Stony Brook University

Connexin50 Intercellular Communication and Its Interaction with Mitogen Activated Protein Kinase Signaling: Implications for Post Natal Lens Growth

A Dissertation Presented

by

Teresa Inez Shakespeare

to

The Graduate School

in Partial Fulfillment of the

Requirements

for the Degree of

Doctor of Philosophy

in

Physiology and Biophysics

Stony Brook University

May 2008

Stony Brook University

The Graduate School

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Abstract of the Dissertation

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In the lens, and almost all tissues gap junction channels are encoded by connexin (Cx) genes and are responsible for the coupling of cells to facilitate the exchange of ions, small metabolites and nutrients. Lens homeostasis depends on these intercellular connections to help coordinate development; as well as supply nutrients to make of for the loss of blood vasculature. In mice, deletion of different connexins alters ocular growth and differentiation in the lens. For example, knockout (KO) of Cx46 reduces lens homeostasis and causes the formation of cataracts. In the Cx50 deficient lens, the rate of postnatal mitosis in the lens epithelial cells is suppressed and lens size is reduced, in addition to mild nuclear cataract formation and defects in fiber maturation. Replacement of Cx50 with Cx46 by genetic knock-in (KI) rescued the cataract phenotype; however the ocular growth defect persisted. Two hypotheses have emerged, the first is that Cx46 is

necessary to maintain lens clarity and the second is that Cx50 is essential for the maintenance of proper ocular growth and lens fiber differentiation. Lens growth and differentiation are regulated by mitogens whose receptors are expressed in the lens. My hypothesis is that Cx50 and mitogen activators may function together to regulate lens cell proliferation and differentiation during postnatal growth. To address this question, dual whole-cell voltage clamp measurements were done on cell co-transfected with either Cx46 or Cx50 and mitogen activated protein-signaling (MAPK). Co-expression of Cx50 with a constitutively active form of MEK (MEK1(E)) caused junctional conductance to increase nearly three fold, without altering Cx50 protein expression. In contrast, MEK1(E) did not alter coupling provided by Cx46. To further address the relationship of Cx50 and MAPK signaling, MEK1(E) transgenic mice were crossbred with Cx50KO, Cx46KO or Cx50KI46 mice. Interbreeding of MEK1(E) transgenic and Cx50KO mice produced lenses that were 20% smaller than wild type with severe nuclear cataract, but a transparent lens cortex. The deletion of Cx50 in MEK1(E) mice, but not Cx46 delayed cataract formation and prevented lens rupture caused by MEK1(E). Histology and lens glucose analysis confirmed that the removal of Cx50 rescued vacuole formation in MEK1(E) lenses. The deletion of Cx50 also reduced postnatal mitosis in the central epithelium of MEK1(E) lenses. These findings support the hypothesis that Cx50 is essential for lens cell proliferation and differentiation, and suggest a role for the interaction of Cx50 and MAPK signaling in order to stimulate proper growth and homeostasis of the lens.

Table of Contents

List of Figures

List of Tables

List of Abbreviations

ATP	Adenosine tri-phosphate
BMP	Bone Morphogenetic Protein
BSA	Bovine serum albumin
Ca^{2+}	Calcium
$CaCl_2$	Calcium chloride
CK1	Casein Kinase 1
cAMP	Cyclic adenosine mono-phosphate
Cav-1	caveolin
cDNA	Complementary deoxyribonucleic acid
cGMP	Cyclic guanine monophosphate
cRNA	Complementary ribonucleic acid
C-terminal	Carboxy terminus
Cl	Chloride
Cx	Connexin
Cx38	Connexin38
Cx43	Connexin43
chCx43	chicken Connexin43
Cx46	Connexin46

zfCx43	Zebrafish Connexin43
zfCx44.1	Zebrafish Connexin44.1
zfCx48.5	Zebrafish Connexin48.5
mCx50	murine Connexin50
DF	Differentiated fiber cells
dpf	Days Post Fertilization
DTT	Dithiothreitol
E1	Extracellular loop 1
E2	Extracellular loop 2
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal Growth Factor
EGTA	Ethylene glycol tetra acetic acid
ERK	Extracellular Regulated Kinase
FGF	Fibroblast Growth Factor
FGFR	Fibroblast Growth Factor Receptor
Ga	Gauge
G_j	Junctional conductance
G_{jss}	Steady state junctional conductance
G_{jmax}	Maximum junctional conductance

G_{jmin}	Minimum junctional conductance
H_2O	Dihydrogen Oxide
HCL	Hydrochloric acid
HEPES	4-(2-Hydroxyethyl) piperazine-1-ethanesulfonic acid
HRP	Horseradish Peroxidase
IACUC	Institutional Animal Care and Use Committee
IgG	Immunoglobulin G
I_j	Junctional current
I_{jss}	Steady state junctional current
I_m	Membrane current
IGF	Insulin-like Growth Factor
IGFR	Insulin-like Growth Factor Receptor
IP_3	Inositol triphosphate
K^+	Potassium
KCl	Potassium chloride
kDa	Kilodalton
KI	Knock In
KO	Knock Out
L-15	Leibovitz Medium

LEC	Lens Epithelial Cells
LEDGF	Lens Epithelia Derived Growth Factor
Lp82	Lens calpain protease
MB	Modified Barth's Medium
MAPK	Mitogen Activated Protein Kinase
MEK	Mitogen Activated Protein Kinase Kinase
MEK1(E)	Constitutively active Mitogen Activated Protein Kinase Kinase
MF	Mature fiber cells
$MgCl_2$	Magnesium chloride
MI	Mitotic Index
MIP	Major Intrinsic Peptide
mm	Millimeter
mM	Millimolar
MS-222	Tricaine methanesulfonate
mV	Millivolt
MΩ	Megaohm
nA	Nano-amperes
Na^+	Sodium
Na_2CO_3	Sodium bicarbonate

Na_3VO_4	Sodium Orthovanadate
NaN_3	Sodium azide
NaOH	Sodium hydroxide
nM	Nanomolar
OR3	Oocyte Ringers Buffer 3
P	Postnatal
PBS	Phosphate buffered saline
PDGF	Platelet Derived Growth Factor
PKA	Protein Kinase A
PKC	Protien Kinase C
PMSF	Phenylmethanesulphonylfluoride
PVDF	Polyvinylidene fluoride
RNA	Ribonucleic acid
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
T	Temperature
TBS	Tris buffered saline
TGF-ß	Transforming Growth Factor Beta
UO126	MEK1/2 Inhibitor
V_j	Junctional voltage

V_m	Membrane voltage
μg	Microgram
μM	Micromolar

Acknowledgements

This dissertation would have not been possible without the support of so many people. I must first give honor to my Lord and Savior, Jesus Christ, who has guided my spirit and helped me to maintain my sanity throughout this intensive process. There have been so many people who have helped me reach this accomplishment and I am grateful for all of them. However, there a few very special people that I must mention by name.

First and foremost, I would like to acknowledge my thesis advisor, Dr. Thomas W. White for all of his support and guidance during my studies in his laboratory. It has truly been an honor to work with such a brilliant individual. I am mostly thankful for his patience, kind spirit and willingness to always provide scholarly and moral support. Over the years, his always calm and collected nature has amazed me. I can only hope that some of his qualities have rubbed off on me. He has truly been a blessing to me and words cannot explain the respect and gratitude that I have for him. I would also like to extend a special thanks to the members of my thesis committee, Dr. Peter Brink, Dr. Richard Mathias, Dr. Raafat El-Maghrabi (my guardian angel), and Dr. Linda Musil for their support throughout this process. I should especially thank Dr. Musil for her constant input and suggestions to make my project better, but most of all, for her travels from Oregon, three times since last year. The members of my committee have truly been beneficial with helping me to improve and complete my thesis and I am forever indebted to you all.

The members of the White laboratory have been a pleasure to work with. Let me start with Dr. Caterina Sellitto, she has been extremely helpful in challenging me to grow

and become a better scientist in the lab. Her candor and friendship have been extremely helpful over the years. I am so thankful for her in this past year; she has been so kind and diligent about providing me with the animals to complete this project. Without the work and talent of both her and Helen, this project would have not been possible. Lastly, I would like to thank my lab mates Gulistan, for always being so nice, Adam for always making laugh even when I wanted to cry and Dwan, for just being Dwan…I am thankful for you all.

There have been many wonderful friends who have been influential in helping me to navigate my way through graduate school. First of all, I would like to give a special thanks to Marjorie BonHomme, who has been a mentor and a friend to me. Next, would be Omar Bakht for always offering advice, and most of all for keeping in contact with me this year, even though he moved to Boston. Lastly, would be Maureen and Arthur Voos, my landlords who have been a surrogate family to me from the very beginning.

Last, but certainly not least, I have to acknowledge my family for being my biggest cheering squad. I am certain that had it not been for there support and that I would not be able to write this dissertation. I am blessed to have a wonderful mother and father (Joseph and Sandra) who have always supported my dreams and aspirations, and encouraged me to always challenge myself and for never allowing me use the words (I can't). Last of all, I owe the biggest thanks possible to my older sister (Wanike) and brother (Joseph) for there continued encouragement. Thank you for always picking me up and dusting me off when I fall, wiping away when I cry and most of all for pushing

me to be my very best self. Oh, and how could I forget, all the financial support!!! You are phenomenal and I am so blessed to have the both of you in my life.

Chapter I

Introduction

Structural and functional organization of gap junctions

Gap junctions are intercellular channels that form a pathway between neighboring cells, allowing the direct exchange and passage of small molecules, ions, and metabolites {Bennett and Goodenough, 1978} {Bruzzone and Ressot, 1997}. These channels are responsible for mediating both electrical and metabolic cell- to- cell communication {Bruzzone and Ressot, 1997}. The structural proteins that form gap junctions are called connexins (Cx). Each connexin consists of four trans-membrane domains with two extracellular loops and one intracellular loop. The N and C termini are located in the cell cytoplasm. The extracellular loops are believed to be involved in aligning gap junctions from neighboring cells and the N and C termini, while the intracellular loop is thought to be a target for post translational modification (Figure I-1) {Bruzzone et al., 1996} {Willecke et al., 2002}. Connexon structures are formed when six individual connexin subunits oligomerize to form a heteromeric or homomeric type hemi-channel. Intercellular gap junction channels are formed when connexons from adjacent cells align in the extracellular space, in order to form a heteromeric, heterotypic or homotypic functional channels (Figure I-2). The diversity of the formed connexons depends on the composition of the connexin subunits that form the channel. Homomeric connexons are formed when a single type of connexin oligomerizes, and heteromeric connexons are made up of different connexin subunits. The complexity of connexons diversity is further established when a full intercellular gap junction channel is formed, as it may be composed of connexons from the same or different connexin subunits. Homotypic channels are channels made of identical connexons, and heterotypic channels are made up of different connexons.

Gap junction communication maintains lens homeostasis

The lens forms when epithelial cells invaginate from the surface ectoderm to form a hollow ball called the lens vesicle. The posterior cells elongate to fill the lumen, forming the primary lens fiber cells which complete their differentiation by accumulating high levels of crystallin proteins and degrading light scattering organelles {Menke et al., 2002}. Cell division occurs within the anterior epithelium and cells migrate toward the equator, where they elongate and differentiate into secondary fiber cells, stretching from the anterior to the posterior poles and ensheathing the underlying primary fibers. This process continues throughout life so that there are always two distinct populations of secondary fiber cells present; a shell of differentiating fibers (DF) near the lens surface and the mature fibers (MF) in the lens core (Figure I-3). The structural organization of the lens is unique in its design. Because the cells in the lens core are devoid of all of their cellular organelles, the process of oxidative phosphorylation is impossible. Therefore a unique system for intercellular transport has been created, in order to allow the delivery of ions and metabolites and the removal of waste products. Three distinct connexin genes have different spatial and temporal expression in the lenses of vertebrates. Cx43, Cx46 and Cx50 share the responsibility to promote proper development and maintain lens homeostasis {White et al., 1992} {Gerido and White, 2004}. Cx43 and Cx50 are expressed between lens epithelial cells on the anterior surface. However, as these cells differentiate into fiber cells that make up the bulk of the lens volume, Cx43 is significantly down regulated. In contrast, Cx50 and Cx46 undergo up regulation to maintain junctional coupling in fiber cells where Cx43 is absent {Gong et al., 1997} {Beyer et al., 1989} {Musil et al., 1990} {White et al., 1992} {Benedetti et al., 2000}.

The synchronized events of up and down regulating levels of connexin protein expression and channel activity are critical for fulfilling the normal physiological role of connexin mediated communication in the lens.

Circulation in the Lens

Although gap junctions play a key role in the maintenance of lens homeostasis; the lens also depends on Na+, K+ and Cl^- conductance to create the circulating current, with sodium as the primary current carrier {Mathias et al., 1997}. Through this model, Na+ coupled with water enters the lens at the anterior and posterior poles and flows inward along the extracellular spaces and is driven by its electrochemical gradient to move into the fiber cells. After reaching the intracellular space, the direction of flow is reversed and transported back to lens surface by gap junctions. Na/K-ATPase activity is concentrated at the equatorial region of the lens, and the intracellular flux transported out of the lens to complete the circulatory loop {Gao et al., 2000} {Candia and Zamudio, 2002} {Tamiya et al., 2003} (FigureI-4). The fluid circulation pattern driven by the Na^+ current enables Ca^{2+} to flow through the lens in a similar manner {Donaldson et al., 2001} {Gao et al., 2000}.

Genetic alterations of lens connexins explain the importance of gap junction communication

Genetically altered mouse models that are deficient in the lens connexins have been used to examine the role of each connexin gene. Many studies have concluded that the expressions of individual lens connexins are necessary to maintain lens clarity and

growth. Studies of Cx43 knockout mice revealed cardiac malformation and neonatal lethality {Reaume et al., 1995}. Prenatal lens development was found to be largely normal; however the studies were impeded because of the lethal outcome {Gao and Spray, 1998} {White et al., 2001}. However this was not the case for Cx46 or Cx50. The deletion of Cx46 resulted in a severe, senile-type cataract, with normal ocular development and growth (Figure I-5) {Gong et al., 1997} {Gong et al., 1999}. Reports revealed that the cataracts observed were initiated in the nuclear region of the lens, and were the result of cleavage and precipitation of γ-crystallin proteins and the activation of calpain protease Lp82, which appeared in three week old mice and was directly related to the degree of opacity seen in the lens {Gong et al., 1997} {Baruch et al., 2001}. Analysis of Cx46's contribution to lens homeostasis was further examined by targeting the replacement of Cx50 with Cx46, thus generating the Cx50KI46 model. In this particular study, mice with the Cx50KI46 genotype were able to maintain lens clarity; but displayed a significant decrease in growth in the eye and the lens. The decreased growth phenotype has previously been reported in the Cx50 knockout animal model, indicating the necessity of specific lens connexins to maintain lens homeostasis {White, 2002} {Rong et al., 2002} {White et al., 1998}.

Cx46 is Important for Ca+ Homeostasis in the Lens

Cx46 is instrumental in the buffering of Ca^{2+} ions in order to maintain the low Ca^{2+} concentration within the fiber cells. The role of intercellular calcium and its impact on cataract formation was examined. Mathias's model proposed that Cx46 in the mature fibers is responsible for facilitating the cycling of Ca^{2+} ions to the lens equator {Mathias

et al., 2007}. When Cx46 is lost, Ca^{2+} cycling is disturbed and intracellular calcium accumulates and leads to cataractogenesis. The model for Ca^{2+} cycling in the lens was supported by measurements of intracellular calcium concentrations in wild type, Cx46 knockout and Cx50KI46 lenses. The findings revealed that intracellular calcium was highest in Cx46 knockout lenses, and was the result of a reduction in coupling caused by the ablation of Cx46 {Gao et al., 2004}. Thus, there was a strong correlation between the magnitude of Cx46 mediated coupling and the intracellular calcium concentration, suggesting that Cx46 was the rate limiting factor in calcium efflux. These results were consistent with the hypothesis of calcium activated proteases initiating the Cx46KO cataract, and further supported by recent studies of a Cx46/Calpain3 double knockout mouse model showing that cataracts were significantly delayed and γ crystallin cleavage was absent{Tang et al., 2007}. These results provided evidence that is consistent with the Cx46 and calcium hypothesis {Mathias et al., 1997} {Baruch et al., 2001}.

Cx50 and Lens Growth

The exploration of Cx46 has provided a great deal of knowledge about lens homeostasis, but Cx50 seems to be equally influential in lens development and homeostasis. Cx50 is initially expressed in the lens epithelial cells and later in the fiber cells with Cx46. The deletion of Cx50 causes a severe growth defect and mild nuclear cataracts, which are less severe than the cataracts seen in Cx46 knockouts {White, 2002} {Rong et al., 2002}. Upon further characterization of Cx50 ablation, a distinct lamellar opacity in the outer regions of the lens was observed {White et al., 1998} {Gerido and White, 2004}. Crystallin protein precipitation was responsible for the observed cataracts

and the growth defect was attributed to fewer fiber cells. This lead to the conclusion that the rate of lens epithelial cell proliferation was negatively impacted {White et al., 1998}.

The mechanism by which microphthalmia and the reduction in fiber cells in the Cx50 knockout lens remains unknown; however several studies have provided correlative data in an attempt to explain this question. The loss or replacement of Cx50 (Cx50KO or Cx50KI46) caused a decrease in the number of dividing cells on post natal days two and three. These results confirmed that Cx50 intracellular gap junction communication is essential for peak mitosis to occur in the post natal lens {Sellitto et al., 2004} (Figure I-6). Analysis of a Cx50/Calpain3 double knockout mouse also supports the above findings. Where, the disruption of calpain 3 was able to prevent cataract formation, but the growth defect was persistent. In addition calpain altered the solubility of crystallin proteins and αß crystallin may possibly be involved in cataract formation {Tang et al., 2007}.

Analyses of a dominant cataractous mouse line (L1) identified a missense Cx50 mutation. The mutation resulted in expression of Cx50-S50P mutant proteins, and it was revealed that expression of mutant Cx50-S50P and Cx50 subunits inhibited the elongation of embryonic lens fiber cells. In addition to this finding, expression of Cx50-S50P and Cx46 subunits resulted in a disruption of differentiation and elongation of postnatal lens fibers {Xia et al., 2006}. In another study, a separate Cx50 mutation, Cx50-G22R demonstrated the necessity of Cx50 and Cx46 interaction in differentiating fibers. Whereby, the expression of Cx50-G22R and Cx46 subunits resulted in dense cataract formation, disruption in nuclear fiber formation and posterior lens rupture {Chang et al., 2002}. Taken together, the results of these two studies suggest that both

Cx50 and Cx46 connexin subunits are responsible for mediating unique mechanisms that influence the developmental fates of lens primary and secondary fiber cell formation.

Most recently, Cx50 has been shown to functionally contribute to epithelial cell coupling during post natal day 2 and 3. In this study it was shown that coupling between lens epithelial cells from wild type lens was suppressed (~90%) in the presence of quinine, an anti-malarial drug that selectively blocks Cx50 gap junctions. Conversely, the junctional conductance of Cx50KI46 lens epithelial cells was not altered. To further confirm that Cx50 coupling in the lens epithelium during the first post natal week is important, P28 lens epithelial cells were treated with quinine and junctional conductance was suppressed by only 24% (Figure 1-7). In correlation, proliferation patterns (via BrdU analysis) of post natal and adult lens epithelial cells suggested that there is a proliferative shift in connexin isoform expression, from Cx50 to Cx43 at P28. Concluding that, optimal proliferation at P2 and P3 requires Cx50, and epithelial cell function in older lenses is not Cx50 dependent {White et al., 2007}.

Further analyses of Cx50 and other contributors of the ocular microenvironment are needed to provide a more complete portrait of Cx50 function. All three connexins are responsible for producing unique phenotypes in the lens. Cx43 is expressed in the lens epithelium and many other tissues and its function in the lens continues to be characterized, Cx46 is largely responsible for the maintenance of lens clarity and Cx50 is imperative for proper lens growth.

Mitogen Activated Signaling Pathways are Functional in Cell Proliferation and Differentiation and Regulate Gap Junction Function

While much is known connexins and growth factors in the lens separately, the implications of how they may function together remain unclear. Members of the fibroblast growth factor (FGF), transforming growth factor (TGF β), insulin-like growth factor (IGF), platelet derived growth factor (PDGF) and epidermal growth factor (EGF) families have been identified for lens epithelial cells (Table I-1) {Lang, 2004}. The assembly and disassembly of gap junction can be regulated by environmental factors such as growth factors, drugs and stress related factors. An example of this is insulin-like growth factor (IGF)-1, which causes the disassembly of surface Cx43 plaques, and the exposure of cells to hydrogen peroxide can cause oxidative stress {Lin et al., 2003} {Hossain et al., 1999}. These effects are due to the activation of protein kinase C (PKC) and subsequent phosphorylation of connexin proteins. Connexins are phosphor-proteins, and phosphorylation is considered to be a regulatory mechanism on gap junction communication {Reynhout et al., 1992} {Saez et al., 1998}. The activation of many kinases can down regulate the formation of gap junction plaques by phosphorylation on serine, threonine, and/or tyrosine at the C terminus of connexin proteins. Cx43 can be phosphorylated on serine 255, 279, 282, 325, 328, 330, 364, 368, or 372 by PKC, PKA, casein kinase 1, or mitogen activated protein kinase (MAPK) {Lampe and Lau, 2000} {Ruch et al., 2001} {Cooper and Lampe, 2002} {Rivedal and Opsahl, 2001} {Reynhout et al., 1992} {Berthoud et al., 2000} {Saleh and Takemoto, 2000}. The recently identified lens epithelial derived growth factor (LEDGF) is a growth, adhesive, differentiation and anti-apoptotic factor that is found at low levels in most actively dividing cells and lens epithelial cells and it is a weak activator of transcription {Khaliq et al., 1999} {Ge et al., 1998}. The growth factor is secreted by the lens epithelial cells

into the extracellular space where it binds to the cell surface, and is then transferred through the cytoplasm into the nucleus and nucleolus; lens epithelial cells that lack LEDGF die within 2-9 days {Singh et al., 1999}. The effects of LEDGF activation on PKC γ and gap junction disassembly in lens epithelial cells have been investigated and it has been demonstrated that LEDGF activates PKC γ and consequently decreases gap junction activity through phosphorylation of Cx43 {Nguyen et al., 2003}. Other studies have reported that PKC γ can regulate the distribution of gap junction plaques within Cav-1 containing lipid rafts. Their results demonstrated that PKC γ co-immunoprecipitated with caveolin (Cav-1) and Cx43 in lipid rafts when the cells were exposed to TPA or IGF-1. Concluding that PKC γ activation is necessary for gap junction redistribution out of plaque clusters, and may have an effect in the redistribution of lipid rafts {Lin et al., 2003}. The disruption of gap junction communication is not solely mediated by PKC γ. PKC α and PKC γ have been reported to have inverse effects on gap junction activity {Wagner et al., 2002}. Therefore further the investigation of the signal transduction of PKC γ in the lens epithelial cell is needed to understand how LEDGF and PKC γ influence gap junction proteins.

