Mukesh Kumar Sahu. THE ROLE OF RAL-INTERACTING PROTEIN OF 76 kDa (RLIP76) IN THE DEVELOPMENT OF OCULAR TISSUES. August 2013. Total number of pages – 114; 17 illustrations; 4 tables; 137 references.

Abstract: RalBP1/RLIP76 is a ubiquitously expressed protein, involved in the promotion and regulation of functions initiated by Ral and R-Ras small GTPases. Presence of multiple domains in its structure enables RLIP76 to be involved in a number of physiological processes such as endocytosis, exocytosis, mitochondrial fission, actin cytoskeleton remodeling, and transport of exogenous and endogenous toxicants. Previous studies in Dr. Awasthi's laboratory have established that RLIP76 provides protection to ocular tissues against oxidative stress by transporting the glutathione-conjugates (GSH-conjugates) of the toxic, electrophilic products of lipid peroxidation (e.g. 4-Hydroxynonenal) generated during oxidative stress. In particular it was demonstrated that by transporting the GSH-conjugates of 4-Hydroxynonenal (GS-HNE), RLIP76 protects ocular tissues against oxidative stress.

Rationale: It was reasoned that lens specific RLIP76 Tg mice due to their enhanced capability to detoxify 4-HNE would be more resistant to HNE-induced cataract formation. We engineered lens specific RLIP76 transgenic mice (RLIP76 Tg) to delineate the role of RLIP76 as a protective mechanism in the lens.

Results: Surprisingly, lens specific RLIP76 Tg mice showed impairment in the development of lens and eye, and a phenotype with small eyes similar to that observed in microphthalmia. I report here for the first time a novel mouse model of human genetic disorder microphthalmia. In this dissertation, I report engineering of lens specific RLIP76 Tg mice, characterization of the associated phenotype, and the possible molecular mechanisms that lead to the impaired

development of lens and eye in these mice. Briefly, the lens specific RLIP76 Tg mice show remarkably small eyes with impaired lens development, disrupted cytoskeleton organization and aberrant fiber cells differentiation. The results of microarray analysis indicated that genes involved in pathways for G-Protein signaling, actin reorganization, endocytosis, and apoptosis are affected in these transgenic mice. The expression of transcription factors, Pax6, Hsf1, and Hsf4b known to be involved in lens development was down regulated in the lens of these Tg mice. The expression of heat shock proteins (HSPs), the downstream targets of Hsfs was differentially affected in the lens showing down regulated expression of Hsp27 and Hsp40, up-regulated expression of Hsp60, and no effect on the expression of Hsp70, and Hsp90. Our results show that the disruption in the organization of actin cytoskeleton of these Tg mice was associated with the inhibition of the activation of Cdc42 that is known to regulate the organization of actin cytoskeleton. Fiber cells differentiation is required for development, growth, and transparency of the lens. Our data suggest disrupted cytoskeleton organization in Tg mice is caused by aberrant differentiation of lens epithelial cells into lens fiber cells.

Conclusions and significance: Present studies show that lens specific RLIP76 Tg mice show phenotype similar to microphthalmia and these mice may provide useful animal model for elucidating the mechanisms of lens development, and etiology of microphthalmia.

THE ROLE OF RAL-INTERACTING PROTEIN OF 76 kDa (RLIP76) IN THE DEVELOPMENT OF OCULAR TISSUES

DISSERTATION

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LIST OF ABBREVIATIONS

AA	Amino acid
A/M	Anophthalmia and/or Microphthalmia
ASC	Anterior subcapsular cataract
AP2	Adaptor protein 2
ATP	Adenosine-5'-triphosphate
BCOR	BCL-6 co repressor
BMP4	Bone morphogenetic protein 4
BMP7	Bone morphogenetic protein 7
Cdc2	Cell division control protein 2 homolog
Cdc42	Cell division control protein 42 homolog
CDE	Clathrin-dependent endocytosis
cDNA	Complementary Deoxyribonucleic acid
CDNB	1-chloro-2, 4-dinitrobenzene
DAVID 6.7	Database for Annotation, Visualization and Integrated Discovery 6.7
DNA	Deoxyribonucleic acid
DNP-SG ATPase	Dinitrophenyl S-glutathione ATPase
DTNB	5, 5-dithio-bis 2-nitrobenzoic acid
ECL	Enhanced chemiluminescence
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid

EGF	Epidermal growth factor
EMT	Epithelial-Mesenchymal Transition
FGF	Fibroblast growth factor
FOXE3	Forkhead box protein E3
GAP	GTPase activating Protein
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GDF6	Growth differentiation factor 6
GDI	GDP dissociation inhibitor
GDP	Guanosine diphosphate
GEF	Guanine nucleotide exchange factor
GGTase	Geranyl-geranyl transferases
G protein	Guanosine nucleotide-binding proteins
GPx	Glutathione peroxidase
GSH	Glutathione
GSSG	Glutathione disulfide
GSTs	Glutathione S-transferases
GTP	Guanosine-5'-triphosphate
HCCS	Cytochrome c-type heme lyase
H&E	Hematoxylin and Eosin
4-HNE	4-Hydroxy-t2-nonenal
HSE	Heat shock element
Hsf1	Heat shock transcription factor 1
Hsf2	Heat shock transcription factor 2

Hsf4b	Heat shock transcription factor protein 4b
Hsps	Heat shock proteins
HLE-B3	Human lens epithelial cells-B3
kDa	kilo-Dalton
LPO	lipid peroxidation
MDA	malondialdehyde
MITF	Microphthalmia-associated transcription factor
MSA	Methanesulfonic acid
NADPH	Nicotinamide adenine dinucleotide phosphate
NCK2	Non-catalytic region of tyrosine kinase adaptor protein 2
OTX2	Orthodenticle homeobox 2
PAK	p21-Activated kinase
PAX6	Paired box gene 6
PBD	p21-binding domain
PCO	posterior capsular opacification
PCR	Polymerase chain reaction
PDK1	Cyclin-dependent kinase 1
PI3 kinase	Phosphatidylinositide 3-kinases
PMSF	Phenylmethylsulfonyl fluoride
POB1	Partner of RalBP1
Ral-GDS	Ral guanine nucleotide dissociation stimulator
RalBP1	Ral-binding protein 1
RIP1	Ral-interacting protein 1 (RIP1)

RIPA buffer	Radioimmunoprecipitation assay buffer
RLIP76	Ral-interacting protein of 76 kDa
RLIP76 Tg Mice	RLIP76 transgenic mice
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RPE	Retinal Pigment Epithelium
RTKs	Receptor tyrosine kinases
RT-PCR	Real time polymerase chain reaction
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SHH	Sonic hedgehog
siRNA	Small interfering RNA
SKAP2	Src kinase-associated phosphoprotein 2
SOD	Superoxide dismutase
SOX2	SRY (sex determining region Y)-box 2
TBS	Tris-buffered saline
TE buffer	Tris-EDTA buffer
TGFβ	Transformation growth factor β
VSX2	Visual system homeobox 2
UV	Ultraviolet
WT	Wild type

CHAPTER I

INTRODUCTION

1.1 Ral binding protein 1 (RalBP1) or Ral-interacting protein of 76 kDa (RLIP76)

Ral binding protein 1 (RalBP1), molecular weight 76 kDa, is an effector protein for two Ras family GTPases; Ral and Rho/Rac [1, 2]. It is known by different names in various species, for example, it has been referred in mouse as the Ral-interacting protein 1 (RIP1) [2], in rat as Ral binding protein 1 (RalBP1) [3] and in humans as Ral-interacting protein of 76 KDa (RLIP76) [1, 4]. While these names of RLIP76 may be interchangeably used in this dissertation, for the sake of consistency the majority of times I have used the name RLIP76. RLIP76 is ubiquitously expressed in most tissues including, lung, heart, brain, kidney, testis, liver, spleen, skeletal muscle and eyes [5, 6]. At the sub-cellular level, it is localized in the cytoplasm, plasma membrane, nucleus and intracellular vesicles [6]. The primary structure of RLIP76 protein can be divided into four distinct domains (Figure 1 & 2). The N-terminal domain spanning the amino acid residues 1-210 (aa 1-210) interacts with the adaptor protein 2 (AP2) that is involved in endocytosis, the internal domain (aa 210-357) has GTPase Activating Protein (GAP) activity for Rac/CDC42 GTPase, the region (aa 402-498) contains the Ral binding domain, and the C-terminal domain (aa 500-647) which regulates interactions of RLIP76 with partner of RalBP1 (POB1), Cdc2 (CDK1), and HSF-1[1, 7, 8]. Interestingly, a cell cycle protein cytocentrin, involved in the regulation of diplosome separation and assembly of mitotic spindle, shares 98.4% amino acids sequence identity with rat RalBP1. Cytocentrin is a splice variant of RalBP1 that has additional 46 amino acids at C-terminal. Cytocentrin shows cell cycle dependent distribution; in interphase it diffuses in cytosol and is localized in centrosome in early prophase where it regulates centrosome functions in mitosis [9]. The necessity of the additional 46 aa for

‘cytocentrin’ activity has been debated. RLIP76 also itself relocate itself from cytosol to membrane under different conditions including heat-shock, oxidative-stress and in a cdc2 (CDK-1) regulated manner during mitosis.

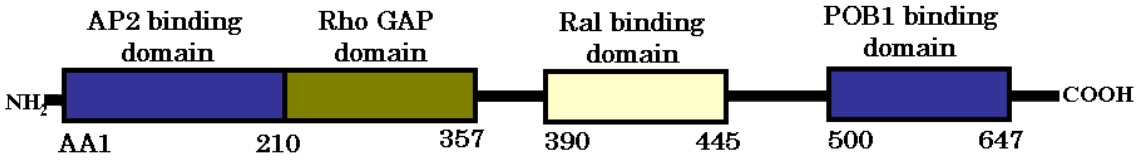
1.2 RLIP76 is a multifunctional protein

Since its discovery, RLIP76 has been shown to be involved in a number of physiological processes including, clathrin-dependent endocytosis [10], exocytosis [11], cell migration [12], mitochondrial fission [13], cytoskeletal organizations [14], developmental pathways [15], and ATP-dependent transport of endogenous and exogenous toxicants outside the cell [7, 16-18]. At the molecular level, RLIP76 is known to mediate and regulate the functions of two small GTPases, Ral and Rho/Rac (Figure 3). The initiation of the signaling cascade is believed to start with the binding of the growth factor to receptor leading to the activation of Ras. Active Ras then binds to and activates Ral-GDS, which in turn activates Ral GTPase via stimulating the exchange of GDP with GTP. Therefore, Ral is a downstream component of Ras signaling cascade [19, 20]. Ral can also be activated through calcium and calmodulin through a pathway independent of Ras [21]. RLIP76 binds to the effector domain of Ral and regulates its downstream functions [1]. Rho subfamily of G proteins is involved in regulations of many cellular processes such as mitogenesis, apoptosis, and response to xenobiotics, membrane trafficking, and cytoskeleton reorganization [22, 23]. It has been suggested that RLIP76 is also involved in some of these functions via regulating GTPase activity of Rho GTPase through its GAP domain and that Ral can regulate Rho GTPase functions through RLIP76, which is a connecting link between these two GTPases [2].

Figure 1: Primary structure of mouse RalBP1. RalBP1 has 648 amino acids in its primary structure. An amino acid sequence presented here is in FASTA format.

>gi|49903314|gb|AAH76636.1| Ralbp1 protein [Mus musculus]
MTECFLPPSSSPSEHRRAEHGSGLTRTPSSEEISPTKFPGLYRTGEPSPPHDVLHEPPDTVS
DDDKDHGKKKGKFKKKEKRTEGYAAFQEDSSGDEAESPSKVKRSKGIHVFKKPSFSKK
KEKDFKIKEKPKEEKHKEEKHKEEKHKEKKSKDLTAADVVKQWKEKKKKKKPIQEPEV
PQMDAPSVKPIFGVPLVDAVERTMMYDGVRLPAVFRECVDYMEKHGMKCEGVYRVS
GIKSKVDELKAAYDREESPNLEEYEPNTVASLLKQYLRDLPENLLTKELMPRFEEACGK
TTEMEKVQEFQRLLRELPECNHLLLSWLIVHLDHVIAKELETKMNIQNISIVLSPTVQISN
RVLYVLFTHVQELFGTVVLKQVTRPLRWSNMATMPTLPETQAGIKEEIRRQEFLLNCLH
RDLQGGIKDLSKEERLWEVQRILTALKRKLREAKRQECETKIAQEIASLSKEDVSKEEMN
ENEEVINILLAQENEILTEQEELLAMEQFLRRQIASEKEEIDRLRAEIAEIQSRQQHGRSET
EEYSSDSESESEDEEELQLILEDLQRQNEELEIKNNHLNQAVHEEREAIIELRVQLRLLQM
QRAKSEQQPQEEEEPERRGGIGPPPCDGVLEVRVAKEQAKASPSKDRKETPI.

Figure 2: RLIP76 domains organization



Legend: RLIP76 has an AP2-binding domain (aa 1-210) at the N-terminus and POB1-binding domain (aa 500-647) at the C-terminus, both domains interact with the proteins that are involved in endocytosis of receptor tyrosine kinase (RTKs). The Rho GAP domain (aa 210-357) has GTPase Activating Protein (GAP) activity towards Rac/CDC42 GTPase, and the Ral-binding domain (aa 390-445) is involved in interaction with Ral GTPase. C-terminus domain also interacts with Cdc2 (CDK1) and Hsf-1.

1.3 Functions of RLIP76 discovered in our laboratory

Our laboratory was the first to show that besides its involvement in Ral and Rho/Rac mediated signaling, RLIP76 also functions as a transporter of glutathione (GSH)-conjugates of exogenous and endogenous electrophilic compounds, and provides protection against electrophilic xenobiotics and oxidant stress [16]. Studies in our laboratory had identified a protein in membrane of human cells, which catalyzed ATP hydrolysis in the presence of the GSH-conjugate of 1-chloro-2, 4-dinitrobenzene (DNP-SG) and hence, this protein was designated as DNP-SG ATPase [24-26]. The molecular identity of DNP-SG ATPase remained elusive for over a decade as this protein was difficult to purify and the purified preparations of DNP-SG ATPase consistently showed multiple peptides of varying chain lengths during SDS PAGE [27, 28]. A 38 kDa band was, however prominent among the bands and polyclonal antibodies were raised in rabbits against this 38 kDa peptide. Immunoscreening of human bone marrow cDNA library using these raised antibodies lead to the cloning and identification of a protein [16, 27, 29] that was eventually shown to be identical to the previously reported protein RLIP76 [1, 2]. Bacterially expressed purified RLIP76 was then subsequently shown to catalyze the ATP-dependent transport of DNP-SG and other glutathione (GSH) conjugates in reconstituted proteoliposomes [16, 30].

Because of its function as ATP-dependent transporter of GSH-conjugates of endogenous and exogenous electrophiles, a protective role of RLIP76 against oxidative stress was proposed and subsequent studies in our laboratory showed that depletion of RLIP76 using siRNA or antisense oligonucleotides and inhibition of RLIP76 function using antibody enhanced the sensitivity of cells to electrophilic stress caused by chemotherapy or radiation [5, 31, 32]. It was demonstrated that the suppression of RLIP76 expression or its transport functions caused accumulation of GSH-conjugates within cells leading to apoptosis of cells [33]. RLIP76 was then shown to be over-expressed in

cancer cells and delivery of recombinant purified RLIP76 by liposome in cells or in animals was shown to cause resistance to cancer drugs such as doxorubicin, sunitinib and sorafenib due to RLIP76 mediated drug transport [5, 31, 32, 34]. In order to find a possible link of RLIP76 to microphthalmia phenotype of lens specific RLIP76 Tg mice reported in this dissertation, a brief discussion of various RLIP76 functions is presented in the following sections.

1.4 Role of RLIP76 in endocytosis and related signaling

RLIP76 has been implicated in the clathrin dependent endocytosis (CDE) of receptor tyrosine kinases (RTKs) leading to the termination of RTK signaling [10]. Binding of the respective ligand to RTKs is known to induce their dimerization and activation of their kinase activity. Active RTKs phosphorylate a number of proteins leading to induction of multiple signaling cascades finally culminating in cellular response such as cell division or migration [10, 35]. Simultaneously, de-sensitization pathways including endocytosis become activated for the termination of receptor signaling. Amplitude and duration of receptor mediated signaling is determined by the balance between positively acting and negatively acting pathways. Alternation in this fine tuning often causes pathogenesis, such as cancer and diabetes. CDE is one of mechanisms for the termination of receptor signaling via internalization and subsequent lysosomal degradation of the receptors [36]. RLIP76 has been shown to be involved in the initiation of clathrin coated pits formation beneath the receptor and facilitates in recognition of receptor that has to be endocytosed. These functions of RLIP76 are accomplished with the help of interacting proteins, μ2 chain of adaptor protein 2(AP2) and partner of RalBP1 (POB1) [4, 37]. Upon activation of growth factor receptor, Ras becomes active and induces Ral activation via Ral-GDS. Active Ral GTPase interacts with and translocates RLIP76 from the cytoplasm (where it is stored bound to Hsf-1, HSP90 and tubulin) to membrane

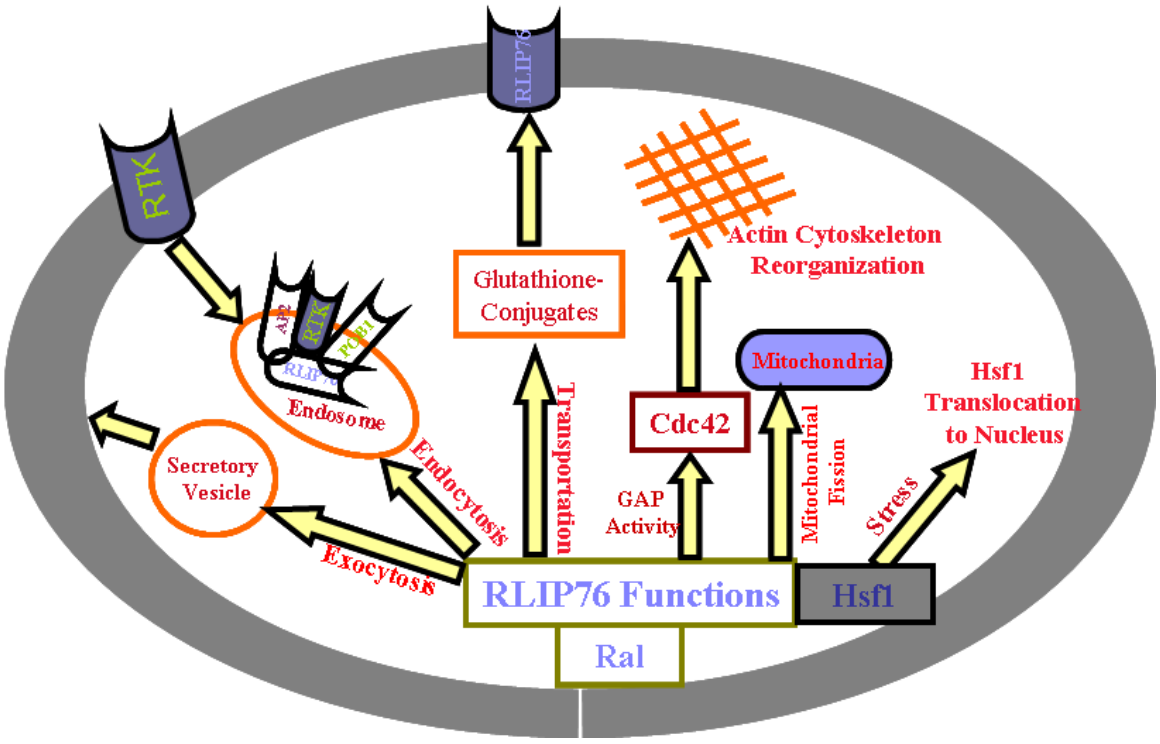
where it transduces signaling and simultaneously, also functions in endocytosis of receptor to terminate signaling [19, 20]. Recent studies from our laboratory have demonstrated that the ATP-dependent transport of GSH-conjugates is obligatory in the process of endocytosis [17]. In view of the role of Ral-Ras-RLIP76 mediated in development as described in the next section, it can be speculated that RLIP76 which is a connective link of Ras-Ral mediated signaling and endocytosis machinery proteins may affect embryonic development.

