has 60% homology to CrkII and is quite similar in biological function and post-translational modifications. For example, CrkL phosphorylation at tyrosine 207 also causes intra-molecular binding and sequesteration of the SH2 and SH3 domain (Figure 4) (53,54).

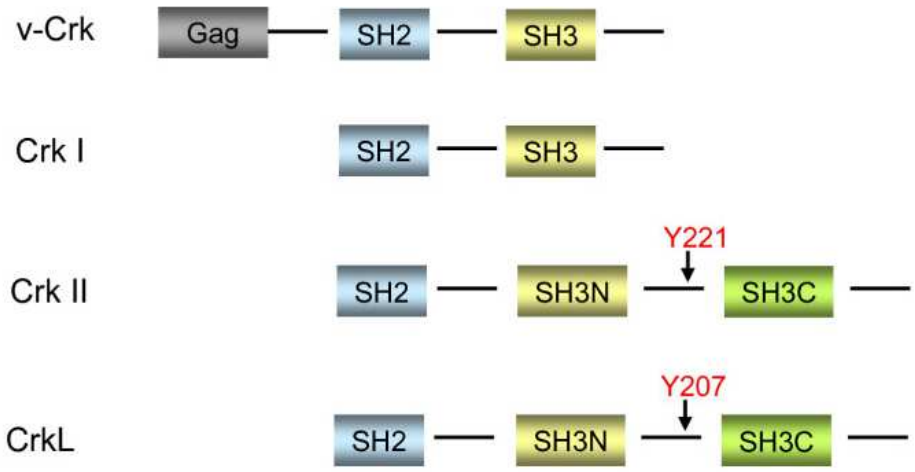

Figure 4. Structure of the Crk family of proteins. All Crk family adaptor proteins are comprised of SH2 and SH3 domains. The domains are boxed: SH2, Src homology 2; SH3, Src homology 3; Gag, viral group specific antigen; Y221 or Y207, negative regulatory phosphorylation site. **Adapted from *Birge et al* (46)**.

All the members of the Crk family carry out their function through their SH2 and SH3 domain. Specifically, to transduce intracellular signaling, Crk acts as a molecular switch by binding to phosphotyrosine residues via its SH2 domain and interacting with proline rich regions of other proteins via its SH3 domain. The first protein that was identified as interacting with the SH2 domain of Crk was paxillin. Paxillin is a substrate for multiple tyrosine kinases such as Src. It can bind to the focal adhesion kinase (FAK) and form complexes with Crk, leading to downstream signaling.

Shortly after the discovery of paxillin, a 130 KDa protein was cloned as a Crk associated tyrosine kinase substrate and thus named p130Cas. It has been well established that p130cas is a part of an important group of multi-site docking and scaffolding proteins. After phosphorylation of p130Cas, Crk and CrkL specifically bind to p130Cas via its SH2 domain. As its name implies, p130cas then becomes a substrate for tyrosine kinases and can be phosphorylated by tyrosine kinases such as Src. Specifically, phosphorylation of p130cas by Src leads to the assembly of a p130cas-Crk-Dock180 protein complex important for lamellipodia formation and cell migration. The phosphorylation of p130cas can also be induced by mechanical stretch, leading to the binding of Crk and activation of the GTPase Rap1 (55,56).

In addition to the SH2 domain, the family of Crk adaptor proteins will employ their SH3 domain to activate effector proteins such as guanine nucleotide exchange factors (GEFs) and kinases to induce downstream signaling. One of the first proteins known to bind to the SH3 domain of Crk is the GEF C3G. The activation of C3G occurs through its recruitment

to the membrane by Crk and phosphorylation on tyrosine 504, leading to repression of a cis-acting negative regulatory domain outside of the catalytic domain (57). Once activated, C3G is responsible for catalyzing the dissociation of GDP from the GTPase Rap1, allowing a GTP molecule to enter and bind in its place. The binding of a GTP to Rap1 leads to its activation, which promotes cell adhesion and cell spreading (58).

Another GEF that binds to the SH3 domain of Crk and induce downstream signaling is Dock180. The family of Dock180 proteins is unique in that, unlike other GEFs, it does not have a catalytic domain but contains a conserved domain named the "Docker" domain. This domain binds to Rho GTPases and is necessary for the nucleotide exchange of Rho GTPases such as Rac. Also the binding of Dock180 to Rac requires association of the protein ELMO to Dock180 SH3 domain. How ELMO aids Dock180 in Rac activation hasn't been fully established, but it can be summarized into three mechanisms. It is first proposed that ELMO helps Dock180 stabilize Rac through its PH domain. It was also established that ELMO prevents the auto-inhibition of Dock180 caused by the binding of the SH3 domain to the "Docker" domain. Lastly, studies have found that the Armadillo repeats in ELMO can guide the Dock180-ELMO complex to the plasma membrane through interaction with active RhoG. Regardless of how the Dock180-ELMO complex comes in contact with Rac1, it will eventually cause activation of Rac1 by the replacement of a GDP by a GTP molecule. After activation, Rac1 will interact with important downstream effectors that are essential for cell proliferation, cell differentiation, and actin cytoskeleton reorganization (59).

As previously mentioned, the SH3 domain of Crk can interact with tyrosine kinases. One of the well-known kinases that bind to the SH3 domain of Crk is Abl (Abelson murine leukemia) kinase. *ABL* was discovered as an oncogene that forms a fusion gene with the Breakpoint Cluster Region (*BCR)* gene. This fusion gene is responsible for the well-characterized human chronic myelogenous leukemia (CML). Although the function of Abl has been primarily studied for its oncogenic activity, it does play a role in multiple cellular events such as actin remodeling and cell adhesion. The binding of Abl to the SH3 domain of Crk and CrkL leads to their phosphorylation which inhibits binding to p130cas and prevents cell migration (60,61). Studies have shown that the lack of phosphorylation of CrkI by Abl is responsible for its transformation activity due to the absence of the Y221 regulatory site found in CrkII. However, recent studies have found that this is only true for CML, but in other cancer related diseases, inhibition of Abl can increase the transformation activity of CrkI. Therefore, the role of Abl in Crk is extremely important but is controversial in its ability to promote or inhibit Crk transformation activity (62).

With its SH2 and SH3 domains, Crk is widely known for its contribution to the integrin signaling pathway. Integrins are a family of transmembrane receptors that mediate cell adhesion, tissue maintenance and repair, and host defense. Integrin is also responsible for relaying signals to control multiple cellular processes such as cell proliferation, survival, and cell migration. This type of signaling is called outside-in signaling. Integrin can also alter its affinity for ligands by undergoing a conformational change in response to signaling from inside of the cell. This is considered inside-out signaling. Both outside-in and inside-out signaling mechanisms are extremely important in integrins' ability to regulate cellular

activity. With respect to integrin inside-out signaling, activation of integrin mainly occurs when talin binds to the integrin tail. This leads to a conformational change which will increase integrin's affinity for its ligand. The binding of the ligand to integrin forms an initial connection between the cytoskeleton and the extracellular matrix in nascent adhesions. On the other hand, mature adhesions consist of complex assembly of multiple proteins at the cytoplasmic side of integrin that strengthen the connection of integrin to the actin cytoskeleton. These multi-protein complexes also transmit signals to the inside of the cells to activate kinases such as the Focal adhesion kinases (FAK). FAK can be phosphorylated by the Src-family protein kinases to recruit paxillin or p130cas. As discussed earlier, paxillin or p130cas have motifs that attract binding of the SH2 domain of Crk, which leads to downstream signaling.

1.5.2 The Role of Crk and CrkL in Embryonic Development

Both Crk and CrkL play multiple roles in embryonic development, which have been revealed with the use of mouse genetics. Global knock-out of CrkL in the mouse cause defects in cranial and cardiac neural crest derivatives such as the aortic arch arteries, the cardiac outflow tract, and the thymus. These mice generally do not survive embryogenesis and Crk cannot compensate for this severe phenotype (63). Conditional deletion of both CrkI and CrkII using the Cre–Loxp system revealed a variety of phenotypes. One of the most obvious phenotypes in the Crk-null embryo at E13.5 was focal and hemorrhagic edema on the snout and nasion. The heart of crk null embryos showed multiple abnormalties, displaying a thin muscular wall in the heart, and a decrease in the tightly packed cells causing the heart to appear extremely dilated. Due to the obvious blood loss,

these embryos had ruptured blood vessels and defects in vascular smooth muscle cells. The defects in smooth muscles cell, indicated by the loss of smooth muscle actin, can inhibit their ability to support blood vessels. Crk null embryos also had defects in nasal development and appeared to have a cleft palate. These observations underscore the importance and distinct roles of both Crk and CrkL in embryonic development (64).

Crk and CrkL support embryonic development mainly through their ability to maintain cell shape and motility. To fully understand this, studies have been done using Cre-loxp systems to conditionally delete Crk and CrkL in fibroblast cells to observe their function on a cellular level. Crk and CrkL deletion in fibroblast cells reduced cell motility and caused loss of focal adhesions. Further observations also showed that Crk and CrkL are responsible for the integrity of the cytoskeleton. These finding indicate the importance of Crk and CrkL in embryonic development through their ability to mediate important biological processes (65).

1.5.3 Crk and CrkL in FGF Signaling

There are only a few proteins that can directly engage with active FGF receptors such as Grb14, Shb, PLCγ, Frs2, Shp2, and Crk (Figure 5.) (66,67). Multiple studies have been done to investigate the interaction between the Crk family proteins and the FGF receptors. For example, using Primary Aortic Endothelial (PAE) cells, *Larsson et. al.,* showed that cells transfected with a chimeric Y463F receptor did not pull down Crk during immunoprecipation as untransfected cells do and phosphorylation of mutant FGF receptor was lost as indicated using phospho-tyrosine antibodies. The chimeric Y463F receptor also

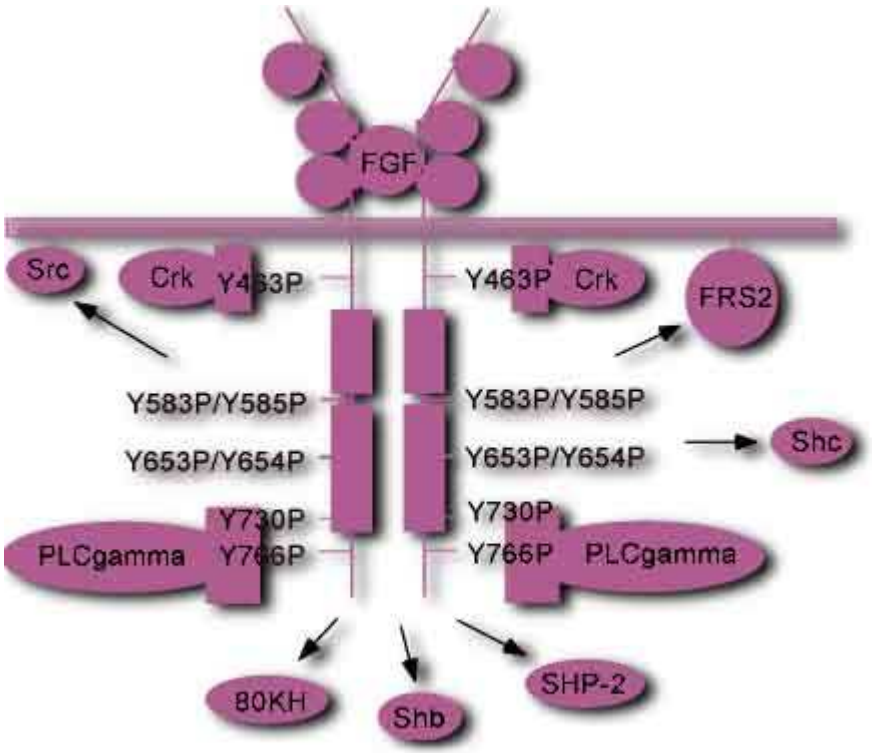

Figure 5. Schematic outline of FGFR-1 signaling transduction molecules. The dimerization and trans-phsophorylation FGFR-1 can associate with moleules through multiple tyrosine phosphorylation sites. The activation of Crk and PLC-gamma and downstream signaling transduction depends on Tyr463 and Tyr766, respectfully. The activated FGF can also interact with membrane bound molecules such as FRS2 and the Src family tyrosine kinases. Other molecules that are not anchored to the membrane include Shc, SHP-2, Shb, and 80 KH. **Adapted from Peter Klint and Lena Claesson-Welsh (60)**.

caused a decrease in downstream effectors of the FGF signaling like Erk2 and JUN kinase. Furthermore, to show that Crk phosphorylation is solely controlled by FGF receptor 1, PAE cells expressing FGF receptor 1 were treated with FGF2 for 7 minutes, which resulted in a remarkable increase in tyrosine phosphorylation of Crk as compared to untreated. Taken together, it was concluded that the interaction between Crk and the phosphotyrosine 463 on FGF receptor 1 is responsible for endothelial cell proliferation (68).

The SH3 domain of CrkL can also interact with FGF receptors to induce downstream signaling. This was shown in a study in which deletion of both FGF8 and CrkL in mice led to severe defects in Cardiovascular, Pharyngeal, and Skeletal development compared to deletion of either FGF8 or CrkL alone. Loss of both FGF8 and CrkL also caused a decrease in cell survival in neural crest cells needed for development of the OFT septum and pharyngeal glands. In-vitro studies using mouse embryonic fibroblast cells showed an increase in FGF receptor 1 and 2 phosphorylation when stimulated with FGF8 and direct binding of both receptors to CrkL (69). Later studies showed direct binding of CrkL to tyrosine 463 of FGF receptor 1 by Molecular dynamic simulation and saturation binding experiment. Importantly, FGF8 induces a CrkL-dependent pathway that activates the GTPases Rac and Cdc42, which acts as a feed-forward loop leading to activation of MAP kinase (Figure 6) (70). There has also been a recently discovered role of CrkL in interacting with Sprouty2 to mediate FGF signaling. Sprouty2 binds to CrkL in response to FGF stimulation and tyrosine phosphorylation. The SH2 domain and the N terminal SH3 domain of CrkL are required for its binding to Sprouty2 and due to the negative regulatory nature of Sprouty2 it was thought to suppress CrkL-dependent Rap1 activity (71).

1.5.4 Human Disorders Associated with Adaptor Proteins Crk and CrkL

Consistent with the role of Abl kinase in the transformation activity of Crk, the family of Crk proteins plays a role in multiple human disorders. Apart from its role in hematopoietic cancers, Crk is also implicated in breast and lung cancer. Crk proteins are involved in multiple cell signaling pathways such as EGF and Integrin pathways that are known to be involved in breast cancer progression. Microarray analysis of breast cancer tumor samples revealed increased levels of CrkI/II and CrkL in high-grade tumors. In addition, increased cell proliferation correlated well with increased levels of Crk in high-grade tumors and triple negative breast cancers. Conversely, knockdown of Crk I/II and CrkL decreased cell proliferation and tumor growth in metastatic breast cancer. It has recently been concluded that the role of Crk in breast cancer is to enhance the response to oncogenic signal in the mammary gland. The cellular mechanisms behind this have not been full elucidated. However, it is known that Crk is required for elevated phosphorylation of p130cas in basal breast cancer cell line and in breast cancer tumor tissues (72).

Similar to breast cancer, elevated levels of CrkI and CrkII correlates with lung adenocarcinomas. In particularly, increased levels of Crk are associated with stage III lung cancer and more invasive tumors. Also, phosphorylation of tyrosine 221 in Crk is highly elevated in lung tumors versus normal lung tissue with the phosphorylation of Crk II being more elevated in poorly differentiated tumors. Using a cDNA microarray-based genomic profiling analysis of lung cancer cell lines and tumors, the CrkL loci was identified to be the most frequently amplified in cancer patients. Consistent with this finding, knockdown of CrkL in lung cancer decreased cell cycle progression, survival, and motility, all

hallmarks of lung tumors. Future studies are needed to understand the specificity of Crk and CrkL in lung tumors and how crosstalk between these two proteins may cause a more severe phenotype (73).

Although the families of Crk proteins are prominent in multiple cancers, it also plays a role in the manifestation of other disease such as DiGeorge Syndrome. DiGeorge syndrome is a developmental disease caused by deletion on chromosome 22 (del22q11). This particular locus is the location of the *Crkl* gene and 90 % of patients with Digeorge syndrome present with this locus deleted. Patients with DiGeorge syndrome exhibit congenital heart disease, delayed speech, immune disorders, etc. Partial DiGeorge syndrome can also present in many patients with multiple clinical abnormalities and a slight defect in T lymphocytes. It was found that expression of CrkL, phosphorylated CrkL, and cell proliferation were decreased in the T cells of patients with partial Digeorge syndrome. This revealed a mechanism for how CrkL may contribute to the phenotype of patients with partial DiGeorge syndrome (74).