FGF is expressed throughout lens development, and both in vitro and in vivo studies conclude that FGFs are important for regulating polarization of the lens, along with epithelial cell proliferation and fiber differentiation. Chamberlain and McAvoy showed that FGF2 could functionally stimulate lens cell differentiation {Chamberlain and McAvoy 1987} {Chamberlain and McAvoy 1989}. It was later documented that if FGF concentrations were altered, different cellular responses could occur. These results led to the hypothesis that an FGF gradient is necessary for polarization of the lens, where

0.15ng/mL of FGF was sufficient to stimulate proliferation of the lens epithelium; and 40ng/mL was required in order for cell differentiation to occur {McAvoy and Chamberlain 1989}. In addition, studies have shown that FGF activity levels are higher in the vitreous than in the aqueous humour {Schilz et al., 1993}. Many in vivo experiments using transgenic mice have shown that a number of FGF members (1, 3, 4, 7and 8) can stimulate fiber cell differentiation {Robinson et al., 1995; Robinson et al., 1998; Lovicu and Overbeek et al., 1998}. The results of these experiments showed that that expression of FGF1 from the alpha- cystallin promotor and secreted from the lens fibers could stimulate lens cell differentiation from the epithelial cells. Many of the results from transgenic models have led to a variety of conclusions; the most affirmative is that FGF inhibition reduces fiber cell differentiation {Chow et al., 1995, Robinson et al., 1995 and Lang 1999}.

The interaction of fibroblast growth factor (FGF) and gap junction-mediated intercellular communication in the lens has been described {Le and Musil et al., 2001}. In primary cultures of embryonic chick lens, FGF was reversibly responsible for up regulating gap junctional intercellular dye transfer without increasing gap junction protein synthesis or assembly. They further demonstrated that FGF was able to induce sustained activation of extracellular regulated kinase (ERK) in lens cells and that FGF-induced activation in the intact lens is higher in the equatorial region than in the polar and core fibers. These data correlate well with the hypothesis that gap junction mediated intercellular coupling is higher in the equatorial region of the lens than at either the anterior or posterior poles of the organ {Baldo and Mathias, 1992} {Mathias et al., 1997}. Thus the analysis of these findings have led to a proposed model that suggests

that regional differences in FGF signaling via the ERK pathway produced the observed asymmetry of gap junctional intercellular coupling in the lens {Le and Musil., 2001}.

Members of the transforming growth factor (TGF) β family are necessary for lens fiber differentiation and maturation {De Iongh et al., 2001} {Faber et al., 2002} {Belecky-Adams et al., 2002}. TGF β can induce development in lenses of opaque sub-capsular fibrotic plaques {Hales et al., 1994} {Liu et al., 1994} {Srinivasan et al., 1998} {Lovicu et al., 2002} {Lovicu et al., 2004}. Transgenic mice with TGF β- induced cataracts had sub-capsular plaques made up of heterogeneous cell populations, myo-fibroblastic cells and lens fiber like cells. In addition they reported that the cells in these plaques did not express lens epithelial markers, Cx43 and Pax6, these findings were similar to human sub-capsular cataracts.

MAP Kinases

MAPKs are abundantly expressed in the lens and thought to be involved in lens development {Lovicu et al., 2005} {Li et al., 2003}. MAP kinase kinase (MEK) is an upstream activator of ERK1/2, and works specifically to activate the MAPK signaling cascade in order to mediate cellular responses to growth and differentiation factors {Mansour et al., 1994}. The kinases are unique because they are dual specific kinases, which mean that they can phosphorylate tyrosine and serine/threonine residues on the same protein. This property gives the protein an advantage over others in the pathway, in that it can allow longer activation states of itself and downstream effectors {Mansour et al., 1994}. In the interest of understanding the signaling pathways involved in cell proliferation and differentiation, the mitogen activated protein kinase (MAPK) signaling

cascade is thought to be involved in the total process of lens development. Because this pathway has been studied in detail and the extracellular regulated kinases (ERKs) are abundantly expressed in the lens {Li et al., 2003}, studying the MAPKs involvement in lens development is essential. More importantly, the FGF signaling cascade is influenced by the activation of the MAPK pathway.

The Raf/MEK/ERK MAPK pathway is particularly important. The activation of growth factor receptor tyrosine kinases can activate Ras proteins that are necessary for signal transduction to occur. In this signaling cascade, MAP kinases, MEK1 and MEK2 are protein kinases that are responsible for phosphorlylating both ERK1 and ERK2 at specific tyrosine and serine/threonine residues. ERK1 and ERK 2 activity have been reported active in the lens epithelium and fiber cells, which suggest that MAPKs are operating in the region of the lens where the circulating current of the lens is maintained. Other studies have shown that activated Ras is able to induce lens epithelial cell hyperplasia, but not premature differentiation {Reneker et al., 2004}. In this study the role of Ras signaling was examined, and analysis of the embryonic lens showed that the N-Ras, the classical Ras protein is expressed at high levels during early postnatal development. Secondly, they revealed that Ras activation induced lens cell proliferation, but not differentiation; and concluded that other signaling pathways are necessary for lens cell differentiation. In a follow-up study, this group expressed a dominant negative Ras in the lens of transgenic mice and reported that lens proliferation was reduced and fiber cell elongation was delayed {Xie et al., 2006}.

Gong {Gong et al., 2001} designed a transgenic mouse model that expressed a constitutively active MEK1 transgene under the control of the αA-crystallin promoter to

examine the effects of the MAPK pathway in lens function. The transgenic model displayed an array of morphological and pathological changes in the lens. Initially, the animals displayed no dramatic differences, but at 2 weeks an observed a change in the eye mass was revealed. At 4 weeks of age, the mass of eye had increased by 50 to 65% in comparison to the wild type animals. In addition, at 5 weeks of age, large vacuoles were present in the cortical regions of the lens. The lenses of transgenic mice also showed a large increase of the glucose transporter 1 (GLUT-1) activity, along with subsequent development of marcophthalmia and cataracts. Interestingly, there were no initial changes in α-, β-, and γ-crystallin distribution in the lenses in 2 to 4 week old transgenic mice. However, 2.5 month old transgenic lenses with a nuclear cataract resulted in a significant loss in the γ-crystallins.

Observation of Mammalian Lens Connexins Phenotypes in other Vertebrates

37 putative connexin genes from the zebrafish (*Danio rerio*) family have been identified. Phylogenetic comparison studies have screened connexin gene families from human (h), murine (m) and zebrafish (zf). The study revealed that of the 37 zebrafish connexin genes, 23 are relatives of 16 mammalian connexins and the remaining 14 are unique to zebrafish {Eastman et al., 2006}. Three zebrafish lens connexins, Cx43, Cx44.1 and Cx48.5 have been identified and characterized. Analyses showed that these three zebrafish connexins have spatial expression patterns that are similar to orthologues in bird and mammals. Amino acid sequence analyses revealed that zfCx48.5, mCx46 and chCx56 are categorized in one subfamily. Zebrafish Cx48.5 and mouse Cx46 share a 61% amino acid sequence identity (Figure IV-1) {Cheng et al., 2003} {Eastman et al.,

2006}. In an initial study, we characterized the electrophysiological properties of zfCx48.5 {Cheng et al., 2004}. The results of this study are discussed in chapter IV. ZfCx48.5 and mammalian Cx46 share many structural and functional features. The identification of zebrafish Cx44.1 and Cx43 show an orthologous relationship to mammalian Cx50 and Cx43, respectively. This study revealed that zfCx48.5 possessed electrophysiological properties that we similar to mammalian Cx46, and that knockdown of Cx48.5 in zebrafish embryos, resulted in the formation of cataract, small eyes and small lenses. In addition, that intercellular gap junction mediated communication is not only essential for development of the mammalian lens, but other vertebrates as well.

Questions addressed in this dissertation

This dissertation will analyze the potential interaction of Cx50 gap junction intercellular communication and the MAPK signaling pathway. Cx50 is influential in supporting proper lens development {Sellitto et al., 2004; White et al., 2002; White et al., 2007}. With the knowledge and apparent importance of distinct connexin expression during development as well as the importance of growth factors during lens development and growth, we hypothesize that lens connexins and mitogen activated factors may cooperate to influence the developmental fates of the lens. Chapter IV focuses on a separate study where we analyzed the electrophysiological properties of Cx48.5, a zebrafish connexin with the highest known sequence identity to mammalian Cx46 {Cheng et al., 2004}. This study provided consistent evidence that intercellular gap junction mediated communication is essential for development of the mammalian lens, as well other vertebrates. The main theme of the dissertation will be addressed in chapter V.

Where, we examined Cx50 and Cx46 and its involvement with mitogens, in particular FGF and MAPK. Here, the experiments conducted first analyzed the electrophysiological properties of gap junction communication in the presence of mitogens, *in vitro*. In an *in vivo* model, we examined the density of lens cell proliferation, cataract progression, histology, mitotic patterning and glucose concentration of the lens to elucidate the impact of connexins and mitogen signaling in the lens. The data presented supports previously documented data that explain the necessity of Cx50 for post natal growth and provides new insights that may explain the mechanism by which proper lens growth is achieved in the presence of Cx50.

Figures and Legends:

Figure I-1. Schematic map of mouse Cx50 protein. This diagram displays the amino acids in the full-length Cx50 protein. The Cx50 protein is comprised of a cytoplasmic N-terminus, intracellular loop and C-terminal domains that are positioned within cell. The extracellular loops 1 and 2 are located in the extracellular space, while the four trans-membrane domains traverse the plasma membrane (illustration from A. DeRosa).

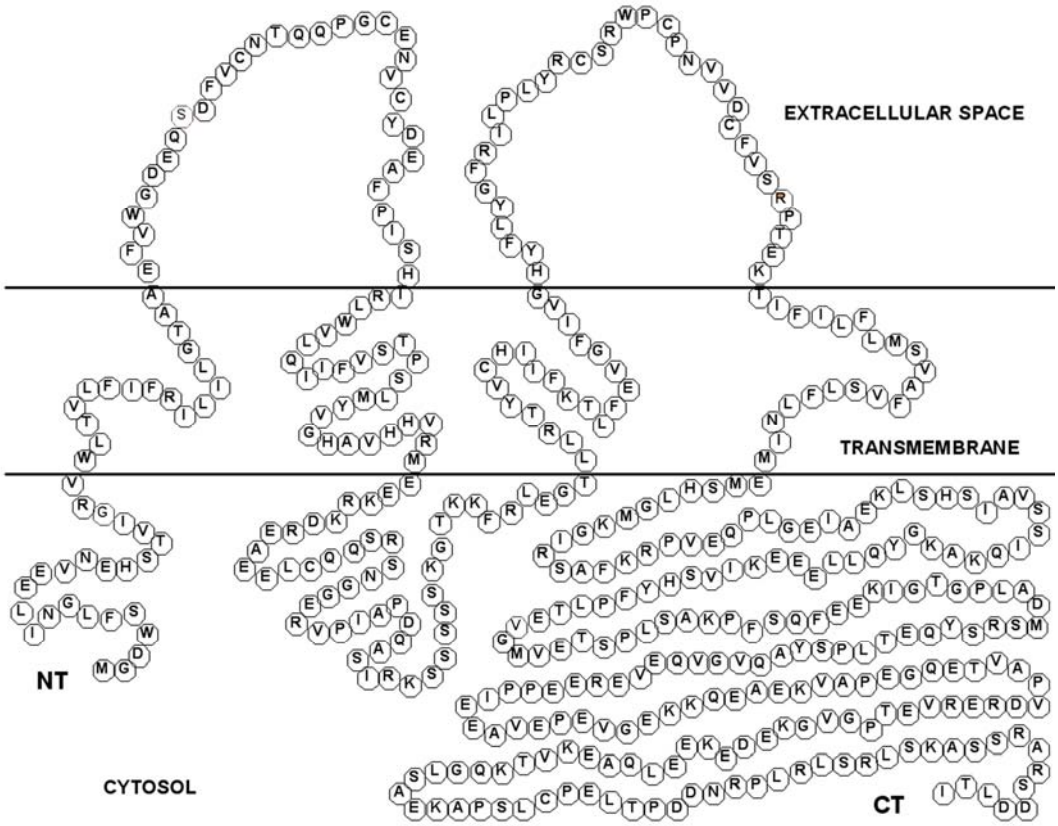

EXTRACELLULAR SPACE
TRANSMEMBRANE
NT
CYTOSOL
CT

Figure I-2. Schematic of a gap junction channel. Six individual connexin subunits oligomerize to form heteromeric or homomeric hemi-channels. Intercellular gap junctions are formed when connexin hemi-channels from adjacent cells align in the extracellular space, and form heteromeric, heterotypic or homotypic functional channels (White et al., 1999).

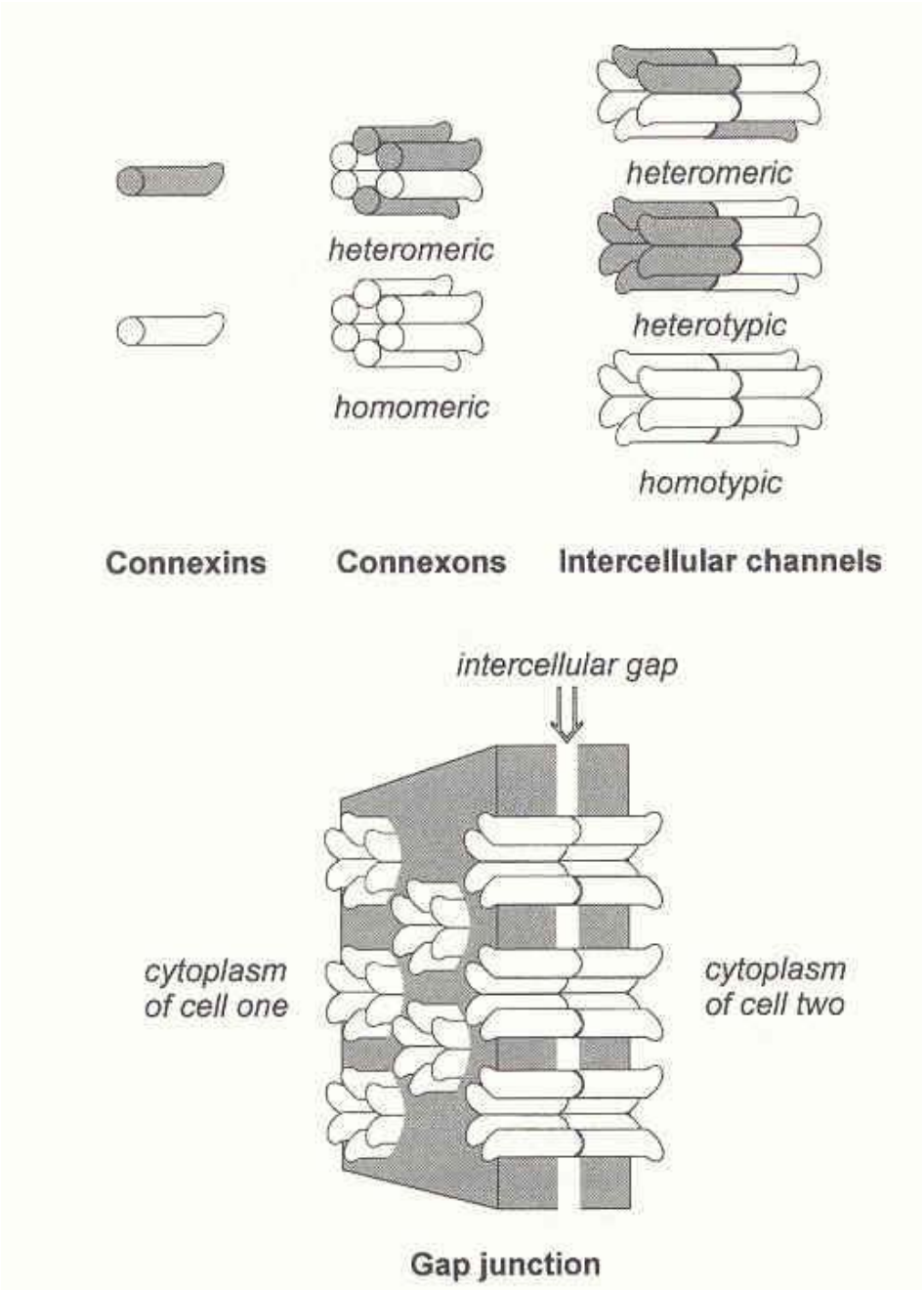

heteromeric
heteromeric
heterotypic
homomeric
homotypic
Connexins
Connexons
Intercellular channels
intercellular gap
cytoplasm of cell one
cytoplasm of cell two
Gap junction

Figure I-3. Vertebrate development of the lens. This figure shows the developmental stages of the lens. The lens starts as a hollow ball of cells surrounded by a lens capsule. The posterior cells elongate to fill the lumen, forming the primary lens fiber cells which complete their differentiation by accumulating high levels of crystallin proteins and degrading light scattering organelles. Cell division occurs within the anterior epithelium and cells migrate toward the equator, where they elongate and differentiate into secondary fiber cells, stretching from the anterior to the posterior poles and ensheathing the underlying primary fibers. This process continues throughout life so that there are always two distinct populations of secondary fiber cells present; a shell of differentiating fibers (DF) near the lens surface and the mature fibers (MF) in the lens core.

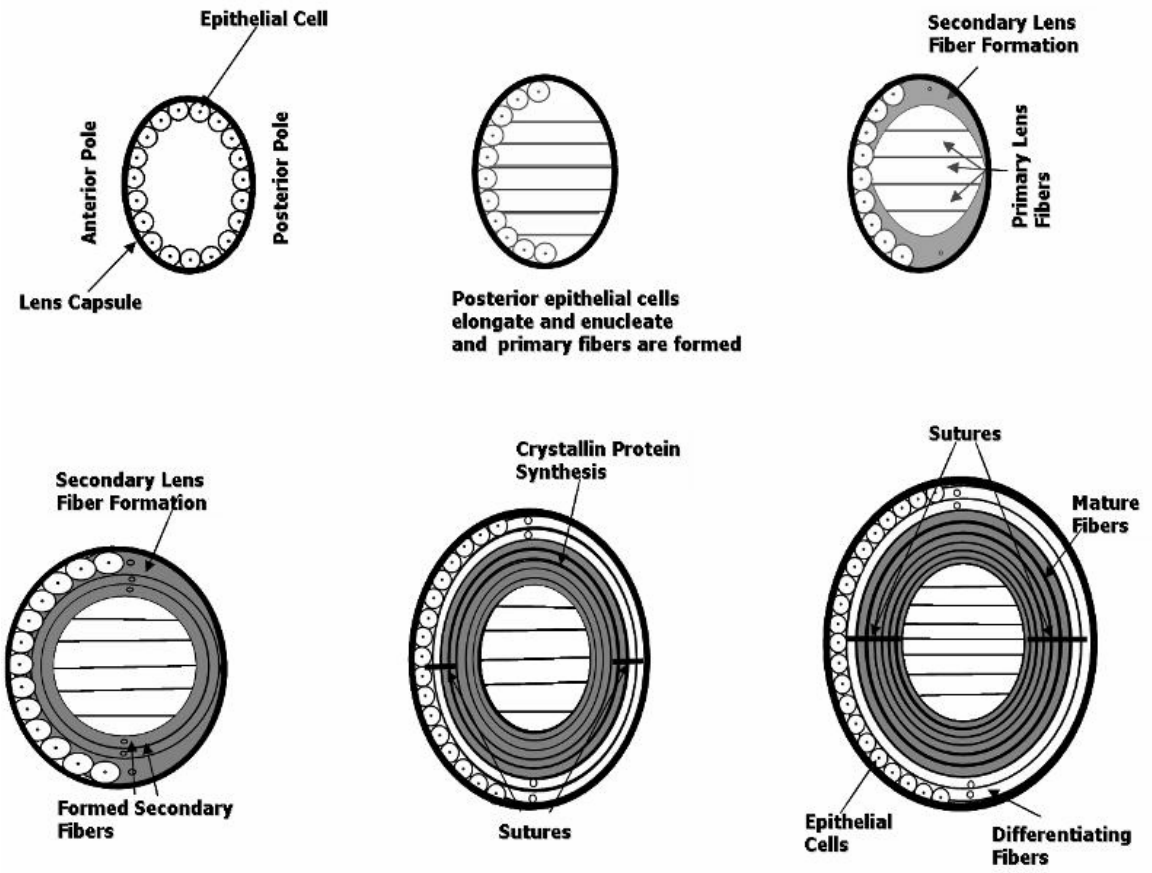

Epithelial Cell
Anterior Pole
Posterior Pole
Lens Capsule
Posterior epithelial cells
elongate and enucleate
and primary fibers are formed
Secondary Lens
Fiber Formation
Primary Lens
Fibers
Secondary Lens
Fiber Formation
Formed Secondary
Fibers
Crystallin Protein
Synthesis
Sutures
Sutures
Mature
Fibers
Epithelial
Cells
Differentiating
Fibers

Figure I-4. Diagram of a vertebrate lens and circulation model. Cross section cut down the anterior/ posterior axis. **A.** There are three physiologically distinct zones. The anterior surface is covered by a simple epithelium expressing Cx43 and Cx50 (light gray). Below the epithelium, the peripheral 20% of the lens is made up of a shell of differentiating fibers which make Cx46 and Cx50 (DF, white). The central 80% of the lens contains the mature fibers lacking organelles and continuing to express Cx46 and Cx50 (MF dark gray). **B.** A model of how the lens internal circulation is generated. In an intact lens, current flows in the pattern indicated by the arrows shown in panel A. The major ion carrier of the circulating current appears to be sodium, which enters the lens along the extracellular spaces between cells, then moves down its electrochemical gradient into fiber cells, where it returns to the surface via gap junctions. The pattern of gap junction coupling in the differentiating fibers directs the intracellular current flow to the lens equator, where the epithelial cells transport it out of the lens using Na/K-ATPase activity (Mathias et al., 1997; modified by Shakespeare et al., 2007).