1.5 Role of RLIP76 in development

RLIP76 seems to play a critical role in the regulation of early development of *Xenopus*, where it has been shown to mediate the regulation of actin cytoskeleton remodeling [14]. Actin cytoskeleton remodeling is a necessary process for migration of cells during gastrulation [38]. Fibroblast growth factors (FGFs) are involved in the marginal zone during gastrulation in the initiation of signaling cascade through Ras for cytoskeleton remodeling [39]. Active Ras can activate Ral through RalGDS which promotes the exchange of Ral bound GDP to GTP. GTP bound active RalB can subsequently interact with different effector proteins and one of these proteins is RLIP76. It has been demonstrated that RLIP76 is a mediator of cytoskeleton dynamic regulation function of RalB [15]. Studies have shown that co-transfection of dominant positive mutants of RalB and RLIP76 in *Xenopus* embryo produce a synergistic effect on the cortical actin disruption and that the expression of Ral-binding domain of RLIP76 has been shown to cause an arrest in gastrulation, as does the expression of constitutively activated form of RalB1[14]. This observation implicates the functioning of RLIP76 in actin cytoskeleton remodeling that involves the interaction of Ral-binding domain with active RalB. Further studies have indicated that interactions of RLIP76 with another GTPase Cdc42, a Rho/Rac GTPase, may mediate the downstream function of RLIP76 for actin

cytoskeleton remodeling [15]. RLIP76 has GTPase Activating Protein (GAP) activity for Cdc42 GTPase and is involved in promotion of hydrolysis of GTP bound to Cdc42 into GDP resulting in its inactivation [2]. It has been shown that both the N-terminal domain and the GAP domain of RLIP76 are required for effectively regulating Cdc42 and that the regulation of Cdc42 by RLIP76 plays an important role in actin cytoskeleton dynamics and in proper gastrulation [15]. It would also be of interest to investigate if Cdc42 expression and activation is affected in the lens/ocular tissues of RLIP76 Tg mice during the present studies.

Figure 3: RLIP76 functions



Legend: RLIP76 is a multifunctional protein that is involved in regulation of clathrin-mediated endocytosis of receptor tyrosine kinase, exocytosis, transport of glutathione conjugates of electrophiles, mitochondrial fission, and regulation of actin cytoskeleton. Initiation of signaling starts with binding of growth factor to the receptor leading to the activation of Ras. Active Ras then binds and activates Ral-GDS, which in turn activates Ral GTPase via stimulating exchange of GDP with GTP. Ral can also be activated through calcium and calmodulin in Ras independent manner. RLIP76 binds to the effector domain of active Ral and appears to promote its downstream functions. RLIP76 is also involved in some of the functions mediated by Cdc42 GTPase. GAP domain in RLIP76 structure regulates activation of Cdc42. It has been suggested that Ral can regulate Rho GTPase functions through RLIP76, which is a connecting link between these two GTPases. RLIP76 also function of RLIP76 to control the activation and translocation of Hsf1 in normal physiological conditions. Exposure to stress causes disruption in binding between RLIP76 and Hsf1, and rescued Hsf1 then forms trimers and translocates to nucleus to induce the expression of Hsp's.

1.6 Eye Morphology and Functions

In order to investigate the possible mechanisms leading to microphthalmia and impaired lens development in lens specific RLIP76 transgenic mice, it is important to understand the morphology and development of eye and lens.

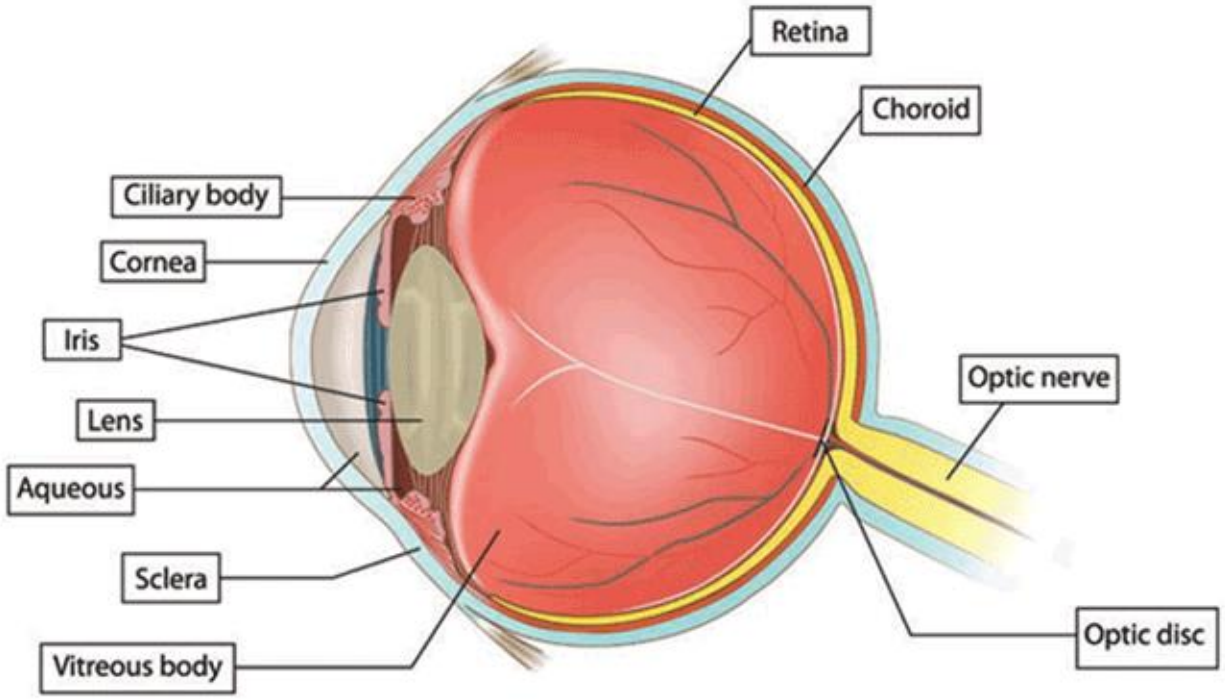
Eyes perceive light and convert it into electro-chemical impulses to neurons. It visualizes objects when light enters in eye through the small hole called pupil and is focused on the retina through optic nerve generated image on retina transmits to the visual cortex and other regions of the brain [40]. The vertebrate eye is a complex structure composed of tissues including pupil, iris, cornea, lens, retina, sclera, choroid, vitreous humor, and optic nerve (Figure 4). Iris, located at the anterior part of lens, controls the amount of light that enters in eye and it closes when light is bright while opens in dark. Lens function is to focus incoming light from different distances on retina and the muscles of ciliary body control focusing of lens. The lens situated in anterior region of the eye has a transparent, biconvex, elastic and accommodative structure that focuses light on the retina with the help of cornea. Lens is held in place by the suspensory ligament of the lens, a fibrous tissue that attaches to the lens at its equator and connects it to the ciliary bodies [40, 41].

Anterior side of lens is more flat than its posterior side. The space between lens and cornea is called anterior chamber filled with aqueous humor produced by ciliary bodies [40]. Aqueous humor supplies nutrients to lens, iris and cornea, controls the balanced production and absorption of nutrients and maintains the desired pressure within eye. Posterior chamber between lens and retina is filled with a jelly like substance called vitreous humor. Lens is surrounded by the capsule, a thick basement membrane containing monolayer of epithelial cells at anterior region and remaining part of the lens is occupied by elongated fiber cells [42, 43]. Size of lens grows with the age. Accommodation property of lens changes optical power of eye so that it can focus on objects at

various distances, thus allowing a sharp real image of the object of interest to be formed on the retina. Ciliary bodies control elasticity of lens. Crystallins are water-soluble proteins that constitute about 90% of total protein within the lens. The α-, β-, and γ-crystallins are three main crystallins found in human lens and these play a pivotal role in maintaining lens transparency. β- and γ-crystallins are present specifically in the lens while α–crystallin has expression patterns in other parts of eye and the body tissues, and is a member of chaperone proteins superfamily [44, 45, 46]. It acts as a chaperone in re-folding of proteins denatured by stress along with heat shock proteins.

Disease and disorders caused by the absence or dysfunction of lens include Presbyopia, Ectopia lentis, Aphakia and Cataracts. In presbyopia, lens losses the accommodation property with age and looses ability to focus incoming light from nearby objects on retina [47]. This could be due to lens becoming hard or undergoing changes in its shape and size. Ectopia lentis is caused by displacement of lens from its original position. Aphakia is condition in which lens is absent in eye it can be due to surgery or injury or it can be congenital. In cataract, lens becomes opaque with age, which can block light from focusing on retina and hinder vision [46, 48]. There are many reasons for the development of cataract such as age related opacity, congenital, injury, and diabetes. Previous studies in our laboratory have shown that the lipid peroxidation product 4-HNE induces cataract in lens [49, 50] and that GSTs and RLIP76 that metabolize and detoxify 4-HNE protect against oxidative stress induced cataractogenesis [51]. These studies provided the rationale for generating the lens specific RLIP76 Tg mice used in present studies. Our surprising novel finding that lens specific RLIP76 mice have impaired eye and lens development and show a phenotype similar to microphthalmia, provide basis for studies presented in this dissertation.

Figure 4: Eye anatomy.



Legend: The vertebrate eye is a complex structure composed of tissues including iris, cornea, lens, retina, sclera, ciliary body, choroid, aqueous, vitreous humor, optic disc and optic nerve. At exterior white covering part of eye is sclera that protects the eye. At the front of eye is the cornea. It protects the eye and allows passage of light into eye. Beneath corneas is iris that controls the amount of light enter into the eye by expanding and contracting the structure. Pupil directs entering light to the lens while the lens functions to focus light onto the retina to generate the image. Nerve fibers in the retina perceived and carry images to the brain through the optic nerve.

Acknowledgement: Glaucoma Research Foundation.
http://www.glaucoma.org/glaucoma/anatomy-of-the-eye.php

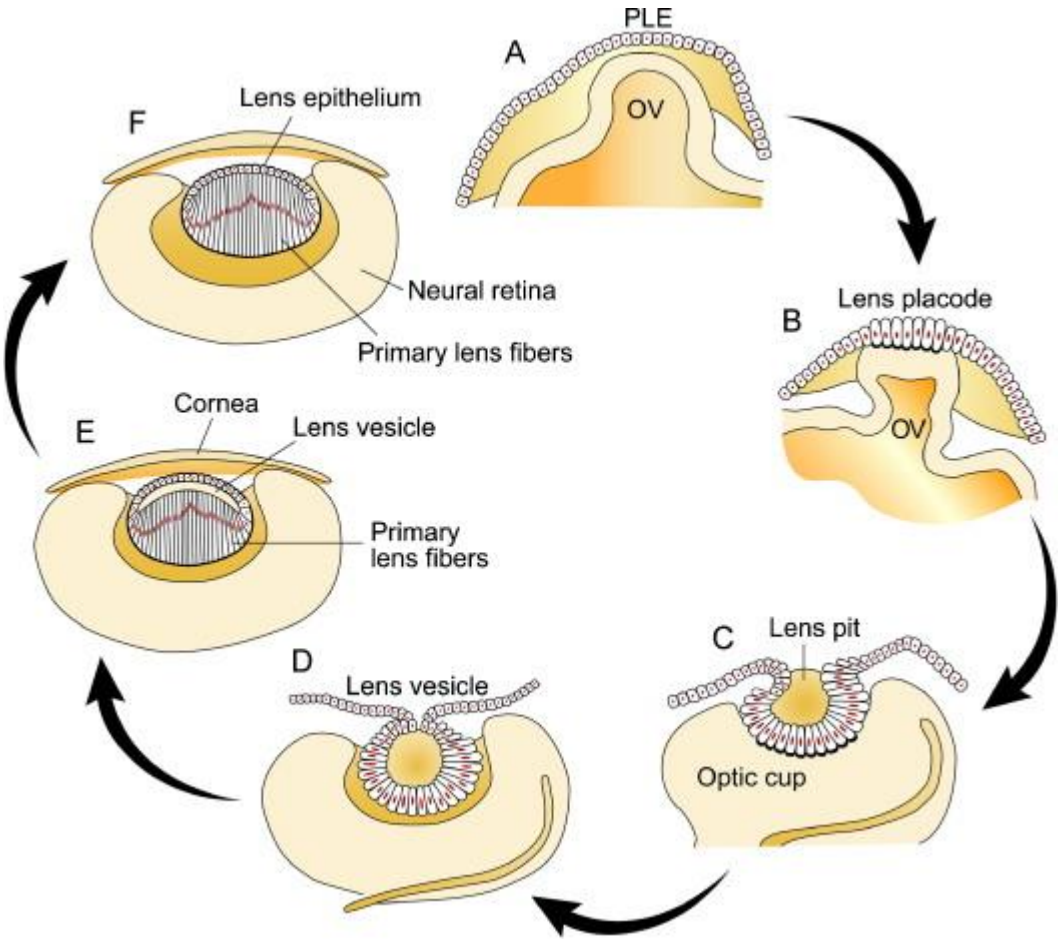
1.7 Development of Eye and Lens

Eye has been an excellent organ to study the developmental process such as tissue induction and the formation of specialized structures such as lens and retina. Eye is formed from three embryonic parts: surface ectoderm, neuroepithelium, and extraocular mesenchyme, through controlled and coordinated interactions between them [52, 53]. Evagination of neuroepithelium of the ventral forebrain leads to the formation of bilateral optical vesicle; distal part of it comes in proximity to overlying surface ectoderm and directs its thickening resulting in the formation of lens placode (Figure 5) [54]. Interaction between lens placode and optic vesicle causes the invagination of the lens placode and optic vesicle leading to generation of a bilayered optic cup. This event takes place around embryonic day 9.5 in mouse. Inner layer of optic cup give rise to neural retina and outer layer develops into retinal pigment epithelium (RPE). Iris epithelium and ciliary body develop from the edge between the two layers and proximal region of optic vesicle becomes the optic fissure [55]. Invaginated lens vesicle finally breaks off from the surface ectoderm and differentiates into the mature lens.

Lens consists of two kinds of cells: epithelial and fiber. After separation of and formation of lens vesicle, posterior region differentiates into fiber cells and the anterior region differentiates into lens epithelium. Differentiated fiber cells at posterior part are known as primary fiber cells and this process takes place in mouse at embryonic day 11.5 [53]. Lens epithelial cells at anterior part proliferate throughout life and differentiate into secondary fiber cells. These newly generated secondary fiber cells migrate and keep adding to the primary fiber cells present in posterior region of lens at embryonic day 15.5 in the mouse. This growth pattern confers polarity to the lens. Differentiation of epithelial cells into fiber cells, migration from equator to outer cortex of lens continues as long as lens grows. Terminally differentiated lens fiber cells start loosing their

organelles such as nuclei and mitochondria and this process completes after a few days of birth [56, 44, 57]. Lens keeps growing through life with continued addition of differentiated fiber cells to the structure.

Figure 5: Development of Lens:



Legend: The vertebrate lens develops from surface ectoderm. Presumptive lens ectoderm (PLE) contacts with optical vesicle and exposure to multiple inductive factors cause thickening of the PLE to form the lens placode. Further that leads to invagination of the lens placode resulting in the formation of the lens pit, which subsequently forms lens vesicle. Anterior part of the lens vesicle differentiates into lens epithelium while posterior part of the lens vesicle forms the elongated primary lens fiber cells. Lens epithelial cells proliferate and differentiate into secondary fiber cells near the equator. The vertebrate lens grows through life by continued addition of lens fiber cells above the older fiber cells [44].

1.8 Anophthalmia and/or Microphthalmia

A/M are defined as absence of and/or reduced in size of eye [58]. A/M is a rare kind of condition with an estimated incidence rate of 1 to 3.2 cases per 10,000 live births. Chromosome abbreviation is perhaps the most prominent causative factor for A/M disease, it has been reported that about 25-30% cases of A/M arise due to genetic mutations [58, 59]. Mutations in a number of genes have been associated this condition but the most frequent mutations are reported in SOX2, STRA6, OTX2, HCCS, BCOR, SMOC1, GDF6, VSX2, SIX6, SHH, BMP4, MITF and Pax6 genes. The mechanisms involved in development of microphthalmia in mutated genes conditions have not been elucidated clearly, but it is postulated that these mutations interfere with the growth of eye [60]. Other factors like fetal alcohol syndrome and infections of herpes simplex virus, rubella during pregnancy can also cause microphthalmia in newborns [61, 62]. Any direct link between lens development and RLIP76 expression or function has not been demonstrated as yet. Our novel findings that lens specific RLIP76 Tg mice show a phenotype similar to microphthalmia are likely to provide significant new information on the etiology of this disorder. During present studies, we have investigated possible mechanism responsible for the impaired development of lens in RLIP76 Tg mice that could provide insight into the mechanisms involved in the etiology of microphthalmia using a lens specific RLIP76 Tg mouse model of this disorder.

Earlier studies have suggested an important role of the transcription factor Pax6 and heat shock transcription factor Hsf4b in development of ocular tissues in *Drosophila and Mouse models*. Therefore, we decided to study the presumptive role of these factors in the impairment of eye development in lens specific RLIP76 transgenic mice.

1.9 Transcription factor Paired box gene 6 (Pax6) and eye development

Transcription factor Pax6 has been shown to be essential for the development of quite some of the organs including the eye, brain and pancreas [63]. It is a member of the Pax family, transcription factor that contains two DNA-binding motifs, the paired domain and paired-type homeodomain [64]. Available evidence indicates that Pax6 function is critical for eye development. The identity of Pax6 was established as a gene associated with *eyeless (ey)* in *Drosophila* and *small eye* (Sey) in mouse [64-66]. Misexpression of Pax6 results in the appearance of developmental abnormalities of eye in *mouse and Drosophila*. Homozygous Sey mutant mouse embryo showed abnormal lateral elongation of optical vesicle and derivation of lens from thickening and invagination of surface ectoderm was failed, aborted eye morphogenesis in mouse. However, in heterozygous mutants the lens was developed but smaller in size and remained attached to cornea [67, 68]. Absence of eyes in humans and mice has been observed if they carry loss of function mutations in Pax6 [69, 70]. Attenuation in Pax6 activity in heterozygotes for Pax6 mutation has been associated with small eye or microphthalmia in mice [71]. Surprisingly, gain-of-functions mutations of Pax6 in *Drosophila* also showed abnormal development of eyes. In an interesting study it has been demonstrated that misexpression of Pax6 results in the formation of ectopic eyes at multiple location in *Drosophila* [72].

1.9.1 Pattern of Pax6 expression in ocular tissues

Expression of Pax6 in mouse first appears at embryonic day 8.0 in the eye region around the surface ectoderm and optic pit [73]. Later its expression is extended to the epithelial layer of optical vesicle and in the developing optic stalk and also around the rim of optic cup where the expression of Pax6 becomes prominent. Pax6 expression in surface ectoderm is transmitted to lens placode and it continues to be present in lens vesicle [73, 74]. Expression is most prominent in epithelium, and it is

reduced at the equator where differentiation of fiber cells starts [74, 75]. This suggests an important role of Pax6 in differentiation of epithelial cells into fiber cells. This idea is also consistent with a weak or reduced expression of Pax6 in older layers of lens fiber cells. During present study, we have examined the effect of RLIP76 over-expression on Pax6 in the Tg mice.