1.6 Elongation

1.6.1 Cell Shape and Function

The shape of a cell is essential to its function in both eukaryotic and prokaryotic cell types. In prokaryotes, cell shape is actually important for the description and classification of bacterial species. Examples of this would be the bacteria spirochete which has a spiral shape. For years, the mechanism behind how cell shape changes occuring in bacteria has not been well understood. However, some recent studies are starting to open doors into the mechanism behind cell shape. It was always clear that the bacterial cell walls and the

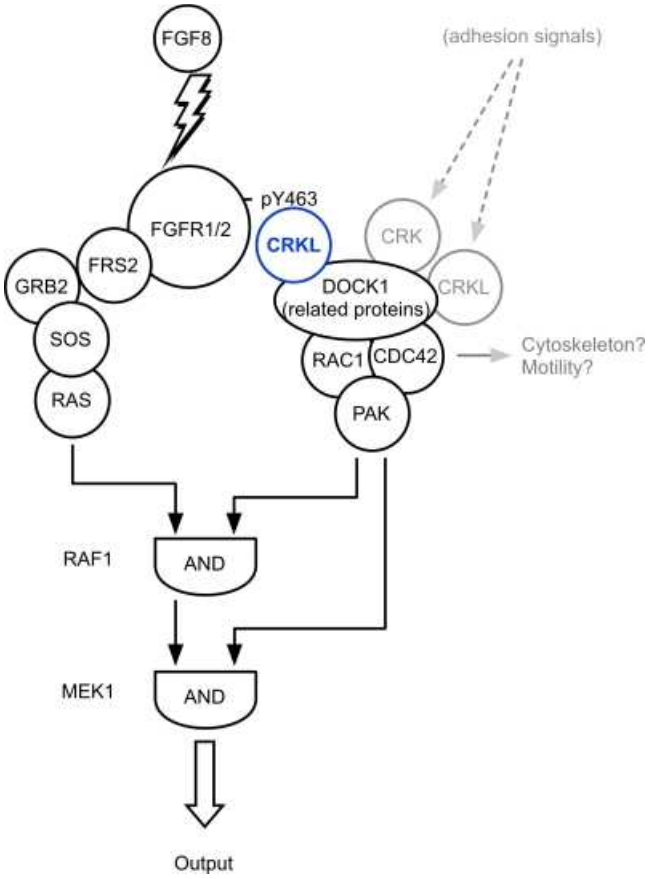

Figure 6. Model of the network that activates the MAP kinase by FGF8. This figure illustrates the role of Crkl in a FGF8 mediated feed forward loop that requires its interaction with FGF receptors 1/2 leading to MAPK activation through Rac1, cdc42, and PAK. Cell adhesion is responsible for creating conditions for cells to respond to growth factors. In the presences of FGF8 Crkl can bypass cell adhesion and create similar conditions for cells to respond to growth factors. **Adapted from Imamoto et al. (70).**

peptidoglycans that constitute the cell wall are important for maintaining the cell shape. More recent genetic studies revealed multiple genes that are important for the rod-shape of E. coli or Bacillus Subtilis such as mre genes and rodA. Consistent with the fact that peptidoglycan is necessary for cell shape, a lot of the genes discovered are involved in peptidoglycan synthesis (75). Lastly, an intermediate filament-like protein called Crestcentin formed a filamentous sheath along the curvature of the cell. This was the first real evidence that bacteria have a form of a cytoskeleton (76).

Unlike prokaryotes, it is well established that eukaryotes have a cytoskeleton that contains actin microfilaments, intermediate filaments, and microtubules that are necessary for various cell shapes. It was also established that focal adhesion assembly is another essential component for cell shape. For mouse embryonic carcinoma cells that lack vinculin, a cytoskeleton protein, cell spreading was inhibited when they are plated on a extracellular matrix (77). Similar to vinculin loss, FAK also had an effect on cell spreading and appears to be controlling this via the Integrin pathway (78). Therefore, cell shape is the balance between the forces exerted from the intracellular components of the cell and the outside environment.

In eukaryotic cells, cell shape changes are essential for development of multiple tissues and organs. One of the most prominent events mediated by cell shape change is ventral furrow formation during Drosophila gastrulation that involves a coordinated apical constriction of progenitors cells located at the ventral-most side of the embryo. Apical

constriction is a form of cell shape change that occurs by pulse contraction of the actomyosin cortical network on the apical side of the mesoderm progenitors (79). Another important biological activity supported by cell shape is development of the epithelia. Epithelial cells maintain various cell shapes such as columnar, cuboidal, and squamous to carry out biological functions like secretion and protection. The cell shapes changes that occur during development of the epithelia are influenced by cell-behavior, mechanical, and molecular factors. For example, the growth of the cell surface of epithelial layers causes the cells to have more of a columnar shape whereas volume reduction due to asymmetric divisions changes the shape into a more squamous morphology. Although research has been done on the cell shape of the epithelia, it is still unclear how the individual factors above come together to form different epithelial shapes (80).

Angiogenesis or formation a new blood a cell is another process that is mediated by the cell shapes changes. Interestingly, the cell shape changes in endothelial cells can result in different response to growth factors. For example, when endothelial cell spreading is limited, treatment with bFGF cause cell differentiation. In contrast, with increasing endothelial cells spreading, bFGF elicits a more mitogenic response and promotes DNA synthesis. Therefore, the mechanical forces shaping a cell can influence its response to cytokines and growth factors (81).

1.6.2 Cell Elongation

The process of cell elongation was first established in plant growth and development. Cell elongation in plants appears as early as germination when seed cells extend in the axial

length of the seed, which is needed for disruption of seed coating. The next step in plant growth where elongation is important is in the elongation of the stem to the surface to reach the source of light. Cell elongation also plays a key role in root expansion, specifically in the lengthening of the root and the elongation of the root hairs necessary for absorption of water. Lastly, elongation is also important for reproductive development of the plant. A great example of this is the apical growth of the pollen tube which elongates so that it can penetrate the style after pollination (82).

Elongation is also significantly important in the embryogenesis of animals. The nematode develops into an elongated worm through contraction of the cytoskeleton and cell movement. The most important cells in this process are the hypodermal or epidermal cells. These cells form three layers consisting of the dorsal, lateral, and ventral epidermal cells that undergo various processes that eventually lead to the formation of the worm. Importantly, cell elongation occurs when the ventral and anterior epidermal cells extend their filpodia. This elongation process is essential for ventral closure during nematode deveopment (83).

1.6.3 Lens Fiber Cell Elongation

The Lens of the eye is a dynamic structure that is composed of two cell types - the anterior epithelial cells and the lens fiber cells. The lens fiber cells make up the bulk of the lens and is crucial to the its role in focuing light on to the retina (84). Primary lens fiber cells arise when the posterior cells of the lens vesicle exit the cell cycle and down regulate the genes specific an epithelial cell fate and up-regulate the genes necessary for developing lens

transparency and its refractive index. Subsequently, these cells undergo a cell shape change from a cuboidal cell to an elongated cell. This is the first stage of cell elongation during lens formation (85).

For the growth of the lens, proliferative cells in the germinative zone of the lens migrating down to the lens equator and differentiating into the secondary lens fiber cells. Secondary lens fiber cell production is a process that happens throughout the life. Similar to primary lens fiber cells, secondary lens fiber cell have an apical and basal polarity with the apical side attaching to the lens epithelium and the basal side attaching to the lens capsule. However, what is unique about the secondary lens fiber cells is that, unlike the primary fiber cells, they maintain their apical connection to the epithelial layer and elongate along the apical side of the epithelial layer. This elongation process stops when the apical sides of fiber cells come in contact with the apical side of another lens fiber cell from the other side of the lens. This same process also happens at the basal end of the lens as the secondary lens fiber cell remains attached to the lens capsule while it elongates and stops when it meets the basal end of another secondary lens fiber cell. The mechanism that governs lens fiber cell elongation is still under investigation (86) (16).

The hypothesized mechanisms for lens fiber cell elongation currently consist of microtubules reorganization, increased cell volume, and actin cytoskeletal dynamics. Microtubules are an essential component of the cytoskeleton and are a part of the underlying membrane in shaping both primary and secondary lens fiber cells. It was

thought that microtubules of the lens are highly organized with the minus end towards the apical tip of lens fiber cells and the plus end facing the basal side of the lens. This observation was also supported by the presence of microtubule organizing centers, at the apical end of the lens fiber cells that are known to interact with the minus end of microtubules. In contrast, there are also studies that suggested microtubules are not necessary for lens fiber cell elongation such as the one done by *Beebe et al* in 1979 showing that inhibitions of microtubules do not effect cell elongation in chick lens explants. In addition, multiple studies have revealed other roles of microtubules in lens development that are independent of cell elongation (87).The complexity and true role of microtubules have yet to be fully elucidated.

David Beebe's group proposed another hypothesis that cell volume is the key factor in lens fiber cell elongation. Several experiment from this group revealed that lower amounts of potassium in the cell increased fluid influx into these cells, causing expansion of cell volume in cultured lens explants (88). Also studies using chicken lens cells demonstrated cell length correlated with the volume of the cell (89). Nevertheless, this model does not appear to be the dynamic processes that are involved in lens fiber cell elongation. The dynamics of the actin cytoskeleton are vital to cell adhesion and cell migration and have been hypothesized to also be a key factor in lens fiber cell elongation. Actin content and actin stress fiber are increased in elongated cells and disruption of actin impairs lens fiber cell elongation. Also, the transition of epithelial lens cells to differentiated fiber cells is closely associated with cytoskeleton reorganization and stabilization of cell-to-cell junctions. Consistent with this finding, disruption of actin stress fibers cause an increase in

differentiation markers and N-cadherin assembly (90). These evidence points to a strong association between lens fiber cell elongation and actin cytoskeleton dynamics.

1.6.4 GTPases and Lens Development

GTPases are a family of proteins that act as molecular switches that control biochemical pathways needed for multiple cellular processes. They control signal transduction by switching between two conformational states, active and inactive. In the inactive state, it is bound to a GDP. These GTPase proteins become active when a Guanine Nucleotide Exchange Factor (GEF) will remove the GDP, allowing a GTP to bind. In the active state, GTPases interact with target proteins to induce downstream signaling until the GTPase Activating Protein (GAP) stimulates hydrolysis of GTP to GDP and the Guanine Nucleotide Dissociation Inhibitor (GDI) removes it from the membrane. There are several GTPases which can be divided into 5 major groups: Ras, Rho, Rab, Arf and Ran (91). GTPase have been studied for years for their roles in developmental processes.

During lens development, members of the Ras and Rho family of GTPase contribute to cell proliferation, cell differentiation, and cell adhesion. Specifically, the small GTPase Ras has been extensively studied for its role in lens cell proliferation. The Ras gene was discovered as a retroviral gene in the 1970s and it can relay signaling after activation by growth factor receptors like the FGF receptor. The activation of Ras is controlled by at least three GEF: SOS, RAS-GRF and RAS-GRP. Multiple studies have established the role of SOS in receptor tyrosine kinase-mediated activation of RAS. SOS will bind the SH3 domain of the adaptor protein Grb2, which is recruited to a receptor kinase via its SH2 domain directly

or indirectly through a multi-protein complex. The association of SOS with the activated receptor brings it in close contact with Ras, turning it into GTP-bound activated Ras (92). *Reneker et. al* (98), have shown the lens of a transgenic mouse expressing a dominant negative form of Ras (dnRas) was dramatically decreased in size and growth compared to the wild type lens. To further confirm this observation, 5-bromo-2′-deoxuridine (BrdU) labeling was done on the both the dnRas lens and the wild type, which showed a two-fold decrease in BrdU labeled cells in the epithelial layer of the dnRas. There was also an increase in the number of apoptotic cells in the epithelial layer and lens fiber cells, indicating that Ras is important for cell survival in the lens. Consistent with this finding, Ras gain-of-function studies also show that cell proliferation was induced in the lens of transgenic mice. Ras can activate multiple downstream effectors and it has been shown that the activation of Erk in the lens is crucial for Ras-induced cell proliferation. Indeed, the lens of dnRas transgenic mice displayed a decrease in Erk activation and (U0126) cell proliferation is decreased in the lens explant treated with the Erk inhibitor. Overall Ras-ERK signaling is crucial to cell proliferation, but more studies are needed to elucidate other roles of Ras in lens development (39,93).

GTPase Rap1 is another member of the Ras family of proteins that has recently been investigated for its role in lens development. Rap1 is activated by several Rap1 GEF, among which C3G is the first one to be discovered. C3G binds to the SH3 domain of Crk and its phosphorylated form can activate Rap1. Other activators of Rap1 include CD-GE, Epac, PDZ-GEF, and DOCK-4. There are two distinct clear roles of Rap1 in cellular functions - cell proliferation and cell adhesion. It was found to attenuate Ras-Erk signaling

by possibly competing with c-Raf1 to inhibit proliferation, but in fibroblast cells it was found to increase cell proliferation maybe through binding of B-Raf independent of Ras. Further studies suggest that the differential roles of Rap1 in cell proliferation is highly cell type dependent. While the role of Rap1 in proliferation was controversial, its role in cell adhesion is clear. Rap1 signaling is known to mediate inside-out activation of integrins through various stimuli. However, even when integrin is stimulated by an activating antibody or Mn^{2+}, cell adhesion still required Rap1 activity. This revealed that Rap1 is required for the entire integrin-mediated cell adhesion process (94). In addition, other studies have uncovered a role for Rap1 in adherence junctions and tight junctions (95,96).

Recently, Rap1 has been investigated for its role in lens development. The lens of the embryonic and neonatal Rap1 mutant mice initially displayed micropthalamia and opacification. Further analysis of various embryonic stages revealed severe defects in cell-to-cell adhesion, cell-ECM adhesion, cell morphology and polarity. In support the loss of adherence junctions, the levels of α-smooth muscle actin and various transcriptional inhibitors of E-cadherin were increased. Lastly, although differentiation was not affected, the Rap conditional knockout lens underwent apoptosis and cell cycle progression. These data are consistent with the role of Rap1 in adherence junctions and integrin mediated cell adhesion (97).

The Rho family of GTPases is well known for their role in cell migration. One of the most extensively studied Rho GTPases is Rac. Rac was discovered as a Ras-related C3

botulinum toxin substrate 1 in 1989. Rac1, Rac2, and Rac3 are the three isoforms of Rac with Rac1 being the most extensively studied. Like all other GTPases, Rac is also activated by GEF, including Dock180. Activation of Rac1 leads to its interaction with downstream effectors such as PAK, IRSp53/WAVE, and IQGAP, all regulators of actin dynamics. Rac1 has been implicated in reorganization of the cytoskeleton, lamellipodia formation, endocytosis and trafficking. Rac1 mediated actin polymerization is necessary for phagocytosis of microorganisms and a plethora of other cellular functions (98,99).

Given the above role of Rac1 in actin dynamics, it is not surprising that Rac1 is responsible for lens shape and cell migration during lens development. Conditional deletion of Rac1 in the lens at embryonic day E14 resulted in abnormalities in cell shape, suture formation, lens fiber cell migration and orientation. The lens size was also decreased in the Racl mutant compared to the wild type. This lens size change was partly due to the apoptosis seen in both the lens epithelium and the lens fiber cells. There was also a defect in actin cytoskeleton organization and down-regulation of Rac1 downstream effector proteins such as WAVE-2 and Abi-2. Adherens junction was also decreased as indicated by a significant loss of E-cadherin (100). Other studies have found that a balance between RhoA and Rac1 is essential for cell shape and lens placode invagination. RhoA is needed for apical constriction of lens epithelial cells during invagination and Rac1 is needed for lens cell elongation, both necessary for the invagination of the lens placode (15). In addition to integrin-controlled cell adhesion and cell migration pathways, growth factors can control reorganization of the cytoskeleton in lens cells (101). These finding has opened up a variety

of ideas of how crosstalk between the integrin and growth factor signaling pathways mediate lens development.

1.7 Central Hypothesis

The murine lens serves as a great model to study cellular processes such as cell proliferation, cell differentiation, cell adhesion, and cell migration because of its simplicity and accessibility to genetic manipulation in model organisms. With one single cross section of the mouse lens, defects in these cellular processes can be observed using immunohistochemistry and other visualization techniques. Using the lens as model, our laboratory has previously studied the role of FGF signaling in lens development and revealed novel mechanisms of how FGF signaling mediates cell processes in the lens. For example, our laboratory established that the Frs2-Shp2 complex is the mediator of FGF signaling in lens development (48). In addition, there are a plethora of studies addressing the important role of FGF signaling in lens cell differentiation and cell elongation (39). However, what mediates cell elongation during lens development is poorly understood. The adaptor proteins Crk and CrkL have been extensively study for their role in cell adhesion, cell migration, and cell shape (65). Therefore, we investigated the role of adaptor proteins Crk and CrkL in lens development and assess the molecular mechanism responsible for FGF mediated lens fiber cell elongation. To explore this, we employed a lens specific Cre to conditionally knockout adaptor proteins Crk and CrkL in the lens at embryonic day 14 and assessed the phenotype using one of most widely used histological stains H&E, immunohistochemistry, immunocytochemistry, and TUNEL analysis. In addition, to further understand the cellular mechanism behind Crk and CrkL mediated lens

fiber cell elongation we conducted western blot analysis and utilized multiple transgenic mouse models.

CHAPTER TWO

Material and Methods

2.1 Mice and Genotyping

Le-Cre (+) mice were crossed with homzygous $Crk^{flox/flox}/Crkl^{flox/flox}$ mice to generate *Le-Cre;*$Crk^{flox/flox}/Crkl^{floxflox}$. *Le-Cre* mice were kindly provided by Dr. Ruth Ashery-Padan, Tel Aviv University, Tel Aviv, Israel and Dr. Richard Lang, Children's Hospital Research Foundation, Cincinnati, OH (102). *Le-Cre;*$Crk^{flox/flox}/Crkl^{flox/flox}$ was crossed with Transgenic (Tg) FGF3 mice to generate *Le-Cre;*$Crk^{floxflox}/Crkl^{flox/flox}$/*Tg-FGF3* mice. Transgenic FGF3 mice were obtained from Dr. Micheal Robinson, Children's Hospital Research Foundation, Cincinnati OH (44). *Le-Cre;*$Crk^{flxo/flox}/Crkl^{flox/flox}$ were crossed with *LSL(Lox-Stop-Lox)*$Kras^{G12D}$ mice to generate *Le-Cre;*$Crk^{flox/flox}/Crkl^{flox/flox}$/*Kras* mice. *LSL-*$Kras^{G12D}$ mice were obtained from the Mouse Models of Human Cancers Consortium (MMHCC) Repository at National Cancer Institute (103). *Le-Cre (+)* mice were crossed with $Rac1^{flox/flox}/Rac2^{flox/flox}$ mice to generate *Le-Cre;*$Rac1^{floxflox}/Rac2^{flox/flox}$. $Rac1^{flox/flox}/Rac2^{flox/flox}$ mice were obtained from Dr. Richard Lang, Children's Hospital Research Foundation, Cincinnati, OH (100). *Le-Cre (+)* mice were crossed with $Rap1a^{floxflox}/Rap1b^{flox/flox}$ mice to generate *Le-Cre;*$Rap1a^{flox/flox}/Rap1b^{flox/flox}$. $Rap1a^{floxflox}/Rap1b^{flox/flox}$ mice were obtained from Dr. Pongugoti V. Rao Department of Pharmacology and Cancer Biology, Duke University School of Medicine, Durham, NC 27710, USA (97). The Constitutively active Rac1 was obtained from the Rajewsky laboratory. This lab has developed an inducible allele of Rac-1 ($R26Stop^{FL}$RACDA), which inserted a loxP flanked transcription stop cassette and a constitutively active G12V mutation in the endogenous Rac-1 locus (104). Cre mediated recombination cleaved the

Stop cassette and activate the expression in a tissue-specific fashion. Therefore, I crossed R26Stop^{FL}RACDA with Le-Cre;Crk/Crkl$^{flox/flox}$ to generate Le-Cre;Crk/Crkl$^{flox/flox}$; R26Stop^{FL}RACDA. In all conditional knockout experiments, mice were maintained on a mixed genetic background and housed in a virus-free facility on a 12-h light-dark cycle and were fed a standard mouse food.