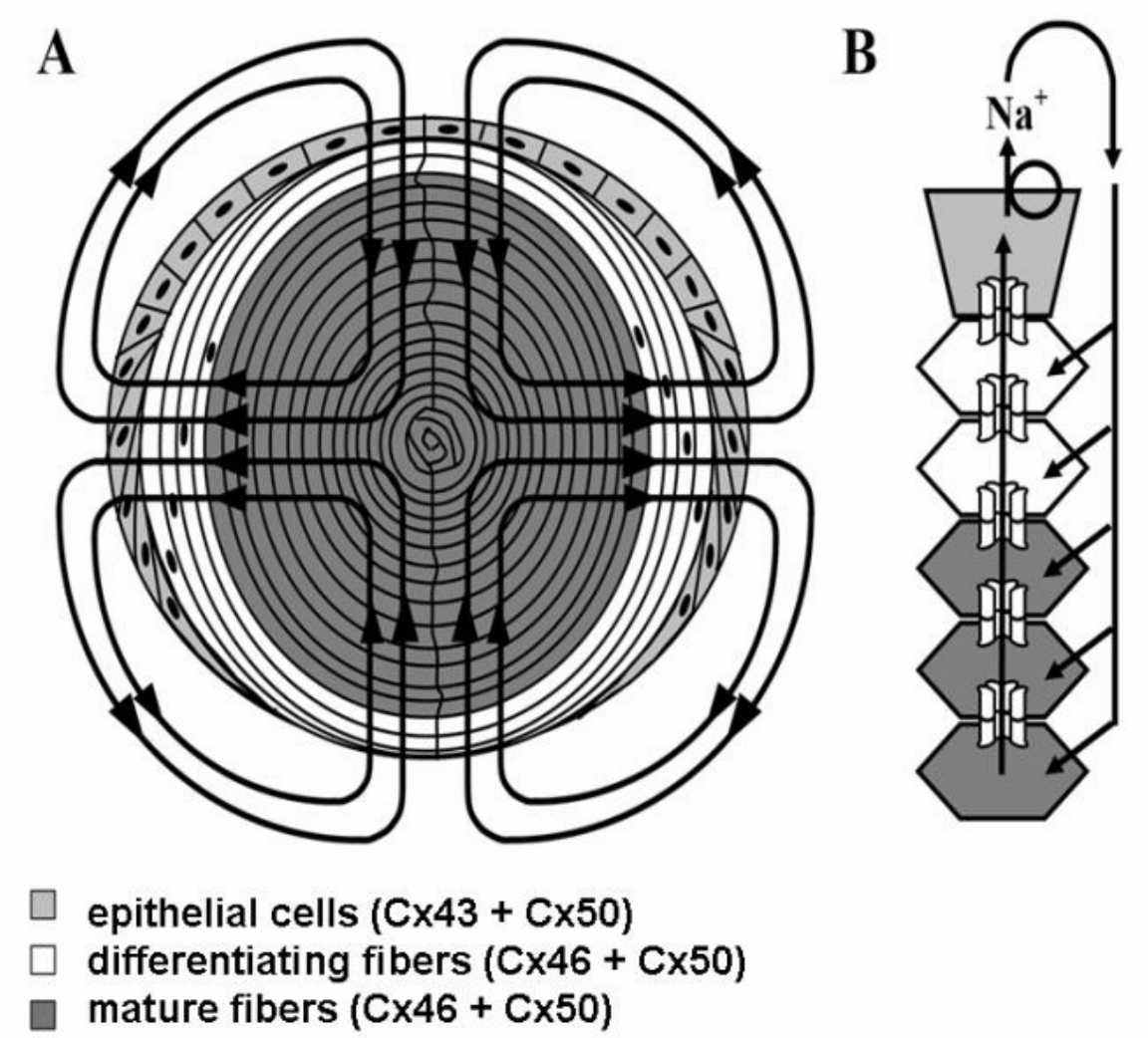

A
B
Na+
epithelial cells (Cx43 + Cx50)
differentiating fibers (Cx46 + Cx50)
mature fibers (Cx46 + Cx50)

Figure I-5. Lens phenotypes of genetically modified mice. Photographs of the anterior surface of wild type, Cx50KO, Cx46KO and Cx46/Cx50 double KO lenses emphasize the importance of connexin genes for lens homeostasis. **A.** Wild type lenses display normal size and transparency. **B.** Deletion of Cx50 results in a mild nuclear cataract and a ~50% reduction in the lens size. **C.** Cx46KO lenses are normal in size with a dense nuclear cataract in addition to cortical opacities. **D.** Double knockout of Cx46 and Cx50 causes a severe cataract and reduced lens size.

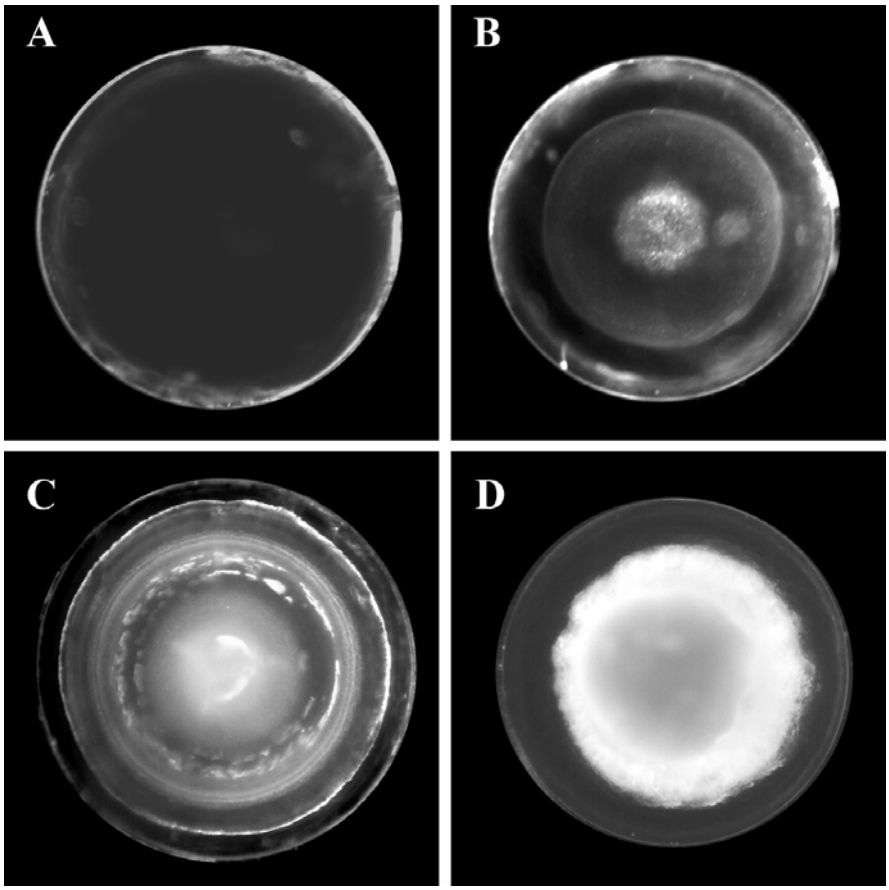

A
B
C
D

Figure I-6. Quantification of Mitotic Indices (MI) in Cx50KO and Cx50KI46 lenses. This figure displays the mean MIs after 1 and 24 hours of exposure to BrdU. The peak mitotic index for the wild type lens occurs on postnatal days two and three. However, both the Cx50 knockout and Cx50KI46 lenses show a significant decrease in mitotic indices in comparison to the wild type on postnatal day two and three (figure from Sellitto et al., 2004).

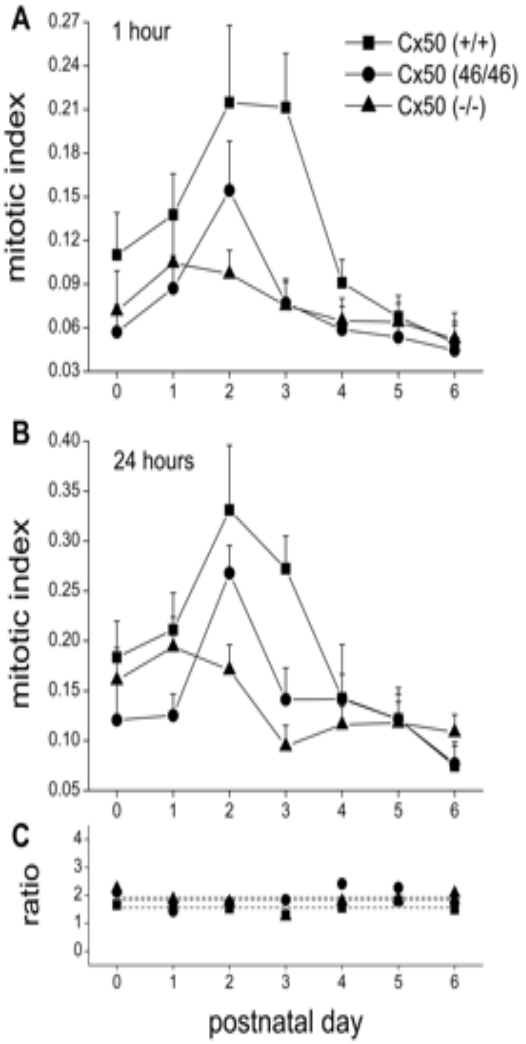
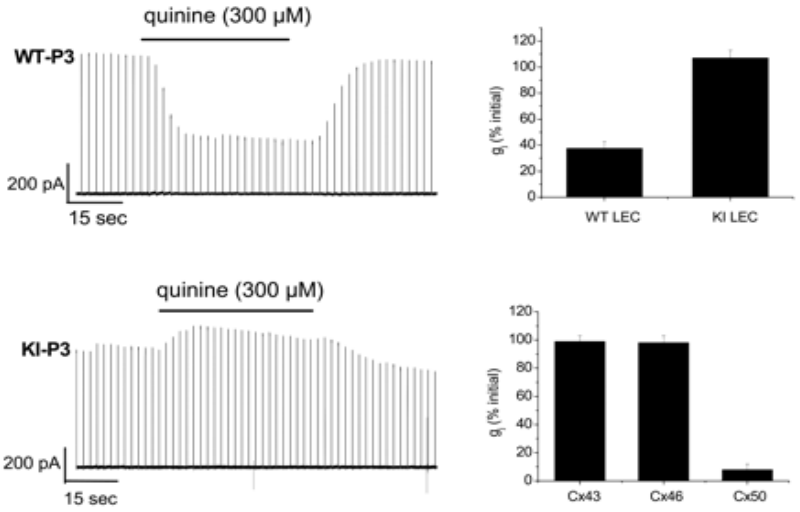

A
1 hour
Cx50 (+/+)
Cx50 (46/46)
Cx50 (-/-)
mitotic index
0.27
0.24
0.21
0.18
0.15
0.12
0.09
0.06
0.03
0 1 2 3 4 5 6
B
24 hours
mitotic index
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0 1 2 3 4 5 6
C
ratio
4
3
2
1
0
0 1 2 3 4 5 6
postnatal day

Figure I-7. Pharmacologic properties of gap junctions in lens epithelial cells. Top panel shows wild type and Cx50KI46 lens epithelial cells on P3. Lower Panel shows coupling of lens epithelial cells on P28. On P3, blockade of Cx50 channels with quinine significantly reduced coupling in wild type lens epithelial cells, but had not effect on Cx43 and Cx46. At P28, magnitude in quinine blockade was reduced to a lesser degree (25%) in lens epithelial cells (figure from White et al., 2007).

Postnatal Day 3



Postnatal Day 28

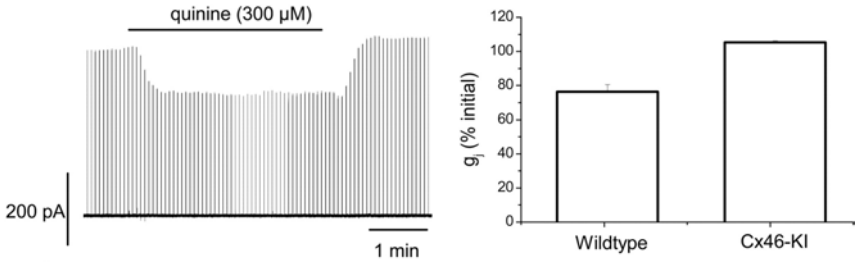


Table 1-1. Growth Factors that Influence Mammalian Lens Connexins. This table summarizes the effect of different growth factors and there interactions with gap junction intercellular communication.

Growth Factors that Influence Lens Connexins

Growth Factors and Kinases	**Lens Expression Pattern**	**Function/Involvement in GJIC**
Fibroblast Growth Factor (FGF) FGFR 1-4 (62)	Entire eye FGF1-3;5;7-10; 11-13; 15 and 19 (numbers are isoforms of FGF	FGF increases GJIC without altering connexin protein synthesis or assembly
Lens Epithelial Derived Growth Factor (LEDGF) (83,64, 119)	Lens epithelial cells	Activates phosphorylation of Cx43 via PKC γ
Insulin-like Growth Factor-1/ IGFR (64)	Lens epithelial cells	Phosphorylates Cx43 via PKC γ to decrease GJIC
Epidermal Growth Factor (EGF)/EGFR (27)	Lens epithelial cells	Activates phosphorylation of Cx43 and enhances GJIC
Protein Kinase C (PKC) α and γ isoforms (58,88,118, 95)	Lens epithelial cells, the γ isoform is more active	Inhibits GJIC via phosphorylation of Cx43
Protein Kinase A (PKA) (27)	Lens epithelial cells	Activator of GJIC via phosphorylation of Cx43
Mitogen Activated Kinase (MAPK)(95)	Lens epithelial cells	Closes hemichannels via phosphorylation of Cx43

Chapter II

Hypotheses and Specific Aims

Specific Aims

The knowledge and importance of distinct connexin expression in the lens is vast. In addition, the importance of many mitogen factors in the lens has been characterized. Therefore, we interested in exploring how connexins and mitogen activated factors might cooperate to influence the developmental fates of the lens and thereby the eye. The overall objective of this dissertation was to further characterize the role of Cx50 and its possible interaction with mitogen activators. We hypothesized that mitogen activators positively interacted with Cx50 to achieve postnatal lens cell proliferation and differentiation. The observations from which this hypothesis was derived that Cx50 is influential in supporting proper lens development and FGF signaling positively influences gap junction intercellular communication {White et al., 2002} {Sellitto et al., 2004} {White et al., 2007}{Le and Musil et al., 2001}. We will examine Cx46 and Cx50 and their interaction with fibroblast growth factor (FGF) and MAP kinase signaling cascades. Two specific aims were designed to investigate how mitogen factors and connexins might cooperate to influence the developmental and behavioral fates of lens cells; along with what Cx50 may contribute to the ocular environment that impacts the growth of the lens.

Specific Aim 1: To determine if there is a functional relationship between Cx50 or Cx46 and fibroblast growth factor (FGF) or mitogen activated kinase kinase (MEK1) *in vitro*.

A. To determine if there is a change in gap junctional conductance in Xenopus oocytes expressing Cx50 or Cx46 and a constitutively active MAP kinase kinase MEK1(E) protein.

MAPKs are expressed in the lens {Lovicu and McAvoy et al., 2005} {Li et al 2003}. MAP kinase kinase (MEK1/2) is an upstream activator of ERK1/2, and works specifically to activate the MAPK signaling cascade in order to mediate cellular responses to growth and differentiation factors {Mansour et al 1994}. The protein is unique because it is a dual specific kinase, giving the protein an advantage over others in the pathway. Because of this dual specificity, which allows the protein to phosphorylate both tyrosine and serine/threonine residues on the same protein. This sometimes allows for longer activation states of itself and its downstream effectors. Thus, the use of a constitutively active MEK kinase mutant may provide insight into interactions between connexins and signal transduction.

B. To determine if there is a change in gap junctional conductance in Xenopus oocytes expressing Cx50 and a Xenopus fibroblast growth factor receptor (XFGFr).

Le and Musil {Le and Musil et al., 2001} unveiled a remarkable finding that provided a link to connexins and FGFs. In an in vitro study, they reported that FGF is capable of up regulating gap junctional intercellular communication. In addition to this finding, their results showed that the increase in intercellular coupling was achieved without increasing gap junction connexin synthesis or assembly. They also reported that treating intact lenses with FGF could regionally sustain the activation of ERK. Whereby, they were able to show that only lens cells in the equatorial region of the lens were responsive to

FGF stimulation, and not the polar and core fibers. This data was striking and suggested a novel role for gap junction communication and FGF signaling {Le and Musil, 2001}. Although these experiments were conducted in avian lens cultures, they resonate to studies in mammalian lens because the avian connexins 43, 45.6 and 56 are orthologues to the mammalian connexins 43, 46 and 50 respectively. In addition to these studies, FGFs have been reported to participate in fiber cell differentiation in addition to several other growth factors {Belecky-Adams et al., 2002}.

C. To determine the biochemical levels and/or activity of Cx50, Cx46 and MAPK proteins and if the phosphorylation state of ERK is altered.

We will determine if there is a difference in protein synthesis of connexin proteins in the presence of MEK1(E).

Specific Aim 2: To characterize the possible interaction between MEK1 and Cx50 or Cx46 *in vivo*.

Gong {Gong et al 2001} designed a transgenic mouse model that expresses a constitutively active mutant of the mitogen activated protein kinase kinase 1 (MEK1(E)). The model displays phenotypes of postnatal cataracts and marcophthalmia. After observing the lens, they report that the lenses were dramatically enlarged, along with swollen fiber cells, increased extracellular space, and vacuole formation. The results from this study are in complete opposition of the phenotypes seen in the Cx50KO animal. Therefore, it would be interesting to use the MEK1(E) animal model as a gain-of-function tool to elucidate the role of Cx50 and its requirement for proper lens growth. In

using these two models we hope to answer questions regarding Cx50 and its relationship with the MAP kinase-signaling pathway.

A. We have characterized the mass of the eye and lens in MEK1(E)-Cx50KO, MEK1(E)-Cx46KO and MEK1(E)-Cx50KI46 animals.

To do this, we crossbred a transgenic mouse that over-expressed a constitutively active MEK transgene with Cx50KO, Cx46KO or Cx50KI46 mice.

B. We have determined the patterns of lens cell proliferation.

Lens cell proliferation in the Cx50KO lens was reduced by 37% in the knockout lens and 31% in the knock-in lens {Sellitto et al., 2004}. Further analysis of mitosis in MEK1(E)-Cx50KO, MEK1(E)-Cx46KO and MEK1(E)-Cx50KI46 animal models will provide insight about the influence of MAPK signaling and Cx50.

C. We have determined if there is a difference in lens histology of MEK1(E)-Cx50KO, MEK1(E)-Cx46KO and MEK1(E)Cx50KI46 mice.

The characterization of the MEK1(E) transgenic mouse revealed that over expression of MEK1 caused vacuole formation in the bow region of the lens {Gong et al., 2001}. Examination of the lens histology of each animal model will provide insight about the contribution of connexin expression and MAPK signaling to vacuole formation.

Chapter III

Materials and Methods

In vitro transcriptions, oocyte microinjection and pairing

The Cx50, Cx46, Cx48.5, Xenopus FGF receptor and constitutively active MEK1(E) coding sequences were sub-cloned into pCS2+ expression vector, gel purified, and used as the template (1μg of DNA) to produce capped RNAs using the SP6 Message mMachine kit (Ambion, Austin, TX). Stage V-V1 oocytes were isolated from Xenopus *laevis* (Nasco, Fort Atkinson, WI), defolliculated by collagenase and hylaronidase digestion, and cultured in MB+ buffer. Cells were injected with a total volume of 40nl of either an antisense oligo-nucleotide (3ng/cell) to suppress the endogenous Xenopus Cx38 or a mixture of antisense plus Cx50 (40ng/cell) using a Nanoject II Auto/Oocyte injector (Drummond, Broomall, PA).

Zebrafish Cx48.5 Experiments

Oocytes were then injected with cRNA for Cx48.5 and stored in modified Barth's medium (MB+) supplemented with 2.9 mM Ca^{2+} and allowed to express overnight. Cells were then immersed in a hypertonic solution to strip the vitelline envelope, transferred to a Petri dish containing MB+ medium supplemented with elevated calcium concentration of 2.9mM, and manually paired with vegetal poles apposed.

Cx50 and Fibroblast Growth Factor Experiments

Oocytes were injected with cRNA for Cx50 and a Xenopus FGF receptor and stored in MB+ medium and allowed to express overnight. Cells were then immersed in a

hypertonic solution to strip the vitelline envelope, transferred to a Petri dish containing MB+ medium, and manually paired with vegetal poles apposed. On the following day, cell pairs were incubated with 15ng/mL of basic FGF ligand (Sigma) for 2h and electrophysiological recordings were made 24h after pairing.

Cx50 and MEK1(E) Experiments

Oocytes were injected with cRNA for Cx50 and stored in OR3 buffer at 14°C overnight. On the following day, cells were injected with MEK1(E) cRNA and allowed to rest for 4h. Cells were then immersed in a hypertonic solution and manually stripped of their vitelline envelope, transferred to a Petri dish containing OR3 buffer, manually paired with vegetal poles apposed and stored at 14°C overnight. Electrophysiological recordings were made 12-16h after pairing.