1.9.2 Regulation of Pax6 expression

Bone morphogenetic protein (BMP) and fibroblast growth factor (FGF) receptor mediated signaling have been implicated in lens in the induction and regulation of Pax6. The bone morphogenetic proteins, BMP4 and BMP7 have shown to be expressed in early tissues and involved in early development of lens [76]. It has been shown that deletion of BMP7 in mouse causes eye developmental defects such as those seen in microphthalmia with the loss of expression of Pax6. The FGF induced signaling plays a role in differentiation of lens fiber cells from lens epithelial cells [77]. Its importance in lens development has been demonstrated recently in studies showing that the inhibition of FGF receptor kinase activity leads to suppression of expression of transcription factor Pax6 that regulates transcription and activation of other transcription factors such FoxE3 and Sox2 that have been shown to be involved in lens development [78].

1.10 Heat shock transcription family (HSF)

Heat shock transcription factors (HSFs) are stress response proteins that are activated after exposure to stressors such as elevated temperature, oxidant, chemicals, and environmental stress agents. In response to stress, HSFs form trimers and bind heat shock element (HSE) at the target genes such as Heat shock proteins 27 (*Hsp27*), *Hsp40*, *Hsp60*, *Hsp70* and *Hsp90*, and thereby activate the transcription of these Hsps [79]. The Hsps then act as chaperones in refolding of accumulated denatured proteins as a result of stress, and protect the cells from the cytotoxic effect of stressors

[80]. HSF family comprises of four members namely *Hsf1, Hsf2, Hsf3* and *Hsf4* in vertebrates, out of these, *Hsf11* and *Hsf2* are ubiquitous among vertebrates, where as *Hsf3* and *Hsf4* are present only in avians and mammals respectively [81, 82]. In yeast, nematodes, and Drosophila only a single Hsf is present [83]. All members of HSF family have similarities in their structure's that include a conserved DNA binding domain, a hydrophobic repeat (HR-A/B) responsible for trimerization, a transactivation domain, and another hydrophobic repeat (HR-C) at the carboxy-terminal [84].

1.10.1 Functions of Hsfs as regulators of development

HSFs have been shown to be involved in the regulation of development. It has been shown that the single Hsf of Drosophila (Hsf1) is required for oogenesis and early development [85]. Similarly, in mouse Hsf1 is also involved in oogenesis, placental development and normal growth. Hsf2 have also shown to be involved in spermatogenesis, oogenesis and brain formation [86]. Fujimoto et al. [87], have shown that HSF4b is expressed in lens where it is involved in the regulation of the expression of FGF receptor and γ-crystallin. γ-crystallin plays a critical role in protein stabilization in dehydrated condition. γ-Crystallin has HSE sequence in its promoter for binding constitutively active HSF4b [87]. Hsf1, Hsf2 and Hsf4 are expressed in both epithelial and fiber cells at early stage of lens [87]. Human families with mutations in *Hsf4* gene in lens show a defect in development of lens and develops cataract in early age [88, 89]. In *Hsf4* knockout mice lens development has been shown to be impaired and consequent develops cataract [87, 90, 91]. Further, examination of lens development process revealed that de-nucleation in fiber cells has been interrupted in the absence of Hsf4.

1.10.2 Regulation of HSFs

Interestingly, HSF4b lacks the characteristic inhibitory hydrophobic repeat (HR-c) of HSFS in its structural organization, which is involved in inhibition of trimer formation and transactivation. This enables HSF4b to remain active at all times [92, 93]. Importantly, RLIP76/RalBP1 has been involved in inhibition of Hsf1 trimer formation and activation [8]. Under normal physiological conditions, RalBP1 forms a heterocomplex in cytoplasm with Hsf1 along with Hsp90 and tubulin [8]. Exposure to stress causes disruption by Ral-GTP in this heterocomplex releasing Hsf1, that leads to it translocation from cytoplasm to nucleus where it functions as the master-transcription factor for chaperones including the Hsps [8]. The mechanism for disruption of this complex is mediated by activated Ral, which has high affinity towards RalBP1 in comparison of Hsf1 and also Hsp90 that exhibits higher affinity towards denatured proteins generated by stress [8]. Since Hsf4b has marked similarity in structural features with Hsf1, it will be insightful to establish whether or not a similar type of regulation of Hsf4b by RLIP76 in involved in the mechanisms of impaired ocular development in RLIP76 Tg mice model.

1.11 Actin cytoskeleton plays a role in the development of lens

The actin cytoskeleton is an extremely dynamic structure that consists of polymers of actin and other associated proteins. Its main functions are to facilitate the cell motility and cell shape changes during the cell cycle and organization of cytoplasm and to generate mechanical forces within the cell in response to extracellular stimuli [94, 95]. Three major components: microtubules, microfilaments, and intermediate filaments together make the cytoskeleton. Actin cytoskeleton originates from the association of actin binding and associated proteins to form microfilaments, the polymers of actin molecules [94, 96]. Actin can switch between its two convertible forms monomeric (G-actin) and

polymeric (F-actin). Actin polymerization can occur at both ends of polymerized actin, however positive end grows faster than negative. ATP bound to actin monomer is hydrolyzed during polymerization and the generated energy enhances polymerization [97]. This switching of actin between its two forms occurs during actin cytoskeleton reorganization and is involved in change in cell shape, and the mechanical movement.

Lens transparency is dependent on the organization of cytosolic, membrane-bound proteins and cytoskeletal proteins perform a crucial role in their organization [95, 98]. During growth of lens, epithelial cells differentiate into secondary fiber cells. Many morphological changes during fiber cells differentiation including cell shape change and cell elongation are dependent on the reorganization of actin cytoskeleton [99]. Therefore, actin cytoskeleton plays a pivotal role in the development and growth of lens. Differentiated fiber cells migrate towards the posterior part of lens and at the suture they detach and finally form contacts with counterparts from the opposite side of the lens. These movements require proper coordination between the actin cytoskeleton reorganization, generation of contractile force, and cell adhesive interactions [100]. Total actin content and ratio of F-actin to G-actin increases in the elongating epithelium, and stress fiber increases during fiber cells elongation [101]. Disruption of actin cytoskeleton causes impairment in lens epithelium elongation and differentiation into fiber cells.

1.11.1 Regulation of actin cytoskeleton organization

The actin cytoskeleton reorganization is regulated by signaling pathways are activated in response to growth factors. Rho GTPase family members are the master regulators of actin cytoskeleton organization and associated processes mediated by them [102]. Beside Rho GTPase, calcium-dependent pathways, PI3 kinase, c-Abl interactor proteins, Cdk5, src kinases, and Wnt signaling

pathways also regulate actin cytoskeleton dynamics [103]. The Rho GTPases is a subfamily of the Ras GTPase superfamily that consists of Rho A, B, and C, Rac 1, 2, and 3, and Cdc42. These GTPases keep switching between their two forms i.e. the inactive GDP-bound form and the active GTP-bound form in response to external stimuli [104]. This reversible conversion from one to another state is controlled by a number of regulators such as guanine nucleotide exchange factors (GEFs), GDP dissociation inhibitors (GDIs) and GTPase activating proteins (GAPs) [105-107]. In response to external stimuli such as growth factors, Rho GTPase translocates to plasma membrane after prenylation by geranyl-geranyl transferases (GGTase). At membrane, Rho GTPase interacts with GEF which induces the release of GDP and provide stability to nucleotide-free Rho proteins. GTP preferentially binds to nucleotide free Rho because of higher intracellular GTP to GDP ratio. Translocation and activation of GEFs are dependent on PI3K signaling, which facilitates the removal of auto-inhibition and promotes translocation to membrane [105]. GTPase activating proteins have preferential binding for GTP-bound Rho and accelerate GTPase activity, thereby converting GTPase into the GDP-bound inactive form [106]. In resting cell GDIs binds with Rho GTPase in cytosol and inhibit their translocation to the membrane, upon activation this inhibition gets removed and Rho protein translocates to membrane [107]. They also hinder the binding of GEF to Rho proteins.

RLIP76 possesses GAP domain in its structure, which is involved in controlling the GTPase activity of Cdc42 GTPase that is a Rho GTPases family member [2]. Activated Rho GTPases interact with specific effector proteins, both RhoA and RhoB bind and activate ROCK family kinases [108], and both Rac and Cdc42 activate the effector p21-activated kinase (PAK) [109]. Both ROCK kinases and PAK kinases activate LIM kinase which phosphorylates Cofilin, thus inactivating its actin depolymerization activity [110, 111]. Thus RLIP76 can regulate the activation of Cdc42 protein and subsequent downstream signaling, though the mechanisms are unclear. We

reasoned that by regulating the activation of Cdc42, RLIP76 may regulate reorganization of actin cytoskeleton and associated signaling. Therefore present studies have investigated the effect of RLIP76 forced expression on activation of Cdc42 and the organization of actin cytoskeleton in lens.

1.12 Epithelial-mesenchymal transition (EMT) in lens tissue

Epithelial-Mesenchymal Transition (EMT) process is comprised of a set of coordinated events which leads to change in epithelial cell phenotype into mesenchymal phenotype, characterized by loss of epithelial cell to cell interactions, cell to basement membrane interactions, and acquisition of elongated mesenchymal phenotype [112]. This phenomenon is featured by switching of E-cadherin to N-cadherin expression, and accompanying morphological changes. These changes are accomplished with the activation of transcription factors such as ZO-1, Snail, Slug and β-Catenin, and repression of some other transcription factors [113]. In lens EMT occurs during pathological conditions: anterior subcapsular cataract (ASC) and posterior capsular opacification (PCO) [114, 115]. In ASC formation, epithelial cells change into spindle-shaped myofibroblast and form fibrotic plaque beneath the lens capsule in response to injury or some disease conditions such as atopic dermatitis. Accumulation of a huge amount of extracellular matrix (ECM) causes loss of lens transparency [116]. Whereas PCO occurs post cataract surgery in 20 to 40 % cases within 2 to 5 years after the surgery. During PCO remaining lens epithelial cells (LECs) migrate to the posterior region of lens where transition of LEC into fibroblast occurs. They produce a large amount of extracellular matrix (ECM) and deposition of ECM leads to loss of lens transparency [117, 118]. Therefore, during present studies, we have examined the status of cadherins expression in RLIP76 Tg lens.

1.13 Oxidative stress and defense mechanisms in lens

Ocular lens is constantly exposed to oxidative stress from radiation, H_2O_2 and other environmental stressors. Lens proteins particularly crystallins have exceptionally long half life and need protection against oxidative stress for maintaining the lens transparency [119]. To protect from the effects of oxidative stress, lens have detoxifying enzymatic pathways and high concentration of ascorbate and reduced glutathione (GSH). Reactive oxygen species (ROS) including H_2O_2, $^{\cdot}O_2$, and $^{\cdot}OH$ are continually generated from various sources such as radiation, UV light, electron transport chain, and cytochrome P450 mediated phase I biotransformation reactions [120]. Lens has enzymatic systems to decompose these ROS generated from various sources. Superoxide dismutase (SOD) can convert reactive $^{\cdot}O_2$ into H_2O_2, and accumulated H_2O_2 can then be converted enzymatically by catalase and glutathione peroxidase (GPx) or non-enzymatically by reduced glutathione into H_2O molecules [119].

Generated ROS can react with proteins, DNA and lipids, and resulting in the formation of various electrophilic toxicants. Lipid peroxidation (LPO) is most deleterious effect of ROS because of the autocatalytic nature of LPO that can continually generate high amount of free radicals, hydroperoxides, and α, β unsaturated aldehydes from polyunsaturated fatty acids to manifest toxicity. Among these reactive α, β unsaturated aldehydes, 4-Hydroxy-*t*2-nonenal (4-hydroxynon-*t*2-enal) the major byproduct of peroxidation of ω-6 unsaturated fatty acids has been shown to be interacting with proteins [121], DNA[122] and lipids [123].

To overcome the deleterious effects of these electrophilic toxicants and free radicals and to maintain homeostasis within the cell, there exists a coordinated detoxification and disposal system that comprises of enzymes working in three phases in tandem. The Phase I enzymes are involved in the introduction/exposure of reactive group on hydrophobic molecules [16] to enable them to

conjugate to hydrophilic molecules such as glutathione (GSH), glucuronate or amino acids. The conjugation process is second step in detoxification system catalyzed by Phase II enzymes: examples of this group of enzymes are Glutathione S-transferases (GSTs) that conjugate the electrophilic compounds such as 4-HNE to GSH to form GS-HNE. Products of this Phase II reaction, the conjugates are then transported outside the cell at the cost of ATP hydrolysis by the Phase III detoxification proteins, known as transporters [16]. RLIP76 acts as the major transporter for the GSH-conjugates of the exogenous and endogenous toxicants such as 4-HNE at the expense of ATP and in conjunction plays a major role in the protection of various tissues including lens and other ocular tissues from oxidative stress [124].

1.14 Role of RLIP76 in protection against oxidative stress in ocular tissues

Previous studies from our laboratory have shown that suppression of RLIP76 expression or inhibition of its transport function causes accumulation of GSH-conjugates leading to oxidative stress and induction of apoptosis in most of the cells including that in HLE-B3 and RPE cells [51, 124]. We have established that lipid peroxidation products particularly 4-hydroxynonenal (HNE) is toxic to ocular tissues and is involved in oxidative stress-induced cataractogenesis [49, 50]. It has been further shown that the accelerated detoxification of HNE through the overexpression of GSTA4-4 and RLIP76 that detoxify of HNE protects against oxidative-stress induced cataractogenesis [51, 124]. Both GSTA4-4 and RLIP76 are induced as an early protective response against oxidative stress in Retinal Pigment Epithelial Cells (RPE) and Human Lens Epithelial Cells (HLE-B3) indicating a role of RLIP76 and GSTs as defense mechanism against oxidants in ocular tissues [125].

1.15 Rationale for engineering lens specific RLIP76 transgenic mice

Reasoning that the lens specific RLIP76 transgenic mice having enhanced capacity to detoxify HNE would be relatively more resistant to oxidative stress-induced cataract, we decided to engineer these Tg mice. The lens specific RLIP76 Tg mice, however showed a phenotype in which the lens development was abnormal and their eyes were smaller in size and weight similar to that observed in microphthalmia, a congenital disorder in humans. As described earlier, anophthalmia and/or microphthalmia A/M are defined as the absence of and/or reduction in the size of eye. A/M is a rare kind of condition with an incidence rate of approximately 1 to 3.2 cases per 10,000 births [58, 59]. As discussed earlier, the role of Ral mediated signaling [126, 127] and a possible role of RLIP76 has been suggested in the development of ocular tissue but direct evidence for the involvement of RLIP76 in microphthalmia or lens development is not known. Our surprising discovery is showing impaired lens development and characteristic small eye in these mice as observed in human microphthalmia thus provided an animal model of microphthalmia and suggest a direct involvement of RLIP76 in the etiology of microphthalmia.

1.16 Specific Aims of the Dissertation

While the role of Ral GTPase in eye development has been suggested, any specific role played by RLIP76 in eye development is not known. Recent studies indicate the role of Ral in development of *Drosophila* eye [126, 127]. These studies showed that the ectopic expression of constitutively activated Ral GTPase disrupted the change in cell shape during eye development. Change in cell shape is necessary for normal ommatidial development in the eye. The abnormalities of eye caused by the expression of activated Ral showed similar phenotype to that of Cdc42/Rho GTPase transgene in *Drosophila* [127]. Co-expression of constitutively active Ral GTPase with Rac GTPase also

showed synergism in disruption of actin cytoskeleton organization [127]. The mechanism for synergism between Ral and Rho/Cdc42 has been suggested to be due to the possible interactions between these two GTPase's through RLIP76. However, any direct evidence for the role of RLIP76 in the eye development has not been demonstrated yet. Our lens specific RLIP76 Tg mice showed a phenotype in which the lens development was abnormal and their eyes were smaller in size and weight similar to that observed in microphthalmia, a congenital disorder in humans. This lens specific RLIP76 Tg mouse model is therefore highly suitable to study the role of RLIP76 in actin cytoskeleton reorganization via the regulation of Cdc42 activation in lens and these studies should also provide insight into mechanisms involved in lens development.

Based on these observations, we hypothesize that **the over expression of RLIP76 impairs lens development through disrupting actin cytoskeleton organization and interfering with the expression of Pax6 and Hsfs**. This will be achieved through the studies in following three specific aims.

Specific Aim 1. : Generation of lens specific RLIP76 transgenic mice and characterization of phenotype.

Specific Aim 2. Effect of RLIP76 over expression on gene expression profile and expression of transcription factors involved in lens development.

Specific Aim 3. Effect of RLIP76 over expression on actin cytoskeleton organization and epithelial cells differentiation.

CHAPTER II

MATERIALS AND METHODS

2. MATERIALS AND METHODS.

2.1 Materials

DNeasy Blood & Tissue kit for genomic DNA extraction, RNeasy mini for RNA extraction, and cDNA first strand synthesis kit were purchased from Qiagen (Valencia, CA). Bradford reagent, bis-acrylamide, and iQ SYBR Green Supermix RT-PCR reagent were obtained from Bio-Rad (Hercules, CA). Enhanced Chemiluminescence substrate (ECL) and Western blot stripping buffer were purchased from Perkin-elmer (Waltham, MA), and Pierce Co. (Rockford, IL), respectively. All other reagents and chemicals were purchased from Sigma-Aldrich (St. Louis, MO).

2.2 Generation of RLIP76 Transgenic (Tg) Mice

The lens specific RLIP76 transgenic mice were generated in the direction of Dr. Wakamiya Maki, Director, at the Transgenic Core Facility of the University of Texas Medical Branch (UTMB) at Galveston, Texas. The use of animals for transgenic mouse production and in the subsequent experiments was approved by the IACUCs of the UTMB, and the University of North Texas Health Science Center (Fort Worth, TX). *Ralbp1* transgene was constructed using δenαA promoter, a heterogenic enhancer/promoter comprising the third intron of the chick δ1-crystallin gene and the mouse αA crystallin promoter; a rabbit β-globin intron; mouse *Ralbp1* cDNA (2,884 bp); and a bovine growth hormone polyadenylation signal (Figure 7A). The δenαA promoter becomes active at the lens pit stage and remains active in all lens cells including both lens epithelial and fiber cells, even at postnatal stages [128]. The cassette containing the promoter and intron sequences was

kindly provided to Dr. Maki by Dr. Paul A. Overbeek. (Baylor College of Medicine). Purified transgene DNA (5.3 kb) was injected into pronuclei of one-cell stage B6C3F2 embryos. Founder mice were identified by Southern blot analysis using a part of *Ralbp1* cDNA as a probe and the results are presented in figure 7B.

2.3 Southern Hybridization

Insertion of transgene of *RLIP76* in genomic DNA of embryos has been confirmed by southern blot using specific probe designed against the transgene. Genomic DNA was obtained from tail biopsy samples [129], and digested by BamHI. Digested DNA was separated in an agarose gel, denatured, and transferred to the Hybond-XL nylon membranes (GE Healthcare Life Sciences, Piscataway, NJ) in 0.4N NaOH. A 346-bp EcoRI-PstI DNA fragment was isolated from mouse Ralbp1 cDNA and used as a probe template. The probe was labeled with p32 via random-primed labeling. The hybridization of the labeled probe to filter-bound DNA was performed overnight at 65 °C in 10% dextran sulfate, 1% SDS, 1M NaCl, 100 μg/ml of denatured salmon sperm DNA. The filter was washed at 65 °C in 0.2X SSC, 0.1% SDS. When a mouse carries multiple tandem copies of the transgene, the probe detects a 5.3-kb band. The probe also hybridizes to a 14.7-kb segment of the endogenous *RalBP1* gene (Figure 7B).

2.4 RLIP76 Transgenic Mice Genotyping

Mice were genotyped by PCR using genomic DNA purified from tail biopsy samples. The primer pair, Ralbp1-F (5'-GCAACGTGCTGGTTATTGTG-3') and Ralbp1-R (5'-CGAGGAAGGGAAGTGGTCTT-3'), was used to amplify a 196-bp DNA fragment corresponding to rabbit β-globin and exon 3-*Ralbp1* region of the transgene. The primer pair, YAint F (5'-

GCTGCCAGCTGTCTTCCGGG-3') and YAint R (5'-GCCACCGCCAAGCAGCCTTC-3'), was also used as a control to amplify a 112-bp DNA fragment corresponding to exon 3-intron 3 junction of the endogenous mouse *Ralbp1* gene. The primers were designed using Primer3 [130].