For embryo generation female mice were checked daily for a semen plug, with morning of detection being considered Embryonic day 0. Pregnant females were sacrificed at the desired stage, and embryos dissected out in cold Phosphate Buffered Saline (PBS). Embryos were placed in 4% paraformaldehyde (PFA) for fixation. Yolk sacs and embryo tails were collected for embryo genotyping. Genotyping was done using polymerase chain reaction (PCR) with various genes specific primers (see table 2.1 for primer sequence). Mouse maintenance and experimentation was performed according to protocols approved by the Indiana University School of Medicine and Columbia University Institutional Animal Care and Use Committee.

Table 2.1 Primer Sequence

Primer Name	Primer Sequence
Cre	Forward: AACATGCTTCATCGTCGGTC Reverse: GAGTTGATAGCTGGCTGGTG
Crk	Forward KO: GGGTGACCTGAGAACTGACC Forward Flox: TCACTTATCCTGGGAATTGGA Reverse WT: CAGCTCGGACTGCAGAATG
CrkL	Forward (CrkL-bPacG1): AGGGTGAGGCGACTTCATAA Forward (CrkL-bAatG1): TCACTTATCCTGGGAATTGGA Forward (CrkL-NeoTk-1): GGAGAGGCTTTTTGCTTCCT Reverse (CrkL-aPacG2): CCACCCCACCTTCATTATTC
K-Ras	Forward: TTGCAGAACTGCTCTGATGG Reverse: GCTCCAACCACCACAAGTTT
Rac1	Forward: TCCAATCTGTGCTGCCCATC Reverse: GATGCTTCTAGGGGTGAGCC
Rac2	Rac2-common: GACGCATGCTCCACCCCCT Rac2-PGKneoF2: TGCCAAGTTCTAATTCCATCAGAAGC Rac2-ex1: CACACACTTGATGGCCTGCAT
Rap1a	Forward: CCAAGGCTCTCAGTTGATTTCTA Reverse: TATCTGCACATAATCTGCATGCC
Rap1b	Forward: CCCTCTCATGCTATTCCTAATGT Reverse: GCCACCTCAGTAGAAAAGACC
391 (Tg-FGF3)	Forward: CCCAGAGGCTCCTGTCTGACTCACT Reverse: GTACTTGGTAGCGCAGTAGAGC

2.2 Cell Culture

Mouse Embryonic Fibroblast (MEF) Cells—Primary MEF cells were isolated from embryos at the E13.5 to E14.5 stage. Briefly, the uterine horns were dissected from pregnant females and rinsed in 70% (v/v) ethanol before transfer into sterilized PBS. After the heads and the internal organs were cut away, the trunks were washed with fresh PBS to remove blood cells, finely minced into small pieces in a minimal amount of PBS, and digested in 1–2 ml of 0.25% trypsin-EDTA for 10 min at 37 °C under gentle agitation. The supernatant was combined with 2 volumes of fresh Dulbecco's modified Eagle's medium

(DMEM) and centrifuged at a low speed (400 g). The cell pellet was re-suspended in DMEM containing 10% FBS (Fetal Bovine Serum) and antibiotics (penicillin G/streptomycin 1:100) and cultured in a humidified 5% CO2 incubator at 37 °C. MEFs from the second passage were infected with Ad5CMVCre (Gene Transfer Vector Core, University of Iowa, IA) overnight at multiplicity of infection 100 plaque-forming units/cell and cultured for 2 more days after replacing with fresh culture medium.

2.3 Histology and Immunohistochemistry

After overnight fixation in 4% paraformaldehyde (PFA), the embryos were dehydrated progressively through 30, 50, 70, 90 and 100% ethanol, cleared in xylene and embedded in paraffin. The paraffin sample blocks were oriented on a Leica 2125 microtome and sectioned at 10 μm. For cryosections, the embryos were incubated in 30% sucrose overnight and cryoembedded using OCT (Optimal Cutting Temperature) compound. Section were cut at 10 μM using a Leica CM 1850 microtome. Paraffin sections were stained with Hematoxylin and Eosin (H&E), and mounted with coverslips, before digital pictures of the sections were taken on a Leica DM3000 compound microscope at 10x and 20x magnification. Lens sizes were measured by the maximum area of the lens using the ImageJ program (National Institute of Health, Bethesda, MD), and the statistical significance was calculated by a two-tailed t-test. Regular immunostaining was performed on the cryosections or paraffin embedded sections (10 μm). Antigen retrieval was performed by microwave heating for 10 minutes at sub-boiling condition in citrate buffer (10 mM sodium citrate, pH 6.0). Non-specific interaction was blocked by 5% goat serum in PBS at room temperature for 1 hour. Sections were incubated with primary antibody at

4°C overnight in a humid chamber and placed in secondary antibody (1:250 Alexa Flour 488; 1:500 Alexa Fluor 555) for one hour the next day. Sections were stained with 4,6-diamidino-2-phenylindole (DAPI) (1:5000) for nuclear staining, pictures were taken with a compound microscope. For phospho-Erk staining, the endogenous peroxidase activity on cryosections was quenched with 3% H_2O_2 in 10% methanol/PBS solution for 10 minutes before blocking for non-specific interactions. Phospho-Erk signaling was amplified using a Tyramide Signal Amplification (TSA 1:50) kit (TSA™ Plus System, PerkinElmer Life Sciences, Waltham, MA). Cell proliferation was quantified by counting the number of Ki67 positive cells versus DAPI-positive cells, and analyzed by two-tailed student t-test. Staining of F-Actin was performed by incubating sections with the Alexa Flour 488 phalloidin (1:100) for one hour. The maximum length of lens fiber cells was measured using the ImageJ program (National Institute of Health, Bethesda, MD), and the statistical significance was calculated by two tailed t test. Antibodies used were: anti-Crkl (Santa Cruz sc-319) anti-phospho-ERK1/2 (cell signaling #4370), anti-P57 (ab75974) (from Abcam, Cambridge, MA), anti-E-cadherin (U3254, Sigma, St Louis, MO), anti-jagged1 (sc-6011 santa cruz) anti-Ki67 (#550609, from BD Pharmingen San Diego, CA), anti-Prox1 (PRB-238C) and anti-Pax6 (PRB-278P) (both from Covance, Berkeley, CA) Laminin (L9393 Sigma St. Louis MO), Active β-Integrin (BD Pharmingen San Diego, CA), Integrin AIIB2 (Developmental Studies Hybridoma Bank, Iowa City, IA), beta-dystrogylcan (Developmental Studies Hybridoma Bank, Iowa City, IA), Anti-Paxillin (BD Transduction Laboratories), Collagen type IX D1-9 (Developmental Studies Hybridoma Bank, Iowa City, IA), anti-Arpc2 (ab11798 Abcam, Cambridge, MA) , anti-phospo-

Paxillin (#2541 cell signaling). The antibody against α-crystallin was kindly provided by Sam Zigler (National Eye Institute, Bethesda, MD) (see table 2.2 for antibody dilution).

2.4 Immunocytochemistry

For immunostaining, MEF cells were cultured on round glass coverslip (corning #1 0211 glass #CLS-1763 Chemglass Life Science) and washed with PBS and fixed with 4% paraformaldehyde and incubated in PBS supplemented 10% NGS and 0.2 % triton X-100 for blocking nonspecific antibody binding. Cells were incubated with primary antibodies (Active β-Integrin (BD Pharmingen San Diego, CA) and anti-phospo-Paxillin (#2541 cell signaling) for 1 h at room temperature, washed in PBS and incubated with Alexa fluor 488 dye-conjugated secondary antibodies (1:250) or phalloidin (1:100) (Invitrogen) for 40 min at room temperature. After extensive wash, coverslips were mounted with Vectashield mounting medium containing DAPI (1:1000) (Vector Laboratories, Burlingame, CA, USA) (see table 2.2 for antibody dilution).

2.5 Cell Protein Extract Isolation and Western Blots

$4.24–6.36\times10^5$ MEF cells infected with Ad5CMVCre were seeded in 60 mm dishes and serum starved (0.5% FBS in DMEM) for 36–48 hours before being stimulated by 50 ng/ml FGF2 (R&D Systems, Minneapolis, MN) for 5 minutes at 37°C. After washing twice in cold PBS, MEF cells were lysed in 160 µl RIPA buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, 1 µg/ml aprotinin, 1 µg/ml pepstatin, 10 mM sodium pyrophosphate, 1 mM PMSF, 0.2 mM Na_3VO_4, 50 mM NaF) and a Bradford protein assay was used to determine the protein concentration from cell

lysates. 6x Laemmli buffer was added to 30-60 ug of protein lysate and then boiled at 100° C for 5 minutes. Equal amount of protein was loaded into the wells of a 10-12% SDS-page gel, along with a molecular weight marker. Gels ran for 1-2 hours at 100 volts in a electrophoresis cell containing 1x running buffer (25mM Tris base, 190 mM glycine, 0.1% SDS). The gel was then transferred to a PVDF membrane for protein analysis using wet elctroblotting (Tank Transfer) containing transfer buffer (25 mM Tris base, 190 mM glycine, 20% methanol). Proteins were visualized by infrared-based western blot analysis using an Odyssey SA scanner (LICOR Biosciences, Lincoln, NE). The signal intensity was quantified using the Odyssey software. The antibodies used were mouse anti-phospho-ERK1/2 (sc-7383, santa cruz), anti-phospho-Crk (tyr221) (cell signaling #3491) (see table 2.2 for antibody dilution).

2.6 TUNEL Analysis

TUNEL staining was performed with an in situ cell-death detection kit (Roche, Indianapolis, IN, USA). Briefly, cryosections were processed for antigen retrieval as described above, incubated with blocking buffer (0.1 M Tris-HCl, pH 7.5, 3% BSA, 20% serum) for 30 minutes at room temperature and then with TUNEL reaction mixture for 2 hours at 37°C. After rinsing with PBS, the sections were blocked again with 0.05% blocking reagent (supplied in the TSA Indirect Tyramide Signal Amplification Kit, Perkin Elmer Life Science, Boston, MA, USA) for 30 minutes and then incubated with TUNEL-POD for 30 minutes at 37°C. Finally, the signal was developed with DAB substrate and detected under a Leica DM500 microscope at 10x and 20x magnification.

Table 2.2 Primary Antibody Dilutions

Primary Antibody Name	Primary Antibody Dilution
anti-Active β-Integrin	1:100
anti-Arpc2	1:100
Collagen type IX D1-9	1:100
anti-Crkl	1:200
Crystallin (α)	1:1000
anti-Dystrogylcan (β)	1:100
anti-E-cadherin	1:200
Anti-ERK 1/2	1:1000 Western Blot
anti-Integrin AIIB2	1:100
anti-jagged1	1:100
anti-Ki67	1:200
anti-Laminin (L9393)	1:1000
anti-Paxillin	1:100
anti-Pax6	1:250
anti-phospho-Crk (tyr221)	1:2000 Western Blot
anti-phospho-ERK1/2	1:200 IHC 1:2000 Western Blot
anti-phospho-Paxillin	1:50
anti-Prox1	1:500
anti-P57	1:100

CHAPTER THREE

Results

3.1 The Role of Adaptor Crk and CrkL in Lens Development

3.1.1 Conditional deletion of Crk and CrkL in the lens

To determine the role of adaptor proteins Crk and CrkL in lens development. Mice that express the lens specific Cre recombinase controlled by the *Pax6* promoter were mated with mice that contained the Crk and CrkL flox alleles. Their progeny will contain conditional deletion of Crk and CrkL in the lens in the presences of Cre recombinase (Figure 7.). To verify deletion of Crk and CrkL using the Cre/loxP system, immunostaining with a CrkL antibody was performed on the wild type and *Le-Cre;Crk*$^{flox/flox}$ */CrkL*^{floxflox} lens. CrkL was deleted as early as E10.5 and was also lost in both the E12.5 and E14.5 lens (Figure 8.). To confirm whether or not Crk and CrkL can compensate for each other during lens development, I deleted each individually in the lens using the Cre/loxP system. Neither Crk nor CrkL had a severe phenotype in the lens, although CrkL appeared to have a slightly more significant phenotype than Crk (Figure 9.). These data suggest that during lens development Crk and CrkL can compensate for each. Therefore, all studies carried out in this thesis had both Crk and CrkL deleted in the lens.

3.1.2 Conditional deletion of Crk and CrkL in the lens cause defects

To begin to understand the role of adaptor proteins Crk and CrkL in lens development, we deleted Crk and CrkL in the lens at embryonic day 14. H&E and immunohistochemistry staining revealed a severe phenotype in the *Le-Cre;Crk*$^{flox/flox}$ */CrkL*^{floxflox} lens compared to wild type. The *Le-Cre;Crk*$^{flox/flox}$ */CrkL*^{floxflox} lens displayed a reduction in lens size,

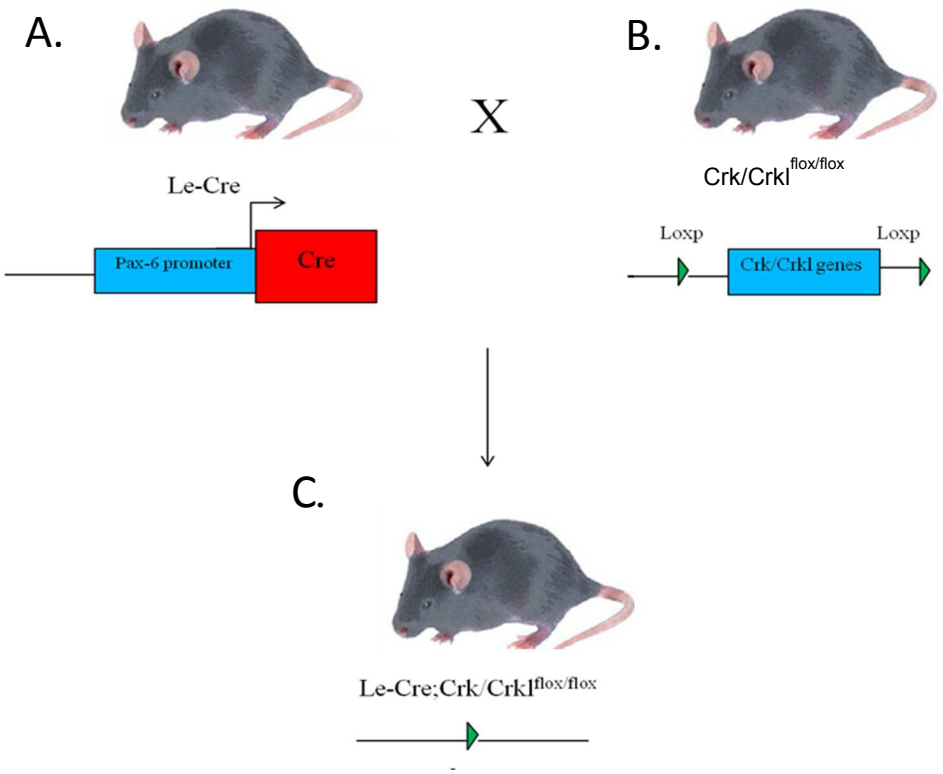

Figure 7. Le-cre/loxP recombination system for Crk and Crkl conditional knockout mice. A) Mouse with Cre recombinase enzyme expressed under the control of a pax6 promoter. B) Mouse with a targeted Crk and CrkL floxed allele. C) Mouse with lens specific deletion of floxed alleles in which cre recombinases is expressed in the lens.

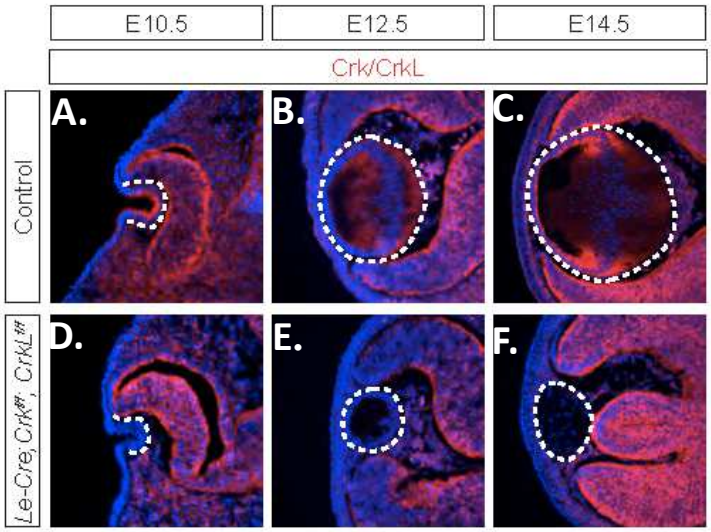

Figure 8. Conditional deletion of CrkL in the developing lens. (A-F) Cross sections of the embryonic mouse lens at three different stages. CrkL immunostaining was markedly reduced at embryonic day 10.5, 12.5, and 14.5 in the *Le-Cre;Crk*$^{flox/flox}$ */CrkL*^{floxflox} lens (D-F arrows) .