Cx46 and MEK1(E) Experiments

Oocytes were injected with cRNA for Cx46 and stored in modified Barth's medium (MB+) supplemented with 4 mM Ca^{2+} and allowed to express overnight at 14°C. On the following day, cells were injected with MEK1 (E) and allowed to rest for 4h. Cells were then immersed in a hypertonic solution to strip the vitelline envelope, transferred to a Petri dish containing MB+ (4 mM Ca^{2+}) medium, manually paired with vegetal poles apposed and stored at 14°C overnight. Electrophysiological recordings were made 12-16h after pairing.

Dual whole cell voltage clamp

The functional properties of the cell-to-cell channels were assessed by dual voltage clamp {Spray et al., 1981}. Current and voltage electrodes were pulled (1.2-mm diameter, omega dot; Glass Company of America, Millville, NJ) to a resistance of 1 –2 mega ohms with a horizontal puller (Narishinge, Tokyo, Japan) and filled with 3M KCL, 10mM EGTA and 10mM HEPES, pH 7.4. Voltage clamping of oocyte pairs was performed using two GeneClamp 500 amplifiers (Axon Instuments, Foster City, CA) controlled by a PC-compatible computer using Digidata 1320A interface (Axon Instruments). pCLAMP software (Axon Instruments) was used to program the stimulus and data collection paradigms. For measurements of junctional coupling (G_j), both cells in the pair were clamped at -40mV to eliminate any transjunctional potential (V_j). Cell one was then subjected to alternating pulses of ±20mV while the current produced by the change in voltage was recorded in cell two. The current delivered was to the second cell was equal in magnitude to the junctional current (I_j), and (G_j) was calculated by dividing the measured current by the voltage difference as follows: $G_j = I_j / (V_1 - V_2)$, where V_1 and V_2 are the voltages in the first and second cells, respectively.

To determine the voltage gating properties, transjunctional potentials (V_j) of opposite polarity were generated by hyperpolarizing or depolarizing one cell in 20-mV steps (over a range of ±120 mV) while clamping the second cell at -40 mV. Currents were measured at the end of the voltage pulse, at which time they approached steady state (I_{jss}), and the macroscopic conductance (G_{jss}) was calculated by dividing I_{jss} by V_j. G_{jss}

was then normalized to the values determined at ±20 mV and plotted against V_j. Data describing the relationship at G_{jss} as a function of V_j were analyzed using Origin 6.0 (Micocal Software, Northhampton, MA) and fit to a Boltzmann relation of the form, $G_{jss}= \{(G_{jmax} - G_{jmin})/1 + \exp[A(V_j-V_0)]\} + G_{jmin,}$ where G_{jss} is the steady- state junctional conductance, G_{jmax} (normalized to unity) is the maximum conductance, G_{jmin} is the residual conductance at large values of V_j, and V_0 is the transjunctional voltage at which $G_{jss} = (G_{jmax}- G_{jmin})/2$. The constant A (= *nq/kT*) represents the voltage through the membrane; *k* is the Boltzmann constant, *T* is the absolute temperature.

To characterize non- junctional hemichannels, single oocytes were assessed with a two electrode voltage clamp procedure {Ebihara et al., 1993}. Cells were initially clamped at -40 mV. Depolarizing voltage steps (-30 to +60 mV at 10-mV intervals) were imposed for a duration of 4.5s, and whole- cell currents were recorded. Mean current values were measured at the end of the pulse and plotted against the membrane potential.

Preparation of oocyte samples for connexin western blot analysis.

Oocytes were collected in 1ml of lysis buffer containing 5mM Tris (pH 8.0), 5mM EDTA, and protease inhibitors and homogenized using a series of mechanical passages through needles of diminishing gauges (20, 22 and 26). Extracts were centrifuged at 1,000g at 4ºC for 5 minutes. The supernatant was then centrifugated at 100,000g at 4ºC for 30 minutes. Membrane pellets were re-suspended in 3X SDS sample buffer (2µL/oocyte) and sample were separated on 10% SDS PAGE gels and then transferred to nitrocellulose membranes. Blots were blocked with 5% BSA in 1X PBS with 0.02% NaN_3 for 1h and probed with polyclonal Cx50 or Cx46 rabbit antibody at 1:1000 dilution

followed by an incubation with alkaline phosphatase- conjugated anti-rabbit secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA).

Preparation of oocytes samples for MAPK western blots analysis.

Oocytes were collected in 10µl of lysis buffer per oocyte containing 137mM NaCl, 20mM Tris pH 8.0, 2mM EDTA, 1% IGEPAL (Sigma), 1mM PMSF (Sigma) and 0.5µM Na_3VO_4 and homogenized by pipetting oocytes up and down. Samples were then centrifuged at 14000g for 10minutes at 4ºC. The supernatants were collected and re-suspended in an equal volume of 2X SDS sample buffer and then separated on 15% SDS PAGE gels and then transferred to nitrocellulose membranes. Blots were then blocked with 5% non-fat dry milk in 1X TBS with 0.1% Tween-20 for three hours and probed with rabbit polyclonal phospho-ERK, total ERK or total MEK antibodies (Cell Signaling Technology, Inc. Danvers, MA.) at 1:1000 dilution, followed by incubation with HRP conjugated goat anti- rabbit secondary antibody (Santa Cruz Biotechnology, Santa Cruz, Ca.).

Interbreeding of Mice

MEK1 (E) mice were obtained from the Gong laboratory at the University of California Berkeley {Gong et al., 2001}. MEK1 (E) transgenic mice have lenses that express a constitutively active mutant of MEK1 under the αA-crystallin promoter and were interbred with Cx50KO, Cx46KO or Cx50KI46 mice {White et al., 1998; Gong et al ., 1997; White TW. 2002} to produce MEK1(E)-Cx50KO, MEK1(E)-Cx46KO or MEK1(E)-Cx50KI46 mice. DNAs isolated from tail biopsy samples were amplified by

PCR (DNA Engine Dyad; MJ Research, Waltham, MA), and amplified products were resolved by agarose gel electrophoresis to confirm the genotype of the animals.

Growth Analysis

Eyes and lenses of age-matched male animals were dissected, blotted dry on tissue paper, and individually weighed.

Microscopy and Photography

Eyes were dissected and immediately transferred to a Petri dish containing 37ºC M199 medium (Sigma) with 10mM HEPES, pH 7.4 on a warm stage. Lenses were dissected and transferred to a pre-warmed poly-lysine-coated Petri dish with a glass bottom (World Precision Instruments) filled with M199 medium. Lenses were visualized and photographed at 12X magnification (model SZX9; Olympus Corporation of America, Lake Success, NY) through a microscope equipped with an Olympus C3030 zoom digital camera.

Histology

Mouse eyes were dissected and fixed in a 4% formaldehyde solution, freshly prepared from para-formaldehyde in phosphate-buffered saline (PBS) for 16 to 24 hours at room temperature. Fixed eyes were rinsed with PBS and dehydrated through an ethanol series and embedded in paraffin. Sections of 2 or 3μm were cut on a diamond knife, deparrafinized, and stained with hematoxylin- eosin. Histological sections were viewed

with 10X and 20X objective on a microscope (model BX51; Olympus) and photographed with a digital camera (MagnaFire; Optronics, Goleta, CA).

BrdU Injection

P1 to P6 mouse pups were injected intraperitoneally with 100 μg/g body weight of 5'-bromo-2'-deoxyuridine (BrdU, Sigma, St. Louis, MO). BrdU at 10mg/mL was dissolved in PBS at 37°C just before use. Injected pups were returned to their mothers. After 1-hour exposure to BrdU at 10mg/mL, pups were sacrificed, and lenses were dissected. The lens capsule was then peeled away from the fiber cell mass using fine forceps and pinned down on an encapsulant (Sygard; Dow Corning) - coated 35-mm Petri dish and fixed for 30 minutes in 2% formaldehyde, made from freshly prepared paraformaldehyde in PBS. Fixed capsules were rinsed with PBS and incubated with 100% MeOH at -20°C for five 5 minutes and mounted on microscope slides and allowed to air dry. BrdU incorporation was immunolabeled with a BrdU in situ detection kit (BD PharMingen, San Diego, CA) according to the manufacturer's instructions with exception that endogenous peroxidase was quenched with 0.3% hydrogen peroxide diluted in absolute methanol, and all antibody incubations were carried at 37°C. BrdU-negative nuclei were counter-stained with aqueous hematoxylin. Stained sections were viewed with 10X and 20X objectives on a microscope (BX51; Olympus) and photographed with a digital camera (MagnaFire; Optronics).

Quantitation of BrdU-Labeled Cells

The diameters of BrdU labeled capsules were individually measured for each genotype at each developmental age (P1-P6). A rectangular area with a length of 3/5 diameter and a height of 1/5 diameter was drawn for each capsule. The rectangle was positioned to cover the area from the lens equator to the center of the lens. BrdU-positive nuclei were counted within the region of the scale and divided by the area to calculate the density of BrdU labeled cells. For analyses of BrdU labeling on P2 and P5, the rectangular area was further sub-divided into three equal squares. The area of each square represented the equatorial, transitional and central regions of the lens capsule. The density of BrdU labeled cells was re-calculated for each region, and the mean densities were plotted. Data were collected from two to twelve capsules on postnatal day 1 to 6 for each genotype. Statistical analysis between wild type, MEK1 (E), Cx50KO and MEK1(E)-Cx50KO lenses was performed using the 3-way ANOVA test.

Measurement of Lens Glucose Concentration

Five week old lenses from wild type, Cx50KO, MEK1(E) and MEK1(E)- Cx50KO mice were assayed for lens glucose concentration. Four lenses from two mice were dissected and pooled for each sample. Lenses were then de-proteinized by homogenization with a 5mm POLYTRAN-Aggregate (Brinkman Instruments, Inc) in 300µL of a 6% perchloric acid ($HClO_4$) solution, followed by centrifugation at 14,000 rpm for 10 minutes at 4°C. The supernatants were then transferred to a fresh eppendorf tube and were neutralized with 70µL of 2M K_2CO_3 followed by centrifugation at 14,000 rpm for 10 minutes at 4°C. Two hundred micro liters of the neutralized supernatant per sample was used to assay for glucose per sample according to the manufacturer's instructions (Glucose (HK) Assay

Kit; Sigma, St. Louis, MO). The lens glucose concentration was calculated from the glucose amount (per lens) and its volume. When calculating the lens volume, we calculated the lens volume based on $1/6\ \pi D^3$, assuming the lens were a sphere, using the equatorial diameter (D).

Chapter IV

Connexin48.5 is required for Normal Lens Development in Zebrafish Embryos

Abstract

Gap junctions are composed of connexin (Cx) proteins and mediate intercellular communication required for many developmental and physiological processes. Here we describe the electrophysiological properties of Cx48.5, a zebrafish connexin with the highest sequence homology to mammalian Cx46. Expression analysis showed that Cx48.5 is expressed in the adult and embryonic lens, heart, adult testis, and transiently in the embryonic otic vesicles. Using the paired Xenopus oocyte system in conjunction with the dual whole-cell voltage clamp assay, we demonstrated that Cx48.5 subunits elicited intercellular electrical coupling with voltage sensitivity similar to mammalian Cx46. In single oocytes, Cx48.5 also induced large outward currents on depolarization, consistent with gap junctional hemichannels. In addition, disruption of Cx48.5 expression in zebrafish embryos with antisense morpholino oligos (morphants) revealed that Cx48.5 has an essential role in the maintenance of lens homeostasis. The morpholino-treated embryos developed small lenses and eyes as well as severe cardiovascular abnormalities. Although Cx48.5 and Cx46 share many structural and functional features, the identification of more zebrafish connexin genes is needed to establish an orthologous relationship between zebrafish and mammalian connexins. However this study provided consistent evidence that intercellular gap junction mediated communication is not only essential for development of the mammalian lens, but other vertebrates as well.

Results

The findings presented in this chapter were done in close collaboration with co-authors from the laboratory of Dr. Gunnar Valdimarsson. This study includes *in vitro* data contributed from our laboratory while the *in vivo* data were provided by the Valdimarsson laboratory. I have included the *in vivo* data (figures IV-1 and IV-5), which correlate with the specific aims for this dissertation. For a more detailed description of the methodology used to attain figures IV-1 and IV-5, please refer to {Cheng et al., 2003; Cheng et al., 2004}. Figures IV-2, IV-3, IV-4 and Table IV-1 are data derived from our laboratory.

Cx48.5 forms functional channels that are gated by transjunctional voltage.

To determine whether Cx48.5 could form junctional channels, we used the paired Xenopus oocyte expression system. Oocytes had resting potentials ranging between -40 and -50 mV and were clamped at -40 mV for measurements of junctional conductance. As shown in figure IV-2A, Cx48.5 induced the development of high levels of electrical coupling 24-48 hours after RNA injection. The mean conductance (G_j) of paired Cx48.5-injected cells was 53.7 ±22.2 micro-siemens (mean ±S.D. n=41). In contrast, the background conductance value, measured between oligo-injected control oocytes, was more than 200-fold lower at 0.15 ± 0.18 micro-siemens (n=36). Thus, the expression of Cx48.5 resulted in the formation of functional gap junction channels. To further characterize the physiological behavior of channels composed of Cx48.5, we analyzed their voltage dependence. A representative family of junctional currents (I_j) evoked by voltage steps of opposite polarities and increasing amplitude (FigureIV-2B) shows that I_j decreased in a time dependent manner for transjunctional voltages >40mV. The rate of

the channel closure, calculated for V_{js} of ± 80mV, yielded a time constant (τ) on the order of 0.3 s, a value intermediate between the slower channels like Cx26 and Cx32 and the more rapidly gating channels such as Cx37 and Cx40 {Barrio et al., 1991; Henneman et al., 1992}. Thus the voltage dependence of Cx48 channels was quite similar to that of rodent Cx46 {White et al., 1994; Ebihara et al., 1993; Hopperstead et al., 2000}.

The voltage dependence of Cx48.5 was further analyzed by plotting junctional conductance (G_j) as a function of transjunctional potential (V_j) (FigureIV-3). G_j values for steady-state junctional conductance (G_{jss}) were normalized to the maximal conductance measured at the lowest V_j (20 mV). No fast gating effects (<5 ms) of voltage on theses channels were observed. In contrast, G_{jss} was dependent on voltage, and this plot was fitted to a Boltzmann relation {Spray et al., 1981} whose parameters are given in TableIV-1. For comparison, the Boltzmann parameters for Cx46 and Cx50 orthologues from chicken, rodent, and zebrafish are shown in TableIV-1. This analysis clearly showed that zebrafish Cx48.5 channels have weak voltage sensitivity. Similar voltage sensitivity has been demonstrated for rat Cx46 and chick Cx56 in both the paired Xenopus oocyte system and in transfected mammalian cells {Hopperstead et al., 2000; Ebihara et al., 1995; White et al., 1995}.

Cx48.5 Forms Non- junctional Hemichannels in Solitary Oocytes

Cx46 was the first cloned connexin shown to be capable of forming voltage-activated hemichannels in single oocytes {Paul et al., 1991; Ebihara et al., 1993; Trexler et al., 1996}. Therefore, we tested the ability of Cx48.5 to induce nonjunctional membrane conductance in single oocytes. Whole-cell currents were recorded in response to depolarizing voltage steps that were sequentially imposed from a holding potential of

-40mV. The responses of oocytes expressing Cx48.5 revealed slowly activating outward currents at membrane potentials of >-10mV that were not seen in water-injected controls (FigureIV-4A). Plotting the current-voltage relationship (FigureIV-4B) demonstrated that the mean currents recorded at the end of the +60mV voltage step from Cx48.5-injected cells (2.28± 1.17μA; n=9) and control oocytes (0.25 ± 0.08μA n=8) were significantly different (p <0.001; Student's unpaired t test). This data demonstrate that zebrafish Cx48.5 also functions as a membrane channel in single, unpaired oocytes. The current-voltage relationship of zebrafish Cx48.5 hemichannels was nearly identical to that of rat Cx46 {Ebihara et al., 1993}, activating at membrane potentials >-10mV in the presence of normal modified Barth's medium ($[Ca^{2+}]$ = 0.9mM). In contrast, Cx50 hemichannels can only be elicited when external Ca^{2+} has been significantly lowered {Zampighi et al., 1999; Beahm et al., 2002}.

Cataract Formation, Microphakia and Microphthalmia in Cx48.5 Morphants

Cx48.5 expression in zebrafish embryos was knocked down by injection of antisense morpholino oligos (morpholinos) in one day old embryos. The center region of the lens in the Cx48.5 morphants became visibly abnormal in the live embryos 3-4 days post fertilization. The lenses in the live Cx48.5 morphants appeared uneven and rough when viewed with differential interference contrast optics, in contrast to the very smooth appearance of the lenses in the control embryos (FigureIV-5, A and B). Sectioning of the morphant eyes also revealed histological abnormalities. In the control lenses at 3 dpf, the primary fibers in the central region were mature and less intensely stained with toluidine blue the cortical fibers. The less intense staining in the central

fibers in the normal control lenses reflects the loss of basophilic materials such as ribosomes and nuclei during the differentiating process. The differentiating secondary fiber cells elongate medially from the equatorial region and form the long, smooth and tightly packed fibers of the normal lens (FigureIV-5, D). However, this differentiation process was disrupted in the Cx48.5 morphants. The primary and secondary fibers in the Cx48.5 morphants did not gain the same long, thin and smooth shape by 3 dpf. Instead, most of the fibers remained nucleated. And the entire core of the lens appeared disorganized (FigureIV-5, C). Cx48.5 morphant and control lenses were dissected from embryos at a series of stages and observed with a microscope equipped with dark field optics. Cataracts first appeared in the Cx48.5 morphant lenses by 5.5-7.5 dpf. The cataractous lens shown in (FigureIV-5, E) was photographed at 9.5 dpf. The size of the cataracts in the lenses of the Cx48.5 morphants also varied.

Previous work has demonstrated that Cx50, but not Cx46, is required for the development of normal lens and eye size in mice {Gong et al., 1997; White et al., 1998; Rong et al., 2002; Martinez-Wittingham et al., 2003}. Measurements of the lens and eye size revealed that both were significantly smaller in the Cx48.5 morphants than in the controls ($p < 0.001$). At 3.5 dpf, when the lens abnormality first became noticeable, the Cx48.5 morphant lenses were 6.3% smaller in diameter than the control lenses (FigureIV-5, G). By using the lens diameter measurements to calculate lens volume (assuming that the lens is a sphere), the lenses in the Cx48.5 morphants were found to be 17.7% smaller in volume than the controls at 3.5 dpf. By 6.5 dpf the morphant lenses were 13.7% smaller in diameter than the controls and 35.7% smaller in volume (FigureIV-5, G). The size of the eye was also significantly smaller in the Cx48.5 morphants (FigureIV-5, H).

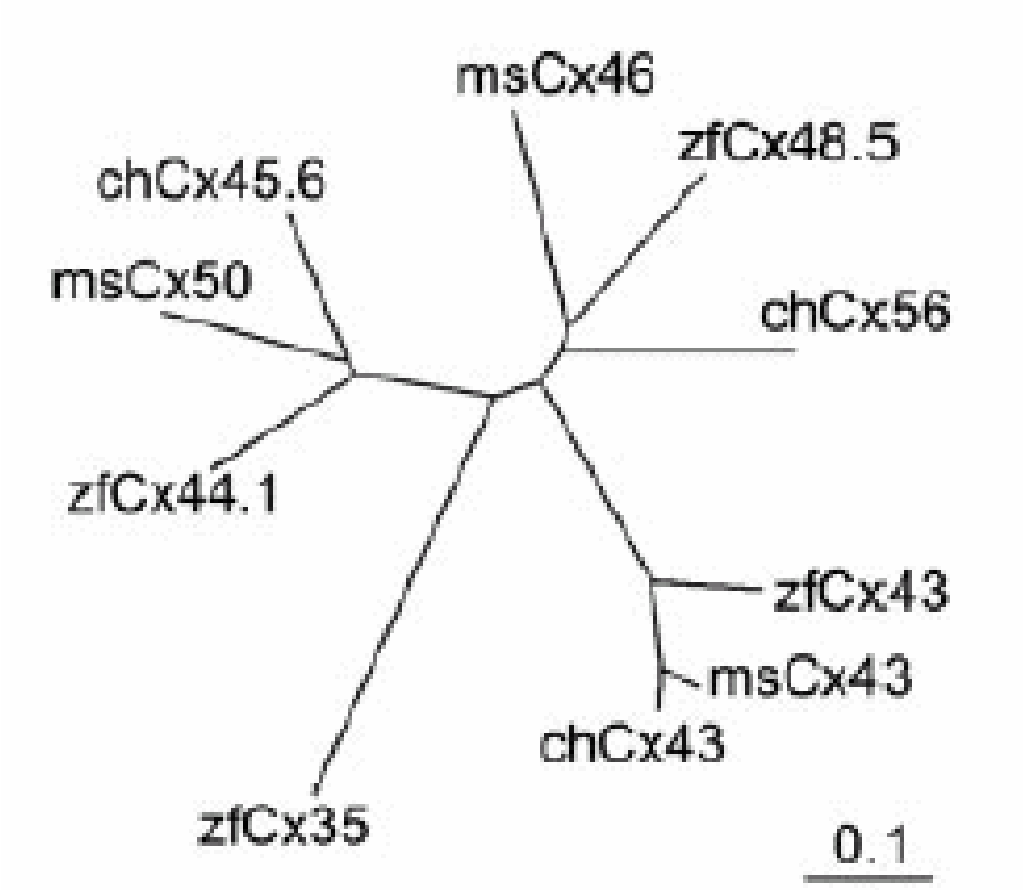
The anterior-posterior diameter of the morphant eyes was 10.5 and 7.2% smaller in diameter of the control eyes at 3.5 and 6.5 dpf, respectively. No significant difference was observed in the length of the whole embryo between the morphants and the control, (FigureIV-5, I) indicating that the smaller lens and eye in the Cx48.5 morphants was not due to undergrowth of the embryo as a whole. Because Cx48.5 expression was observed in the otic vesicles early on, the size was measured at 2.5 and 3.5 dpf. No significant difference was found in the size of the otic vesicles between the Cx48.5 morphants and the controls (FigureIV-5, J).