2.5 Cell Culture and treatment with oxidants

Human Lens Epithelial Cells-B3 (HLE-B3) were grown and maintained in Dulbecco's modified minimal essential medium supplemented with 20% fetal bovine serum. Cells were kept in a humidified incubator containing 5% CO2, at 37°C. Two days before the treatment, cells were harvested and equal number of cells was plated in 100mm Petri dishes. When cells confluency reached at 90%, treatment was performed. 50μM Hydrogen peroxide or 10μM 4-hydroxynonenal were added for 2 hours and radiation UV-C for 2 minutes. Then fresh medium was added and cells were left, harvested after 24 hours of incubation. Lysate was prepared in RIPA buffer and after quantification of protein, equal amount of proteins was subjected to SDS-PAGE and western blot analysis was performed as described in western blot section.

2.6 Western blotting

For Western blot analyses, lens tissues were lysed on ice in RIPA buffer containing PMSF, 1 mM sodium orthovanadate, and protease inhibitors (Santa Cruz, USA). The lysates were centrifuged at 12000 g for 10 min at 4°C and clear supernatants were suspended in SDS sample buffer, boiled for 5 min, and subjected to electrophoresis on SDS-polyacrylamide gel (SDS-PAGE) and then transferred to a nitrocellulose membrane (Bio-Rad Laboratories, CA, USA). The membrane was blocked with milk or BSA, probed with specific primary antibodies, washed with TBS, and then probed with secondary antibodies conjugated to horseradish peroxidase. Primary antibodies RalBP1,

Pax6, Hsf4b, SOD, GAPDH, α- crystallin and γ-crystallin were purchased from Santacruz (Santacruz, CA) while primary antibodies Hsf1, Hsp27, Hsp40, Hsp60, Hsp70, Cdc42, E-cadherin, N-cadherin, vimentin, β-catenin, phospho cofilin, and Hsp100 were obtained from Cell Signaling Technology (Danvers, MA). Antibody for GSTA4 and GPx were raised in Dr. Yogesh Awasthi's lab. GAPDH was used as the loading control. Immunoreactive bands were visualized by enhanced chemiluminescence (ECL) with Western Blotting Reagent (Perkin elmer, CA, USA).

2.7 Hematoxylin & Eosin Staining and Immunofluorescence

To examine lens tissue organization of RLIP76 Tg and wild-type mice, we performed H&E staining following the protocol described by Schmitz et al., 2000 [23]. Slides were examined and the images were captured using Nikon inverted microscope with 10X objective. In brief, the tissue was de-paraffinized, washed with xylene, and subjected to hydration in serially diluted ethanol and finally in water. Tissues were permeabilized and incubated with goat serum for 1hr. Appropriate primary monoclonal antibody was then added to slides for overnight and the next day after washing, flourophore labeled secondary antibody was added. Primary antibodies Hsf4b, RalBP1, Pax6, α-crystallin, γ-crystallin and actin were purchased from Santacruz (Santa Cruz, CA) and antibody for Hsf1 E-cadherin and N-cadherin was obtained from Cell Signaling Technology (Danvers, MA) Slides were visualized and images were acquired by LSM 510 Confocal Laser Scanning Microscope.

2.8 Microarray studies and data analysis

Eyes from 3 week old RLIP76 transgenic and wild type mice were snap freezed in liquid nitrogen followed by dissection of lenses. Six lenses from each group were pooled and homogenized in lysis buffer. Total RNA was purified using RNeasy mini kit (Qiagen) according to manufacture's

guidelines. Quantity of RNA was determined by NanoDrop and quality of RNA was determined using Agilent Bioanalyzer.

Microarray experiment procedures hybridization and data acquisition were performed by Phalanx Biotech Group, Inc., (Belmont, CA). Briefly, cDNA was reverse transcribed from 10 μg of each RNA sample and labeled with Cynanine 5 (Cy5) dye using Ambion MessageAmp aRNA kit. Fragmented aRNA was hybridized with Onearray MOA2.0 chip in triplicates for the wild type and Tg samples. Data was acquired from chip and normalized in context of housekeeping gene expression, and average of triplicate was calculated.

For gene ontology analysis genes having 1.5 fold modulated expression and p-value <.05 were considered. The expression of total 6348 genes was either up-regulated or down-regulated in transgenic lens compare to the wild type according to above defined criteria. The gene lists were analyzed using Database for Annotation, Visualization and Integrated Discovery 6.7 (DAVID 6.7) [131] tool for segregating genes according to functional categories. Statically significance for pathways was calculated using p-value <0.05.

2.9 Real-Time Polymerase Chain Reaction

Total RNA was isolated from pool of six lenses of 3 week-old mice from each group using RNeasy Mini Kit (Qiagen, Valencia, CA) according to the manufacture's protocol. The cDNA was synthesized from 1μg of total RNA by using QuantiTect Reverse Transcriptase kit (Qiagen, Valencia, CA) as described by the manufacture. Synthesized cDNA was amplified for gene expression analysis by RT-PCR using iQ SYBR Green Supermix (BioRad, Hercules, CA) and primers specific for the desired gene. Sequences of primers used for RT-PCR are listed in table 4. Specificity of primers was confirmed by running the melting temperature curve.

2.10 Cdc42 activation assay

Cdc42 activation assays were performed using Cdc42 Activation Assay Kit from CELL BIOLABS, INC (San Diego, CA) according to manufacture's instructions. Briefly, lens was dissected from eye and lysed in buffer provided in the kit. The lysate proteins (800 μg) were incubated with PKA conjugated beads at 4 ˚C for 2 hr. The beads were washed three times with lysis buffer before elution and eluted proteins were subjected to western blot analysis as described above using mouse Cdc42 primary antibodies provided in the kit.

CHAPTER III

RESULTS

3.1 **Specific Aim 1:** Generation of lens specific RLIP76 transgenic mice and characterization of phenotype

Up regulation of RLIP76 expression upon exposure to oxidants: We have previously shown that RLIP76 levels get increased in response to exposure of oxidants that cause oxidative stress [124]. Lens is exposed to UV-C radiations, xenobiotics, and hydrogen peroxide that induced lipid peroxidation which generates 4-HNE. Up regulated RLIP76 can protect cells from the toxicity of oxidative stress by transport out the generated electrophiles including 4-HNE. In order to provide evidence in support for generating Tg mice we confirmed these findings. For these studies, Human Lens Epithelial Cells-B3 (HLE-B3) was treated with hydrogen peroxide, 4-HNE, and UV-C radiations. All three toxicants caused substantial up regulation of RLIP76 compared to the untreated sample (Figure 6). This effect was observed even 24 hr after the treatment which indicates that RLIP76 respond to oxidative stress and cells have maintained levels of RLIP76 for a long time period. These results confirmed our previous finding that RLIP76 was an oxidative stress-responsive protein and further provided rationale for the generation of RLIP76 Tg mice.

Engineering of lens specific RLIP76 Tg mice: Five lines of RLIP76 transgenic mice designated as # 3, 4, 24, 41, 47 were generated as detailed in methods section. To make expression lens specific vector used which have a promoter sequence from αA crystallin gene and enhancer from chick δ1-crystallin gene to drives expression in both lens epithelial and fiber cells (Figure 7B). Mouse 1885 base pairs long *RLIP76* gene was successfully cloned in the vector. Using restriction enzymes

ASP718 and NotI, 5.3 Kb transgene digested from the vector and successfully injected into the pronuclei of B6C3F2 fertilized eggs. We were able to transfer these injected eggs into the surrogate mothers and above mentioned five founders were generated.

Confirmation of *RLIP76* transgene insertion into the founders by southern blot: We then confirmed that mouse *RLIP76* gene had successfully been inserted into the genome and this represent an extra copy of gene other than endogenous present *RLIP76* by Southern blotting. The results presented in the Southern blot figure show the transgene *RLIP76* band at 5.3 kb and endogenous *RLIP76* gene band at 14.7 kb in pups originated from five different lines (Figure 7B). Loading control showing nearly similar amount of DNA was used for Southern blot. Transgene was transmitted to the offspring in all five lines. Southern blot (Figure 7B) the highest numbers of transgene copies were found in Lines 24 and 47, followed by Line 4, and then Line 41. Line 3 appeared to have low-copy transgene integration in two separate loci, and we have not yet examined this line. We have currently generated and used the colony of one of these five RLIP76 Tg mice (Line 47) showing a selective overexpression of RLIP76 in lens and all the subsequent studies with Tg mice generated in this colony.

Identification of RLIP76 Tg mice by genotyping: Identity of offspring mice were confirmed by the genotyping. For breeding purpose, we used wild type either male or female with RLIP76 Tg either gender and produced offspring's were heterozygous in the expression of RLIP76. Genotyping were performed using genomic DNA purified from mice tail and primers specifically designed against regions spanning rabbit β-globin and RLIP76 exon3 integrated into the genome by the RLIP76 transgene. For genomic DNA purification 3 weeks old mice tail were collected after

sacrifice. DNA was purified using Qiagen kit as guided, and eluted in the TE buffer. Purification quality and quantity of DNA was determined by Nanodrop spectrometer which also gives ratio of 280/260 ratio and all DNA sample passed that criteria. In genotyping 200 ng of DNA was used for each sample. We also ran polymerase chain reaction (PCR) for Yaint primers along with primers for RLIP76 Tg. Yaint primers facilitates in determining the successful run of PCR and DNA quality used for the run.

Product of PCR reaction was analyzed by running samples on DNA-agrose gel and was visualized using imager. The band of RLIP76 transgene appeared on distance of 200 bps and Yaint primers product at 100bp. Representative picture presented in Figure 9 samples that were used for genotyping. These results show that sample 2, 3, and 6 showing band at 200 bp originated from RLIP76 Tg mice while the remaining samples originated from the wild type.

Confirmation of lens specific RLIP76 overexpression in Tg mice: To determine that Tg mice are over expressing RLIP76 only in lens compared to that of wild type and that this over expression is specific for lens, we have dissected lens from other eye tissues from both the wild type and Tg mice and separately pooled these fractions. Western blot shown in figure 10A indicated that more RLIP76 protein was present in Tg lens as compared to lens of WT. On the other hand, the remaining ocular tissues of Tg mice showed similar level of RLIP76 expression to that was observed in the ocular tissue and lens of wild type mice. These results confirmed that the over expression of RLIP76 protein in Tg mice and that this over expression occurred only in lens and not in other ocular tissues. These results confirmed lens specific over expression of RLIP76 in Tg model and validated the functions of the promoter used to facilitate lens specific over expression of RLIP76.

Lens-specific over expression of RLIP76 in Tg mice was further confirmed by immunofluorescence studies performed on the sections of eye from the WT and Tg eyes. The results of these studies presented in Figure 10B showed overexpression of RLIP76 in epithelial and fiber cells both in lens of Tg mice. RLIP76 expression was however, relatively more pronounced in the fiber cells of Tg mice. RLIP76 was remarkably less in lens of wild type mice. These results further validated lens specific over specific over expression of RLIP76 in our Tg mouse model.

Characterization of RLIP76 Tg Mice Eye Phenotype:

RLIP76 Tg mice have comparatively smaller eyes: Preliminary examination of RLIP76 Tg mice showed had smaller eyes as compared to that of the wild type mice. A picture of the eyes from the same age group siblings of the Tg and WT mice is shown in the representing figure 8A that clearly showed small eye in Tg mice. This small eye phenotype was persistent and consistent in three lines 24, 41, 47, which were reproduced for further experiments. Consistently appearances of the phenotype in different lines suggest that this effect is RLIP76 protein plays a role in the development of the eye.

Weight of the eye in Tg mice is lesser: Further, we measured weight of eye dissected from three lines 24, 41, and 47. . We, however, found that lines 24 and 47 exhibited noticeably small eyes (Figure 8B). We measured body weight and eye weight of a small number of mice ranging from 4 weeks to 6 weeks old (Table 2). Their age and parents were similar which ruled out possibility that difference in weight could be the result of diversity of age and parents. We have also taken body weight body in account to consider the fact that eye size can depend on the overall mouse size and weight. Data shown in the table 2 is presented in adjusted eye weight (mg) format, containing

standard deviation and numbers in bracket indicating number of animals were used. Adjusted eye weight numbers are divided using formula in which individual eye weight is divided by overall body weight and multiplied by hundred. A fourth column in table is showing the ratio of adjusted eye weight of Tg against that of wild type. These data indicated that eye weight/body weight ratios were reduced 40-45% in line 24/47, and 20-25% in line 41 compared to their wild-type (WT) littermates (Figure 8B).

Effect of RLIP76 expression on eye morphology and development: To study the tissue organization of eye tissues in RLIP76 Tg mice we have performed Hematoxylin and eosin staining. The eye of Tg mice exhibited remarkably smaller and disorganized lens as compare to the WT. All other eye tissues including retina, cornea were present but their architecture and position seems to be distorted perhaps because of small lens (Figure 11B). Thus smaller size of lens and gross morphological abnormalities were characteristics of RLIP76 Tg mice. As expected, the eye of WT mouse was normally developed showing organized structure of lens and the surrounding tissues (Figure 11A). We also compared lens development of RLIP76 Tg with WT mice by taking picture of eye using stereomicroscope. Results of these studies (Figure 11A) also showed that, the lens development was severely impaired in Tg mice as compared to that in WT mice.

Expression of major constituent of lens protein: Crystallin gene promoter was used to generate RLIP76 Tg mice but crystallins constitute a major portion of lens protein. Therefore, we examined whether or not the expression of major constitutive lens crystallins was altered due to decreased availability of trans factors driving endogenous crystalline genes expression in the Tg mice. The results of both Immunofluorescence and western blot experiments shown in figure 12 showed the both groups have similar expression of α-crystallin and γ-crystallin proteins in the lens of Tg and

WT mice that excluded the possibility of the crystallin promoter being the causative factor for the observed phenotype.

Expression of antioxidant enzymes in transgenic mice. Since RLIP76 has been known be involved in providing protection against oxidative stress and prime interest of engineering RLIP76 transgenic mice was to study the protection of ocular lens. Therefore, we examined whether or not the expression of antioxidant enzymes was altered in the Tg mice. The results of western blot experiments shown in figure 13 showed the both groups have similar expression of GSTA4, catalase, SOD, and GPx enzymes in the lens.

Taken together the results presented in this specific aim 1 show that we successfully generated lens specific RLIP76 Tg mice. The over expression of RLIP76 in these Tg mice was lens specific. The Tg mice had a characteristic phenotype in which the eyes were comparatively smaller in size and weight. These mice also showed impaired development of lens and eye. Studies into the mechanisms that may lead to this phenotype similar to that observed in humans with microphthalmia are described in succeeding aims.

Figure 6: RLIP76 expression induced in HLE-B3 cells subjected to oxidative stress.

HLE-B3 cells were treated with 50μM hydrogen peroxide and 10μM 4-HNE for 2 hours, and with radiation UV-C for 2 minutes. Fresh medium was added to the treated cells and followed by recovery period of 24 hours. Western blot analysis was performed with total cellular extracts using the RLIP76 antibody. GAPDH was used as a loading control.

Figure 6.

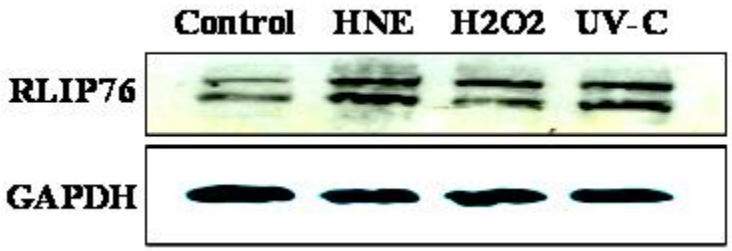


Figure 7: Generation of lens specific RLIP76 transgenic mice.

(A) Schematic presentation of vector used for RLIP76 Tg mice generation. To over express RLIP76 gene specifically in lens we used the vector comprises of δenαA promoter, a heterogenic enhancer/promoter comprising the third intron of the chick δ1-crystallin gene and the mouse αA crystallin promoter; a rabbit β-globin intron; and a bovine growth hormone polyadenylation signal. The δenαA promoter becomes active at the lens pit stage and remains active in all lens cells including both lens epithelial and fiber cells, even at postnatal stages [128].

(B) Southern blot of five lines of RLIP76 Tg mice. Mouse 1885 base pairs long *RLIP76* gene was cloned in the vector. Transgene 5.3 Kb in size was digested out from the vector using restriction enzymes, ASP718 and NotI, and injected into the pronuclei of B6C3F2 fertilized eggs. Later, the injected eggs were transferred into the surrogate mothers and five lines of RLIP76 transgenic mice were produced. Insertion of transgene in all five lines was confirmed by southern blot using a pair of primers designed against RLIP76 transgene. RLIP76 transgene bands visualized at 5.3 kb distance and endogenous RLIP76 bands appeared at 14.3 kb. Loading control is showing equal amounts of DNA used for southern blot analysis.

Figure 7A.

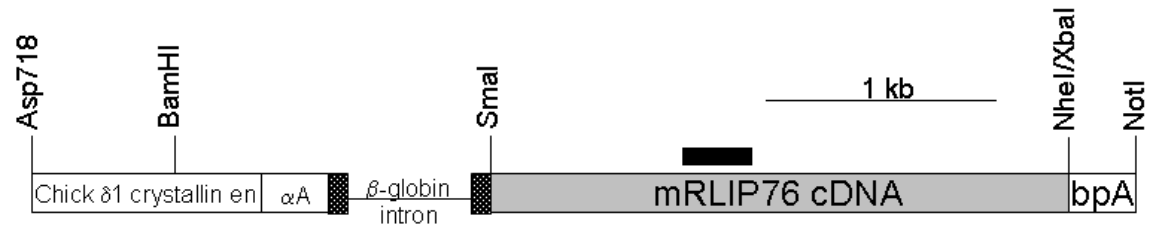


Figure 7B.

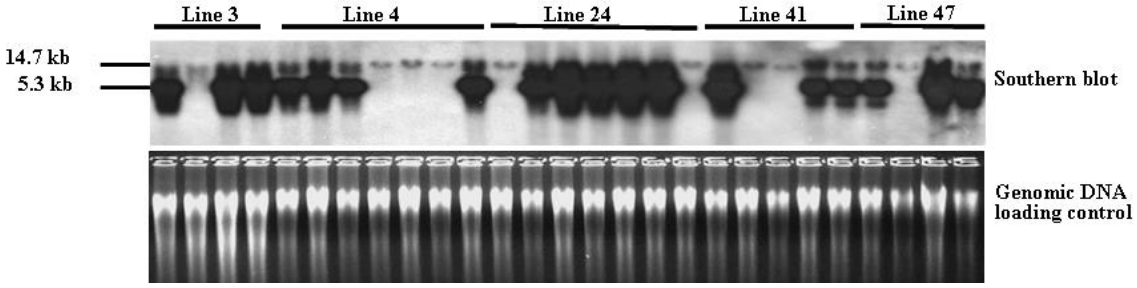


Figure 8: **RLIP76 Tg mice possess eyes smaller in size and weight**.

(A) A representative photograph of WT and Tg mice showing difference in eye size.

(B), Bar graph showing wet eye weight ratios of Tg/WT mice.

Average weight of eyes adjusted by body weight. (Adjusted weight= Eye weight (mg)/body weight (g) x 100*)

Figure 8A.

Figure 8B.