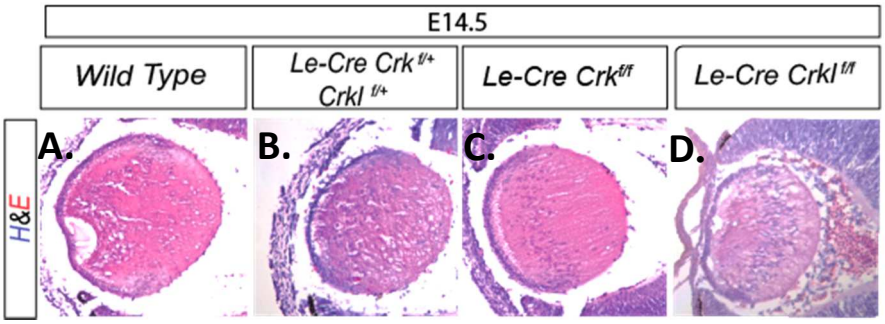

Figure 9. Crk and CrkL can compensate for each other during lens development. (A-D) H&E staining at embryonic day 14.5 of the mouse lens. B) The *Le-Cre;Crk*$^{flox/+}$*/CrkL*$^{flox/+}$ lens exhibits a very mild phenotype. (C-D) *Le-Cre;Crk*$^{flox/flox}$ and *Le-Cre;CrkL*^{floxflox} lens display a mild phenotype with the *Le-Cre;CrkL*^{floxflox} being slightly more severe.

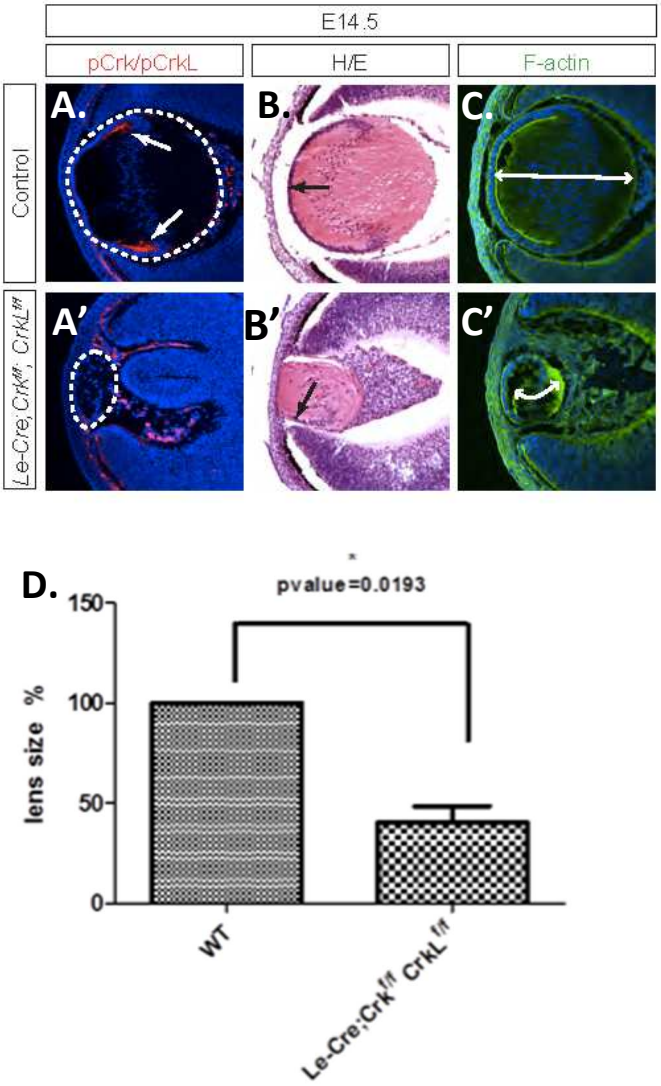

Figure 10. Phenotype of *Le-Cre;Crk$^{flox/flox}$ /CrkL^{floxflox}* lens. (A-A') The *Le-Cre;Crk$^{flox/flox}$ /CrkL^{floxflox}* lens size is reduced and the phosphorylation of Crk is also lost. (B-B') The anterior epithelium of the lens is rotated in *Le-Cre;Crk$^{flox/flox}$ /CrkL^{floxflox}* lens (indicated by arrow). (C-C') The *Le-Cre;Crk$^{flox/flox}$ /CrkL^{floxflox}* lens also displayed disorganization of the lens fiber cells as well as a decrease in lens fiber cell length (indicated by arrow). D) Lens size measurements of the *Le-Cre;Crk$^{flox/flox}$ /CrkL^{floxflox}* compared to wild type. A student t-test was preformed on three different lens for each phenotype.

rotation of the lens epithelial layer, and disorganization of the lens fiber cells. Lastly, one of the most striking phenotypes was a decrease in lens fiber cell length. The wild type and *Le-Cre;Crk$^{flox/flox}$/CrkL^{floxflox}* lens was stained with an Alexa flour 488 conjugated phalloidin. Phalloidin is a member of phallotoxins derived from poisonous mushrooms and is used to observe actin and cell shape because it has the ability to bind and stabilize actin filaments. In this study it was specifically used it as a guide to measure lens fiber cell length. The length of lens fiber cells appeared to be decreased in the *Le-Cre; Crkflox$^{/flox}$ /CrkL^{floxflox}* lens. The wild type and *Le-Cre;Crk$^{flox/flox}$ /CrkL^{floxflox}* lens size was also quantified to confirm reduction in lens size (Figure 10. D).

To further analyze the role of Crk and CrkL in lens development, we begin by staining the *Le-Cre;Crk$^{flox/flox}$ /CrkL^{floxflox}* lens with differentiation markers. We stained the *Le-Cre;Crk$^{flox/flox}$ /CrkL^{floxflox}* lens with Prox1, Pax6 and multiple forms of Crystallin. Prox1 is a homeodomain protein that is essential for lens fiber cell differentiation and elongation (105). Pax6 is a transcription factor that is essential for ocular development and deletion of Pax6 using the lens specific Cre recombinase resulted in the absence of the lens (102). Similar to Prox1, Crystallin is present in lens fiber cells, with α-Crystallin making up about 40% of the lens protein with another 50% being β and γ crystallin (106). Staining with these specific markers revealed that the *Le-Cre;Crk$^{flox/flox}$ /CrkL^{floxflox}* lens displayed no changes in Prox1, Pax6, or the three forms of Crystallin , indicating that Crk and CrkL are not essential for lens fiber cell differentiation (Figure 11. A-G').

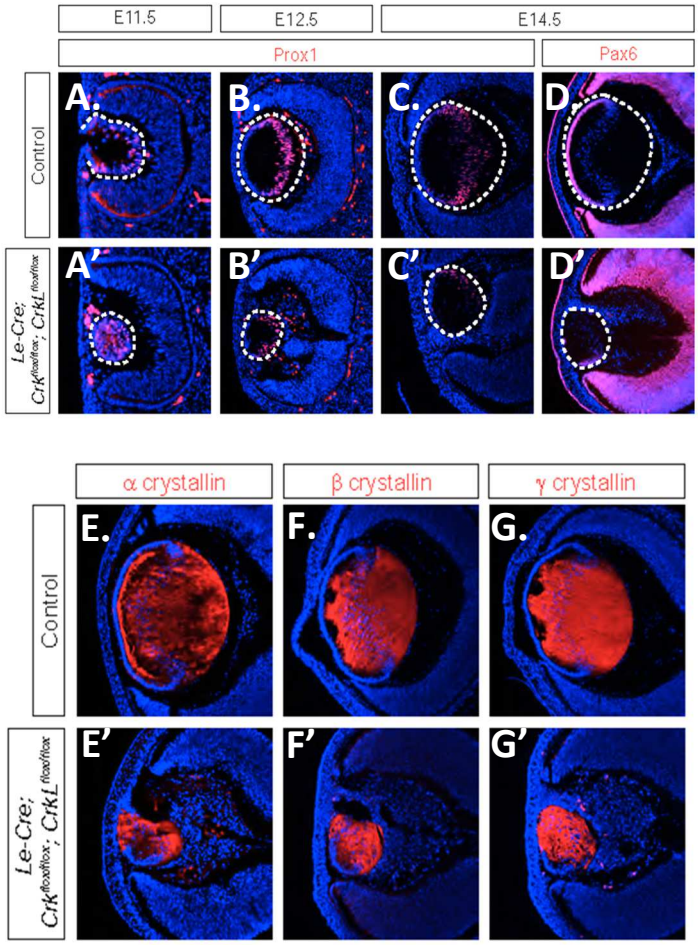

Figure 11. Conditional deletion of Crk and CrkL in the lens does not have effect on lens determination and differentiation proteins. (A-D') Prox-1 and Pax-6 was performed on the control and *Le-Cre;Crk*$^{flox/flox}$ */CrkL*^{floxflox} lens. There are no significant changes in staining intensity with the lens determination marker Pax-6 and the lens differentiation marker Prox-1. (E-G') Immunohistochemistry training with three different forms of Crystallin (α, β, γ). All three forms of Crystallin did not display any changes in staining intensity at embryonic day 14.

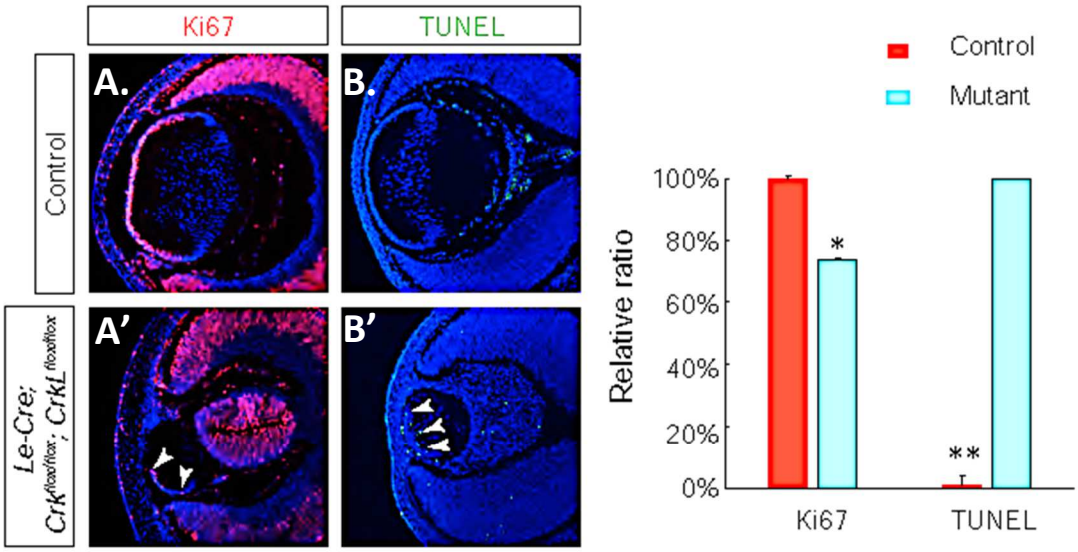

Figure 12. Conditional knockout of Crk and CrkL in the lens cause a decrease in lens cell proliferation and an increase in apoptosis during lens development. (A-A') Ki67 was decreased in the *Le-Cre;Crk$^{flox/flox}$ /CrkL^{floxflox}* lens compared to the wild type at embryonic day 14. (B-B') TUNEL staining was present in the *Le-Cre;Crk$^{flox/flox}$ /CrkL^{floxflox}* lens at embryonic day 14. (C) Relative ratio percentage of proliferation and apoptosis in the wild type and *Le-Cre;Crk$^{flox/flox}$ /CrkL^{floxflox}* lens. A student t-test was preformed on 3 different lens for each phenotype.

In addition to differentiation lens cell proliferation is essential for embryonic lens development. Therefore, we stained the lens with Ki67 which is considered a proliferation marker because its expression is detected in the nucleus during interphase and is present in all active phases of the cell cycle except the resting cell G (0) (107). When stained with Ki67, the *Le-Cre;Crk$^{flox/flox}$ /CrkL^{floxflox}* lens revealed a decrease in lens cell proliferation (Figure 12. A-A'). The number of proliferating cells was counted and analyzed using the student's t-test.

To investigate whether the small lens seen in the *Le-Cre;Crk$^{flox/flox}$ /CrkL^{floxflox}* lens was due to lens epithelial cells undergoing apoptosis, TUNEL staining was performed on the lens at embryonic day 14. Comparing the wild type and *Le-Cre;Crk$^{flox/flox}$ /CrkL^{floxflox}* lens, there was a significant increase in TUNEL staining in the *Le-Cre;Crk$^{flox/flox}$ /CrkL^{floxflox}* lens (Figure 12. B-B'). Therefore, the small lens observed is at least partly due to lens epithelial cell apoptosis. To confirm are analysis we performed a student t-test to observed the relative ratio of cell proliferation and apoptosis (Figure 12. C).

3.1.3 Deletion of Crk and CrkL causes extracellular matrix and cell adhesion defects of the lens.

Adaptor proteins Crk and CrkL play an important role in the formation of the extracellular matrix. Therefore, it is imperative to observe whether or not deletion of Crk and CrkL in the lens has any extracellular matrix defects. To explore this possibility, I conducted immunohistochemistry staining with cell surface receptors and basement membrane markers. There are two cell surface receptor that are important for connecting the

extracellular matrix to the cytoskeleton, Integrin and Dystroglycan. Integrin is a bidirectional cell surface receptor that is important for basement membrane assembly, cell shape, cell migration, and cell survival (108). Similar to Integrin, Dystroglycan was also thought to be important for basement membrane assembly. Dystroglycan is a transmembrane protein that is a member of the Dystrophin Glycoprotein Complex and is known as the link between the extracellular matrix and the cytoskeleton (109). At embryonic day 14, both B1 integrin and Dystrogylcan was reduced in the Crk and CrkL conditional knockout lens (Figure 13. A-D). The connection of the extracellular matrix to the cytoskeleton is also maintained by the binding of integrin to the components of the basement membrane (110). Immunostaining with components of the basement membrane like Collagen and Laminin was also decreased in the Crk and CrkL conditional knockout lens (Figure 13. E-H). From this data, it is concluded that during lens development Crk and CrkL play a role in maintaining the extracellular matrix.

Both Crk and CrkL are also essential for the motility and adhesion of the cell. One of the most important events in cell adhesion occurs when Integrin binds to its ligand such as Laminin and Collagen. Binding of Integrin to its ligands leads to activations of proteins like FAK and Src (111). FAK or Src will phosphorylate Paxillin and recruit Crk (112). Crk actives Rac1 which leads to the phosphorylation of the effector p21-activated kinase (PAK). PAK then binds to the actin nucleator complex Arp2/3. The Arp2/3 is responsible for actin nucleation and lamellipodia formation (15). Lamellipodia are membrane protrusion that facilitates the clustering of and binding of transmembrane cell surface receptors to the substratum components or formation of cell adhesions. To investigate if

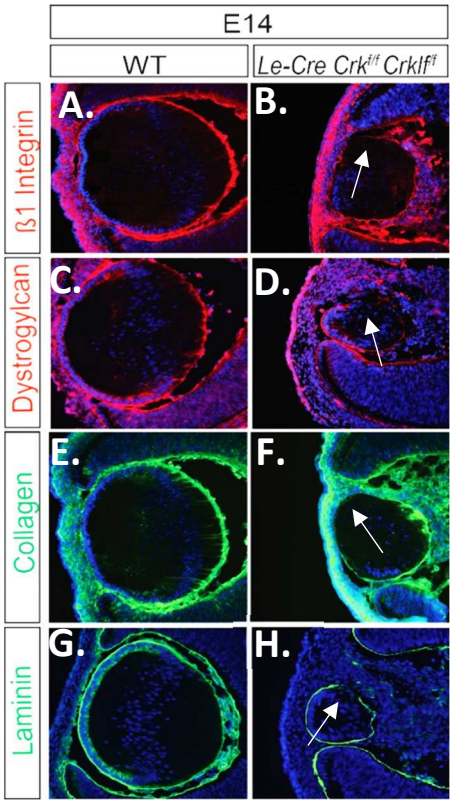

Figure 13. Deletion of Crk and CrkL causes extracellular matrix defects of the lens. (A-D) The *Le-Cre;Crk*$^{flox/flox}$ */CrkL*^{floxflox} lens exhibits a significant loss of the cell surface receptors Integrin and Dystroglycan compared to wild type. (E-H) The *Le-Cre;Crk*$^{flox/flox}$ */CrkL*^{floxflox} lens display a significant loss of basement membrane markers compared to wild type.