Discussion

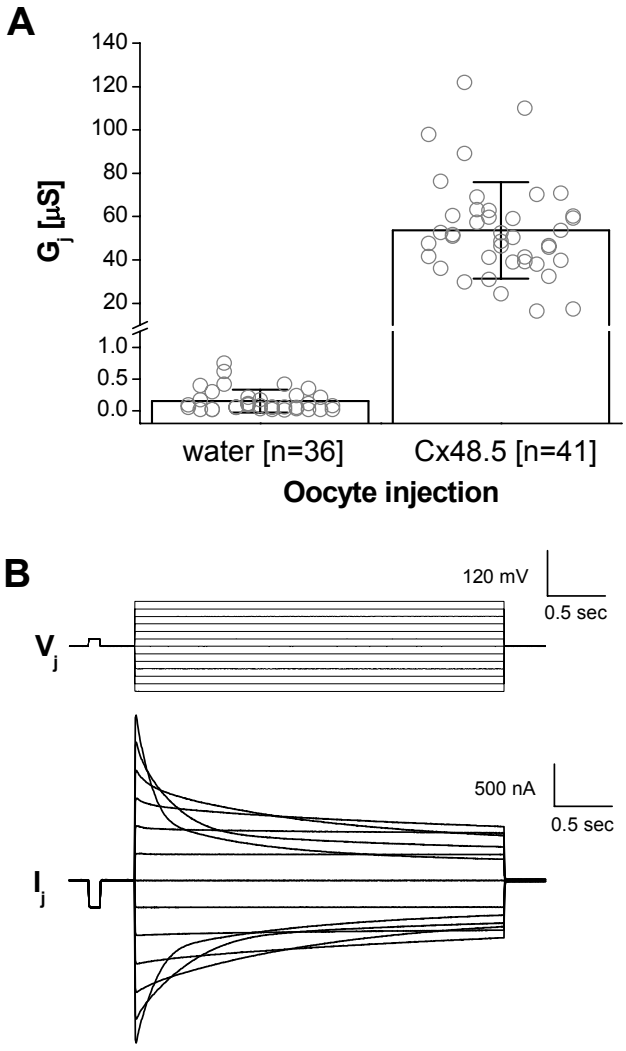
In this study, a novel member of the zebrafish connexin gene family, Cx48.5 was characterized. The amino acid sequence of Cx48.5 was shown to be relatively homologous to the mammalian connexin family members, including predicted structures such as four α-helical transmembrane segments and two extracellular loops with three conserved cysteine residues in each loop. After comparing sequences, it was confirmed Cx48.5 shared the highest degree of sequence identity with the Cx46 orthologous group of connexins. Data have shown that Cx44.1 is expressed earlier than Cx48.5 in the developing lens of zebrafish. Thus, it might be assumed that Cx44.1 is required for growth, whereas Cx48.5 is necessary for fiber homeostasis, similar to mouse. The overall sequence homology of Cx48.5 is shared with Cx46, however, the phenotypes of Cx48.5 knock down embryos resembles the Cx50 orthologous group of connexins more than the Cx46 subgroup, in some respects. Although Cx48.5 and Cx46 share many structural and functional features, the identification of more zebrafish connexin genes is

needed to establish an orthologous relationship between zebrafish and mammalian connexins. However, this study provides consistent evidence that intercellular gap junction mediated communication is essential for development the mammalian lens and other vertebrates.

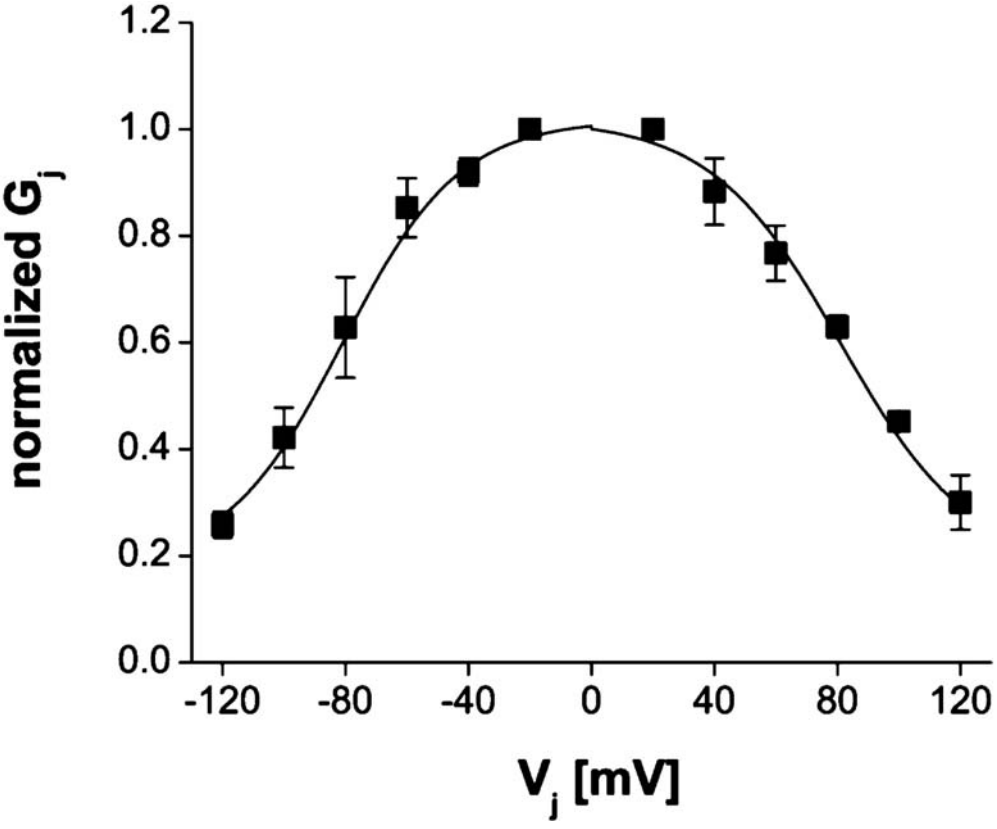
IV-1: Lens Connexin Phylogeny. This phylogeny chart categorizes several lens connexins from different species into subfamilies. zfCx48.5, chCx56 and msCx46 are grouped into one subfamily. Through the use of sequence analysis, it has been reported that zfCx48.5, Cx44.1 and Cx43 share 61%, 62% and 80% amino acid identity with their mouse orthologues. Zebrafish Cx35 is a non- lens connexin and was an outlier. (Figure adapted from Cheng et al., 2003)


msCx46
zfCx48.5
chCx45.6
msCx50
chCx56
zfCx44.1
zfCx43
msCx43
chCx43
zfCx35
0.1

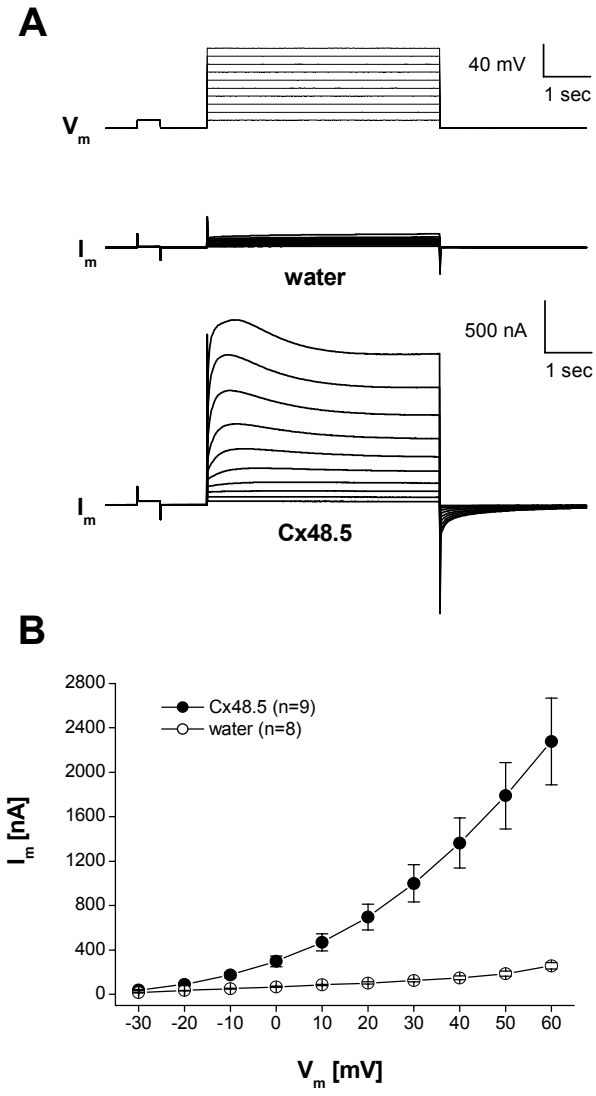
IV-2: Cx48.5 forms functional channels that are gated by transjunctional voltage. A. Junctional conductance (G_j) developed between pairs of Xenopus oocytes as measured by dual voltage clamp. Oocytes were co-injected with the Cx48.5 cRNA and an oligonucleotide antisense to mRNA for Xenopus Cx38 to eliminate the possible contribution of endogenous coupling to the recorded conductance. Antisense treated water-injected cells were used as negative controls. Cells were then stripped of the vitelline envelope in hypertonic medium and paired with the vegetal poles facing each other 24–48 h before electrophysiological measurements. Bars show the mean ± S.D. of the number of pairs indicated, and symbols show the clustering of all data points. **B.** Voltage gating behavior of gap junction channels formed by Cx48.5.A time-dependent decay of junctional currents (I_j) was induced by transjunctional voltage (V_j) steps. At V_j steps ±40 mV, I_j decayed symmetrically over the time course of the voltage step. The voltage gating of intercellular channels composed of zebrafish Cx48.5 displayed a high degree of conservation to GJA3 orthologues such as chicken Cx56 and rat Cx46.


A
140
120
100
80
60
40
20
1.0
0.5
0.0
G_j [µS]
water [n=36]
Cx48.5 [n=41]
Oocyte injection
B
120 mV
0.5 sec
V_j
500 nA
0.5 sec
I_j

IV-3: Quantitation of Cx48.5 voltage gating. Shown is the relative relationship of Vj to steady state junctional conductance (G_{jss}) normalized to the values obtained at ±20mV for Cx48.5. The solid line represents the best fit to the Boltzman equation, whose parameters are given in Table IV-1. The voltage gating of intercellular channels composed of zebrafish Cx48.5 displayed a higher degree of conservation to the Cx46 orthologues than the Cx50 orthologues.


normalized G_j
1.2
1.0
0.8
0.6
0.4
0.2
0.0
-120
-80
-40
0
40
80
120
V_j [mV]

IV-4: Cx48.5 forms voltage-gated hemichannels. A. Single Xenopus oocytes injected with Cx48.5 cRNA or water were studied by voltage clamp. Cells were initially clamped at ±40 mV. Depolarizing voltage steps of 10, 20, 30, 40, 50, 60, 70, 80, 90, and 100 mV were imposed, and the whole-cell currents were recorded. Cx48.5 induced rapidly activating outward currents that exhibited a slower partial inactivation at higher membrane potentials (>10 mV). In contrast, oligo-injected control cells showed negligible membrane currents. **B.** Current-voltage relationships in Cx48.5 and oligo-injected oocytes. Whole-cell membrane currents (I_m) were measured at the end of a voltage step. At membrane potentials (V_m) >-10 mV, Cx48.5 injected cells displayed whole-cell currents not seen in oligo-injected controls. Results are shown as the mean ± S.D. of the indicated number of cells.


A
40 mV
1 sec
V_m
I_m
water
500 nA
1 sec
I_m
Cx48.5
B
Cx48.5 (n=9)
water (n=8)
I_m [nA]
2800
2400
2000
1600
1200
800
400
0
-30
-20
-10
0
10
20
30
40
50
60
V_m [mV]

IV-5: Cx48.5 morphants developed cataracts and small lenses and eyes. A. Microscopic observation of the Cx48.5 morphants with differential interference contrast (DIC) optics revealed marked irregularities and roughness in the lens core by 3 days post fertilization (dpf), which was a in contrast to the smoothness of the control lenses **B.** Sections of those lenses revealed that lens fiber differentiation was abnormal and immature in the Cx48.5 morphants **C**, whereas the lens fibers in the core region of the control lenses had fully differentiated by this time **D.** Note also that the retina in the morphants **C**. is smaller than in the controls. Lenses were dissected from 9.5 dpf emybros and observed with a dark field microscope. The morphant lenses developed nuclear cataracts **E**, whereas the control lens is transparent **F**. In figures **G, H** and **I.,** the lens, eyes and body length were compared between the Cx48.5 morphants and controls. The Cx48.5 morphant has significantly smaller lenses and eyes at 3.5 and 6.5 dpf. However, there was no difference in the whole body length and the otic vesicle size of the control and morphant cohorts **J**.

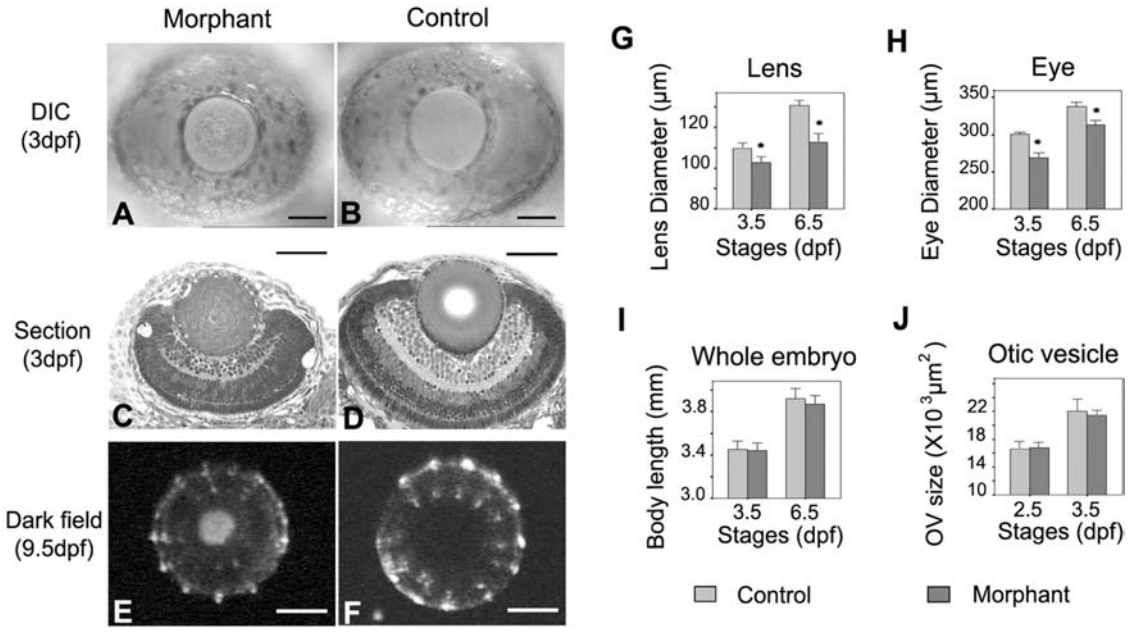

Morphant
Control
DIC
(3dpf)
Section
(3dpf)
Dark field
(9.5dpf)
A
B
C
D
E
F
G
Lens
Lens Diameter (µm)
120
100
80
3.5
6.5
Stages (dpf)
H
Eye
Eye Diameter (µm)
350
300
250
200
3.5
6.5
Stages (dpf)
I
Whole embryo
Body length (mm)
3.8
3.4
3.0
3.5
6.5
Stages (dpf)
J
Otic vesicle
OV size (X10 3µm2)
22
18
14
10
2.5
3.5
Stages (dpf)
Control
Morphant

Table IV-1: Boltzmann parameters of zebrafish, chicken and rodent lens connexins. Junctional conductance (G_j) developed between Xenopus oocytes was measured by dual voltage clamp in response to increasing transjunctional potentials (V_j) of opposite polarity and normalized to the conductance measured at a V_j of ±20mV (G_{jmax}, set as unity), as described under the methodology section. Data were fit to a Boltzmann fit, and V_0 is the voltage at which a half- maximal decrease of G_j is measured. The cooperativity constant (A), reflecting the voltage sensitivity of the channel, reflects the equivalent number of electron charges moving through the transjunctional field.

Connexin	A	V_0	G_{jmin}
Zebrafish Cx48.5	0.05	81	0.18
Chick Cx56[a]	0.07	50	0.13
Rat Cx46[b]	0.09	67	0.10
Zebrafish Cx44.1[c]	0.30	21	0.19
Chick Cx45.6[d]	0.38	25	0.18
Mouse Cx50[b]	0.34	18	0.14

[a]Values from {Ebihara et al., 1995}
[b]Values from {White et al., 1994}
[c]Values from {Cason et al., 2001}
[d]Values from {Jiang et al., 1995}

Chapter V

Potential Interactions between Connexin50 Intercellular Communication and Mitogen Activated Protein Kinase Signaling in Lens Growth

Abstract

In mice, deletion of Connexin50 (Cx50KO) resulted in smaller lenses and a reduction in the number of epithelial cells undergoing mitosis during the first postnatal week. Thus, Cx50 is essential for normal postnatal growth and lens cell differentiation, although the mechanisms whereby it influences these events are poorly understood. Several growth factors are known to stimulate lens cell proliferation and predominantly function by activating the mitogen activated protein kinase (MAPK) signaling cascade. We hypothesized that Cx50 and growth factors may interact during postnatal lens growth. We used the paired Xenopus *laevis* oocyte expression assay to examine the biochemical and electrophysiological properties of Cx50 and MAPK signaling in vitro. To further determine the relationship of Cx50 and MAPK signaling in vivo, we crossbred transgenic mice over expressing a constitutively active form of mitogen activated protein kinase kinase (MEK1(E)) under the αA-cystallin promoter with Cx50KO mice and analyzed the mass of the lens. Expression of MEK1(E) in oocytes stimulated ERK phosphorylation. Co-expression of Cx50, but not Cx46 with MEK1(E) in oocytes caused junctional conductance to increase three fold without altering Cx50 protein expression. Cx46 junctional conductance was not altered when co-expressed with MEK1(E). In addition, Cx46 protein expression remained unchanged. Transgenic MEK1(E) lenses were ~1.7 times larger than wild type by 7 weeks of age and had severe nuclear and cortical cataracts. Cx50KO lenses were 40% smaller than wild-type lenses with a mild nuclear cataract. Interbreeding of MEK1(E) transgenic and Cx50 knockout mice produced lenses that were 20% smaller than wild type with severe nuclear cataract, but a transparent lens cortex. Deletion of Cx50, but not Cx46 eliminated marcophthalmia, delayed

cataractogenesis and prevented lens rupture caused by MEK1(E). In addition, we observed that the removal of Cx50 repaired enlarged vacuoles in the equatorial region of the lens and reduced glucose accumulation in MEK1(E) lenses. Lastly, we observed that deletion of Cx50 reduced postnatal mitosis in the central and transitional epithelium in MEK1(E) lenses. These results were consistent with an interaction between MAPK signaling and Cx50 being necessary for the proper regulation of lens growth and osmotic homeostasis.

Results

Co-expression of an active form of MEK 1 and Cx50 increases junctional conductance in Xenopus oocytes

We have used the Xenopus oocyte expression system {Dahl et al., 1987} and the dual whole- cell voltage method {Wagner et al., 2000} to determine whether the expression of a constitutively active MAP kinase kinase, {Mood et al., 2004; Gong et al 2001} would influence Cx50 gap junctional conductance. Oocytes were injected with an anti-sense oligonucleotide to reduce the background conductance of endogenous Xenopus Cx38. The oocytes were then injected with Cx50 and expression was allowed for 24h. We then injected the oocytes with MEK1(E) cRNA, the cells were paired 3h later and junctional coupling was measured 16-18h later. Oocytes injected with water or MEK1(E) were used as negative controls. As shown in Figure V-1A, wild type Cx50 expressing pairs produced junctional coupling (G_j) that was 50-fold higher than water and MEK1(E) injected cell pairs. Oocytes that co-expressed Cx50 and MEK1(E) had

conductance (G_j) values that were 3.4-fold higher than wild type expressing pairs (mean ±SE, n = 17-24; P <0.05, Student's t- test). These results suggest a possible interaction for Cx50 and MAPK signaling, however, the mechanism by which this interaction occurs remains unknown.

Activation of MAPK signaling does not alter Cx50 protein levels

Since an increase of junctional conductance is often times associated with an increase in connexin protein synthesis {Spray et al., 1985}, we examined whether the increase in Cx50 junctional coupling was due to an increase in Cx50 protein levels. Homogenates were prepared from oocytes injected with water, MEK1(E), Cx50 or Cx50 and MEK1(E) and were analyzed for protein expression by western blotting. We used a polyclonal antibody with specificity for the central cytoplasmic loop of mouse Cx50 {White et al., 1992} to detect protein expression of Cx50. A 60kDa band, which corresponds to Cx50, was visualized and confirmed equal levels of protein expression in oocyte samples injected with Cx50 alone or Cx50 co-expressed with MEK1(E) (Figure V-1B, 1st panel). Thus, allowing us to conclude that the increase in Cx50 mediated junctional conductance was not due to an increase in Cx50 protein levels.

MEK1(E) expression stimulates ERK phosphorylation in oocytes

To examine MAPK protein activity, western blotting analysis was conducted with anti- ERK (2rd panel) and anti-phospho-ERK (3rd panel) antibodies (Figure V-1B). We found that expression of an active form of MEK1 does not alter the endogenous pool of ERK expression. However, we were able to show activation of the downstream ERK

signaling pathway for oocytes injected with the active construct. There was a large increase in p-ERK expression in MEK1(E) injected or Cx50 and MEK1(E) expressing cells, compared to water or Cx50 injected oocytes

Co-expression of Cx46 with MEK1(E) does not alter Cx46 mediated junctional conductance

To determine whether the expression of a MEK1(E), {Mood et al., 2004; Gong et al 2001} would influence Cx46 gap junctional conductance. We used the same method of injection described for Cx50. As shown in (Figure V-2A), wild type Cx46 pairs formed functional channels with junctional conductance that were 43.4-fold higher than control water and MEK1(E) pairs. Oocytes that co-expressed Cx46 and MEK1(E) were able to form junctional channels that produced conductance values that were equivalent to the Cx46 alone expressing pairs (mean ±SE, n= 12-19; P >0.05, Student's t-test).