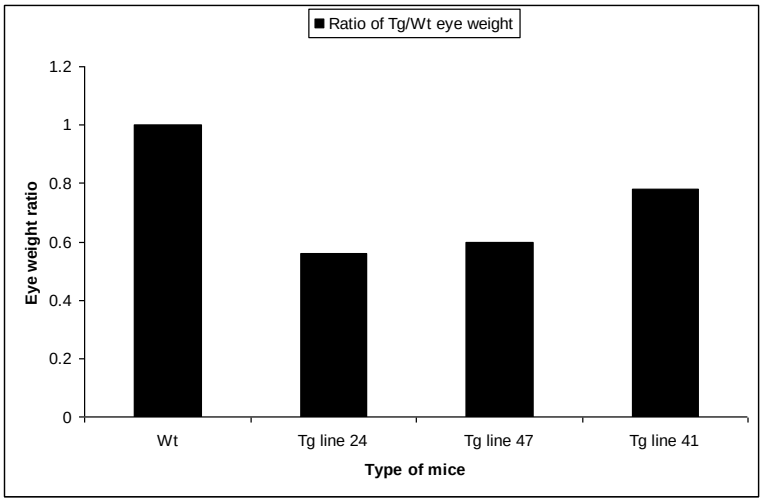


Table 1. Representing eyes weight of wild type and RLIP76 transgenic mice.

Tg line	Tg adjusted eye weight (Avg±SD (n))*	Wt adjusted eye weight (Avg±SD (n))*	Ratio of Tg/ Wt eye
24	55±5 (4)	98±12 (3)	0.56
47	67±4 (4)	111±2 (2)	0.60
41	80±12 (6)	103±9 (2)	0.78

Figure 9: Identification of transgenic mice by genotyping. After sacrificing mice, tail biopsy was performed. Genomic DNA was purified from the mouse tail and quality of purified DNA was determined. DNA concentrations ranging from 200ng to 500ng were used in Polymerase Chain Reaction (PCR) for amplification of RLIP76 transgene. Primers (table 2) used for transgene amplification were designed for amplification of specific RLIP76 transgene and their amplified product was 198 bp long. Yaint primers were used as PCR control and their amplified products was 100 bp in size.

Figure 9.

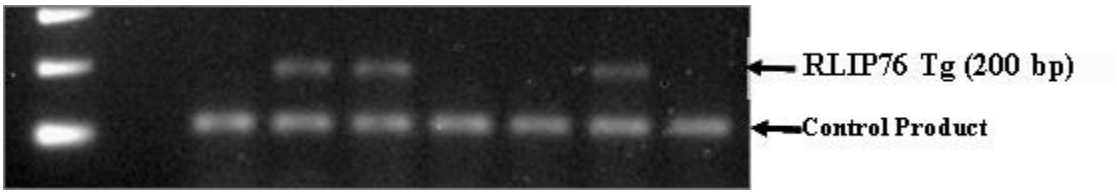


Table 2: Primers used for genotyping.

RLIP76 Transgene (Forward)	GCAACGTGCTGGTTATTGTG
RLIP76 Transgene (Reverese)	CGAGGAAGGGAAGTGGTCTT
Yaint (Forward)	GCTGCCAGCTGTCTTCCGGG
Yaint (Reverse)	GCCACCGCCAAGCAGCCTTC

Figure 10: Lens specific over expression of RLIP76 in Tg mice.

(A) Western blot analysis of lens and remaining ocular tissues showing selective over expression of RLIP76 in the lens of Tg mice. Lenses were dissected from eyes of three weeks old WT and Tg mice. Isolated lens and remaining ocular tissues were homogenized and lysed in RIPA buffer. Western blot was performed with lens and ocular tissues lysates using the RLIP76 antibody. GAPDH was used as a loading control.

(B) Immunofluorescence localization of RLIP76 in WT and Tg lenses. Three weeks old mice eyes were fixed and sectioned. Sections were stained with RLIP76 antibody and imaged with confocal microscopy. FITC-conjugated secondary antibody was used to detect RLIP76 expression. RLIP76 was visualized as green fluorescent signals in the lens epithelial and lens fiber cells. Nuclei staining with DAPI were visualized in blue. Arrows showing lens epithelial cells; arrowheads, showing lens fiber cells.

Figure 10A.

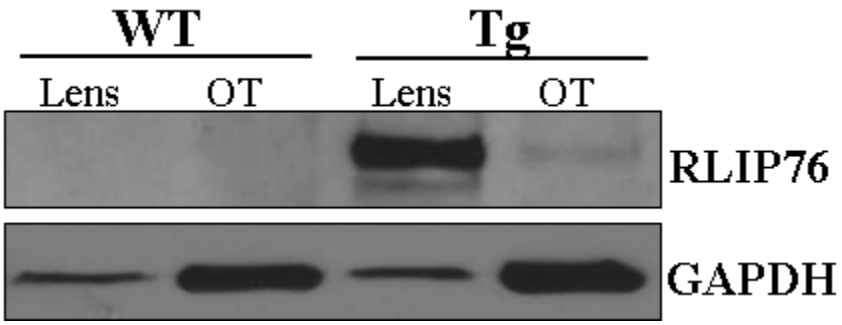


Figure 10B.

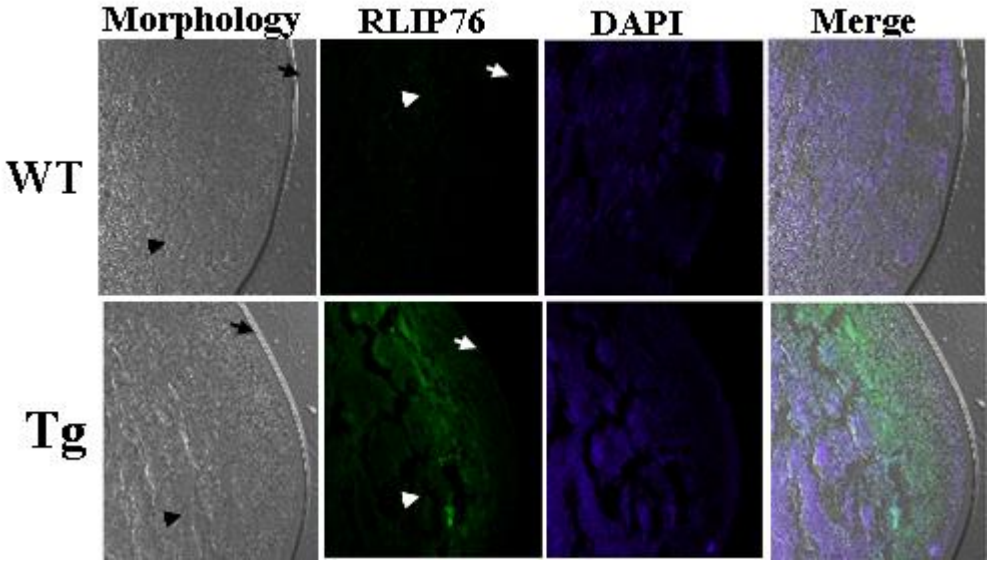


Figure 11: **Ocular tissue organization is disrupted in lensRLIP76Tg mice.**

(A) Comparison of lenses of WT and Tg mice. Mice were euthanized. Eyes were dissected, weighed, and kept in DMEM on ice. Pictures of the eyes were taken using stereomicroscope. Picture on left: eye from WT showing normal lens development. Picture on right: eye from lensRLIP76Tg (labeled as mutant) photographed at double the magnification showing poor development of lens.

(B) Hematoxylin and Eosin staining of eyes from WT and Tg mice showing anterior and posterior regions of eye. Figure showing proper developed eye and lens in WT while in Tg eye, ocular tissues are disorganized and lens becomes small in size. Abbreviations: C, cornea; Ir, iris; LE, lens epithelial cells; LF, lens fiber cells; R, retina.

(C) Hematoxylin staining of eye from day one old mice. Showing organ free zone (OFZ) is more in Tg lens as compared to that of WT. Abbreviations: L, Lens; arrow, lens epithelial cells; arrowhead, fiber cells.

Figure 11A.

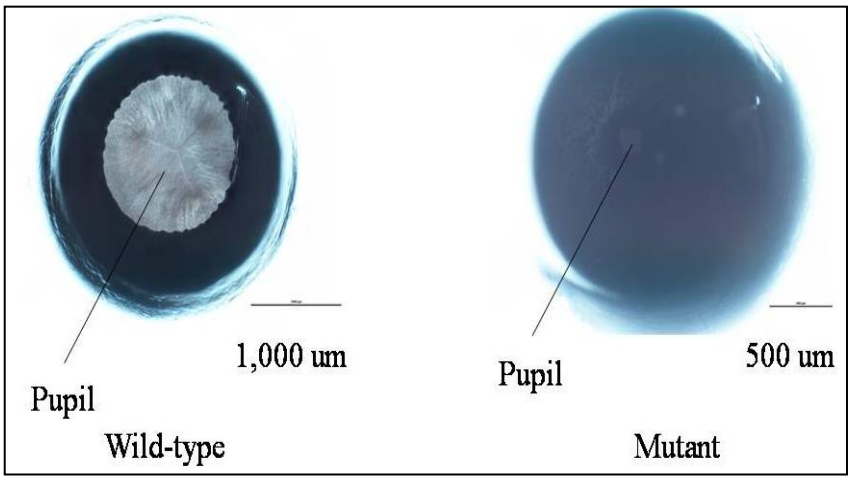


Figure 11B.

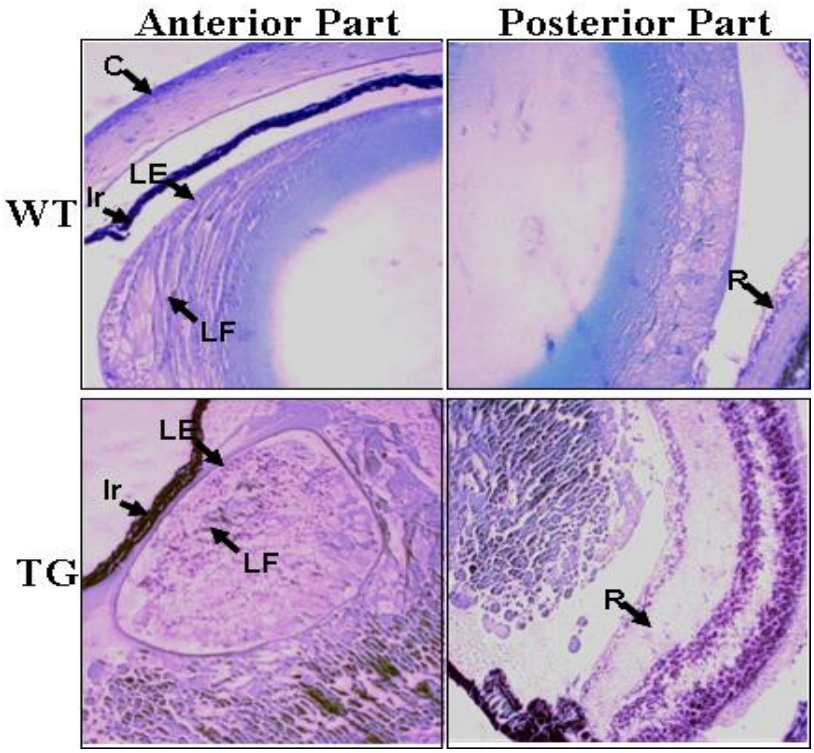


Figure 11C.

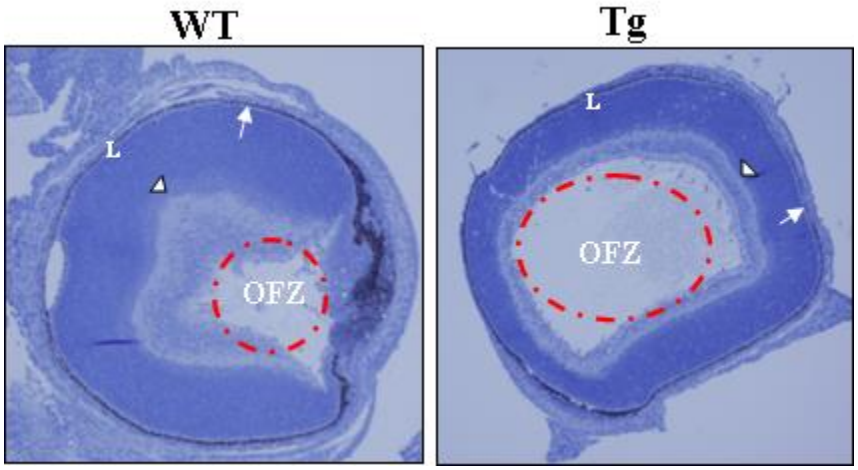


Figure 12: Similar expression of crystallins in Tg and WT mice.

(A) Western blot analysis showing similar expression of α-crystallin and γ-crystallin proteins. Lenses were dissected from eyes of three weeks old wild type and transgenic mice. Lens was homogenized and lysed in RIPA buffer. Western blot analysis was performed with lens extracts using the α-crystallin and γ-crystallin antibodies. GAPDH was used as a loading control.

(B) Immunofluorescence image showing expression of α-crystallin and γ-crystallin in lens of WT and Tg mice.

Three weeks old mice eyes were fixed and sectioned. Eye sections were stained with α-crystallin and γ-crystallin antibodies and imaged with confocal microscopy. FITC-conjugated and Rhodamine Red-x secondary antibodies were used to detect α -crystallin and γ-crystallin expression. α –crystallin was visualized as red fluorescent signals and γ-crystallin as green in the lens.

Figure 12A.

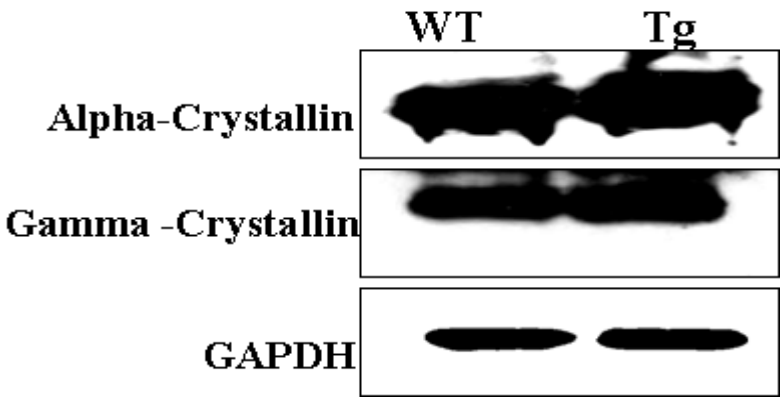


Figure 12B.

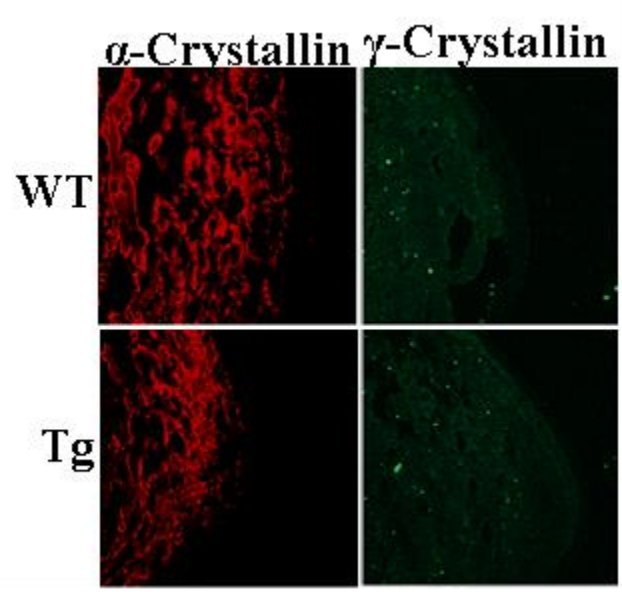
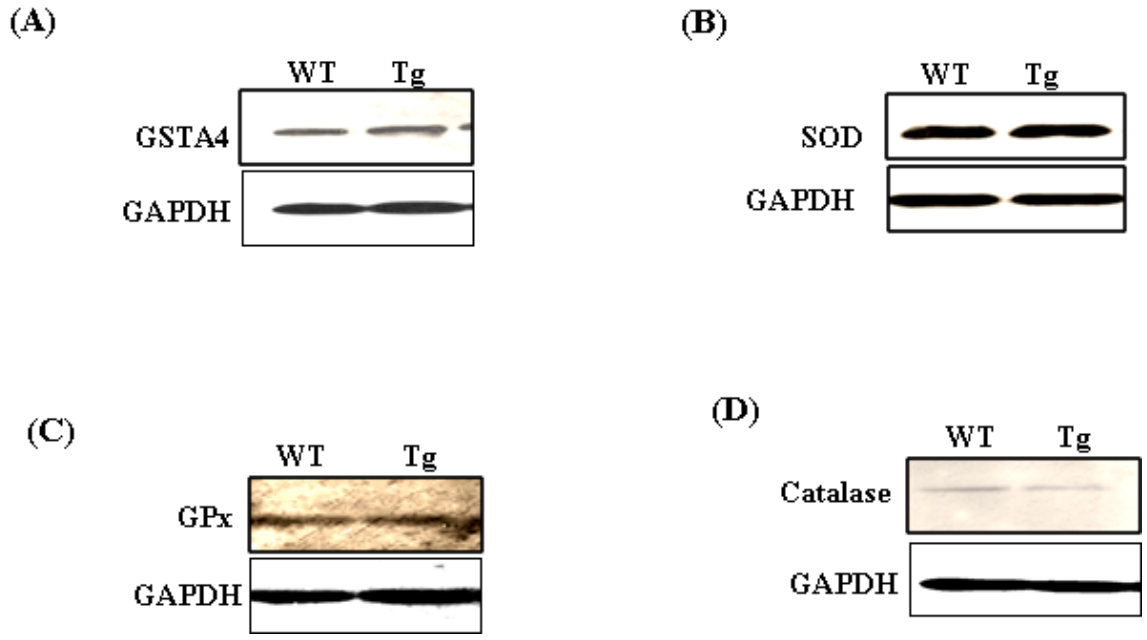


Figure 13: Determination of antioxidant enzymes expression in transgenic mice. Lenses were dissected from eyes of three weeks old wild type and transgenic mice. Isolated lens was homogenized and lysed in RIPA buffer. Western blot analysis was performed with lens extracts using GSTA4, SOD, Catalase and GPx antibodies. GAPDH was used as a loading control.

Figure 13.


(A)
WT
Tg
GSTA4
GAPDH
(B)
WT
Tg
SOD
GAPDH
(C)
WT
Tg
GPx
GAPDH
(D)
WT
Tg
Catalase
GAPDH

3.2 **Specific Aim 2.** Effect of RLIP76 Over Expression on Gene Expression Profile and expression of transcription Factors Involved in Lens Development.

Changes in gene expression profile in lens of RLIP76 Tg mice: To better understand the effects of RLIP76 over-expression on lens morphology, we analyzed changes in the gene expression profile in Tg lens by microarray technology. Microarray analysis was performed by Phalanx Biotech Group, Inc., (Belmont, CA), using Onearray MOA2.0 chip. Total RNA was purified from lenses of 3 weeks old WT and Tg mice. Data generated from microarray experiments was showing modulation of total 9000 genes expression in lens of RLIP76 Tg mice. Further consideration was given to genes, whose expression altered by 1.5 fold at least after normalization with the confidence level of 95%. In Tg lens, 6348 genes were found modulated, (either induced or suppressed) as compared to the WT. These modulated genes were then organized according to their known involvement(s) in biological process using the Database for Annotation, Visualization and Integrated Discovery 6.7 (DAVID 6.7) [131]. A preliminary examination of these results presented in Table 3 suggested that the transcriptional profile of many of the genes induced by RLIP76 over expression was related to changes in visual perception, eye and cell morphogenesis, cell-cell signaling, cell cycle, actin cytoskeleton organization, small G proteins signaling, and RNA splicing and processing. While suppressed genes profile was associated with changes in induction and regulation of apoptosis, transcription and brain development.

Expression of Pax6 is downregulated in transgenic lens:

It is well established that Pax6 is a major transcription factor involved in the initiation and further development of lens. We examined the expression levels of Pax6 in lenses of WT and Tg mice. The

overall expression level of Pax6 is downregulated in Tg lens compared to wild type as shown by western blot analysis (Figure 14A).