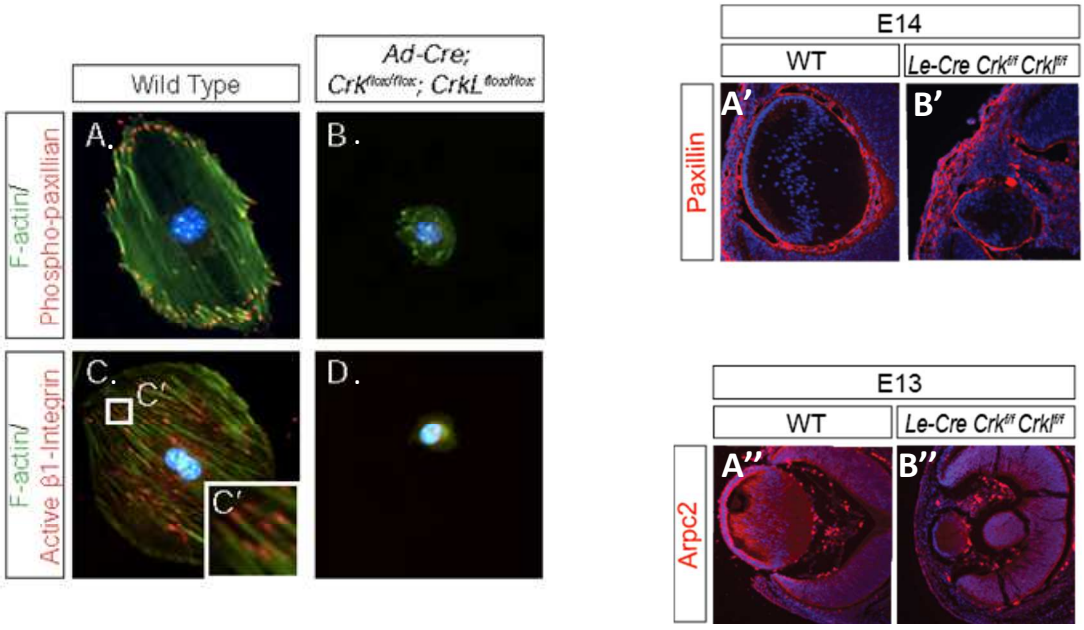

Figure 14. Cell adhesion is disrupted in the Crk and CrkL conditional knockout lens. (A-D) Mouse Embryonic Fibroblast (MEF) cells lose focal adhesion and cell spreading ability. (A′-B′) Arpc2 is decreased in the lens epithelial layer (indicated by errors) of the *Le-Cre;Crk*$^{flox/flox}$ */CrkL*^{floxflox} lens at embryonic day 13. (A″-B″) In the *Le-Cre;Crk*$^{flox/flox}$ */CrkL*^{floxflox} mutant lens, there was a reduction in Paxillin staining at embryonic day 14.

this process is interrupted by the deletion of Crk and CrkL during lens development, I conducted immunofluorescence staining with activated Integrin, phospho-Paxillin, and Arpc2 (member of the Arp2/3 complex). Deletion of Crk and CrkL in Mouse Embryonic Fibroblast (MEF) cells revealed a loss of activate Integrin, phospo-Paxillin, and cell spreading (Figure 14 A-D). In the Crk and CrkL conditional knockout lens there was also a decrease in Arpc2 and Paxillin (Figure 14.). This data taken together reveal that Crk and CrkL are important for cell adhesion and cell spreading.

3.1.4 Deletion of Crk and CrkL cause a decrease in the downstream effector phospho-ERK of FGF signaling pathway.

It is clear that both Crk and CrkL can interact with FGF receptors to induce downstream signaling (68) (69). However, the downstream effectors that are activated by the interaction between the FGF receptor and Crk/CrkL is poorly understood. To begin investigating the role of Crk and CrkL in FGF signaling during lens development, I accessed whether or not Crk and CrkL can cause activation of phospho-ERK. ERK (Extracellular signaling regulated kinase) or MAPK (Mitogen-activated protein kinase) is a member of a three protein cascade that eventually leads to its activation (113). ERK is activated by its phosphorylation and is a major component of pathways controlling cell proliferation, cell differentiation, and embryonic development (114). One of the known pathways that ERK play a role in is the FGF signaling pathway. FGF signaling and the activation of ERK are both essential for lens development. Therefore, we hypothesize that Crk and CrkL mediates activation ERK during lens development. Stimulation of phosphorylated ERK by FGF2 in Crk and CrkL deleted MEF cells appeared to be decreased compared to wild type MEF

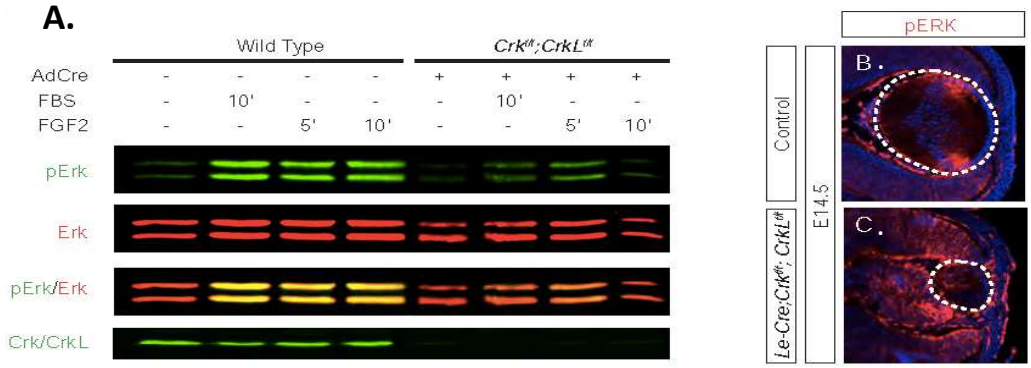

Figure 15. Deletion of Crk and CrkL cause a decrease in the downstream effector phospho-ERK of FGF signaling pathway. (A) Mouse Embryonic fibroblast (MEF) cells were obtained from Crk/Crkl flox embryos at embryonic day 14. Using an adenovirus cre, Crk/Crkl was deleted by the cre-lox method, a western blot was performed to assess the quantity of phospho-ERK following deletion of Crk/Crkl. In the presence of FGF2 there was a sharp reduction of phospho-ERK stimulation in the *Cre;Crk/Crkl* MEF cells. (B,C) The *Le-Cre;Crk*$^{flox/flox}$ */CrkL*^{floxflox} lens displayed a drastic decrease in phospho-ERK staining compared to the wild type.

cells (Figure 15. A). In addition, conditional deletion of Crk and CrkL in the lens at E14 resulted in a reduction in phosphorylation of ERK (Figure 15 B-C). These data explain that Crk and CrkL are mediating FGF signaling via ERK activation during lens development.

3.1.5 The Tg-Fgf3 gain of function phenotype is lost in the presences of the Crk/CrkL conditional KO mutation.

To further confirm that Crk and CrkL is contributing to FGF signaling during lens development, we used a transgenic mouse that contains a αA-crystallin promoter to target expression of FGF3 in the developing lens. Expression of FGF3 in the lens at E14 results in a gain of function phenotype with premature differentiation and ocular proptosis (44). Therefore, transgenic FGF3 mice were mated with Crk and CrkL conditional knockout mice to assess if there was an epistatic relationship between Crk/CrkL and the FGF receptor. Deletion of Crk and CrkL in the presences of transgenic FGF3 led to a phenotype very similar to Crk and CrkL deletion alone (Figure 16. A-H). In addition, the deletion of Crk and CrkL in the presences of the transgenic FGF3 also displayed a decrease in lens fiber cell length similar to the Crk and CrkL conditional knockout (Figure 16. I). Therefore, it was concluded that Crk and CrkL are epistatic to the FGF receptor.

3.1.6 Constitutive Kras signaling can compensate for the loss of Crk and CrkL in lens development.

Downstream FGF signaling can be carried out through activation of the GTPase Ras. Therefore, it is hypothesized that Ras signaling could be the target of Crk and CrkL during

lens development. In a genetic rescue experiment, the *Le-Cre*; *Crk*$^{flox/flox,}$ *Crkl* $^{flox/flox}$ mutants were crossed with the *LSL-Kras*^{G12D} allele, which can be induced to express a constitutively active *Kras*^{G12D} mutant by Cre-mediated recombination. TUNEL staining was performed on the *Le-Cre*; *Crk*$^{flox/flox}$*; Crkl*$^{flox/flox}$ *LSL-Kras*^{G12D} and consistent with our previous report (115), abnormal cell apoptosis, as indicated by TUNEL staining, was not rescued by activated Kras signaling (Figure 17 A-A''). Also double staining with proliferation and differentiation markers Ki67, E-cadherin, p57 and jagged-1, respectively. Ki67 has been previously discussed and E-cadherin is a cell-to-cell adhesion molecule important for the behavior of epithelial cells and the function of multiple tissues such as the lens (116). p57 is a cell cycle inhibitor that is presents in cells that have exited the cell cycle. E-cadherin is only found in the proliferating epithelial layer of the lens. Jagged-1 is a ligand that binds to the Notch receptor and is essential for lens fiber cell differentiation (117). Double staining with the various markers revealed there was no significant difference in staining intensity (Figure 17. B-C''). However, Results show that despite the loss of Crk and CrkL in the *Le-Cre*; *Crk*$^{flox/flox}$*;Crkl*$^{flox/flox}$ *LSL-Kras*^{G12D} mutant, Lens size was rescued and it was accompanied by induced ERK phosphorylation (Figure 17. A-D, A′-D′, E).

To begin understanding what cellular processes are mediated by Crk and CrkL activated Ras, immunohistochemistry staining was done with β1 Integrin, Dystrogylcan, Laminin, and Paxillin. *Le-Cre*; *Crk*$^{flox/flox}$ *;Crkl*$^{flox/flox}$ *LSL-Kras*^{G12D} mutant still displayed a loss of the cell surface receptors, cell membrane, and cell adhesion markers similar to *Le-Cre*; *Crk*

flox/flox ;*Crkl*$^{flox/flox}$ alone (Figure 18. A-L). The ability for Kras to rescue some but not all of the Crk and CrkL mutant phenotypes reveals that Crk and Crkl mediates other pathways

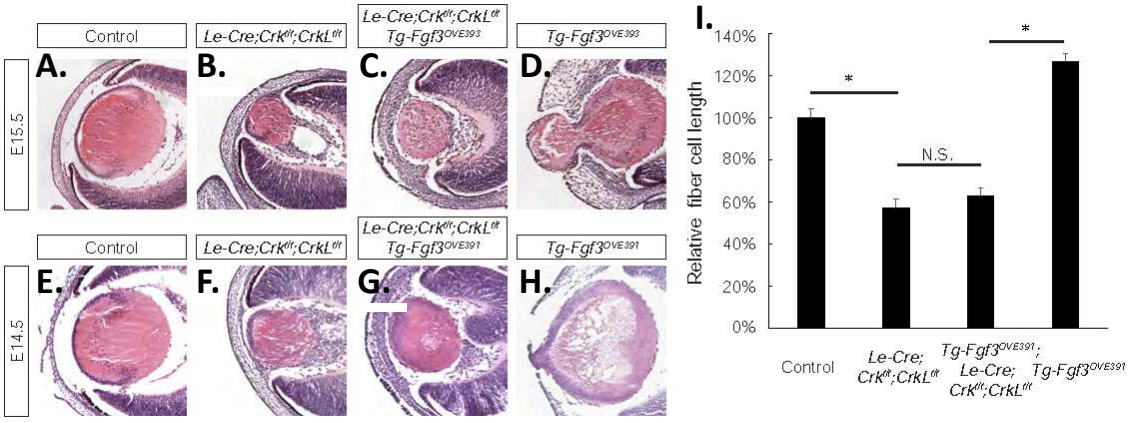

Figure 16. The Tg-Fgf3 gain of function phenotype is lost in the presences of the Crk/Crkl conditional KO mutation. (A,B,C,D) In the presences of the *Tg-Fgf3* the Crk/CrkL KO lens has a similar phenotype to the Crk/CrkL conditional KO indicated by the small lens size. (E,F,G,H) A less severe strain of the Tg-FGF3 391, was also observed in the presences of the Crk/CrkL knockout lens and also showed a similar phenotype to the Crk/Crkl conditional KO. I) Relative lens fiber cell length in the wild type, Crk/Crkl conditional KO, the Crk/Crkl conditional KO;Tg-Fgf3, and *Tg-Fgf3* lens revealed a drastic decrease in lens fiber length in the Crk/Crkl conditional KO and the Crk/Crkl conditional KO;Tg-Fgf3 lens. A student t-test was preformed on three different lens for each phenotype.

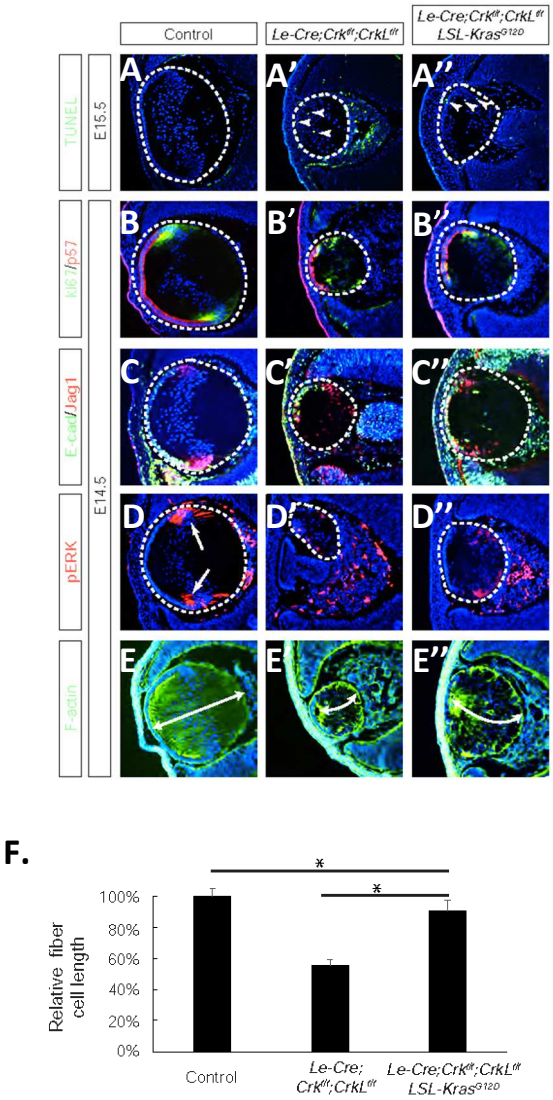

Figure 17. Constitutive Kras signaling can compensate for the loss of Crk and CrkL in lens development. (A-A'') TUNEL staining was present in the *Le-Cre;Crk*$^{flox/flox}$*;CrkL*^{floxflox} lens at E14 remained significantly elevated in *Le-Cre;Crk*$^{flox/flox}$*;CrkL*^{floxflox}*;LSL-Kras*^{G12D} lens. (B-B'' and C-C'') There was no significant difference in staining intensity when the lens was stained with proliferation and differentiation markers. (D-D'') Despite the loss of Crk and CrkL, ERK phosphorylation recovered in *Le-Cre; Crk*$^{flox/flox}$*; CrkL*$^{flox/flox}$; LSL-Kras^{G12D} mutant lens. (E-E'') The lens fiber cell length observed in the *Le-Cre;Crk*$^{flox/flox}$ *;CrkL*^{floxflox} lens appeared to be rescued in the *Le-Cre; Crk*$^{flox/flox}$*; CrkL*$^{flox/flox}$*; LSL-Kras*^{G12D} mutant lens. F) Fiber cell length was measured and quantified. A student t-test was preformed on three different lens for each phenotype.

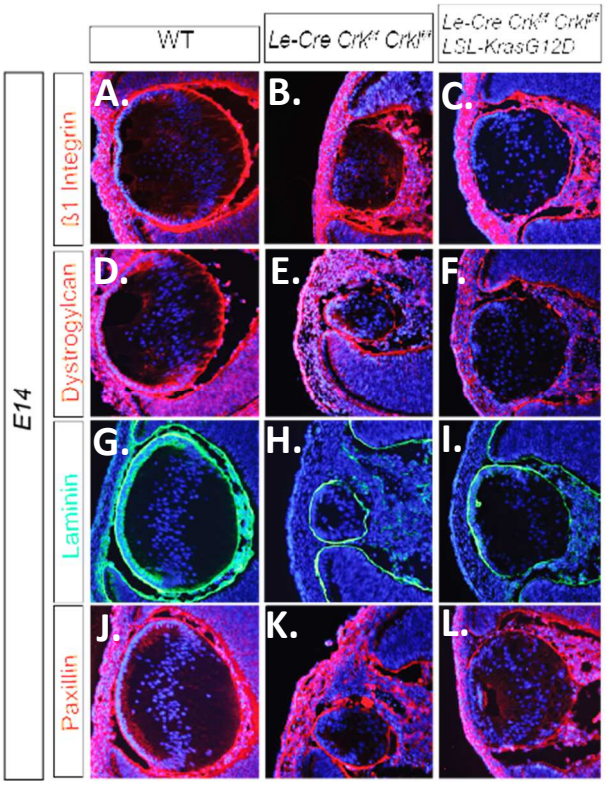

Figure 18. Activated Ras did not rescue the ECM and cell adhesion defect in the Crk and CrkL mutant lens. (A-F) The *Le-Cre;Crk$^{flox/flox}$;CrkL^{floxflox} ; LSL-Kras^{G12D}* lens exhibits a significant loss in the staining of the cell surface receptors Integrin and Dystroglycan compared to wild type. (G-I) The *Le-Cre;Crk$^{flox/flox}$;CrkL^{floxflox} ; LSL-Kras^{G12D}* lens display a significant loss of the basement membrane marker laminin compared to wild type. (J-L) In *Le-Cre;Crk$^{flox/flox}$;CrkL^{floxflox} ; LSL-Kras^{G12D}* mutant lens, there was a reduction in Paxillin staining at E14.

during lens development in addition to Ras signaling pathway. The Ras-MAPK pathway is known to promote cell survival, therefore the rescue of the Crk and CrkL mutant lens could be due to suppression of apoptosis by Ras. This scenario is plausible because Kras similarly cannot rescue some of the Crk and CrkL phenotype such as the lens epithelial layer rotation and the ECM defects.