Activation of MAPK signaling does not alter Cx46 protein expression

To analyze protein expression by western blotting, we used a polyclonal antibody raised against the extreme carboxyl terminus of rat Cx46 {Paul et al., 1991} to detect protein expression of Cx46. A ~46kDa band which corresponds to Cx46 was visualized and confirmed equivalent levels of protein expression in oocyte samples injected with Cx46 alone or Cx46 co-expressed with MEK1(E) (Figure V-2B, 1st panel). We then examined MAPK proteins; western blotting analysis was conducted with anti- ERK (2nd panel) and anti-phosho- ERK (3rd panel) antibodies (Figure V-2B). We found that expression of MEK1(E) did not influence the endogenous pool of total ERK. Phospho-

ERK expression was greatly increased in oocytes that expressed MEK1(E), whether or not they co-express Cx46. Bands for p-ERK show that we were able to activate the MAPK signaling cascade.

FGF signaling also increases Cx50 mediated junctional coupling

We expressed a Xenopus fibroblast growth factor receptor (XFGFr) with Cx50 in order to activate the MAPK signaling pathway via receptor ligand interaction (Figure V-3, A). Oocytes were injected with Cx50 and XFGFr, after 16-18h of expression, cells were paired and incubated with 15ng/ml FGF and intercellular coupling was analyzed. Cx50 expressing pairs produced junctional coupling that were 50-fold higher than water injected pairs, and FGFr co-expressing pairs produced junctional conductance that were 4.6-fold higher than Cx50 pairs after FGF treatment (Figure V-3, B) (mean ±SE, n= 12-19; $P < 0.05$, Student's t-test).

Analyses of Animals

To characterize the interaction of MAPK signaling and gap junctional communication in the lens, we crossbred a transgenic MEK1(E) mouse {Gong et al., 2001} with Cx46KO, Cx50KO, or Cx50KI46 mice {Gong et al., 1997, White et al., 1998; White 2002}. All animals were fertile and viable. We plotted total body mass of as a function of time, and there were no statistical differences in the body masses of all animals (Figure V-4).

Deletion of Cx50 delays the progression of cataracts

The deletion of Cx50 was reported to perturb lens homeostasis and induced a mild nuclear cataract and an abnormality in overall growth of the lens {White et al., 1998}. To determine the impact of Cx50 in response to a cataract induced by MEK1(E), we observed the progression of the cataract phenotype of lenses from MEK1(E)-Cx50KO and control littermates. The lens phenotype was documented by photography at 1, 3, 5 and 7 weeks of age. Wild type lenses were transparent and normal in size (Figure V-5, 1st panel). MEK 1 transgenic lenses at P7 displayed a severe nuclear cataract and cortical cataracts. At three and five weeks of age, the lenses were significantly larger in size and the cataract phenotype was more severe (Figure V-5 2nd panel). However, in the event that Cx50 was deleted, at P7 the lens maintained the mild nuclear cataract (Figure V-5, 4th panel). But interestingly, at one and three weeks of age, the central and the cortical cataract was less severe in comparison to the MEK1 transgenic, however, by five weeks the central cataract was similar to the MEK1(E) transgenic lens, but there was a noticeable improvement of the cortical cataract in the MEK1(E)-Cx50KO lens (Figure V-5, 4th panel). These results suggest that Cx50 influences the rate and severity of cataractogenesis of the lens.

MEK1(E)-Cx50KO and MEK1(E)-Cx50KI46 mice do not display macrophthalmia

The phenotypes of MEK1(E) eyes and lenses were previously described {Gong et al., 2001}, and displayed severe nuclear and cortical cataracts and macrophthalmia (Figure V-5). Cx50 null mice have been described and have eyes and lenses that are

smaller in mass compared to wild type mice {White et al., 1998}. To further investigate the growth of the eye and the lens, we plotted eye and lens mass as a function of age. As shown in (Figure V-6 A and B), by seven weeks of age the eyes and lenses of MEK1(E) animals were ~1.3 and ~1.7 times larger than their wild type littermates, respectively . We observed that MEK1(E)-Cx50KO eyes and lenses were ~20% smaller than wild type ($P < 0.05$, Student's *t* test). In the Cx50KI46 animals, where Cx50 was replaced with Cx46, knock in eyes and lenses have been reported to have a 25% and 34% reduction in growth, respectively in comparison to wild type {White et al., 2002}. We found that the mass of MEK1(E)-Cx50KI46 eyes and lenses were not statistically different from wild type at seven weeks of age (Figure V-6 C and D) ($P = 0.3$ and 0.4, respectively, Student's *t* test). However, the decrease in eye and lens mass for MEK1(E)-Cx50KO and MEK1(E)-Cx50KI46 in comparison to MEK1(E) mice suggest that MAPK signaling may positively interact with Cx50 to promote postnatal growth of the lens.

MEK1(E)-Cx46KO mice exhibit macrophthalmia, cataract and lens rupture

The lenses of Cx46KO mice have a severe, senile-type cataract, with normal ocular development and growth {Gong et al., 1997}. We cross bred MEK1(E) transgenic mice with Cx46KO mice to further examine the interaction of Cx50 and MAPK signaling, in the absence of Cx46. On P7, we observed that the mass of the eye and lens in the MEK1(E)-Cx46KO animal was increased (Figure V-7) compared to wild type ($P < 0.05$, Student's *t* test). As early as P7, we saw that the MEK1(E)-Cx46KO lens had a nuclear and cortical cataract that was similar to the MEK1(E) lens. As the lens aged, the severity of the cataract become more prevalent, and by 5 weeks of age, macrophthalmia

was visibly apparent (Figure V-5). In addition, by seven weeks of age, ~45% of the MEK1(E)-Cx46KO lenses were ruptured (Figure V-8). Here we show that deletion of Cx46 did not prevent macrophthalmia and lens rupture caused by MEK1(E). Taken together, the results from the MEK1(E)-Cx50KO, MEK1(E)-Cx50KI46 and MEK1(E)-Cx46KO animal models further support the previous findings that Cx50 is necessary for growth {Sellitto et al., 2004} {White et al., 2007} and that a specific interaction between MAPK signaling and Cx50, but not Cx46, may regulate lens cell growth and differentiation.

Removal of Cx50 prevents lens rupture

The macrophthalmia phenotype in MEK1(E) lenses appeared to be coupled with lens rupture {Figure V-5}, therefore we were interested in further examining the role of Cx50 and MAPK signaling in regards to this problem. When observing the phenotypes of the MEK1(E) transgenic lens, we observed that from three to seven weeks of age, the transgenic lenses underwent an increasing incidence of lens rupture, from ~4% to 57%, respectively (FigureV-8). Like the MEK1(E) lenses, MEK1(E)-Cx46KO lenses frequently ruptured (~45% at seven weeks of age). Interestingly, MEK1(E)-Cx50KO did not display the rupture phenotype during the seven week observation, and at seven weeks 1 out of 14 MEK1(E)-Cx50KI46 lenses ruptured (FigureV-8). Thus, we were able to conclude that the deletion of Cx50 rescues lens rupture induced by MEK1(E).

Histology analysis of MEK1(E), Cx50KO, MEK1(E)-Cx50KO and wild type lens

The differences in lens transparency and frequency of lens rupture in MEK1(E) lenses were indicative of changes in the morphology in the lens. Several *in vitro* and *in vivo* studies have documented changes in the lens epithelial layer in the presence of ERK inhibition and over stimulation {Lovicu et al., 2001; Robinson et al., 1995}. To observe the pathologic conditions of the lens, histologic sections from mouse eyes were stained with hematoxlyin and eosin. Sections of P9 wild type eyes reveal normal ocular development without cellular pathology, particularly in the core and bow region of the lens (Figure V-9 A), where cataracts and vacuoles are visible in Cx50KO and MEK1(E) lenses (Figure V-9 B, F and C). Vacuole formation in the lens cortex was previously described in the MEK1(E) lens {Gong et al., 2001}. In contrast, sections from MEK1(E)-Cx50KO lenses (Figure V-9 D and H), maintained a disruption of the staining in the core of the lens because of the cataract, however vacuoles at the lens equator were absent. Thus, the histologic differences in the MEK1(E) and MEK1(E)-Cx50KO lenses suggest that over stimulation of MAPK signaling may alter the circulating current at the lens equator {Baldo and Mathias., 1997} {Le and Musil et al., 2001} therefore, if MAPK signaling were to influence Cx50 coupling, the maintenance of homeostasis maybe altered.

Deletion of Cx50 reduces MEK1 (E) stimulated lens glucose accumulation in the lens

Glucose is used by the lens to support growth and homeostasis {Berman 1991}. The lens is an avascular organ, and although the epithelial and differentiating cells are still able to undergo oxidative phosphorylation, the mature fibers at the core of the lens are dependent mostly on glycolysis for energy production {Bassnett 2002}. Studies have

shown that membrane transporters facilitate the uptake of glucose and other amino acids in the lens {Merriman-Smith et al., 2003; Donaldson et al., 2004; Mathias et al., 2007}. Up regulation of the glucose transporter 1 (GLUT-1) was suggested to cause an elevation of glucose uptake in MEK1 (E) transgenic lenses {Gong et al., 2001}. Based on the results of our lens histology sections, we examined the effects of Cx50 and MAPK signaling in response to elevated glucose uptake in the MEK1(E) lens. In a preliminary observation, we found that the deletion of Cx50 reduced the glucose concentration in MEK1(E) lenses (Figure V-10). Our results suggest that the removal of Cx50 in the presence of MAPK over stimulation eliminates the vacuole phenotype seen in the equatorial region of MEK1(E) lenses. However, further analyses by glucose assay as well as examination of glucose transporter regulation are needed to determine the underlying mechanism associated with the formation of vacuoles in the lens.

Deletion of Cx50 reduces MEK 1(E) stimulated mitosis in MEK1(E) and MEK1(E)-Cx50KO lenses

During the first postnatal week, rodent lenses do not grow at a constant rate {Brewitt et al., 1988}. In Cx50 and Cx50KI46 lenses, the pattern of lens mitosis during the first postnatal week is altered {Sellitto et al., 2004} {White et al., 2007}. In order to better understand the interaction between Cx50 and MAPK signaling during postnatal growth of the lens, we assayed lens capsules for BrdU incorporation. As shown in the graph (FigureV-11 G), we plotted the density of BrdU labeled cells as a function of age. On P2, the day of peak postnatal mitosis, wild type and MEK1(E) positive BrdU incorporation was similar, while MEK1(E)-Cx50 epithelial cells have fewer BrdU

labeled cells across the epithelial zone. By P5, cellular mitosis in wild type epithelial cells is dramatically decreased and densities of BrdU labeling densities are similar to the MEK1(E)-Cx50KO lenses. In contrast, MEK1(E) lenses display a sustained stimulation of mitosis at P5. Upon further analysis of the BrdU incorporation on P2 and P5, we divided the lens capsule into sub-regions (equatorial, transitional and central zones) and found that the density of BrdU positively labeled cells were significantly reduced in the central epithelium of MEK1(E)-Cx50KO lens, but to a lesser degree in the lens equator (Figure V-12). These results suggest that Cx50 and activation of MAPK signaling are functional during postnatal peak mitosis especially in the central epithelium.

Discussion

To date, the literature has provided a plethora of data to describe the physiological role of gap junction communication in the mammalian lens; however, little attention has been focused on the underlying mechanisms or mitogen factors that may interact with connexins to regulate postnatal lens growth and development. Studies report that active forms of ERK 1 and ERK 2 are expressed in the lens epithelium and the differentiating fibers of the lens in rat, human and bovine {Li et al., 2003}. In addition, FGF signaling has been shown to up regulate gap junctional coupling, without increasing gap junction assembly; and may influence the angular gradient of intercellular coupling in the lens {Le and Musil 2001}. Cx50, which is expressed in the lens epithelium and differentiating fibers influences lens cell proliferation and is necessary to achieve peak mitosis and lens cell differentiation during postnatal development {Sellitto et al., 2004; White et al 2007}. Taken together, all of these findings support the idea that MAPK signaling may be active

at the lens equator, where junctional coupling is highest {Gong et al., 2001; Baldo and Mathias 1997; Le and Musil, 2001}. We hypothesized that Cx50 and mitogen activators may function together to regulate the postnatal growth of the lens. Therefore, one might predict that expressing mitogens with lens connexins in an *in vivo* model may provide valuable understanding to this question. In this study we analyzed Cx46 and Cx50 in the presence of FGF or MEK1(E) to identify the functionality of the channels. We found that co-expression of Cx50 with a FGF receptor and incubation with FGF caused an increase in Cx50-mediated junctional conductance. While we were able to show a positive correlation with Cx50 junctional communication, our experimentation was hampered by poor cell viability, and biochemical analyses was limited. In an attempt to better understand this effect, we activated the MAPK signaling cascade downstream of FGF by co-expressing a constitutively active MEK1 transgene with Cx50. We found that MEK1(E) caused a similar increase in Cx50 mediated junctional conductance, as previously shown in FGF experiments. However, Cx46 mediated junctional conductance in the presence of MEK1(E) was not changed. Our biochemical analysis showed that protein synthesis of both Cx46 and Cx50 in the co-expressing cells were similar to the cells expressing Cx46 or Cx50 protein levels alone. Thus, an increase in connexin subunits was not likely a cause of the increased junctional conductance. Although we report an increase in Cx50-mediated junctional conductance, the mechanism by which this increase occurs is unclear. Because Cx46 is not expressed in the lens epithelium like Cx50, we can speculate that Cx50 may possess a specialized property that responds to mitogens in the lens epithelium. However, further experimentation will be required to determine the underlying mechanism.

Many studies have focused on protein- protein interactions of connexins and mitogens, and a wide array of explanations have been documented {Hales et al., 1994; Hossian et al., 1999; Lang et al., 2004; Le et al., 2001; Li et al., 2003; Saez et al., 2003}, but the data are limited in regards to Cx50 and its interaction with mitogens. Some possibilities to consider may include an increase in unitary conductance of single channels, an increase in channel open probability, and or direct phosphorylation of the connexin. Investigation of these parameters may help explain the mechanism by which Cx50 junctional conductance was increased as well as the failed response of Cx46.

Toward that goal, we examined the interaction of MAPK signaling, Cx50 and Cx46 in an *in vivo* system. Knockout of Cx50 in mice results in a lens that displays a mild nuclear cataract and microphthalmia, which was due to reduced proliferation and a delay in lens cell differentiation {White et al., 1998; Rong et al., 2002}. Conversely, the transgenic MEK1(E) mouse developed lenses that were cataractous and macrophthalmic {Gong et al., 2001}. In this study, our data demonstrates that the removal of Cx50 in MEK1(E) lenses was able to restore many of the pathologies caused by MEK1 over expression, they include a delay in cataract formation, recovery of macrophthalmia and lens rupture, rescue of vacuole formation in the equatorial region of the lens, reduction in MEK1(E) stimulated mitosis in the central and transitional epithelium of the lens and restoration of glucose concentrations in the lens.

The cataract and macrophthalmia phenotype seen in the MEK1(E) lens is thought to be associated with an influx of water and ions; which could lead to altered osmotic pressure, and cause the lens to swell and eventually rupture {Gong et al., 2001; Merriman-Smith et al., 2003; Donaldson et al., 2004}. However, the absence of Cx50 in

MEK1(E) lenses resulted in a delay in cataract formation and the cortical cataract in the outer cortex of the lens showed improvement. When measuring lens glucose concentration, we report that the deletion of Cx50 in MEK1(E) lens restored the concentration of lens glucose to milli-molar concentrations that were similar to wildtype and rescued the enlarged vacuoles in the bow region of the lens. Perhaps, these results suggest a role for Cx50 and ERK activation to support osmotic regulation. However, in order to attain positive confirmation of this assumption, in future studies it would be useful to investigate the channel transporter expression of the GLUT-1, GLUT- 3, as well as the water and chloride transporters in the MEK1(E)-Cx50KO lens, because each of these transporters are expressed at different stages of development and have been shown to be up regulated in lenses with sugar cataract {Donaldson et al., 2004; Merriman- Smith et al., 2003}.

However, if MEK activation were responsible for setting up the angular gradient for junctional coupling and the circulating current, it is reasonable to assume that over stimulation of MEK may perturb circulation and disrupt osmotic homeostasis. If that were the case, our results would concur with the proposed model, where FGF stimulated sustained ERK activation which caused an increase junctional coupling in a gradient dependent manner without increasing gap junction assembly {Le and Musil 2001}. This would also explain our results where we observed an increase in Cx50-mediated junctional conductance when co-expressed with MEK1(E) or FGF in oocytes. Studies have revealed that replacement of Cx50 with Cx46 by genetic knock in was not able to restore central epithelial proliferation in comparison to wild type. In addition, recent results suggest that simple ionic coupling is not enough to stimulate mitosis in the lens

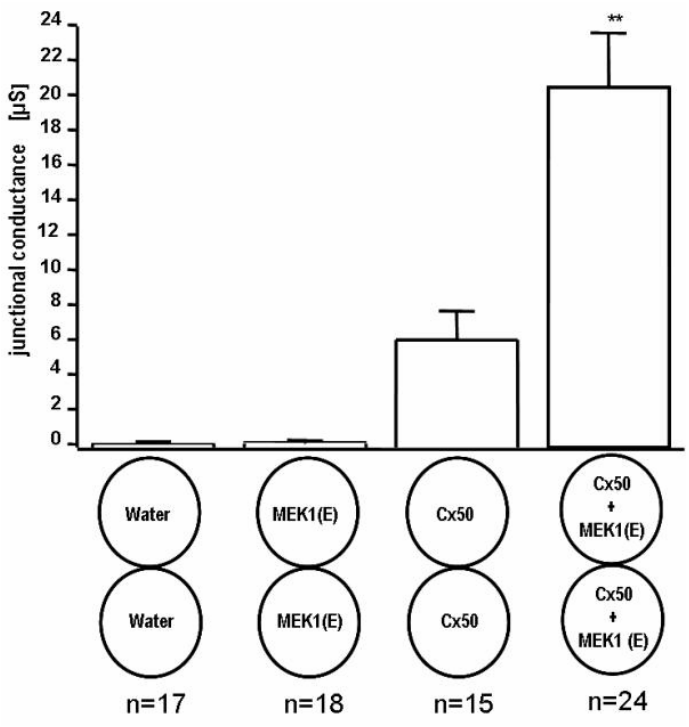
{White et al., 2007; Sellitto et al., 2004}. We show that Cx46 mediated junctional conductance in oocytes is non-responsive in the presence of MEK1(E). Hence, we can speculate that Cx50 selectively mediates intercellular propagation of the second messengers and mitogens to activate intercellular signaling cascades in the central epithelium. However, more experiments will be needed to determine the mechanisms that are influencing Cx50 junctional communication.

Previous studies have shown that Cx50 promotes postnatal lens development and until recently, no studies had shown that Cx50 functionally contributes to gap junction communication between lens epithelial cells {Sellitto et al., 2004; White et al., 2007}. White et al., 2007 recently demonstrated that Cx50 provides most of the functional coupling between epithelial cells during the first postnatal week and is progressively decreased with age. We show that the deletion of Cx50 reduces MEK1(E) stimulated mitosis in the central epithelium of P2 and P5 lenses. While the concentration of MEK1 in the equatorial and central region of the lens is unknown, our western blot analyses show that ERK1 and ERK2 are expressed and phosphorylated in MEK1(E) lens capsule (data not shown). In addition, ERK 1 and ERK2 have been shown to be expressed in the capsular epithelium and the cortical layer of the rat lens {Li et al., 2003}.

If we were to speculate that MAPK signaling were propagated by Cx50 to the central epithelium, this might explain the reduction in the density of mitotic division in the central epithelium in MEK1(E)-Cx50KO mice. Future *in vitro* experiments to examine the single channel properties of Cx50 in the MEK1(E) and MEK1(E)-Cx50KO lens epithelial cells could provide novel insights to better understand the mechanism of MAPK signaling and Cx50 channel activity during postnatal growth.

Figure V-1. Cx50 and MEK1(E) co-expression increases junctional conductance. A. Junctional conductance measurements recorded from Xenopus oocyte pairs injected with wild-type Cx50 and MEK1(E) transcript alone or in combination. Cell pairs expressing water or MEK1(E) do not form functional intercellular channels, while wild type Cx50 and co-expressing pairs form functional gap junctions with mean conductance values of approximately 6 and 21 μS, respectively ($P < 0.05$, Student's t-test). **B.** Immuno-blot analysis of oocytes showed equivalent levels of wild-type Cx50 and total endogenous ERK protein expression for the conditions tested. Expression of MEK1(E) stimulated phospho- ERK in oocytes.

A.


junctional conductance [µS]
24
22
20
18
16
14
12
10
8
6
4
2
0
**
Water
Water
MEK1(E)
MEK1(E)
Cx50
Cx50
Cx50 + MEK1(E)
Cx50 + MEK1 (E)
n=17
n=18
n=15
n=24

B.