This observation was further confirmed by immunofluorescence studies of eyes demonstrating a selective suppression of Pax6 in fiber cells of the lens of Tg mice as compared to the WT mice. The lens fiber cells of the Tg mice showed a remarkable decrease in the levels of P*ax6* (Figure 14B). However, in the lens epithelium of the Tg mice the expression of *Pax6* was not altered significantly. These data suggest that cell specific alterations in the expression and functions of Pax6 may contribute to poor eye/lens development in RLIP76 Tg mice.

To determine when the promoter used for engineering of lens specific RLIP76 Tg mice starts expression of RLIP76 in the lens and to examine the affects of RLIP76 overexpression on lens morphology and Pax6 expression, we performed immunofluorescence studies at E10.5, day 1 and three weeks old mice eye sections. Pax6 was used as a marker of lens development. Results of these studies showed that the promoter starts expressing RLIP76 at lens pit formation stage in E10.5 embryo (data not presented). In the lens of Tg mice at day 1, RLIP76 over expression was notable (Figure 14C), the expression of Pax6 was down regulated, and the size of the lens was smaller as compared to that of wild type mice (Figure 14D). These results suggest that over expression of RLIP76 affected Pax6 expression from the starting of lens development.

Hsf1 and Hsf4b expression is remarkably suppressed in lens of Tg mice:

Heat shock factors (Hsfs), particularly Hsf4b have been suggested to be involved in the mechanisms of lens development through inhibition of fiber cells differentiation [87, 93]. Since previous studies have shown that RLIP76 interacts with Hsf1 and keeps it inactive in the cytosol in physiological

condition [8]. Since Hsf1 and Hsf4b share the most of structural domains, we thought it would be interesting if the expression of these two transcription factors was examined. We compared the expression of Hsf1and Hsf4b in the lens of WT and RLIP76 Tg mice. The results of Western blot analysis presented in Figure 15A showed that both, Hsf1 Hsf4b were remarkably down regulated in the lenses of Tg mice as compared to those of the WT. Suppression of the expression of these Hsfs in the lenses of Tg mice was further confirmed by the results of immunofluorescence studies conducted with the WT and RLIP76 Tg lenses presented in figures 15B and 15C.

Altered expression of Hsps in the lens of RLIP76 Tg mice:

Since the expression levels of HSFs were decreased in transgenic mice and HSPs are downstream targets of HSFs, we examined the effect of RLIP76 overexpression on lens Hsps. Hsps play an important role in lens development and act as chaperones to provide stability to proteins. The results of Western blot analysis (Figure 15D) showed that while the expression of Hsp27 and Hsp40 was down-regulated and that of Hsp60 was up-regulated; the expression of Hsp70 and Hsp90 was not significantly altered in the lens of Tg mice. These results suggest that RLIP76 over expression in lens may affect the functions mediated by Hsfs.

Table 3. Induced or suppressed genes in RLIP76 (RalBP1) Tg lens distributed into biological categories[1]. Upper panel showing organization of induced genes in RLIP76 Tg lens compare to wild type into Gene Ontology (GO) categories and the number of significantly changed genes in the various categories. Lower panel showing suppressed genes in various Gene Ontology categories.

[1]GO analysis was performed using NIH server DAVID http://david.abcc.ncifcrf.gov.
[2]GO ID numbers were obtained from http://www.ebi.ac.uk/GOA
[3]% of total induced or suppressed genes.
[4]T-test p-value with confidence of 95% representing gene enrichment for that pathway

GO ID[2]	GO Category	Genes	%[3]	P-value[4]
Induced genes according to pathways				
GO:0007601	visual perception	63	1.0%	2.4E-11
GO:0050953	sensory perception of light stimulus	63	1.0%	4.4E-11
GO:0016192	vesicle-mediated transport	198	3.3%	1.4E-9
GO:0046907	intracellular transport	184	3.0%	3.6E-9
GO:0016071	mRNA metabolic process	132	2.2%	1.4E-7
GO:0006897	endocytosis	85	1.4%	6.0E-6
GO:0006396	RNA processing	171	2.8%	1.1E-5
GO:0048592	eye morphogenesis	33	0.5%	6.2E-3
GO:0000902	cell morphogenesis	123	2.0%	8.5E-5
GO:0016567	protein ubiquitination	35	0.6%	1.3E-4
GO:0007267	cell-cell signaling	113	1.9%	4.4E-4
GO:0007049	cell cycle	218	3.6%	5.5E-4
GO:0008380	RNA splicing	82	1.3%	6.0E-4
GO:0007155	cell adhesion	200	3.3%	9.5E-4
GO:0042981	regulation of apoptotic process	195	3.2%	2.0E-3
GO:0030029	actin filament-based process	71	1.2%	2.0E-3
GO:0007010	cytoskeleton organization	121	2.0%	2.3E-3
GO:0007264	small GTPase mediated signal Transduction	98	1.6%	2.7E-3
GO:0030036	actin cytoskeleton organization	65	1.1%	6.0E-3
GO:0033554	cellular response to stress	137	2.3%	3.5E-2
GO:0007179	TGF beta receptor signaling pathway	21	0.3%	4.7E-2
GO:0006915	apoptotic process	160	2.6%	1.4E-2
GO:0007169	tyrosine kinase receptor signaling pathway	71	1.2%	2.1E-2
Suppressed genes according to pathways				
GO:0042981	regulation of apoptotic process	12	4.6%	3.6E-2
GO:0045944	positive regulation of transcription From RNA polymerase II promoter	9	3.4%	3.9E-2
GO:0043066	negative regulation of apoptotic process	7	2.7%	4.4E-2
GO:0051338	regulation of transferase activity	7	2.7%	2.1E-2
GO:0030900	forebrain development	6	2.3%	3.4E-2
GO:0006936	muscle contraction	4	1.5%	2.7E-2
GO:0008015	blood circulation	5	1.9%	3.2E-2
GO:0097190	apoptotic signaling pathway	3	1.1%	2.9E-2
GO:0006917	induction of apoptosis	6	2.3%	3.4E-2
GO:0002449	lymphocyte mediated immunity	4	1.5%	4.8E-2

Figure 14: Immunofluorescence and western blot studies for expression of RLIP76 and Pax6 in lens.

(A) Expression of Pax6 is downregulated in RLIP76 over expressed lens tissues. Lenses were dissected from eyes of three weeks old WT and Tg mice. Isolated lenses were homogenized and lysed in RIPA buffer. Western blot analysis was performed with lens extracts using Pax6 and RLIP76 antibodies. GAPDH was used as a loading control.

(B) Suppression in Pax6 expression in Tg lens of three weeks old mice was confirmed by immunofluorescence studies. Eyes from three weeks old mice were dissected and after fixing, sections were made. Eye sections were stained with Pax6 antibody and imaged with confocal microscopy. FITC-conjugated secondary antibody was used to detect Pax6 expression. Pax6 expression was visualized as green fluorescent signals in the lens epithelial and lens fiber cells. Nuclei staining with DAPI were visualized in blue. Arrows showing lens epithelial cells; arrowheads, showing lens fiber cells.

(C) Showing the expression of RLIP76 is up regulated in day 1 old lens of RLIP76 Tg mice as compared to that of WT. Eyes from day one old mice were dissected and after fixing, were sectioned. Eye sections were stained with RLIP76 antibody and imaged with confocal microscopy. Rodamine Red-x conjugated secondary antibody was used to detect RLIP76 expression. RLIP76 expression was visualized as Red fluorescent signals in the lens. Nuclei staining with DAPI were visualized in blue. Abbreviations: L, lens; arrows showing lens epithelial cells; arrowheads, showing lens fiber cells.

(D) Showing the expression of Pax6 is down regulated in day 1 old lens of RLIP76 Tg mice as compared to that of WT. Eyes from day one old mice were dissected and after fixing, were sectioned. Eye sections were stained with Pax6 antibody and imaged with confocal microscopy. Rodamine Red-x conjugated secondary antibody was used to detect Pax6 expression. Pax6 expression was visualized as Red fluorescent signals in the lens. Nuclei staining with DAPI were visualized in blue. Abbreviations: L, lens; arrows showing lens epithelial cells; arrowheads, showing lens fiber cells.

Figure 14A.

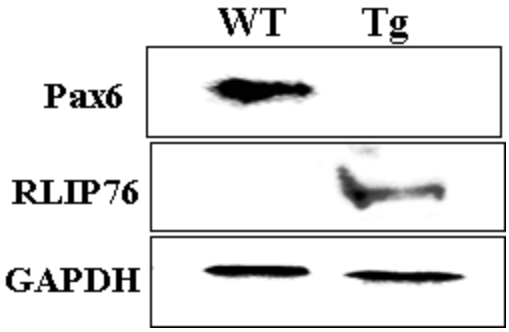


Figure 14B.

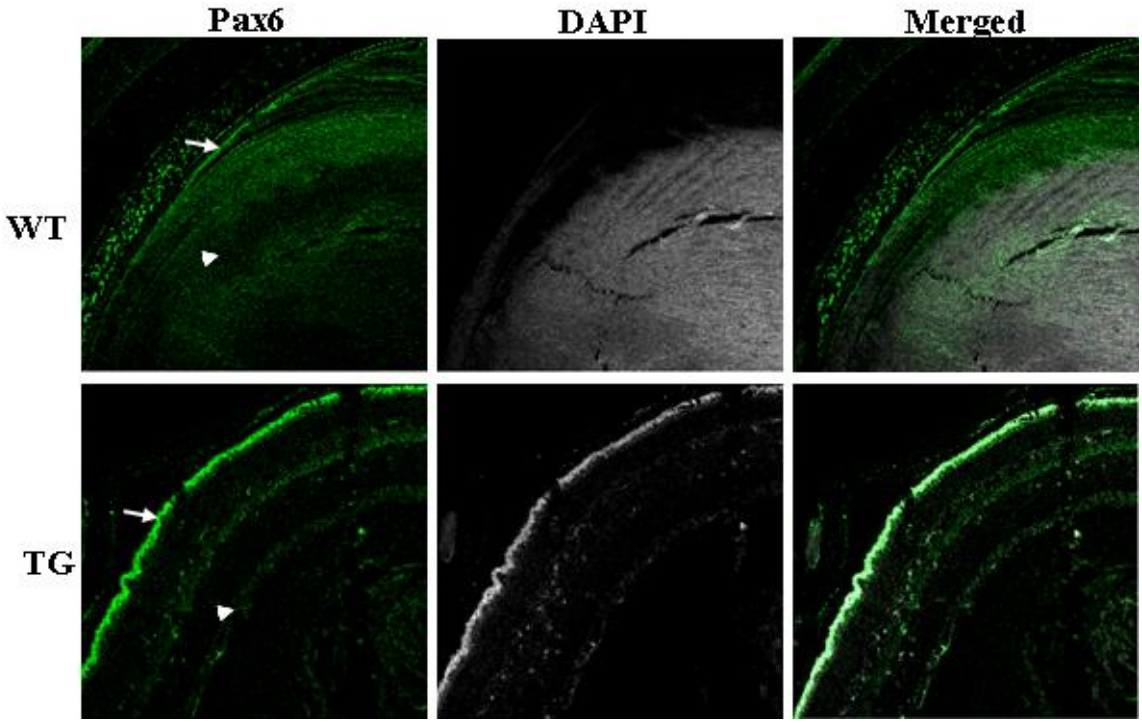


Figure 14C.

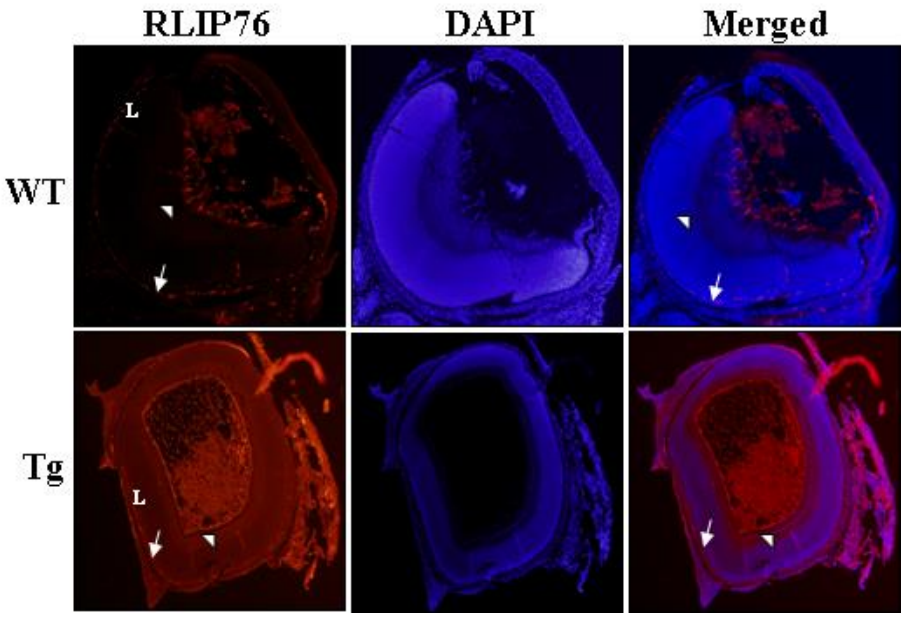


Figure 14D.

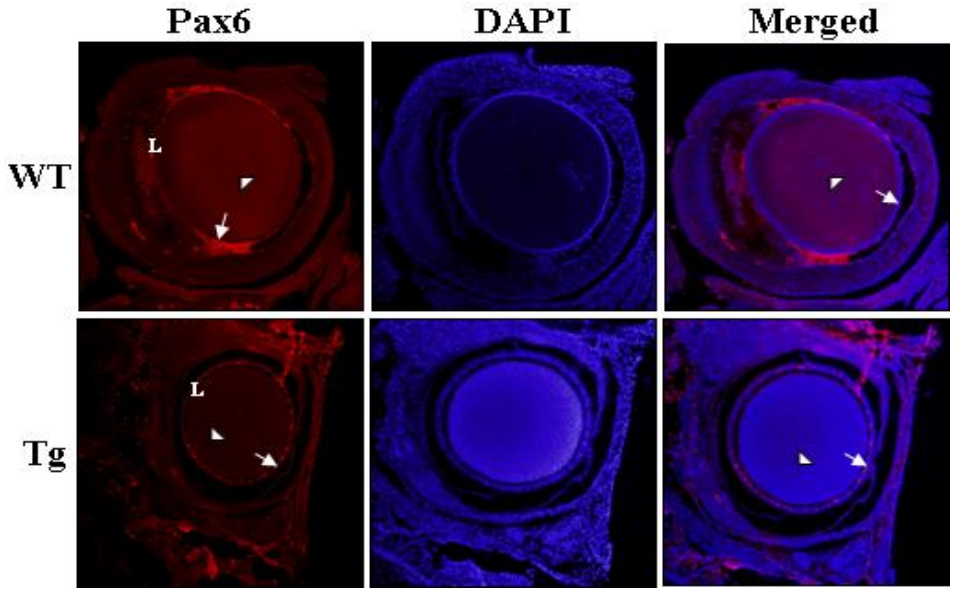


Figure 15: Suppression in Hsf1, Hsf4b, and alterations in Hsps expression in transgenic mice.

(A), Western blot analysis showing down-regulation of the expression of Hsf1 and Hsf4b in Tg lens as compared to the WT. Lenses were dissected from eyes of three weeks old WT and Tg mice. Isolated lenses were homogenized and lysed in RIPA buffer. Western blot analysis was performed with lens extracts using Hsf1, Hsf4b and RLIP76 antibodies. GAPDH was used as a loading control.

(B) Immunofluorescence of Hsf1. showing suppressed expression of Hsf1 in Tg lens as compared to the WT lens. Eyes from day one old mice were dissected and after fixing, were sectioned. Eye sections were stained with Hsf1 antibody and imaged with confocal microscopy. FITC-conjugated secondary antibody was used to detect Hsf1 expression. Hsf1 expression was visualized as green fluorescent signals in the lens. Nuclei staining with DAPI were visualized in blue. Abbreviations: C, lens capsule; arrows showing lens epithelial cells; arrowheads, showing lens fiber cells.

(C) Immunofluorescence showing suppressed expression of Hsf4b in Tg lens as compared to the WT lens. Eyes from day one old mice were dissected and after fixing, were sectioned. Eye sections were stained with Hsf4b antibody and imaged with confocal microscopy. FITC-conjugated secondary antibody was used to detect Hsf4b expression. Hsf4b expression was visualized as green fluorescent signals in the lens. Nuclei staining with DAPI were visualized in blue. Abbreviations: arrows showing lens epithelial cells; arrowheads, showing lens fiber cells.

(D) Western blot comparing the expression of Hsp27, Hsp40, Hsp60, Hsp70 and Hsp90 in WT and Tg lenses. Lenses were dissected from eyes of three weeks old WT and Tg mice. Isolated lenses were homogenized and lysed in RIPA buffer. Western blot analysis was performed with lens extracts using Hsp27, Hsp40, Hsp60, Hsp70 and Hsp90 antibodies. GAPDH was used as a loading control.

Figure 15A.

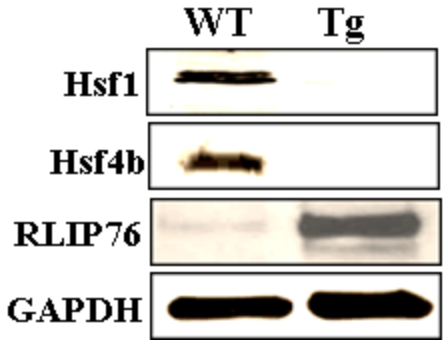


Figure 15B.

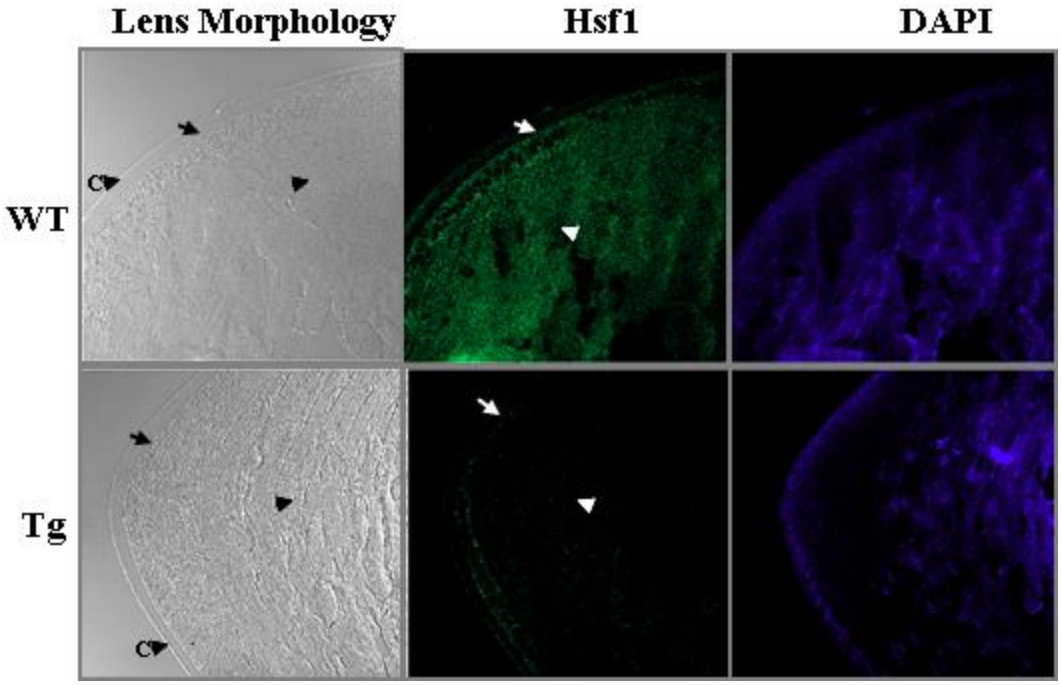


Figure 15C.