3.2 Crk and CrkL are required for lens fiber cell elongation

3.2.1 Crk and CrkL and required for lens fiber cell elongation but not differentiation

Cell differentiation and cell elongation are two dynamic processes that appear to proceed sequentially. During lens development, secondary lens fiber cells will differentiate first and then elongate along the epithelial layer and the lens capsule. Although cell elongation happens after cell differentiation, are they controlled by the same pathway? To answers this question, we conducted immunohistochemistry staining using three sets of markers that specifically stain the lens epithelium and the lens fiber cells on the *Le-Cre; Crk*$^{flox/flox;}$ *CrkL*^{floxflox}*, Le-Cre;Crk*$^{flox/flox}$*;CrkL*^{floxflox}*;Tg-Fgf3*, and the Tg-Fgf3 mice. What is unique about the Tg-Fgf3 is that this transgene causes premature differentiation of lens epithelial cells and an increased in lens size compared to the wild type. Interestingly, Crk and CrkL can inhibit the lens size but not the premature differentiation indicated by the precocious appearance of differentiation markers p57, Jagged-1, and C-Maf in the epithelial layer of the lens in both the Tg-Fgf3 lens and the *Le-Cre;Crk*$^{flox/flox}$*;CrkL*^{floxflox}*;Tg-Fgf3* lens (Figure 21. A-K). These data indicate that Crk and CrkL is not necessary for lens fiber cell differentiation but they are required for fiber cell elongation.

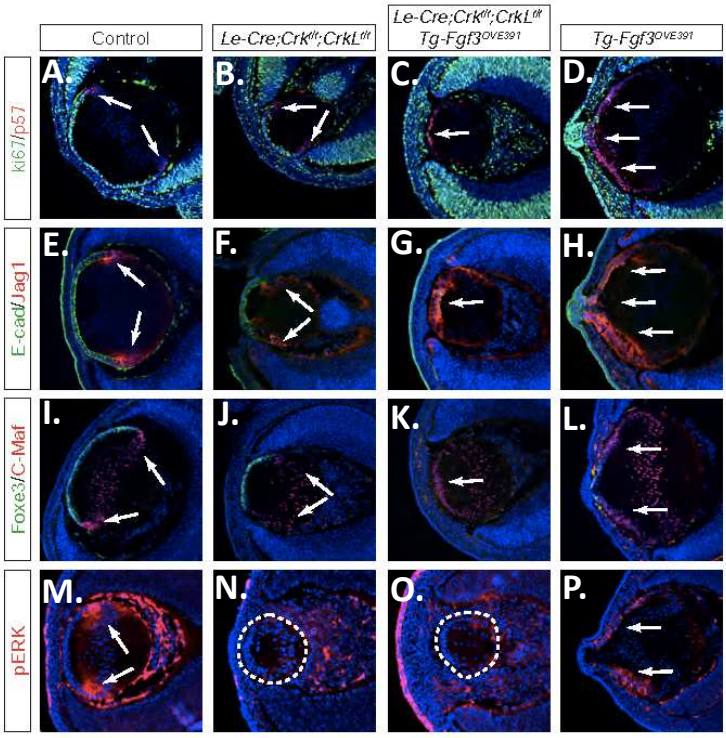

Figure 19. Crk/CrkL deletion prevents FGF-induced cell elongation without affecting differentiation. (A-K) The deletion of Crk and CrkL did not prevent the premature differentiation phenotype of the *Tg-Fgf3* lens indicated by the presences of three differentiation markers (p57, Jagged-1, and C-Maf) in the presumptive epithelial layer of the *Tg-Fgf3* and *Le-Cre;Crk*$^{flox/flox}$*;CrkL*^{floxflox}*;Tg-Fgf3* lens (see arrows).

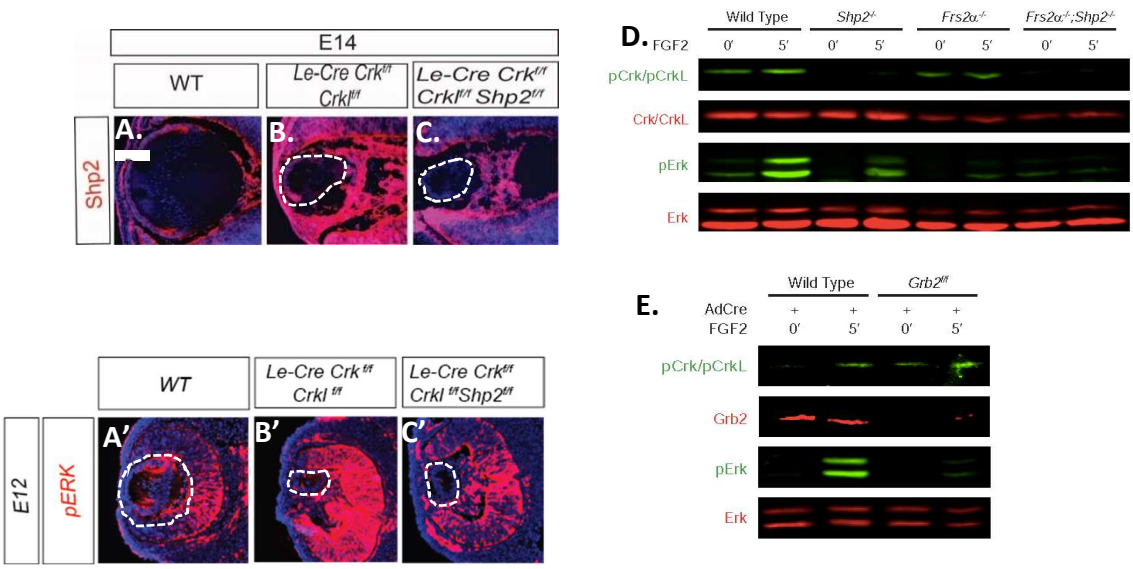

Figure 20. Crk/CrkL, Shp2, and Frs2 interact with each other during lens fiber cell elongation. (A-C) Shp2 was successfully deleted in the Le-Cre;Crk$^{flox/flox}$;CrkL^{floxflox}; Shp2 ^{floxflox}. D) phospho-Crk was drastically reduced in Shp2 deleted MEF cells. (E-G) Phospho-Erk staining was loss in the Le-Cre;Crk$^{flox/flox}$;CrkL^{floxflox}; Shp2 ^{floxflox} compared to the Le-Cre;Crk$^{flox/flox}$;CrkL^{floxflox} and the wild type.

3.2.2 Crk/CrkL and Shp2 have a synergistic relationship during lens fiber cell elongation

To address whether Crk is acting alone or synergistically with other proteins to mediate FGF signaling in regulating lens fiber cell elongation, I deleted Crk, CrkL, and Shp2 together in the lens at embryonic day 12 and 14. Complete deletion of Shp2 in the lens was confirmed by immunohistochemistry staining with Shp2 (Figure 22. A-C). Shp2 is a protein tyrosine phosphatase intimately involved in multiple cellular activities including cytoskeletal maintenance and cell differentiation (118). Shp2 is also a key factor in regulating the FGF signaling pathway during lens development (115). It was previously shown that conditional deletion of Crk and CrkL in the lens resulted in a decrease in phospho-ERK signaling at embryonic day 14 (Figure 15. C). This drastic loss of phospho-ERK was also present at embryonic day 12 (Figure 22. B'). Surprisingly, deletion of Crk, CrkL, and Shp2 together led to a complete loss of phospho-ERK staining (Figure 22. C'). Supporting this data, deletion of Shp2 in MEF cells prevents FGF signaling from stimulating phospho-Crk (Figure 22. D). This indicates that Shp2 controls the activity of Crk downstream during FGF signaling to mediate lens fiber cell elongation.

In addition to Shp2 being one of the main mediators of FGF signaling, Frs2 is another protein that is known to be essential for FGF signaling. Frs2 acts as a scaffolding or docking protein that is activated via its phosphorylation by receptor tyrosine kinases such as the FGF receptor. Once Frs2 get phosphorylated it contains binding sites for proteins like Shp2 that aid in the induction of essential cell signaling pathways (119). Therefore, I investigated whether deletion of Frs2 in MEF cells can also prevent phospho-Crk stimulation. Frs2

deleted MEF cells were stimulated with bFGF for five minutes and similar to Shp2 it also prevented phospho-Crk stimulation. Interestingly, although there was no phospho-Crk stimulation the basal level phospho-Crk still remained in Frs2 deleted MEFs cells but not in Shp2 deleted MEF cells. This could be partly due to Shp2 having another important role in mediating Crk that may lead to the loss of the basal level of phospho-Crk.

As mentioned previously, upon phosphorylation by a receptor tyrosine kinase, Frs2 has a docking site for Shp2 and other proteins such as Grb2 (Growth factor receptor bound protein 2). The role of Grb2 in FGF signaling has been extensively studied and it has been established that upon FGF stimulation Frs2 binds to the Grb2/SOS complex to initiate downstream signaling (120). Therefore, it is possible that Grb2 could also interact with Crk to induce FGF mediated lens fiber cell elongation. To investigate this, I conditionally deleted Grb2 in MEFs using the adenovirus Cre . Grb2 deleted MEF cells did not have an effect on phospho-Crk but as expected showed a drastic decrease in phospho-ERK. These data taken together reveal a novel pathway in which Crk, Shp2, and Frs2 work together to initiate FGF mediated lens fiber cell elongation.

3.2.3 Conditional Deletion of Rac1 in the Lens has a cell elongation defect

It has been established that Ras and Rho GTPases play a significant role in cell adhesion, cell spreading, and cell shape (121)(122). For example, Rap1 is a known Ras GTPase that is downstream of Crk and is targeted by the Crk SH3 binding domain guanine nucleotide exchange releasing factor C3G (123). In addition, activation of Rap1 is known to play a

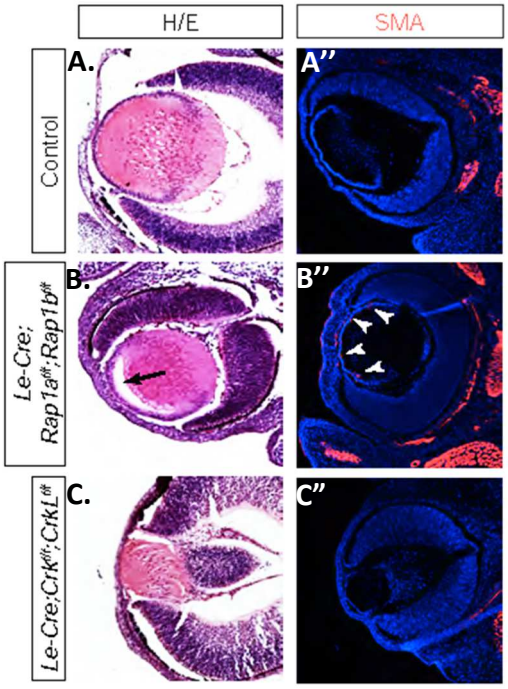

Figure 21. The GTPase Rap1 is not essential for lens development and lens fiber cell elongation. (A-C) H&E staining of the control lens, conditionally deleted Rap1a/Rap1b lens, and conditionally deleted Crk/CrkL at embryonic day 14. The Rap1depleted lens displayed a detachment of the lens fiber cells to the apical side of the lens epithelial layer. (A"-C'') Smooth Muscle Actin (SMA) staining of the control lens, conditionally deleted Rap1a/Rap1b lens, and conditionally deleted Crk/CrkL at embryonic day 14. SMA was present in the lens epithelial layer of the Rap1 depleted lens (indicated by arrow) but not in the control lens or the Crk/CrkL depleted lens.

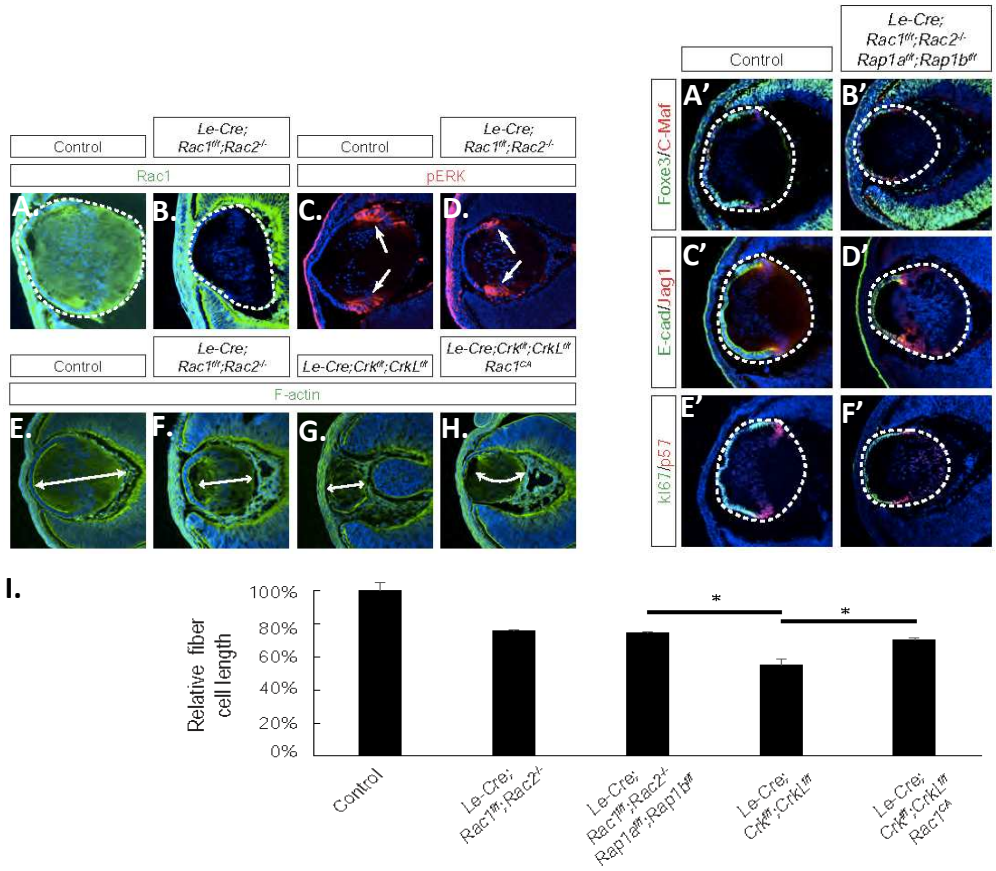

Figure 22. Conditional Deletion of Rac1 in the Lens has a cell elongation defect. (A-B) Staining of the lens at embryonic day 14 with Rac1 antibody. Rac1 appeared completely deleted in the Rac depleted lens. (C-D) Staining of the Rac depleted lens with phospho-Erk antibody showed no significant difference in staining intensity. (E-H) Phalloidin (F-actin) staining of the control, deleted Rac1 (conditionally using the cre-lox system) and Rac2, conditionally deleted Crk and CrkL, and condtionally deleted Crk and CrkL in the presences of constitutively active Rac1 lens at embryonic day 14. Lens fiber cell length was measured to observe fiber cell length (indicated by arrows). (A'-F') Immunohistochemistry staining of the Rac and Rap1 depleted lens with proliferation (Foxe3, E-cadherin, Ki67) and differentiation (C-Maf, Jagged 1,p57) markers. (I) Graph of relative fiber cell length. A student t-test was preformed on 3 different lens for each phenotype.

role in ERK activation and is also one of the main activators of the Integrin pathway (94). This motivated us to investigate whether deletion of Rap1 may also affect lens fiber elongation. Therefore, I conditionally deleted both Rap1a and Rap1b in the lens at embryonic day E14. The Rap1depleted lens had a slight reduction in size and displayed a defect in fiber cell attachment to the apical side of the lens epithelium (Figure 23. A-B). However, the Rap1depleted lens had none of the fiber cell elongation phenotypes observed in the Crk/CrkL mutant lens (Figure 23. B-C).

Previously, it was found that the Rap1 mutant displayed a phenotype that altered the epithelial plasticity of the the Rap1mutant lens indictated by the presences of an epithelial to mesenchymal (EMT) biomarker SMA (smooth muscle actin) in the lens epithelial layer (97). I used this phenotype to further assess if the role of Rap1 in lens development is mediated by Crk/CrkL. As previously reported when Rap1 was conditionally deleted in the lens at embryonic day 14 and SMA was present in the lens epithelial layer. Interestingly, in the Crk and CrkL conditional knockout lens SMA was not present in the lens epthelial layer. This data appears to rule out the idea that Rap1 has an essential component in lens development and lens fiber cell elongation.

As mentioned previously, Rho GTPases are essential for cell adhesion and cell morphology. One of the main Rho GTPases that is essential for the above cellular processes is the GTPase Rac. Rac is an important downstream mediator of Crk signaling and is essential for cell adhesion, actin polymerization, lamellipodia extension, and

differentiation (98). Therefore, it is possible the Rac could also play a significant role in lens fiber cell elongation. To address whether or not Rac also play a role in lens fiber cell elongation, I observed the phenotype of the lens depleted of Rac1 and Rac2 isoforms together. Staining with a Rac1 antibody confirmed complete deletion of Rac1 in the Rac depleted lens (Figure 24 A-B). Unlike the conditionally deleted Crk and CrkL lens, the Rac1 deleted lens did not display a decrease in phospho-Erk (Figure 24. C-D). However, Rac deletion resulted a lens fiber cell elongation defect, albeit milder than that of deletion of Crk and CrkL in the lens (Figure 24. E-G). This data was also confirmed by statistical analysis of the lens fiber cell length (Figure 24. I). To further explore the role of Rac1 in lens fiber cell elongation I employed a constitutively active Rac1. In the presence of the constitutively active Rac1, the Crk and CrkL conditional KO partially rescued the lens fiber cell defect. This data reveals a possible role of Rac1 in lens fiber cell elongation.