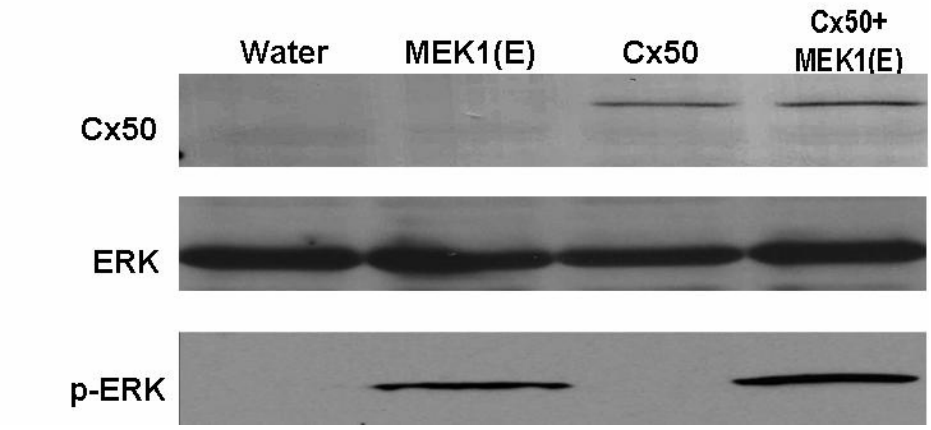
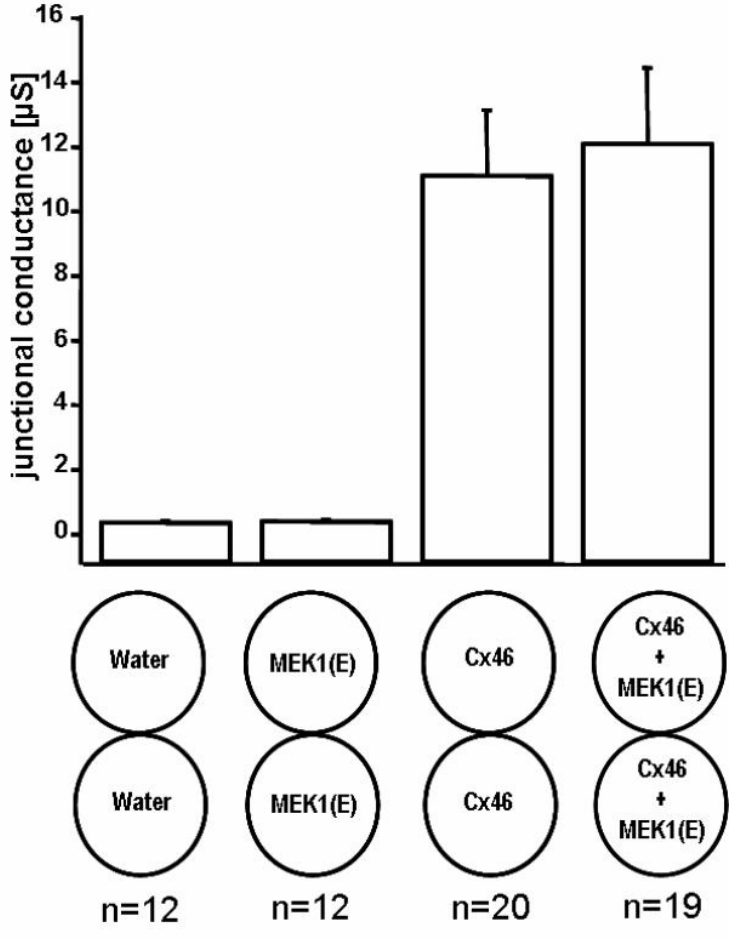

Water
MEK1(E)
Cx50
Cx50+ MEK1(E)
Cx50
ERK
p-ERK

Figure V-2. Cx46 and MEK1(E) co-expression does not increase junctional conductance. A. Junctional conductance measurements recorded from Xenopus oocyte pairs injected with wild-type Cx46 and MEK1(E) transcript alone or in combination. Cell pairs expressing water or MEK1(E) do not form functional intercellular channels, while wild-type Cx46 and co-expressing pairs form functional gap junctions with mean conductance of equal value (mean ±SE;$P > 0.05$, Student's t-test). **B.** Immuno-blot analysis of oocytes showed equivalent levels of wild-type Cx46 and total ERK protein expression for the conditions tested. Expression of MEK1(E) stimulated phosho-ERK activation in oocytes

A.


junctional conductance [µS]
16
14
12
10
8
6
4
2
0
Water
MEK1(E)
Cx46
Cx46 + MEK1(E)
Water
MEK1(E)
Cx46
Cx46 + MEK1(E)
n=12
n=12
n=20
n=19

B.

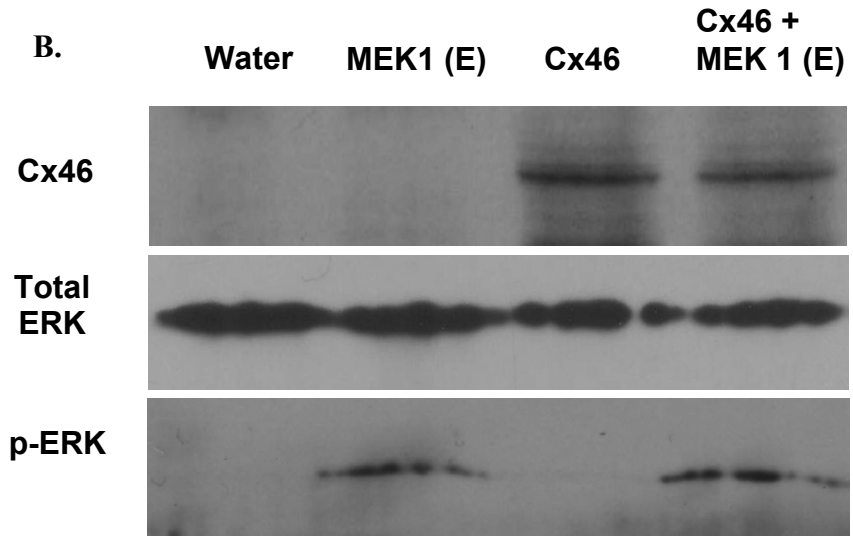
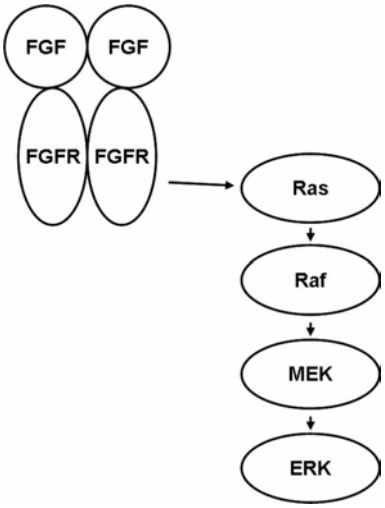

Water
MEK1 (E)
Cx46
Cx46 + MEK 1 (E)
Cx46
Total ERK
p-ERK

Figure V-3. Fibroblast growth factor (FGF) increases Cx50 junctional conductance. **A.** Cartoon of fibroblast growth factor signaling shows that when FGF binds to its receptor, downstream mitogen factors are activated via phosphorylation. **B.** Junctional conductance measurements recorded from Xenopus oocyte pairs injected with wild-type Cx50 and Xenopus FGF receptor transcripts, followed by incubation with FGF ligand. Cell pairs expressing water do not form functional intercellular channels, while wild-type Cx50 and co-expressing pairs form functional gap junctions with mean conductance values of approximately 5 and 23 μS, respectively (mean ±SE; $P < 0.05$ Student's t-test).

A.


FGF
FGF
FGFR
FGFR
Ras
Raf
MEK
ERK

B.

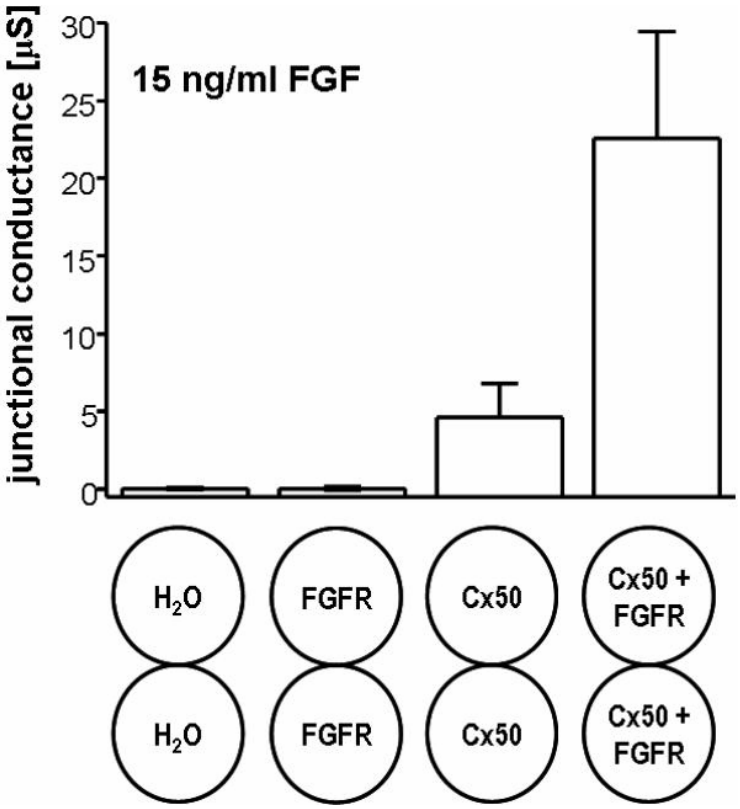

junctional conductance [µS]
30
25
20
15
10
5
0
15 ng/ml FGF
H2O
FGFR
Cx50
Cx50 + FGFR
H2O
FGFR
Cx50
Cx50 + FGFR

Figure V-4. Growth analyses of animal body mass. The body mass of all mice were plotted as a function of age. There was no difference in the body masses of all animals when compared to their wild type littermates ($P > 0.05$, Student's *t* test).

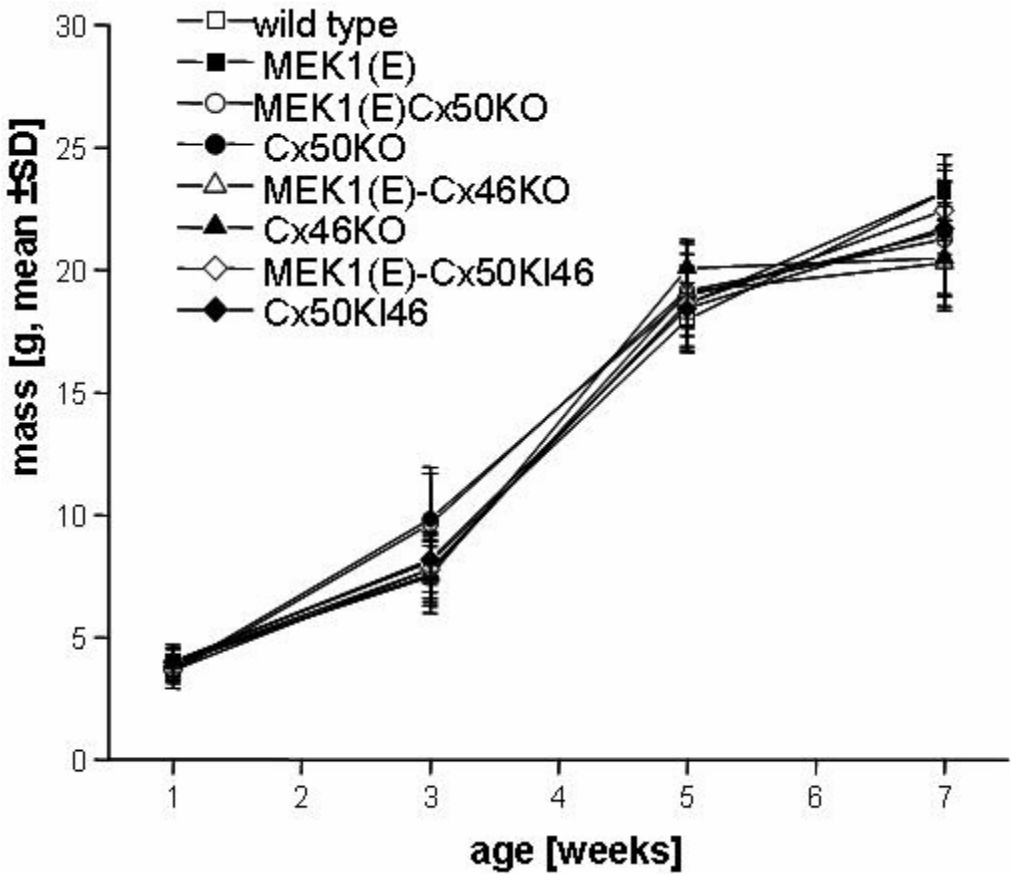
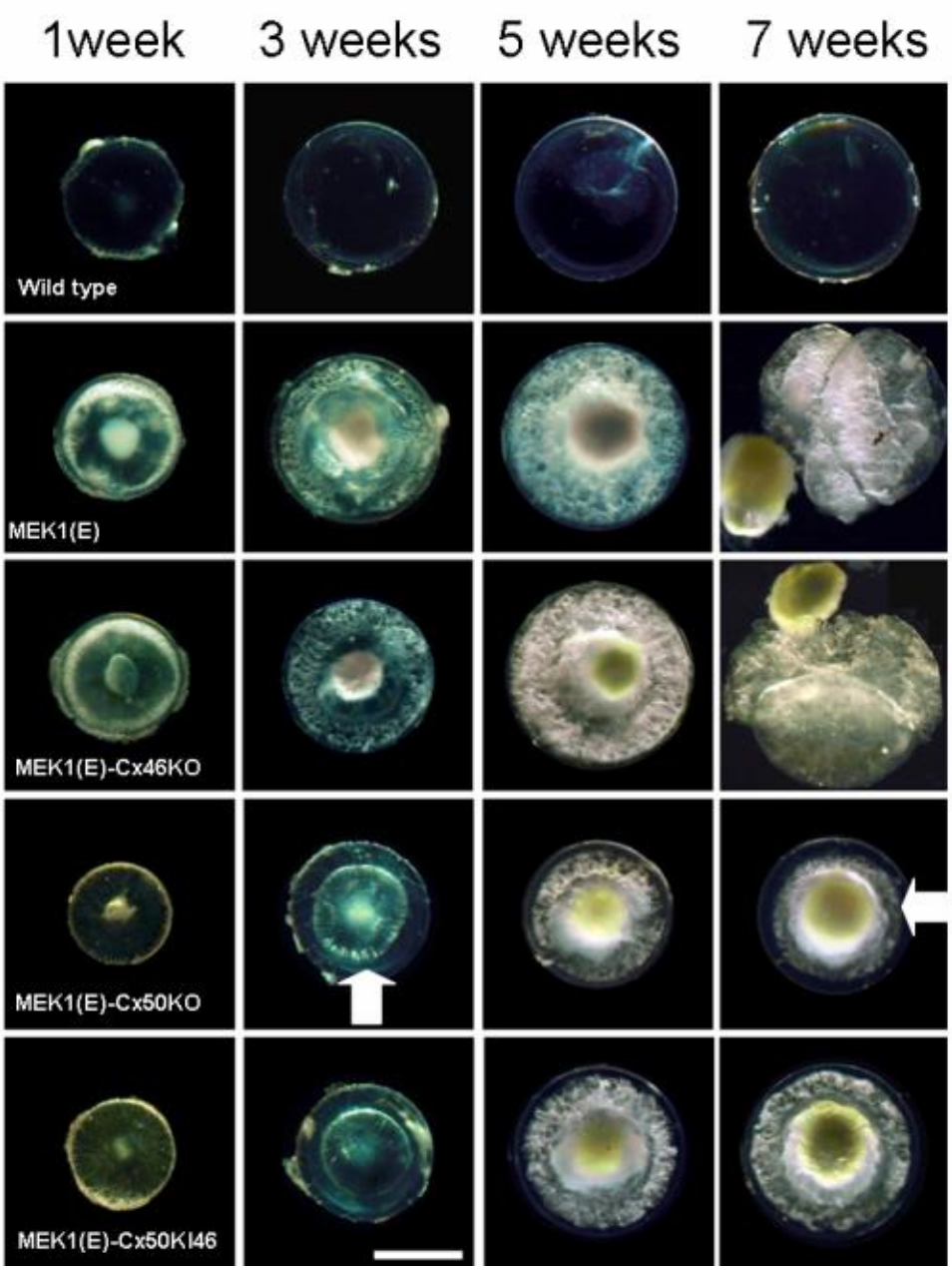
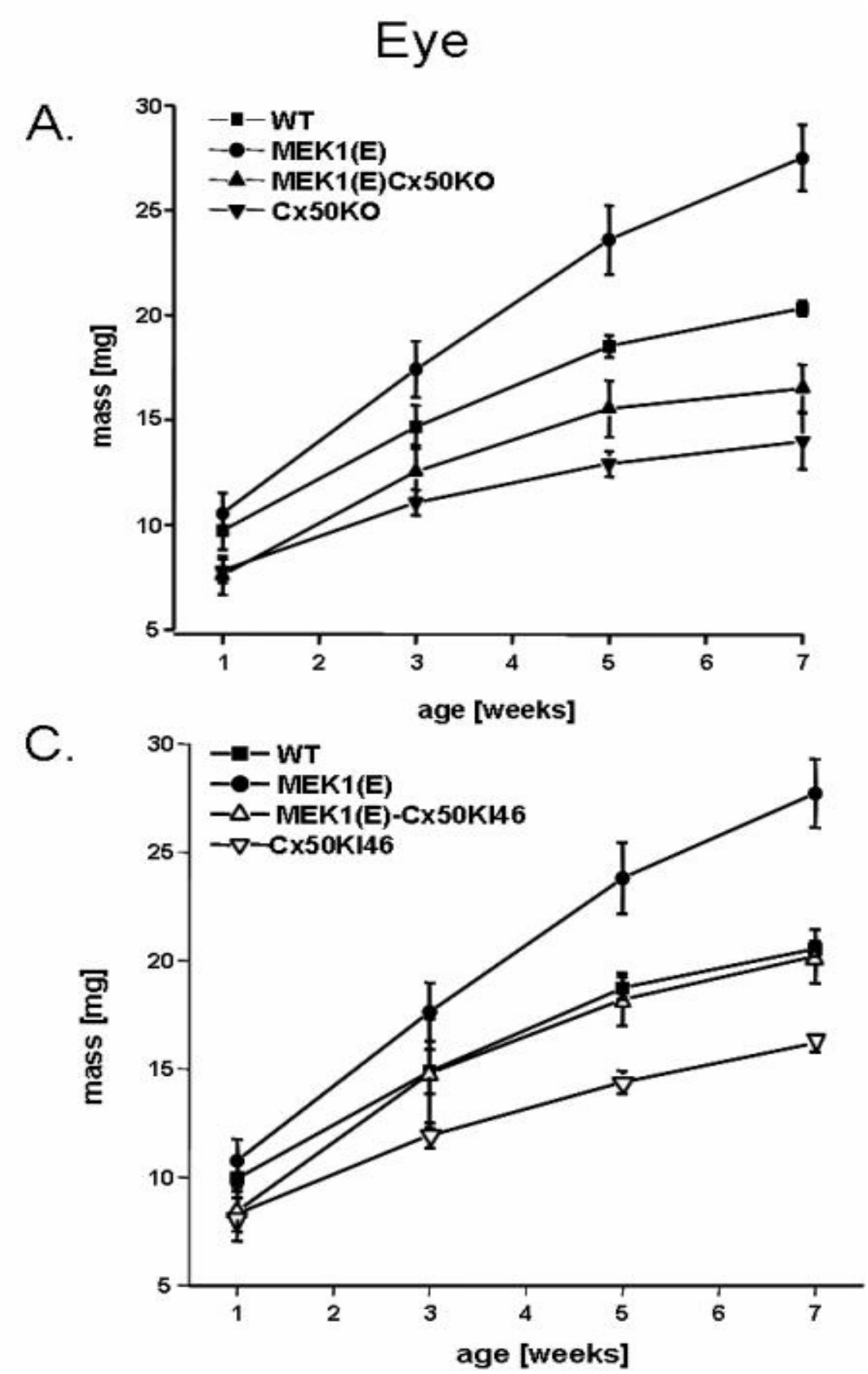

wild type
MEK1(E)
MEK1(E)Cx50KO
Cx50KO
MEK1(E)-Cx46KO
Cx46KO
MEK1(E)-Cx50KI46
Cx50KI46
mass [g, mean ±SD]
age [weeks]
0
5
10
15
20
25
30
1
2
3
4
5
6
7

Figure V-5. Lens phenotypes of MEK1(E), MEK1(E)-Cx50KO, MEK1(E)-Cx46KO and MEK1(E)-Cx50KI46 mice. Photographs were taken to visualize the cataract phenotype induced by MEK1(E) expression. (First panel) Wild type lenses from one to seven weeks of age grow normally and are transparent. (Second Panel) MEK1(E) lenses develop a nuclear and cortical cataract as early as P7 that continue to progress in severity. By seven weeks of age, the lens displays marcophthalmia and often time undergoes rupture. (Third Panel) Cx46KO-MEK1(E) lenses have phenotypes that are similar to MEK1(E) lenses, and have a high incidence of rupture also. Knockout of Cx50 or replacement of Cx50 with Cx46, results in a milder onset of the central cataract in the first postnatal week. As the lens ages, the cortical cataract (🡅) is apparent but less severe, and by seven weeks of age the MEK1(E)-Cx50KO lens still show improvement in cortical cataract and no rupture (🡄).


1week
3 weeks
5 weeks
7 weeks
Wild type
MEK1(E)
MEK1(E)-Cx46KO
MEK1(E)-Cx50KO
MEK1(E)-Cx50KI46

Scale = 1mm

Figure V-6. MEK1(E) eye and lens mass are affected by deletion and or replacement of Cx50. Eye mass as a function of age (panels a and c). By seven weeks of age, MEK1(E) eyes are 1.3 times larger than wild type. As previously reported, Cx50KO and Cx50KI46 eyes were 32% and 25% smaller, relative to wild type. While lens mass of Cx50KO and Cx50KI46 was 46% and 34% reduced, in comparison to wild type. At seven weeks of age lens mass of MEK1(E) (panels b and d), was 1.7 times larger than wild type. MEK1(E)-Cx50KO lenses were ~20% smaller than wild type and MEK1(E)-Cx50KI46 lenses grew as well as wild type. (Values are the mean ±SD, n= 4-16 for each genotype). Thus, MAPK signaling slightly recovered the lens growth defect in Cx50 deficient lenses.