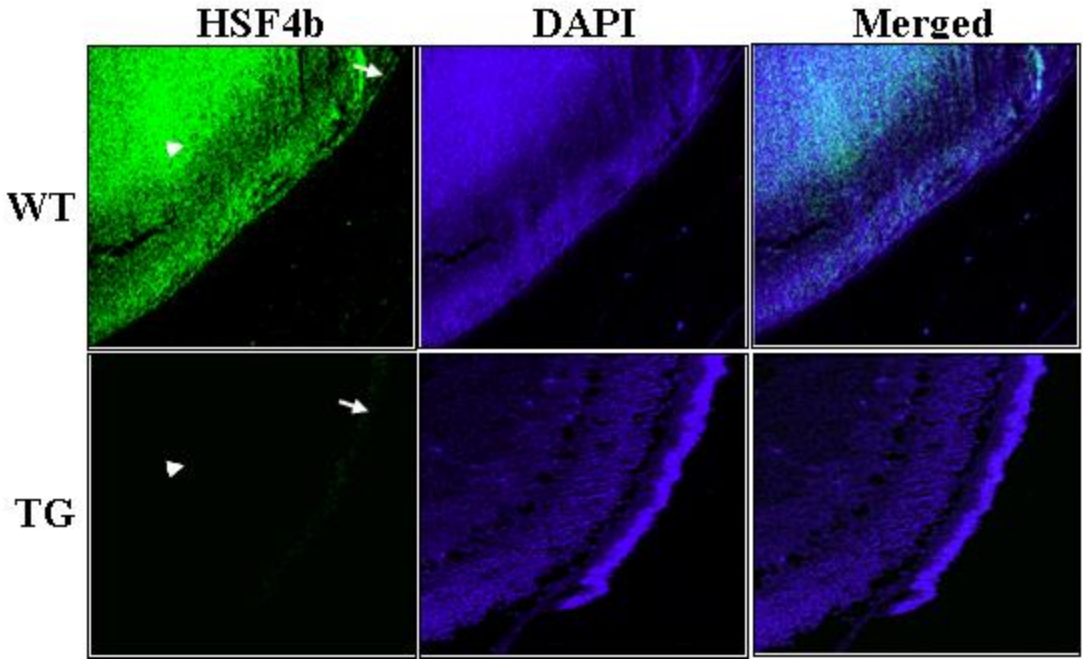
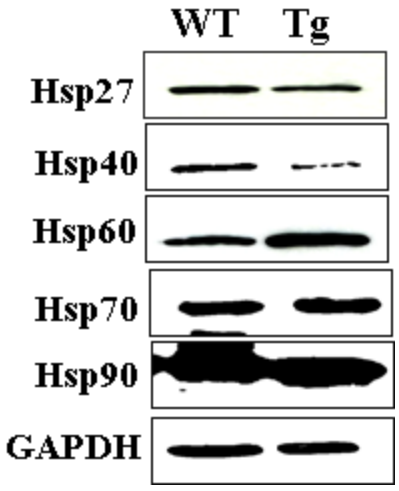


Figure 15D.



3.3 **Specific Aim 3.** Effect of RLIP76 over expression on actin cytoskeleton organization and epithelial cells differentiation.

Overexpression of RLIP76 causes disruption of lens actin cytoskeleton organization:

An actin cytoskeleton structure provides physical support on which lens architecture builds on. A proper actin cytoskeleton organization is required for the normal development of lens and differentiation of fiber cells. Previous studies have suggested that overexpression of Ral GTPase, an upstream molecule to RLIP76, caused disruption of actin cytoskeleton organization of *Drosophila* eye but the mechanism leading to disruption of actin organization and the effect of RLIP76 overexpression were not deciphered. Therefore, we examined the effect of RLIP76 over expression on actin cytoskeleton organization in the lens of Tg mice. We performed Immunofluorescence studies using actin antibody. The results of these studies presented in Figure 16A clearly showed that the lens cytoskeleton in Tg mice was profoundly disrupted while the actin cytoskeleton in wild type lens was intact and well organized.

Activation of Cdc42 is decreased in Tg lens:

It has been documented that Cdc42, a member of Rho GTPase family, plays a pivotal role in the regulation of actin cytoskeleton dynamics. This dynamic of actin cytoskeleton is an important phenomenon necessary for the differentiation of epithelial cells into fiber cells and consequently for the growth of lens. Since RLIP76 has GTPase Activating Protein domain in its structure and organization of cytoskeleton was disrupted in Tg lens, we have examined the activation level of Cdc42 and endogenous expression level. Results of GTPase activation assay presented in figure 16B clearly showed decreased activation levels of Cdc42 in RLIP76 Tg lens compared to the wild type

lens, while the endogenous expression levels of Cdc42 in both group were same. These results suggested that RLIP76 was regulating only the activation of Cdc42 and not the expression of Cdc42. GTPase activation assay kit uses the Cdc42 property of selective interaction in activated state with p21-binding domain (PBD) of p21-activated protein kinase (PAK). Therefore, experiments were conducted in which the lysates from the WT and Tg mice lenses were incubated with agarose beads conjugated with PAK to specifically bind the GTP-bound Cdc42. After washing off the unbound and non specific proteins, bounded Cdc42 were eluted and western blot was run and data shown in the figure 16B. These results indicate that RLIP76-mediated suppression of Cdc42 activation is associated with the disruption of cytoskeleton organization in the lens of the Tg mice.

Phosphorylation of cofilin is decreased in Tg lens:

Activated Cdc42 regulates the phosphorylation of cofilin via the activation of LIM kinase. It is known that un-phosphorylated cofilin binds to actin polymers to depolymerize them into monomers and the phosphorylation of cofilin inhibits its depolymerizing activity [111]. Therefore, we compared the expression and phosphorylation of cofilin in lenses of WT and Tg mice. As presented in figure 16C, a significant decrease in the phosphorylation of cofilin was observed in Tg lens that was consistent with the decreased activation of Cdc42 (figure 16B). However, similar to that observed for Cdc42, the total expression of cofilin was also comparable in WT and Tg lens. Together, these results suggest that RLIP76-mediated suppression of Cdc42 activation in Tg lens results in the suppression of actin depolymerizing activity of cofilin that contributes to the disorganization of actin cytoskeleton in the lens of RLIP76 Tg mice.

Expression of SKAP2 and NCK2:

SKAP2 expression is regulated by Hsf4b and that is involved in the actin cytoskeleton reorganization. It interacts with NCK2, which along with PAK forms a complex involved in the regulation of actin cytoskeleton reorganization. Since Hsf4b expression is suppressed in Tg lens, we have examined the expression of SKAP2 and NCK2 at mRNA levels. RT-PCR examination of these genes revealed expression of SKAP2 mRNA was down-regulated by 50% and that of NCK2 was down regulated by 40% in the Tg lens as compared to the WT lens (Figure 16D). Result presented in figure 16D show the suppression of SKAP2 is consistent with the results showing suppression of Hsf4b. Furthermore, these results suggest that the suppression of Hsf4b and its downstream target SKAP2 are also involved in observed disruption of actin cytoskeleton organization in lens of Tg mice.

Expression of Epithelial-Mesenchymal Transition (EMT) Markers:

We have examined the expression of EMT markers in Tg lens by western blot and Immunofluorescence. When epithelial phenotype changes and acquires phenotype of mesenchymal cells, expression of E-Cadherin switches to N-Cadherin and expression of other proteins such as vimentin, β-catenin and fibronectin get up regulated. Result presented in figure 17A-D showed that in the lens of RLIP76 Tg mice, expression of E-cadherin was up regulated while the expression of N-cadherin, Vimentin, and β-catenin was down regulated as shown in figure 17 A-D. These results suggest that the epithelial phenotype was more prominent in Tg lens as compared to WT lens and that the overexpression of RLIP76 suppresses the transition of epithelial cells into Mesenchymal cells.

Figure 16: Effect of RLIP76 over expression in lens on actin cytoskeleton organization, regulatory genes SKAP2 and NCK2 expression and activation of Cdc42.

(A), Staining of actin cytoskeleton with actin antibody showing the myriad disrupted cytoskeleton organization. Eyes from three weeks old mice were dissected and after fixing, sections were made. Eye sections were stained with actin antibody and imaged with confocal microscopy. Rhodamine Red X-conjugated secondary antibody was used to visualize actin organization. Actin expression was visualized as red fluorescent signals in the lens. Nuclei staining with DAPI were visualized in blue. Lens is indicated as L.

(B) The activation of Cdc42 is attenuated in Tg lens compared to WT.

The lysates from the WT and Tg mice lenses were incubated with agarose beads conjugated with p21-binding domain (PBD) of p21-activated protein kinase (PAK) to specifically bind the GTP-bound activated Cdc42. Western blot analysis of eluted proteins showing decreased amount of GTP bound activated Cdc42 in Tg lens as compare to the WT lens. The expression of endogenous Cdc42 is, however, not changed in Tg lens as compared to the WT lens. GAPDH was used as a loading control.

(C) Phosphorylation of cofilin is decreased in Tg lens as compared to WT.

Lenses were dissected from eyes of three weeks old WT and Tg mice. Isolated lenses were homogenized and lysed in RIPA buffer. Western blot analysis was performed with lens extracts using cofilin and phosphor cofilin antibodies. GAPDH was used as a loading control. A significant decrease in the phosphorylation of cofilin was observed in Tg lens. However, the total level of cofilin is similar in the lens of both WT and Tg mice.

(D) Expression of mRNA of SKAP2 and NCK2 that are known to be regulators of actin cytoskeleton is down-regulated in Tg lens. Lenses were dissected from eyes of three weeks old WT and Tg mice. Isolated lenses were homogenized and lysed in lysis buffer for RNA purification. Purified RNA's quality and quantity was determined by Nanodrop spectrophotometer. cDNA was synthesized and equal amount of cDNA was used for the Real Time Polymerase Chain Reaction(RT PCR). Gene specific primers were used for the amplification and primer sequences are mentioned in Table 4. GAPDH primers were as a housekeeping control for normalization of data.

Figure 16A.

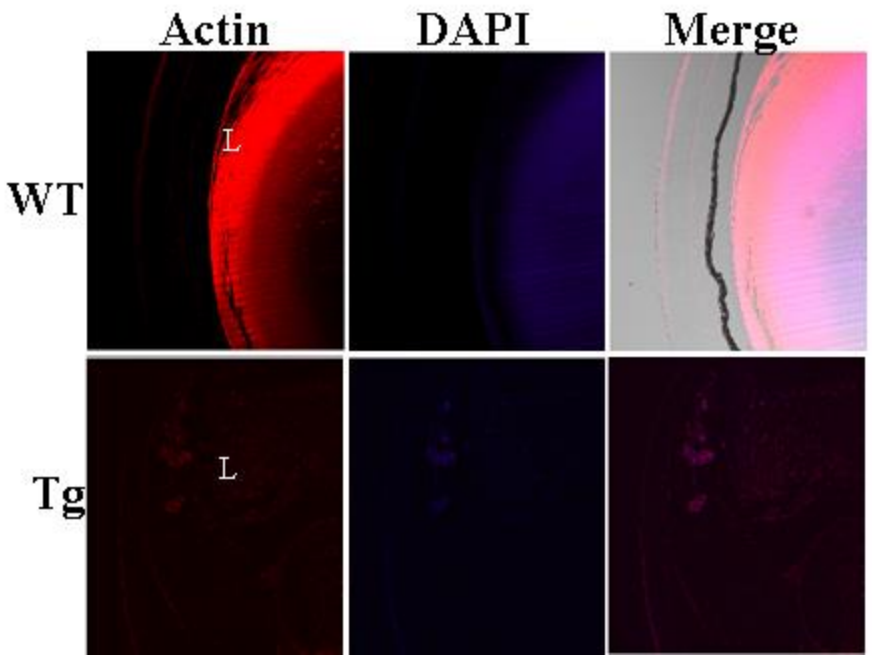


Figure 16B.

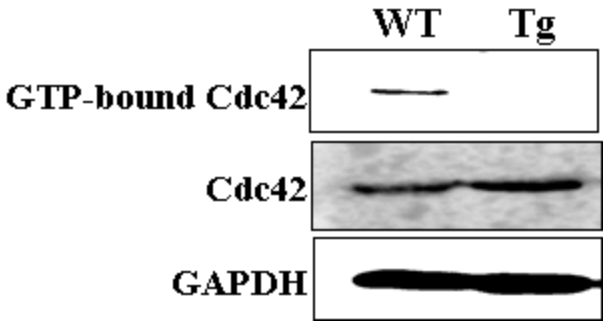


Figure 16C.

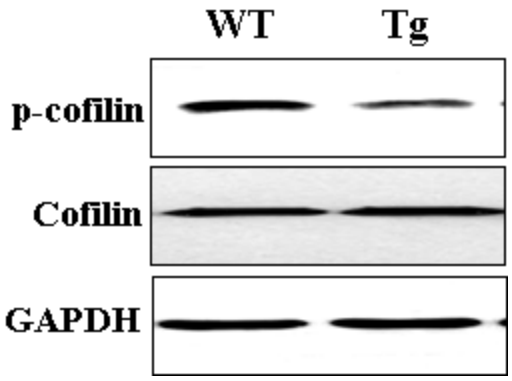


Figure 16D.

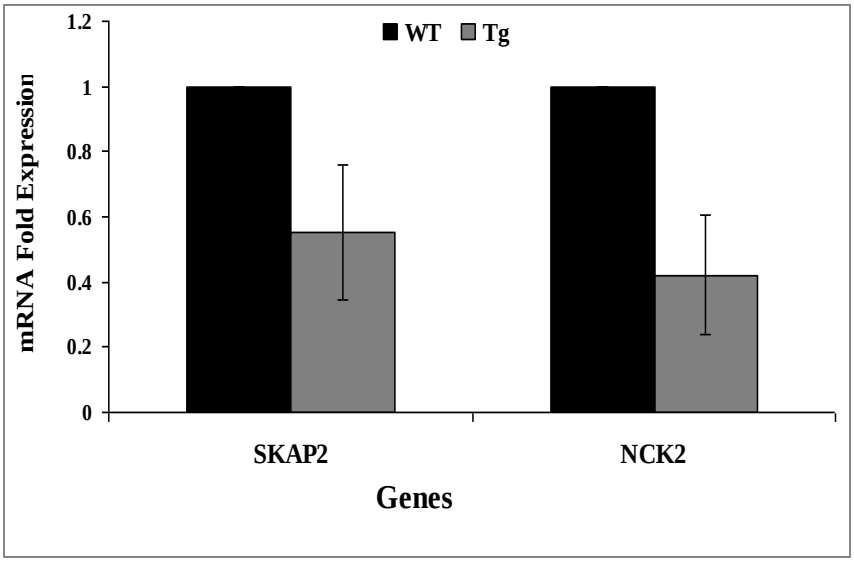


Table 4: Primers used for the RT PCR.

SKAP2 (Forward)	TAGGAACCTGTTGGCAGATGT
SKAP2 (Reverse)	TCTGAGGCTAAGGAAATCGTGT
NCK2 (Forward)	AAGCACTGATGCGGAGTACC
NCK2 (Reverse)	TCATCCTCTCGTTCTGCTACG
GAPDH (Forward)	AGGTCGGTGTGAACGGATTTG
GAPDH (Reverse)	TGTAGACCATGTAGTTGAGGTCA

Figure 17: Effects of RLIP76 overexpression on the expression of epithelial-mesenchymal transition (EMT) markers such as E-Cadherin, N-Cadherin, Fibronectin, Catenin and Vimentin.

(A) Western blot showing decreased expression of Mesenchymal markers N-Cadherin, vimentin, beta-catenin and increased expression of epithelial marker E-Cadherin in Tg lens. Lenses were dissected from eyes of three weeks old WT and Tg mice. Isolated lenses were homogenized and lysed in RIPA buffer. Western blot analysis was performed with lens extracts using E-cadherin, N-cadherin, β-catenin and vimentin antibodies. GAPDH was used as a loading control.

(B, C &D) Immufluorescence study showing suppressed expression of N-Cadherin and Fibronectin and upregulated expression of E-cadherin in Tg lens.

Eyes from three weeks old mice were dissected and after fixing, sections were made. Eye sections were stained with E-cadherin, N-cadherin and fibronectin antibodies and imaged with confocal microscopy. FITC-conjugated secondary antibody was used to detect E-cadherin, Rhodamine Red X-conjugated secondary antibody for N-cadherin and fibronectin expression. E-cadherin expression was visualized as green fluorescent signals, N-cadherin and fibronectin as red in the lens. Nuclei staining with DAPI were visualized in blue.

Figure 17A.

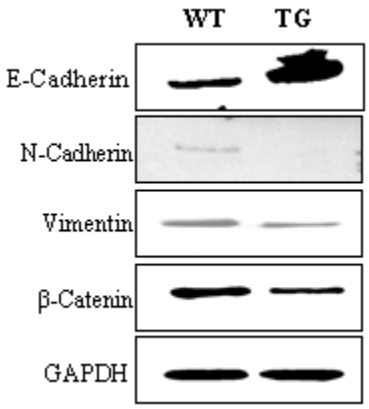


Figure 17B.

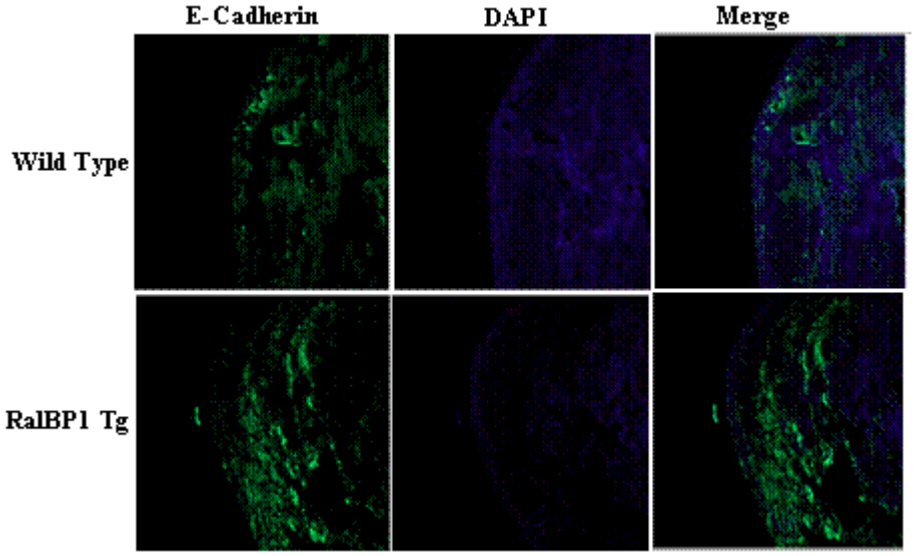


Figure 17C.