It is clear that both Rac1 and Rap1 are downstream effectors of Crk and or CrkL and have two separate phenotypes. However, it is possible that Rac1 and Rap1 can partly compensate for each other during lens development and could mask the true phenotype. Therefore, we condtionally deleted both Rac and Rap1 in the lens at embryonic day 14. The Rac and Rap1 lens was double stained with proliferation and differentiation markers to observe any changes that might have occurred as a result of both GTPases being deleted. Interestingly, deletion of both Rap1 and Rac1 in the lens had no additional phenotypes other than the phenotypes seen in both Rac and Rap1 alone. Deletion of both Rac1 and Rap1 seems to have an additive effect on the lens, displaying a phenotype similar to Rac1 alone with the addition of the apical attachment phenotype seen in the Rap1 mutant alone. There were

also no significant differences in the staining intensity or location for the proliferation and differentiation markers (Figure 24. A'-F'). The relative fiber cell length also confirms that deletion of both Rac and Rap1 in the lens was still signifcantly larger than the Crk and CrkL deleted lens. There was also no difference in the relative lens fiber cell length comparing the Rac deleted lens to the Rac1 and Rap1 deleted lens (Figure 24. I).

CHAPTER FOUR

4.1 Discussion and Future Direction

The role of FGF signaling in human development has been studied for many years because of the detrimental affect it has on fetal development during the gain or loss of function of FGF ligands and FGF receptors. For example, loss of the ligand FGF10 in the cause of Lacrimo-auriculo-dento-digital syndrome (LADD). LADD is an autosomal dominant disorder characterized by multiple phenotypes such as hypoplasia of the lacrimal and salivary gland, hearing loss, and dental anomalies (124). In addition , the loss of function of another ligand FGF3 is the cause of an autosomal recessive syndrome that causes congenital deafness due to a complete loss of the inner ear structure (125). Although, loss of function mutations is common in FGF ligands, gain of function mutations can also occur. A gain of function mutation in FGF23 causes autosomal dominant hypophosphataemic rickets (ADHR). Patients with ADHR have rickets due to the disruption of phosphate homeostasis caused by phosphate wasting (126).

As mentioned previously, mutations in FGR receptors also contribute to multiple development disorders. For example, loss of function mutations in FGFR1 is the main cause Kallmann Syndrome (127). Named after the geneticist who discovered it, Kallmann syndrome is associated with patients not being able to reach puberty and usually lack the ability to sense smell. A gain of function mutations in FGFR2 is the main cause of craniosynostosis, which is described as the inability of the fibrous sutures in the skull to fuse properly. Lastly, a Gly380Arg point mutation in FGFR3 can promote non-covalent

interactions between transmembrane helices and is present in most cases of achrondroplasia, one of the most common genetic forms of dwarfism (128).

To fully understand how the above disorders, mouse models have been one of the most useful tools in helping scientist understanding the role FGF signaling in human developmental disorders. For examples, a mouse model was generated for achrondroplasia by isolating the mouse FGFR3 gene and introducing a point mutation by changing Gly (GGG) to Arg (AGG) in codon 374 (the ortholog of human codon 380), which is located in exon 10. Mice with the Gly374Arg point mutation displayed a phenotype that was similar to that of the human Gly380Arg point mutation. Interestingly, mice homozygous for this mutation displayed kinky tails, exhibited overgrowth of the long bones, and contained a greatly expanded growth plate, unlike the heterozygous mice which only exhibited a form of dwarfism. Therefore, it was concluded that the expression of the achondroplastic phenotype depends on the expression of mature mRNA transcribed from the mutant allele. Using this same mouse model it was confirmed that achrondroplasia is caused by ligand-independent activation of the FGFR3 (129). Using models have also paved the way for understanding how to treat multiple genetic disorders like Huntington's disease (HD). HD is a neurological disorder causes by a polyglutamine expansion in the N terminus of the protein huntingtin (Htt). It is known that FGF2 deficient mice display impaired neurogenesis (130). Therefore, FGF2 treatment was administered to an established HD mouse model. Treatment with FGF2 induced neurogenesis and expanded life of the HD mice (131). Therefore, creating mouse models is excellent source of

information for investigator when it comes to understanding and eradicating developmental diseases.

Our laboratory and other laboratories have used mouse models to understand the FGF signaling pathway. In this study, I investigated the role of adaptor Crk and CrkL during FGF mediated lens development using various mouse models. The connection between Crk and CrkL and FGF signaling has been addressed, but not extensively. It is currently known that Crk is phosphorylated by the FGFR1 when it binds to tyrosine 463 which is dependent on FGF2 stimulation. It also understood that Crk is essential for FGFR-1 cell proliferation (68). Studies have also revealed a role for CrkL in Digeorge syndrome and other human disorders. Using a CrkL and FGF8 knockout mouse model, it was found that they both genetically interact with each other and more importantly FGF8 can induce phosphorylation of FGF receptors and their binding to CrkL. These study have begun to address the role of Crk and CrkL in development, however the molecular mechanism is still not clear. Crk and CrkL both play an essential role in cell adhesion, cell morphology, cell spreading, etc. Most of the above cellular activities are connected to ability of Crk to mediate the Integrin pathway. I aimed to understand the molecular mechanism and cellular processes associated with the interaction between Crk/CrkL and the FGF receptor.

To begin, I employed mouse genetics and conditional deleted Crk and CrkL in the mouse lens. The lens serves as a great model for studying cellular mechanisms and developmental processes due to its simplicity. The lens lacks blood vessels and is contains only one cell type that undergoes proliferation and differentiation to form the mature lens. In addition, the lens is derived from a single common cell called the lens precursor cell and will only

differentiate into lens epithelial cells and lens fiber cells which cause it to have a simple morphology. This simple morphology leads to the lens being one of the most useful organs to study developmental biology (12). One of the most valuable lens based studies was the establishment of the first line of transgenic mice that contained a tissue-specific promoter. In these particular studied it was a α-Crystallin promoter. Crystallin is considered the main protein present in the ocular lens (132). Lens based studies have also been crucial in understanding multiple aspects of cancers. A recent studied done by Jean Jiang group used the lens to understand the role of connexins in cell growth, cell differentiation, and tumorgenesis. From this study it was found that connexins play a role in regulating cell cycle modulators and eventually cell growth and differentiation (133).

Although the lens serves as a great model for studying developmental processes because its simplicity, there are some caveats to using the lens as a model. Using the lens as a model for development, scientist often run the risk of their data being lens specific when it comes to important cellular mechanism. An example of this is a study done by the Soriano lab in which they found the FGFR1 is mediated by Frs in some developmental contexts but other developmental processes like gastrulation and somitogenesis are controlled by FGFR1 independent of Frs (67). So it is possible that in this study Crk/CrkL plays a role in FGF signaling during lens development but not in other developmental processes.

There are also other organs that can serve as great models to study FGF signaling and development. FGF signaling has been implicated in being extremely important in heart development. To investigate how morphogenetic events are controlled by secreted

signaling, loss of function of FGFR1 and FGR2 in mouse was used to understand the role of FGF signaling in heart outflow tract development (134). This study is a great example of trying to understand signaling events using a mouse model and the heart as the organ of choice. Another example of a great organ that's used for study development is the development of the inner ear. In a mice that contained a null FGF3 and hypomorphic FGF8 fail to undergo otic induction. Indicating the importance of FGF signaling in otic induction (135). Lastly, FGF signaling is essential for another rigid organ called bone. FGF signaling has been studied many years for its role in skeletal development making it a great model to study developmental processes. A recent study was done to understand the role of FGF18 in autophagy. Chondrocyte autophagy is essential for bone growth. Chondrocytes are related to bone in that they are embedded in cartilage and secrete the matrix for cartilage. Cartilage is the essential connective tissue found in between bones. From this study it was found that FGF18 is a regulator of chondrocyte autophagy which was observed by mice lacking FGF18. The mice that lacked FGF18 displayed low levels of chondrocyte autophagy compared to wild type mice (136).

In addition to there being multiple organs that could have been use to study development and FGF signaling there are multiple model organisms that could have been used to investigate FGF signaling. In particular, there are other organisms that could have been used to study eye development. One widely used organism other than mice is the zebrafish. Zebrafish have been used to study eye development for many years. Similar to mice zebrafish exhibit multiple characteristic that make it a great model for studying development including the large number of offspring, its rapid embryonic growth, and

transparency of its embryos. When it comes to eye development in the zebrafish, the eye and retina are visually oriented similar to humans. This allows scientist to study the ocular development and various diseases of the eye (137). A recent study investigated the role of melanosomes in zebrafish lens development. Albino zebrafish, that completely lack melanosomes, developed abnormal lens reflection and increased oxidative stress. Suggesting that melanosomes in pigmented epithelial cells play a major role in protecting the lens from oxidative stress (138). Zebrafish have also been used to investigate the earliest development of the lens, as early as the pre-placodal cells. Zebrafish were used in identifying pre-placodal cells using cell-tracing experiments (139).

There is also another model organism that has been used frequently which is the drosophila melanogaster or the fruit fly. The compound eyes of the drosophila have been used to study various cellular activities including cell proliferation, differentiation, polarization, and migration. In addition, the drosophila has been use for understand multiple human diseases such as cancer and neurodegenerative disorders (140). There are several drosophila cancer models that have been established to understand metastasis. The Shearn group used an allograft experiment to understand the role *lgl* and *brat* in tumor metastasis. Metastic tumors mutant larvae where allograft into the abdomen of wild type adult drosophila. This resulted in rapid tumor and was the first evidence of proving that scientist are able to use drosophila to study metastasis (141).

As mentioned early drosophila has been a great model organism for understanding neurodegenerative disease. Alzheimer's diseases (AD) have been one of the main

neurodegenerative disease that has been investigated using a drosophila model. More importantly, most of the genes associated with AD have a drosophila homolog. The Amyloid Precursor Protein (APP) has a drosophila homolog APPL (APP-like). Drosophila with the APPL deleted displayed abnormal behavior and could be resued by a human APP transgene (142). Lastly, drosophila has been a great model for scientist who study the development of the eye. Drosophila has been used to investigate targets for Pax6 to carry out the function of eye development because although the structure of the drosophila eyes is very different from vertebrates, the molecular mechanism underlying eye pattern is similar. Therefore, it was established that drosophila could be used to study mammalian ocular development (143).

Lastly, chick embryos have been implicated as a great model in studying eye development. Similar to mice and drosophila, the chick embryo is an excellent model because of rapid development and its ability to be easily manipulated for experimentation. In particular chick embryos have mainly been used to understand the retina and retina development. For example, the chick embryo has been used to investigate the molecular mechanism responsible for retina regeneration. The chick embryo lends itself as a great model for studying retina regeneration mainly because of how accessible the embryo is for microsurgery and the ability of the retina to be replaced if damaged (144).

The chick embryo has also been used to investigate the formation of the ciliary body. It was found that FGF is responsible for mediating the induction of the ciliary body in the eye of the chick embryo (145). Lastly, the chick has been used to study lens development.

Specifically, using the lens of the chick embryo it was found that all pre-placodal cells are specified as lens, until FGF expressed in migrating neural crest cells leading leads to the promotion of the olfactory fate (146). Therefore, the chick would have also been a great model to use for this study. However, our established mouse models was an excellent choice for this study.

In this study, I demonstrated that during lens development Crk and CrkL individual mutants did not have a severe phenotype and these two protein could compensate for each other. The thought that Crk and CrkL can compensate for each other as been controversial. Although the homology between Crk and CrkL is similar in terms of the SH2 and SH3 domain they have distinct roles in multiple cellular processes. In addition, mice that have either Crk or CrkL deleted systemically die but from very different causes (53). In embryonic fibroblast cells that have Crk II deleted, CrkL was not overexpressed, indicating that there is no compensation by CrkL. This was also evident for cells that have CrkL deleted (147). There is also evidence that Crk and CrkL can compensate for each other. Crk adaptors have been implicated in playing a role in the reelin pathway. Stimulation of the reelin pathway enhances dendritogenesis in the hippocampal neurons of mice in a Src dependent manner. It was found that this stimulation was only blocked by decrease expression of both Crk and CrkL. Indicating that Crk and CrkL can compensate for each other during dendritogenesis (148).

However, deletion of both Crk and CrkL in the lens results in a severe phenotype consisting of defects in lens morphology, cell proliferation, cell adhesion, the extra-cellular matrix, and cell elongation. However, one of the most striking phenotypes in this study

was the rotation of the lens epithelium caused by the deletion of Crk and CrkL in the lens. How or why Crk and CrkL is causing the rotation of lens epithelium hasn't been addressed. There are several theories that could explain this phenotype. One theory could be that Crk and CrkL plays an important role in planar cell polarity. Planar cell polarity is the polarization of cells with in the plane of a cell sheet and is essential for the function of multiple biological processes. In particular, planar cell polarity is mostly seen in epithelial tissues in which cells within an epithelium often display polarity across the plane of the epithelium (149). To investigate whether disruption of the planar cell polarity is the cause of the lens epithelium rotation immunohistochemistry could be perform on the Crk and CrkL knockout lens with planar cell polarity markers such as c-Jun terminal kinase (JNK) which is known to be downstream of the GTPases Rac and Rho in the β-catenin independent planar cell polarity pathway (150). Other possible markers can include disheveled 1/2/3, and Daam-1, both key factors in the planar cell polarity pathway.

Another reason that the lens epithelium is rotated in the Crk and CrkL knockout lens could be because the lens epithelial cells have a cell migration defect. Specifically, lens epithelial cells may not be able to migrate properly and completely form the lens epithelium. Crk activated Rac is important for the formation of membrane protrusions by actin polymerization during cell migration (151). To investigate this a scratch assay and or an in vitro cell migration assay can be done using lens epithelial cells such as TN4α, presumable with Crk and CrkL knockdown in these cells.

Deletion of Crk and CrkL also attenuated a gain-of-function phenotype caused by overexpression of FGF3. The gain-of-function phenotype, seen in the lens by the overexpression of FGF3, was first discovered by Micheal Robinson's group on their quest to find the role of FGFs and FGF receptors during lens development. It was shown that the overexpression of FGF3 in the developing lens of transgenic mice, driven by a αA-crystallin promoter, caused abnormal cell elongation and differentiation of lens epithelial cells. The phenotype of embryos beginning at E12.5 also consisted of ocular proptosis and eventually the rupturing of the cornea (44). Taking advantage of this unique phenotype our laboratory recently addressed the role of heparan sulfates in FGF signaling during lens development. Disruption of Ndst-mediated heparan sulfate modifications led to a small lens and attenuation of phospo-ERK, indicating the down regulation of FGF signaling. Interestingly, the overexpression of FGF3 in the embryonic lens leads to ERK hyper-phosphorylation. However, in an embryonic lens with both the deletion of Ndst and overexpression of FGF3, the ERK hyper-phosphorylation was decreased and abnormal differentiation of lens epithelial cells was abolished. These results indicate that the Ndst mutation is epistatic to FGF3 overexpression and the function of Ndst is essential for FGF3 signaling during lens development (46). Using this same approach our lab was the first to show that Crk and CrkL is also important for FGF3 mediated lens development indicated by the decrease in ERK hyper-phosphoryation and loss of abnormal differentiation in the embryonic lens. However, the question of whether or not the activity of Crk and CrkL is specific to the FGF3 still remains. There is another transgenic mouse that was developed by Micheal Robinson and colleagues, that overexpresses human FGF1 in the developing lens. Similar to the overexpression of FGF3, at embryonic day 15 the lens exhibited

abnormal lens epithelial cell elongation, lens vacuolization, and cataract. Therefore, it would be interesting to see if deletion of Crk and CrkL could attenuate the phenotype displayed by the overexpression of FGF1.

Deletion of Crk and CrkL in the lens led to a decrease in activity of the downstream effector ERK. This data is not surprising in that it has been shown that deletion of Crk in MEF cells show a decrease in ERK activity in the presences of FGF8 (69). However, it would be interesting to see what happens if ERK is conditional deleted in the lens. It is expected that conditional knockout of ERK in the lens would have a cell elongation defect similar to the Crk and CrkL knockout lens. However, it is also possible that the activation of ERK by Crk is not important for lens fiber cell elongation but could be important for other cellular processes like cell proliferation. In this study I did show that deletion of Crk and CrkL in the lens led to a decrease in lens epithelial cell proliferation.

The deletion of Crk and CrkL could be rescued by constitutively active Kras. It is not surprising that Crk and CrkL can interact with Ras. However, what is surprising is the fact tha Crk and CrkL is upstream of Ras during FGF mediated lens fiber cell elongation. The Crk adaptor proteins are known to be upstream of various GTPases so to reveal specifically that it is upstream of Ras during lens fiber cell elongation is novel. Also, deletion of Crk and CrkL in the lens displayed several phenotypes that could not be rescued by the constitutively active Kras such as the rotation of the lens epithelium, defects in the extracellular matrix, and abnormal cell adhesion. This data suggests the idea that the Crk and CrkL have additional roles in lens development independent of Ras signaling pathway.

Crk and CrkL have been extensively studied for its role in the Integrin pathway. Crk and CrkL are responsible for integrin mediated cell adhesion, actin polymerization, cell migration, and cell polarity (52,55). The known roles of Crk and CrkL in Integrin signaling is consistent with the phenotypes seen in Crk and CrkL mutant lens, explaining why these phenotypes could not be rescued by Kras.

The involvement of Crk and CrkL in multiple processes made it imperative that in this study I narrowed down the role of Crk and CrkL in lens development. Lens fiber cell differentiation is a large part of lens development and is essential for the function of the lens. What is very interesting is how Crk and CrkL can control ERK activity but have no effect on differentiation. Especially since lens fiber cell differentiation is characterized by lens fiber cell elongation and ERK is essential for lens fiber cell differentiation (152). This is could be possible, through a mechanism in which Crk and CrkL are recruited to the FGF signaling pathway after lens fiber cell differentiation to promote lens fiber cell elongation. This is possible because when Crk and CrkL is deleted in the lens, the lens fiber cell differentiation isn't affected indicated by the abundant staining intensity of multiple differentiation markers.

Concerning the regulation of Crk adaptor proteins in FGF signaling during lens development, Crk activity is surprisingly controlled by the phosphatase Shp2, indicated by the loss of phospho-Crk when Shp2 is deleted in MEF cells. However, how exactly Crk and CrkL interact with Shp2 is still not understood. Therefore, to further confirm the interaction with Shp2 and Crk it would be interesting to know what are the specific binding sites of Crk and CrkL on Shp2. Shp2 has two tyrosine phosphorylation sites Tyr-542 and

Try-580 that are known to activate ERK in FGF stimulated MEF cells (153). It would be nice to explore whether or not mutations of both of these tyrosine phosphorylation sites on Shp2 in MEF cells cause a decrease in the activation of Crk similar to the loss of observed in this study when Shp2 was deleted in MEF cells. A caveat to this is that Crk and CrkL may not directly bind to Shp2 but could bind to another protein downstream or upstream of Shp2. Proteins such as Grb2 are known to interact with Shp2 to activate downstream Ras. In a recent study to identify CrkL binding proteins in myotubules using a tagged form of CrkL and spectrometric analysis, Grb2 was ranked number eight in the a group of sixty-one proteins that are considered the most abundant proteins bound to CrkL in myotubules (154). Therefore, Grb2 is a strong candidate for the connection between Crk and Shp2 in FGF mediated cell elongation. However, deletion of Grb2 in MEF cells reveal that when stimulated with FGF there is no significant differences in phospho-Crk between wild type MEF cell and the Grb2 deleted MEF cells. This supports the idea that Shp2 could directly binds to Crk.

However, there is a candidate protein that could be upstream of Crk and CrkL in addition to Shp2, called Frs2. Frs2 is a docking protein that is responsible for the recruitment of multiple molecules to the FGF receptor (155). In addition, our lab has shown that deletion of both Shp2 and Frs2 in the lens causes a severe defect in the lens compared to either alone (48). This study also shows that phospho-Crk was not stimulated by FGF2 in Frs2 deleted MEF cells. Therefore, it would be interesting to investigate the genetic interactions between Crk, CrkL, and Frs2. Conditional deletion of all three molecules in the lens could

further explain whether or not they interact indicated by a more severe phenotype than Crk and CrkL alone and loss of downstream FGF effectors such as phospho-ERK.

The idea that Shp2 can heavily control the activity of Crk promotes the idea that Shp2 is really important for lens fiber cell elongation and deletion of Shp2 alone in the lens would produce a phenotype similar to the Crk and CrkL deleted lens. It is important to note that, Shp2 has been known to play an important role in Focal Adhesion (FA) formation and cell morphology (156). It would also be a great idea to take advantage of cell biology techniques by observing cell morphology and focal adhesion in Shp2 deleted MEF cells. The cells could also be stained with proteins that are known to be in the focal adhesion complex such as phospo-Paxillin and Integrin.

Crk and CrkL are known to activate other GTPases such as Rac1 and Rap1 to induce downstream signaling. In my study, conditional deletion of both Rac and Rap1 in the lens displayed phenotypes less severe than Crk and CrkL mutant lens. It is unlikely that Rap1 is downstream of FGF signaling because it does not have any of the Crk and CrkL mutant lens phenotypes such as a defect in cell elongation. In addition, the Crk and CrkL mutant lens doesn't display the key phenotypes seen in Rap1 mutant lens. This is definitely possible because Rap1 is important for regulating the ERK/Ras pathway possible by trapping Raf in an active complex thereby inhibit ERK activation. ERK activity would still be functional when Rap1 is deleted which why I might not see the lens fiber cell elongation defect seen in the Crk and CrkL condtional knockout lens. This could also mean that deletion of Rap1 in the lens could rescue the Crk and CrkL deleted phenotype.

What is definite is that Rap1 does play a significant role in lens development (97). In our laboratories quest to investigate lens development it would interesting to find another novel pathway in which Rap1 is important for other cellular processes that are related to is phenotype like cell adhesion. Although, the role of Crk in Rap1 signaling has been extensively investigate that are other stimulators or environmental factors that could activate Rap1 during lens development (157). On the other hand, although less severe, Rac1 mutant lens does have a cell elongation defect and exhibits a reduction in lens size similar to Crk and CrkL mutant lens. Previous studies done by the Rao group show that growth factor can induce activation of Rho and Rac GTPases in human lens epithelial cells (101). It would be interesting to see if the Rac mutant lens could be rescued by Kras. Notably, Lambert and colleagues recently showed that the guanine nucleotide exchange factor Tiam mediates Ras activation of Rac during PI3K signaling (158).

I also employed a constitutively active Rac1 to see if the Crk and CrkL deleted lens fiber cell elongation defect could be rescued. It is clear that the Crk and CrkL lens fiber cell elongation defect could partly be rescued but not fully. This was also true in the presences of a constitutively active Kras. This indicates that both of these GTPases are essential for lens fiber cell elongation. What isn't clear is the cellular mechanism. Are they both control by the FGF signaling pathway? To answer this question there is a plethora of experiments that could be done. I could first see if Ras could rescue the the Rac1 conditional deleted lens phenotype. This was possible because as mentioned previously Ras can activate Rac.

It would also be important to create a mouse model with the Crk and CrkL conditional knockout lens in the presences of both the constitutively activate Kras and Rac.

Once Crk and CrkL is activated our study predicts the binding of a GEF to Crk and CrkL leads to the activation of the GTPases Ras. The GEF that leads to GTPase activation in our novel pathway is unknown. Crk and CrkL is known to bind to several GEF including C3G, DOCK180, and SOS. Knowing the GEF that is responsible for Ras activation could add to the specificity of the proposed pathway. Conducting a Co-Immunoprecipation assay with Crk/CrkL and the GEFs above would be the possible approach to answering this question. Specifically, this study has confirmed that the FGF signaling pathway is mediating lens fiber cell elongation with the help of Crk and CrkL. However, we cannot rule out the possible of crosstalk between the FGF signaling pathway and the integrin pathway. Crk and CrkL are essential for integrin signaling and plays a role in cell morphology, cell adhesion, cell migration, etc (65). Although in the presences of a constitutively active K-ras, the lens size and elongation defect of the Crk and CrkL knockout lens was rescued but it still wasn't complete rescued compared to wild type. This opens up ideas for the involvement of other pathways, like the integrin pathway, in lens fiber cell elongation. Contributing to this idea conditional deletion of Rac in the lens showed a phenotype similar to Crk and CrkL. Rac is known to be downstream of the Integrin pathway and is activated when Crk binds to the GEF DOCK180 (53). Thus, the idea that the Integrin pathway could be contributing to lens fiber cell elongation should be explored possible through deletion of the various Integrin's that are expressed in the lens. The proposed experiment would be to observe if there is a cell elongation defect when Integrin is deleted in the lens.

Lastly, data from this study strongly supports the role of Crk and CrkL in FGF mediated lens fiber cell elongation. To support this theory in vitro studies using lens epithelial explants is worth exploring. Using lens epithelium explants will give us the opportunity to observe lens fiber cell elongation in the presence of FGF in wildtype and Crk and CrkL knockout lens epithelial explants. It is expected that in the presences of FGF the wild type lens epithelial cells in the explant will differentiate into lens fiber cells and elongate. On the other hand, in the Crk and CrkL knockout lens explant, the epithelial cells will maintain their cuboidal shape and not elongate in the presences of FGF. Immunocytochemistry with epithelial and differentiation markers will confirm the differentiation of epithelial cells to lens fiber cells. Lens fiber cell length would also be quantified. In conclusion, data from this studies strongly support a novel pathway that is important for mediating lens fiber cell elongation, a mechanism that has not be extensively explored until now. However, there are still multiple experiments that can be done to support this study.

CHAPTER FIVE

5.1 Summary

Cell shape is essential to the function of multiple cells types and is associated with multiple disease such as sickle cell disease and cancer. Patients with sickle cell disease present with abnormal shaped red or sickled blood cells that is caused by a mutation in the hemoglobin gene. This cell shape change causes red blood cells to stick to the wall of the blood vessel and lead to a blockage in blood flow preventing the distribution of oxygen to multiple tissues. Along these same lines, changes in cancer cell morphology due to environmental cues is associated with tumor metabolism and or metastasis spreading. Therefore, it is imperative that the molecular mechanism behind cell shape changes is fully understood.

Cell shape plays a major role in the development of the lens during lens fiber cell elongation. During lens development, lens epithelial cells will migrate down from the germinative zone of lens, differentiate into lens fiber cells and elongate along the anterior and posterior of the lens. The elongation of the lens fiber cell is an important cell shape change that is valuable for the development of the lens. However, the molecular mechanism that controls this is poorly understood. The FGF signaling pathway is required for lens development but its involvement in lens fiber cell elongation is not clear. In addition, adaptor Crk and CrkL are likely candidates for mediating cell elongation because of their known roles in cell adhesion, cell migration, and cytoskeleton reorganization.

Therefore, in this study we investigated the role of adaptor proteins Crk and CrkL in FGF signaling during lens development. We employed the cre-lox system to conditional delete Crk and CrkL in the lens. At E14 the lens had a severe phenotype consisting of a small lens, rotation of lens epithelium, and disorganization of the lens fiber cells. Immunostaining with the proliferation marker Ki67 revealed a decrease in proliferating cells indicating a cell proliferation defect. TUNEL analysis also showed an increase in cell apoptosis which partly explains the small lens phenotype. The Crk and CrkL knockout lens also displayed a defect in cell adhesion and the extracellular matrix indicated by loss in prominent cell adhesion and cell membrane markers such as paxillin and collagen, respectively.

To confirm a role of Crk and CrkL in FGF signaling during lens development I stained the Crk and CrkL knockout lens with activated Erk (phospho-Erk), a downstream effector of the FGF signaling pathway. There was a clear reduction of phospho-Erk in the knockout lens compared to wild type. To support this data using western blot analysis, deletion of Crk and CrkL in MEF cell stimulated with FGF2 had a decrease in phospho-Erk. In addition, Crk and CrkL is epistatic to the FGF receptor and the lens size and cell elongation defect in the Crk and CrkL mutant lens could be rescued by a constitutively active K-Ras. Data from this study also showed that during lens development cell elongation is independent of differentiation.

To observe whether there is a synergistic relationship between Crk and other proteins downstream of the FGF receptor, Crk, CrkL, and the phosphatase shp2 was conditionally

deleted in the lens. Loss of all three molecules exhibited a complete loss of phospho-Erk in the lens. This data along with loss of phospho-Crk in Shp2 deleted MEF cells stimulated with FGF2 shows that Shp2 can control Crk activity during FGF signaling. Lastly two GTPases, Rac and Rap1 that are known to be downstream effectors of Crk and CrkL were analyzed for their role in lens fiber cell elongation. Conditional deletion of Rac and Rap in the lens exhibited a very different phenotype in the lens, with Rac having a phenotype similar to the Crk and CrkL mutant lens. Rap however, had a less severe phenotype compared to Crk and CrkL consisting of a slightly smaller lens and disruption in the attachment of the apical end of lens fiber cells to the lens epithelium. Although Rap1 is excluded for being involved in lens fiber cell elongation, Rac is still under investigation do to its similarities to Crk and CrkL when its deleted in the lens. Therefore, this data taken together reveals a novel Crk-Shp2-Ras pathway important for FGF mediated lens fiber cell elongation.

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Curriculum Vitae

Tamica N. Collins

Education

Indiana University, Indianapolis, IN — November 2016
Doctor of Philosophy, Medical and Molecular Genetics

Jackson State University, Jackson, MS
Master of Science, Biology — August 2009
Bachelor of Science, Biology — May 2007

Teaching Experience

Presenter and Volunteer, BIOBUS New York, NY — 2015-2016

- An educational not-for-profit project based in New York City dedicated to inspire students to love science
- Presented current scientific research on the BIOBUS to the general public at Figment NYC
- Helped conduct scientific experiments with elementary students at the home facility for the BIOBUS, the BIOBASE

NYAS Teaching Fellow, Afterschool Mentoring Program,
New York, NY — Fall 2014

- Nationally recognized program geared towards mentoring K-8th grade students in Science and Technology
- Conducted scientific experiments at PS165 elementary school once a week for 60–90 minutes
- Managed a classroom with 15-20 students

Organizer, College Preparatory Academy/St. Vincent MICI-AHE,
Indianapolis, IN — Spring 2013

- Organized visit to IUPUI School of Medicine for student in the College Preparatory Program
- Coordinated scientific experiments and organized lab tours with students during visit to IUPUI School of medicine
- Served as a moderator for a graduate and medical student panel for students during visit to IUPUI School of Medicine

Teacher, Imagine Indiana Life Science Academy Indianapolis, IN — 2011-2012

- Presented short lectures on various scientific topics to students grades K-8 for weekend science program
- Engaged students with fun a creative scientific experiments
- Mentored underrepresented Minority Students

Teacher, Hoosier Health Academy Health Career Opportunity Program, Indianapolis, IN Spring 2010

- Lectured high school students on basic genetics and genetic traits
- Organized a scientific experiment to help students understands that basis of genetic traits
- Established a Mentor-Mentee Relationship with multiple students

Lab Instructor, Jackson State University, Jackson MS 2008-2009

- Taught genetics laboratory course to 25-30 undergraduate students
- Created quizzes and test to assess student progress in the genetic lab course
- Collaborated with genetics teacher to create a curriculum that was cohesive with the general genetics course

Research Experience

Indiana University School of Medicine, Indianapolis IN 2009-2016
Columbia University, New York
Doctoral student in Dr. Xin Zhang Laboratory
Ph.D. Thesis Project: The Role of Adaptor Proteins Crk and CrkL in Lens Development

Jackson State University, Jackson MS 2008-2009
Graduate Student in Dr. Stephen Ekunwe Laboratory
Master Thesis Project: Bioactivity and Mechanism of Action of Fraction P_2 and $P_{3\text{-}1}$ of *Ocimum Gratissimum (Og)* Leaf Extract in Prostate Cancer Cells

Indiana University School of Medicine, Indianapolis IN 2008
Summer Graduate Student in Dr. Alexander Dent Laboratory
Summer Project: The Regulation of p53 In Breast Cancer and Estrogen

Indiana University School of Medicine, Indianapolis IN 2007
Summer Graduate Student in Dr. Maureen Harrington Laboratory
Summer Project: Signal Induces Changes in Subcellular Location of IRAK-1 Integrates LPS and TNFR1 Signaling Pathways

Jackson State University, Jackson, MS 2005-2007
Undergraduate Student in Dr. Naomi Campbell Laboratory
Research Project: Effects of 1-Butyl-3-methylimidazolium Chloride on Protein Unfolding

University of California San Francisco, San Francisco, CA 2006
Summer Undergraduate Student in Dr. William Weiss Laboratory
Summer Project: The Role of Tumor Suppressors RIN and CCK in Neuroblastoma

Scientific Contributions

Hongge Li, Chenqi Tao, Zhigang Cai, Kristina Hertzler-Schaefer, **Tamica N Collins**, Yu Shao,Fen Wang, Gen-Sheng Feng, Noriko Gotoh, and Xin Zhang. (2014) Frs2α and Shp2 signal independently of Gab to mediate FGF signaling in lens development. *J Cell Sci* 127: 571-582.

Tamica Collins, Xin Zhang. Crk and CrkL Mediate FGF signaling in Lens Cell Elongation (2016). Manuscript in preparation submitting to *Development.*

Honors/Awards

Columbia University

Faculty of 1000 specialist of the year 2015

Gordon Research Conference: Carl Storm Underrepresented Minority Fellowship 2014

New York Academy of Science (NYAS) Teaching Fellowship and Credential 2014

IU School of Medicine

Edward T. Harper Scholar IU School of Medicine 2012

Jackson State University

NIH Bridge to the Doctorate Degree Fellowship 2007-2009

NIMH-COR Research fellowship 2005-2007

ABRCMS Travel Grant Awardee 2006

MS Academy of Sciences-2nd place Poster Winner 2006

Valedictorian /Salutatorian Scholarship/ Jackson State University 2003-2007

Services

Toastmasters' club "T.I.C. Toastmasters" 2015-Present

Secretary

Keep track of new and old memberships and record minutes during meetings

Faculty of 1000 2015-Present

Specialist contact

Answer any questions related to use F1000Prime, F1000Workspace, F1000Research for Columbia University Faculty and Students. Arrange workshop and seminars about Faculty of 1000.

Women in Science at Columbia (WISC)/ Curiosity Machine 2014-2015

Co-chair

Organized visit to elementary schools for Curiosity Machine. Mentored students around the United States online through Curiosity Machine

IU School of Medicine 2012

Managed and mentored undergraduate student Dr. Xin Zhang Laboratory

Presentations

Columbia University Cell Biology Research Group 2015
Oral: Crk and CrkL Mediate FGF signaling in Lens Cell Elongation.

Columbia University Neuroscience Outreach "Late Night Science Talk" 2015
Oral: How Cells Communicate During Lens Development

Gordon Research Conference: Signaling by Adhesion Receptors 2014
Poster: The Role of Adaptor Proteins Crk and CrkL in Lens Development.

Mississippi Academy of Science 2007
Poster: Signal Induces Changes in Subcellular Location of IRAK-1 Integrates LPS and TNFR1 Signaling Pathways.

NIMH-COR National Conference 2006
Poster: Effects of 1-Butyl-3-methylimidazolium Chloride on Protein Unfolding.

ABRCMS National Conference 2006
Poster: Effects of 1-Butyl-3-methylimidazolium Chloride on Protein Unfolding.

Professional Training

Course on "An Introduction to Evidence-Based Undergraduate STEM teaching" 2015
Received Statement of Accomplishment

Preparing Future Faculty 2012-2013
Attended workshops on academia and how to prepare yourself for a faculty positions.

Course on "Rhetorical principles in preparing and delivering written and Oral presentations" 2011

Course on "Experimental Design and Grant Writing" 2010

Organizations and Professional Memberships

Toastmasters International T.I.C. Club 2015-Present
Metro NY Association for Women in Science 2015-Present
WISC (Women in Science at Columbia at Columbia University 2014-Present
The New York Academy of Science 2014-Present
The Underrepresented Professional and Graduate Student Organization at IUPUI 2011-2013