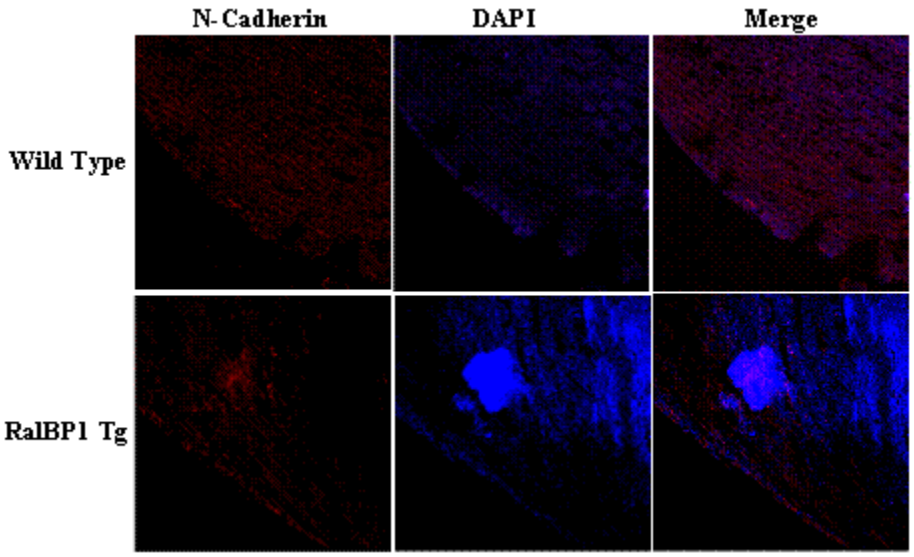
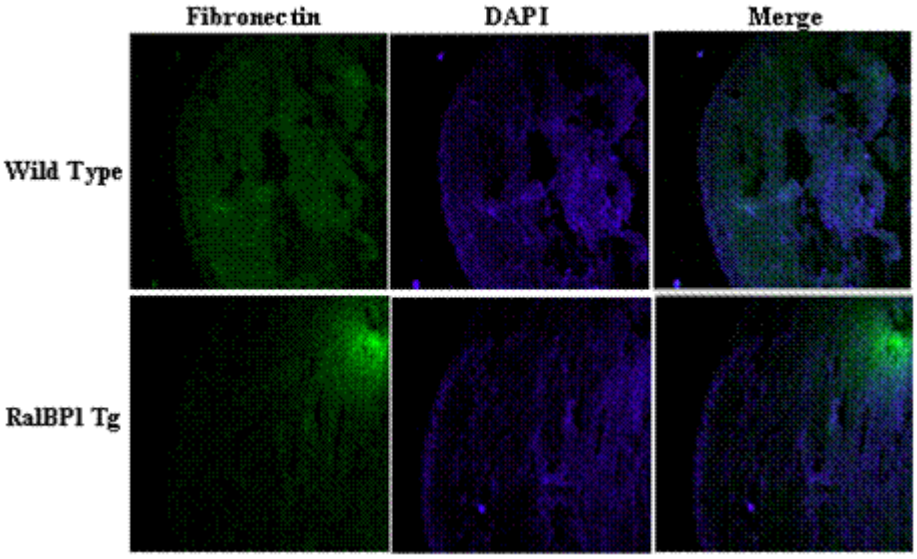


Figure 17D.



CHAPTER IV

DISCUSSION

In this study, we have used eye of mouse as a model system to elucidate functions of RLIP76 in the development of lens. To study the functions of RLIP76 in lens development, we engineered RLIP76 transgenic (Tg) mice using mouse RalBP1 transgene cloned in the vector containing a promoter which provides specificity to gene expression in lens. RLIP76 Tg mice were successfully engineered and five different lines of these RLIP76 Tg mice were produced. Initial genotypical and phenotypical characterization of RLIP76 Tg mice confirmed the integration of mouse RalBP1 transgene in mouse genome and integrated transgene was able to over express RLIP76 specifically in the lens. Breeding between RLIP76 Tg and WT mice was successful and they produced offspring's which carries RLIP76 transgene in the genome. It showed that the integration of transgene had not affected the reproduction in the Tg mice and produced progeny have been able to transfer the integrated exogenous transgene generation to generation. These observations confirmed and verified the RLIP76 Tg model that has been employed in the present study. The results of phenotypical characterization demonstrated that RLIP76 Tg mice appear to possess the smaller eye phenotype having impaired lens and eye tissues development. We report for the first time that lens specific over expression of RLIP76 leads to impaired normal lens development and smaller eye size phenotype similar to that of microphthalmia. Microarray data showing changes in gene expression and relevant pathways in Tg mice presented in the result section (table 3) may be helpful in providing a platform for future mechanistic studies. This data will be submitted in the Gene Expression Omnibus Public Repository at National Center for Biotechnology Information.

Further investigation of biological process and molecules that are affected by the overexpression of RLIP76 showed that the expression of some transcription factors particularly Pax6, Hsf1, and Hsf4b was suppressed in RLIP76 Tg mice. These transcription factors are important players in normal development of lens. Moreover, activation of Cdc42 was decreased and subsequent phosphorylation of cofilin was attenuated in transgenic lens. It can be concluded that deceased activation of Cdc42 is culminating in the disruption in organization of actin cytoskeleton of lens/eye tissues in Tg mice. It is known that actin cytoskeleton reorganization plays an important role in differentiation of fiber cells from epithelial cells via facilitation of cell elongation and cell migration towards the axis of lens and then finally to the core of lens [98, 99]. Our results showed that the expression of epithelial cell markers such as E-cadherin is more prominent in Tg lens as compared to the lens of wild type mice. Up regulation of E-cadherin and presence of more organ free zone (OFZ) in Tg mice suggest that differentiation of epithelial cells into fiber cells is inhibited resulting in reduced number of fiber cells in Tg lens required for growth in to a compact and transparent lens. Abrupt differentiation of fiber cells may therefore be causing a problem with tightly packaging of secondary fiber cells that is required for growth of the lens and for maintaining its transparency. These observations suggest that down-regulation of Pax6, Hsf4b and Hsf1, altered expression of Hsps, increased inactivation of Cdc42, attenuate phosphorylation of cofilin and disrupted cytoskeleton organization are contributing to aberrant differentiation of fiber cells and impaired lens development in RLIP76 Tg mice lens.

Presence of smaller size lens in Tg mice is may be subsequently affecting the positioning, organization, and development of other eye tissues. This could be a reason for appearance of smaller eye phenotype in RLIP76 transgenic mice. RLIP76 Tg mice seem to mimic microphthalmia, a human syndrome that is characterized with variably diminished eye size [59,

67]. The mouse model reported for the first time in this study could be a valuable asset for studying the biochemical and genetic basis of microphthalmia. Since our results suggest a direct correlation between the severity of small eye phenotype of Tg mice and RLIP76 expression, it may be speculated that gain of function mutations of RLIP76 may be involved in the etiology of microphthalmia.

It has been suggested that microphthalmia in newborns may be associated with fetal alcohol syndrome, and certain infections during pregnancy [132]. This seems to be consistent with present findings showing the over-expression of RLIP76 in lens leads to small eye phenotype. Previously, it has been shown that in HLE-B3 and RPE cells, RLIP76 is rapidly induced by several folds by even a minimal oxidative stress [124]. We have confirmed these findings during the present studies. It is possible that a persistent up-regulation of RLIP76 expression due to oxidative stress caused by frequent alcohol consumption or infections could contribute to microphthalmia in newborns. Lens specific RLIP76 transgenic mouse model reported in this dissertation could be a suitable vertebrate model for investigations into the mechanisms involved in lens and eye development.

Role of Pax6:

Our data shows that Pax6 expression is suppressed in Tg lens. It has been known that Pax6 function is critical for eye development including ocular lens. In lens, its role starts from lens specification and subsequent formation. It has been shown that absence of eyes can result in humans and mice if they carry loss of function mutations in Pax6 [63]. Attenuation in Pax6 activity in the heterozygotes for Pax6 mutations also leads to small eye syndrome in mice [64]. Pax6 is important to differentiation epithelial cells to fiber cells [75]. Change in Pax6 levels is

associated with disturbed fiber cells differentiation. Any association between RLIP76 and Pax6 had however not been demonstrated previously and present studies for the first time show that the expression of Pax6 is down regulated upon RLIP76 over expression in lens. Furthermore, our findings indicate that down regulation of Pax6 expression by RLIP76 may be differential and cell type specific. Immunofluorescence studies suggest that it was differentially affected in the lens fibers and epithelial cells. While pax6 expression was suppressed in lens fibers, but in the lens epithelial cells it seemed to be slightly up regulated in the Tg mice. Regulation of Pax6 expression by RLIP76 may be through Cdc42 GTPase. An embryonic study showed that activated Cdc42 induces expression of Pax6 though the activation of mTOR [133]. Since RLIP76 Tg mice have deceased activated Cdc42, therefore it could be attenuating Cdc42 mediated induction of Pax6 through mTOR pathway resulting in the decreased expression of Pax6 in Tg lens as compared to WT. The significance of this suppressed expression and differential regulation of Pax6 by RLIP76 and its relevance to lens development should be further investigated.

Role of HSFs:

Our data also show that expression of Hsf1 and Hsf4b is decreased in Tg lens. Decreased level of Hsf's in Tg lens was corroborated by corresponding reduction in expression of their downstream targets such as Hsp25, Hsp40, and SKAP2. However, the expression of Hsp60 is upregulated, and there is no change in Hsp70 and Hsp90 levels. Clinical significance of Hsf4b in human lens development and formation of cataract has been reported. It has been observed in humans mutations in DNA binding domain of Hsf4b cause formation of early childhood cataract, called

lamellar cataract [88, 89]. We believe changed levels of Hsf's are contributing to impaired lens development through alteration in expression of downstream targets mentioned above.

Hsf's were identified as inducers of Hsp genes and other target genes those have HSE sequence in their promoters. Hsfs have also been shown to play a role in the regulation of development. The single Hsf of *Drosophila* is required for oogenesis and early development [85]. Similarly in mouse, Hsf1 is involved in oogenesis, placental development, and normal growth [86]. Hsf2 is also involved in spermatogenesis, oogenesis, and brain formation [134]. Hsf4b is selectively expressed in lens, lungs, brain and pancreas. However, in lens development only Hsf4b role has been suggested. In lens, it is involved in the expression of γ-crystallin that is involved in protein stabilization in dehydrated conditions [87]. Now it is believed that Hsf4b may behaves like a repressor and compete with Hsf1 as an activator. In fact in lens Hsf4b behaves differently depend on the cell type. For example, it activates the expression of Hsp27 and γ-crystallins in lens fiber cells while it represses the expression of Hsps and FGF in lens epithelial cells [87].

Crystallins and Hsp's are important to maintain and to stabilize lens proteins present in the center. These proteins can not turnover and must remain in stable condition through life to maintain lens transparency and to focus incoming light on the retina. In addition to induction of Hsps expression, Hsf4b also induces the expression of γ-crystallins. These γ-crystallins have heat shock element (HSE) in their promoter for the binding of Hsf4b. However, these HSE sites can also be occupied by Hsf1 but it is argued that Hsf1 remains in monomeric form in the absence of stress [87]. However, trimmers of Hsf4b can be available in normal physiological conditions and Hsf4b binds to the promoter of γ-crystallins at HSE and induces its expression. Thus, Hsf4b, not Hsf1, plays a role in the development of lens. Hsf4b and Hsf1 can regulate proliferation and differentiation of lens epithelial cells via controlling the expression of fibroblast growth factor

(FGF). FGF is documented as a proliferation stimulator at low dose while at high dose it stimulates differentiation of epithelial cells in lens [87, 135]. Hsf4b and Hsf1 competitively regulate FGF expression, Hsf1 acts as an inducer for the FGF expression while Hsf4b as a repressor. It has been reported that knockdown of Hsf4b in lens caused increased proliferation and premature differentiation of epithelial cells in to fiber cells while double knockdown of Hsf1 and Hsf4b had restored normal proliferation and differentiation [87, 90]. Our findings showing profound suppression of Hsf4b and Hsf1 in Tg mice confirm the role of Hsf4b signaling in lens development and demonstrate for the first time the regulation of Hsf4b by RLIP76.

We noted that Hsp25 and Hsp40 expression is decreased and Hsp60 expression is increased in RLIP76 Tg mice. Altered Hsps expression is due to suppressed expression of Hsf1 and Hsf4b in the lens of RLIP76 Tg mice. The function of Hsps is to refold denatured proteins into their native confirmation. Thereby, they provide protection to cells from stress induced apoptosis and promote cell survival [83, 84]. In exposure to stress, cell responds by drastic modification of cytoskeleton and a rapid induction of Hsps synthesis. Many Hsps are reported to interact with different cytoskeleton components, large Hsps such as Hsp70 and Hsp90 bind to microtubule network and centrosome while sHsps (small Hsps) are involved in maintaining the integrity of actin polymers and intermediate filaments [136]. Hsp27 binds at the end of actin filament and prevents further addition of actin monomer, thus prevents polymerization of actin polymers. This function of Hsp27 is depending on its phosphorylation status. During stress, binding of Hsp27 to actin polymer provides stability to actin filaments, moreover, it helps in reorganization of actin cytoskeleton to recover from deformities induced by stress. Since, expression of Hsp27 is down regulated in Tg lens and it may be possible that suppressed expression of Hsp27 is contributing to disrupt cytoskeleton organization phenotype of Tg lens [136].

We also observed suppressed expression of SKAP2, a downstream target of Hsf4b, which is involved in reorganization of actin cytoskeleton [137]. Results showed that SKAP2 mRNA expression is downregulated in RLIP76 Tg lens. Interestingly, mRNA expression of NCK2 is also downregulated, which interacts with SKAP2 during the reorganization of actin molecules. Together these two proteins form a complex in response to FGF that is involved in promotion of actin cytoskeleton reorganization during differentiation of fiber cells. It has been shown that knockdown of Hsf4b causes reduced expression of SKAP2 and disrupts actin cytoskeleton, which leads to aberrant differentiation of fiber cells. We have observed disruption of actin cytoskeleton and defect in fiber cells differentiation in RLIP76 Tg lens, that can be attributed to reduced expression of Hsf4b and down regulation of SKAP2 that lead to disruption of actin cytoskeleton and the inhibition of fiber cells differentiation.

Role of Cdc42:

Our results show that the activation of Cdc42 is compromised in the lens of RLIP76 Tg mice, however total expression levels of Cdc42 is not affected. RLIP76 promotes GTP hydrolysis activity of Cdc42 which results in the inactivation of it. Cdc42 acts as a regulator of actin cytoskeleton reorganization, so we further examined the phosphorylation status of cofilin. Our data show that phosphorylation of cofilin is decreased in lens of Tg. However, the level of total cofilin expression is same in both wild type and Tg lens. Reduction in cofilin phosphorylation was expected as it is also regulated by activated Cdc42. In activated state Cdc42 regulates cofilin phosphorylation through the activation of PAK which subsequently activates LINK to phosphorylate cofilin and thus inactivates it [111]. De-phosphorylated cofilin has preferential binding with actin polymers to convert them into monomer subunits. Presence of more de-

phosphorylated cofilin in RLIP76 Tg lens is expected to affect the organization of actin cytoskeleton.

It has been suggested that RLIP76 acts as a connecting link between two GTPase, Ral and Cdc42. Ral, the upstream protein to RLIP76 (RalBP1), has been implicated in development of *Drosophila* eye, where constitutively active Ral was inhibiting normal cell shape changes, a step necessary for normal eye morphology [127]. Inhibition of change in cell shape was due to disruption in actin cytoskeleton organization upon expression of constitutively active Ral. It was speculated that this outcome was possibly because of regulation of Cdc42 activity by Ral through its effector RLIP76, but direct evidences were not presented in the study. Our study confirms this speculation that RLIP76 is regulating Cdc42 activation and is acting as a connecting link between Ral and Cdc42 GTPase. Present studies provide direct evidence for the role of RLIP76 in activation of Cdc42 in ocular tissues and are consistent with earlier studies showing a developmental role of RLIP76 in the embryonic development of *Xenopus* at gastrula stage, where it affects actin cytoskeleton organization [14, 15]. Actin cytoskeleton reorganization plays an important role in lens fiber cells differentiation from epithelial cells for the growth and maintaining the transparency of lens. Further studies are needed to understand the mechanism via which RLIP76 over expression disrupts cytoskeleton organization.

CHAPTER V

CONCLUSIONS

RLIP76 is known as a responder to oxidants and as a protective agent to ocular tissues from oxidative stress. It provides protection from toxicants via their transport out the cell. Thereby, RLIP76 prevents lens from oxidative stress induced cataractogenesis. We had engineered RLIP76 transgenic (Tg) mice with the purpose to study whether enhanced transporting capacity of lens provides more protection to lens from oxidative stress in transgenic mice. Surprisingly, these transgenic mice appeared to have smaller eye phenotype and impaired lens development; they have phenotypical similarities with microphthalmia. Following conclusions can be derived from the present study:

- RLIP76 transgenic mice possessed small eye phenotype and lens development was impaired in these mice. Smaller eye phenotype is similar to that of a congenital pathological condition known as microphthalmia in humans. Thereby, RLIP76 Tg mice can serve as a model to study the mechanisms involved in human microphthalmia.
- Presence of undeveloped and small lens in eye affected the development and positions of other ocular tissues. That could lead to small size phenotype. Examination of changes in gene expression profile of RLIP76 Tg mice, suggested that the expression of a number of genes was significantly affected in these mice. These altered genes are known to be involved in the pathways in several biological processes such as actin cytoskeleton reorganization, brain development, G-coupled protein signaling, transportation, endocytosis, and apoptosis. Alteration in these pathways in RLIP76 Tg mice could contribute to impairment of lens development and microphthalmia.

- Expression of the transcription factors such as Pax6, Hsf1, Hsf4b and their down stream targets Hsps was suppressed in Tg lens. These proteins are key regulators of normal lens development and functioning. Suppressed levels of these proteins could contribute to the aberrant development of lens in RLIP76 Tg mice.
- Morphological examination of lens of these transgenic mice showed that actin cytoskeleton organization that plays an important role in differentiation of lens epithelial cells into lens fiber cells was profoundly disrupted in Tg lens. Differentiation of fiber cells is required for the growth and maintenance of the transparency of lens. Disruption in actin cytoskeleton organization could be contributing to the impaired lens development through inhibition of the differentiation of epithelial cells into fiber cells.
- Our mechanistic studies suggest that the disruption in cytoskeleton organization in Tg lens was due to the decreased activation of Cdc42 GTPase, an important regulator of actin cytoskeleton organization. We demonstrate that the inactivation of Cdc42 leads to attenuated phosphorylation of cofilin that is necessary to stop the depolymerization of actin polymers into monomers, which inhibits depolymerization activity of cofilin.
- Taken together, the findings of this study suggest a major role of RLIP76 in the development of lens/ eye through the regulation of transcription factors such as Pax6, Hsf4b and Hsf1, and organization of actin cytoskeleton. Earlier studies had suggested Ral GTPase, an upstream to RLIP76, was regulating *Drosophila* eye development through inhibition of the actin cytoskeleton organization via Cdc42. However, role of RLIP76 had not been explored in these studies. Present study fills that gap in the information and shows that RLIP76 acts a connecting link between Ral and Cdc42 GTPases and plays an important role in the development of lens.

CHAPTER VI

FUTURE DIRECTIONS

We have demonstrated that over expression of RLIP76 leads to impaired lens development and a small eye phenotype similar to that of microphthalmia. Also, in RLIP76 Tg lens the organization of actin cytoskeleton was disrupted and a regulator of cytoskeleton, Cdc42, activation and subsequent phosphorylation cofilin was attenuated. Transcription factors such as pax6, Hsf1, Hsf4b and their downstream targets Hsps expression was also downregulated. These factors are necessary for the normal development of lens.

We observed that lens development in RLIP76 Tg mice was impaired in day one and three weeks old mice. Lens became smaller in size and over all eye size was reduced. For understanding the effect of RLIP76 over expression on lens development, it would be interesting to examine the effects of RLIP76 over expression in various stages of lens development in mouse embryo. These studies should provide insight about the stage at which RLIP76 over expression causes defects in lens development. Future studies in this direction should be conducted.

In present study we showed Pax6 expression was downregulated. It is a major transcription factor involved in determination of fate of lens development. Mechanisms involved in RLIP76-mediated down regulation of Pax6 have not been studied yet and should be studied in future. Earlier brain development studies have suggested the regulation of Pax6 expression by Cdc42 through mTOR. In future studies we can elucidate the role of RLIP76 in activation of mTOR. Since, we have shown RLIP76 to be a regulator of Cdc42 activation future studies on mechanisms of the regulation of Cdc42 must be conducted. Furthermore, the regulation of Hsf4b and Hsf1 by RLIP76 in Tg lens can be studied to delineate the mechanisms involved in the regulation of expression of Hsf4b and Hsf1 also needs to be studied.

Since our Tg mice show microphthalmia phenotype, it would be interesting to test the hypothesis that "gain of function mutations of RLIP76 may be the genetic basis of microphthalmia". Microphthalmia in newborns is associated with fetal alcohol syndrome. Since RLIP76 transgenic mice are showing a phenotype similar to microphthalmia and RLIP76 is also responsive to oxidative stress, it would be an interesting study to investigate whether RLIP76 does play a role in the etiology of alcohol-induced microphthalmia in newborns.

CHAPTER VII

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