Eye
A.
WT
MEK1(E)
MEK1(E)Cx50KO
Cx50KO
mass [mg]
age [weeks]
C.
WT
MEK1(E)
MEK1(E)-Cx50KI46
Cx50KI46
mass [mg]
age [weeks]

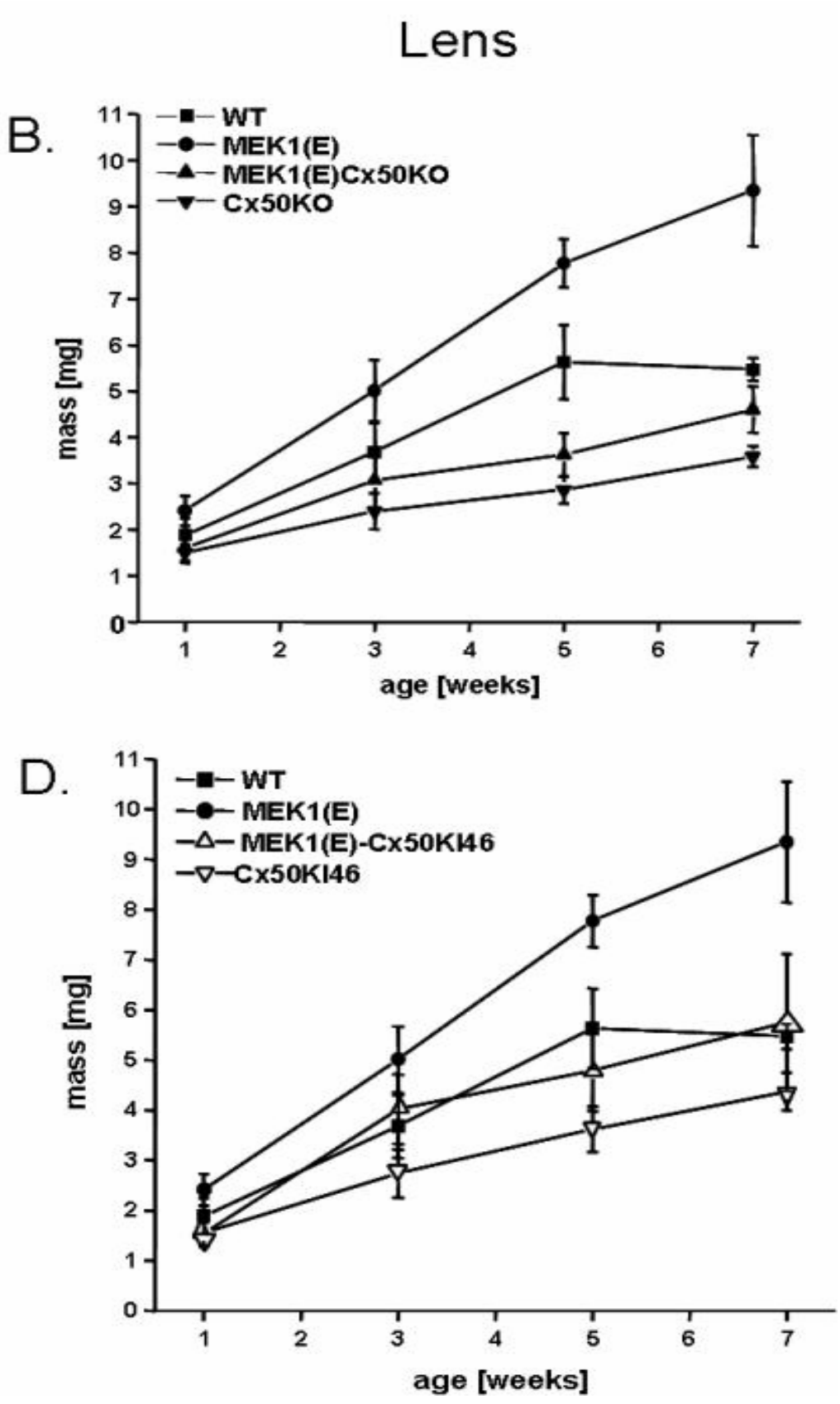
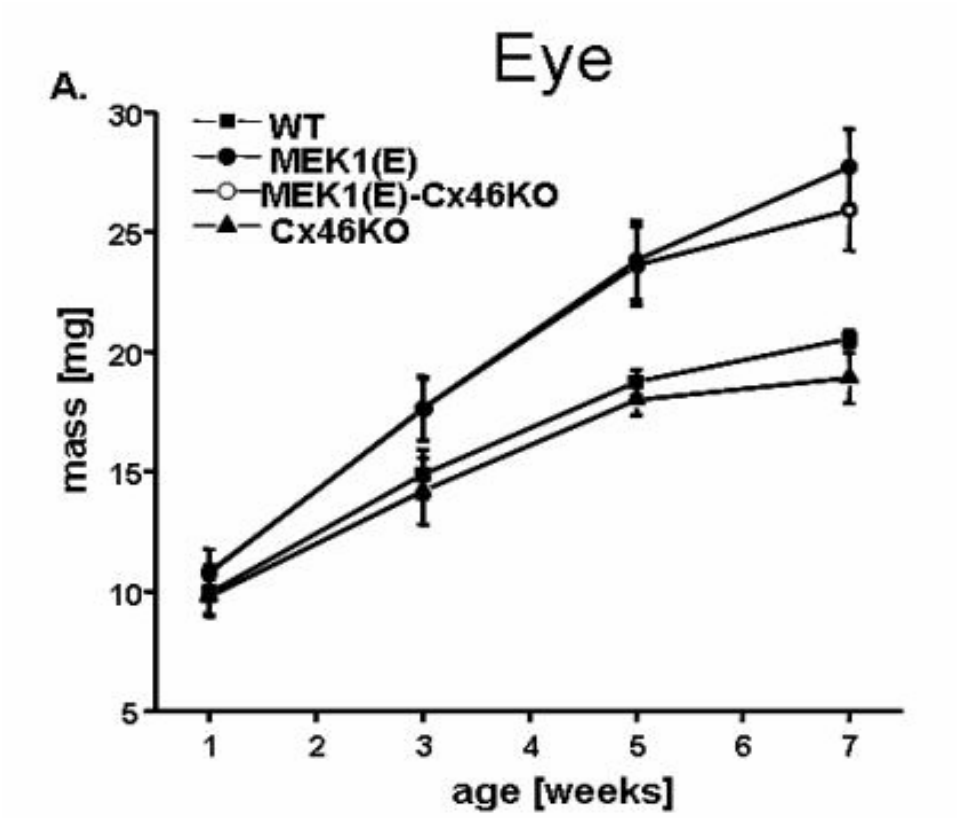

Lens
B.
WT
MEK1(E)
MEK1(E)Cx50KO
Cx50KO
mass [mg]
age [weeks]
D.
WT
MEK1(E)
MEK1(E)-Cx50KI46
Cx50KI46
mass [mg]
age [weeks]

Figure V-7. Eye and lens growth are affected by MEK1(E), but not by deletion of Cx46. Eye mass as a function of age (panels a and b). By seven weeks of age, MEK1(E) eyes and lenses are 1.3 times larger than wild type. As previously reported, Cx46KO eyes and lenses grow normally compared to wild type. At seven weeks of age lens mass of MEK1(E) (panels a), was 1.7 times larger than wild type. MEK1(E)-Cx46KO lenses grew as well as MEK1(E) lenses as early as P7. The greater variability in lens mass at seven weeks was due to the high incidence of ruptured lenses in both sample pools. ($P < 0.05$, Values are the mean ±SD, n= 4-16 for each genotype). Thus, MAPK signaling and Cx46 do not interact to regulate post natal lens growth and cell differentiation.


A.
Eye
WT
MEK1(E)
MEK1(E)-Cx46KO
Cx46KO
mass [mg]
age [weeks]

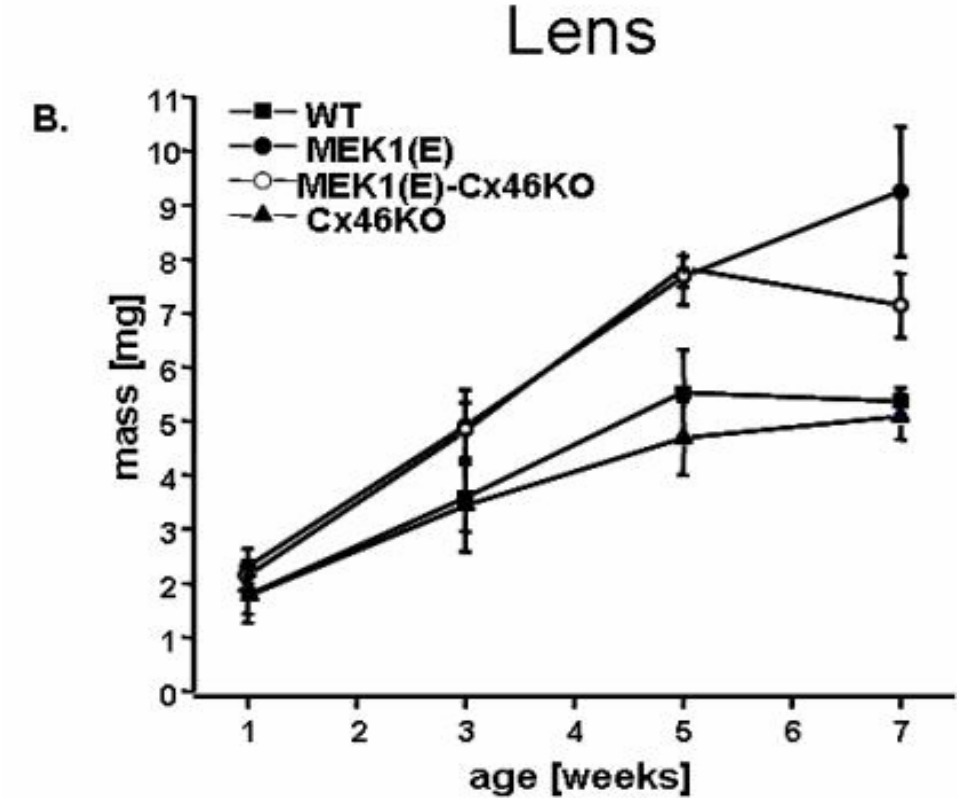
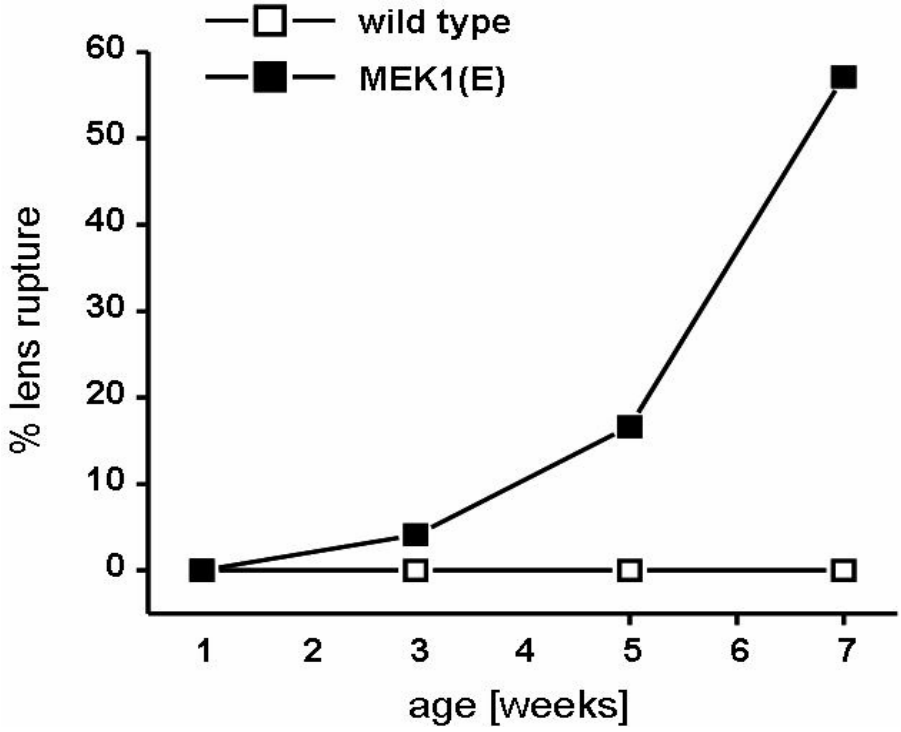

B.
Lens
WT
MEK1(E)
MEK1(E)-Cx46KO
Cx46KO
mass [mg]
age [weeks]

Figure V-8. Deletion of Cx50 rescues lens rupture. Interbreeding of MEK1(E) and Cx50KO or Cx50KI46 mice resulted in recovery of the lens rupture phenotype. Graphs show the percentage of rupture for wildtype, MEK1(E)-Cx50KO, MEK1(E)-Cx46KO and MEK1(E)-Cx50KI46 lenses from P7 to seven weeks of age. MEK1(E) transgenic lenses rupture from three to seven weeks of age, ~4% to 57%, respectively. Deletion of Cx46 causes lenses to rupture from three to seven weeks of age, ~6% to 45%, respectively. Removal of Cx50 caused lenses to recover from rupture, thus the interaction of Cx50 and MAPK signaling may influence osmotic homeostasis in the lens


wild type
MEK1(E)
% lens rupture
60
50
40
30
20
10
0
1
2
3
4
5
6
7
age [weeks]

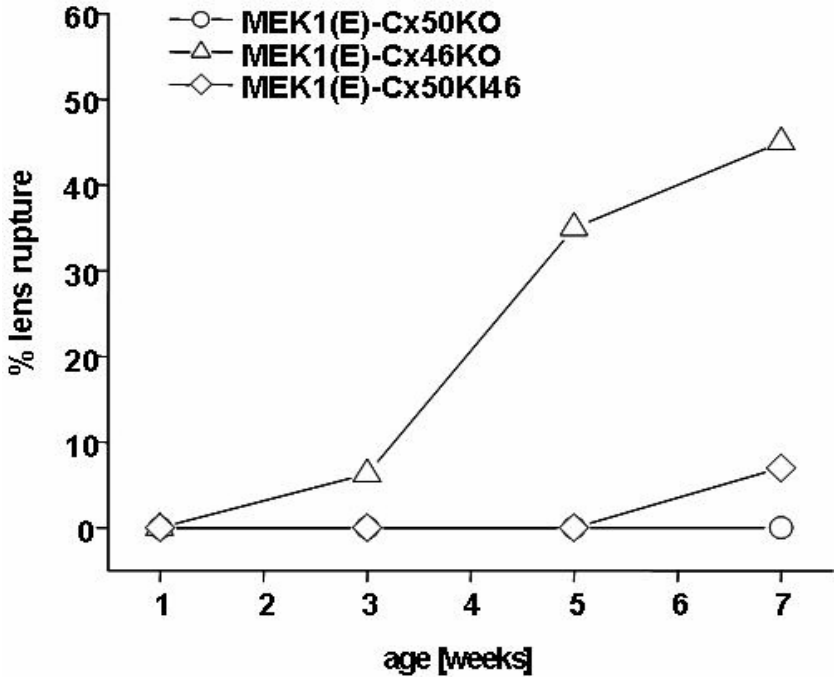

MEK1(E)-Cx50KO
MEK1(E)-Cx46KO
MEK1(E)-Cx50KI46
% lens rupture
60
50
40
30
20
10
0
1
2
3
4
5
6
7
age [weeks]

Figure V-9. Histological analyses of wild type, MEK1(E) and Cx50KO and MEK1(E)-Cx50KO lenses. Hematoxylin-eosin stained sections from P9 lenses. Sections of the visual axis at 10X magnification, **A.** section of wild type lens appear normal and uniform. **B.** MEK1(E) lens display a nuclear cataract and vacuoles in the bow region of the lens. **C.** Cx50KO lens have a nuclear cataract and no vacuole formation in the bow region of the lens. **D.** Deletion of Cx50 in MEK1(E) lenses removes the vacuoles seen in the MEK1(E) transgenic lens, however the cataract remains. Panels **E, F, G** and **H**, are sections focused on the equatorial region of the lens at 20X magnification. The epithelial layer in all lenses appears normal and uniform.

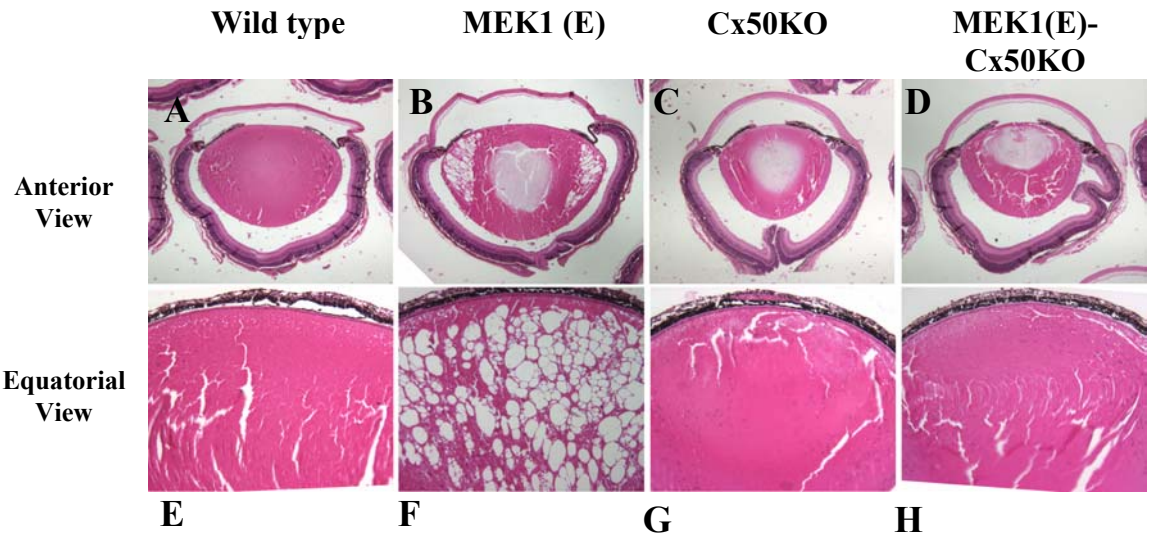

Wild type
MEK1 (E)
Cx50KO
MEK1(E)-Cx50KO
Anterior View
Equatorial View
A
B
C
D
E
F
G
H

Figure V-10. Quantitation of glucose in the lens. Lens glucose concentrations in five week old wild type, MEK1(E), MEK1(E)-Cx50KO and Cx50KO lenses. Wild type lenses have a glucose concentration of 0.9mM. MEK(E) lenses display an increased glucose concentration, ~1.5mM. Deletion of Cx50 restores the glucose concentration of MEK1(E) to glucose concentrations similar to wild type.

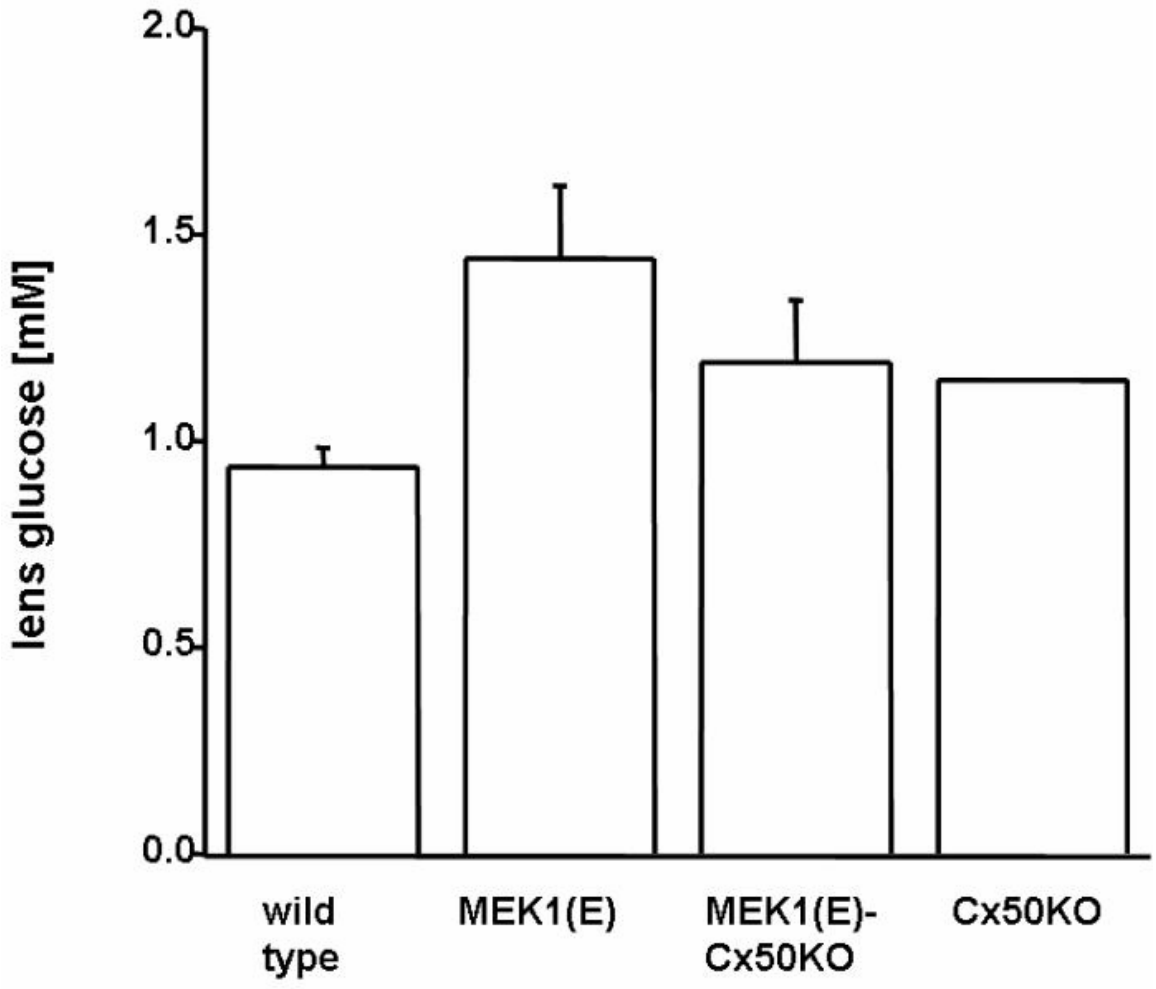
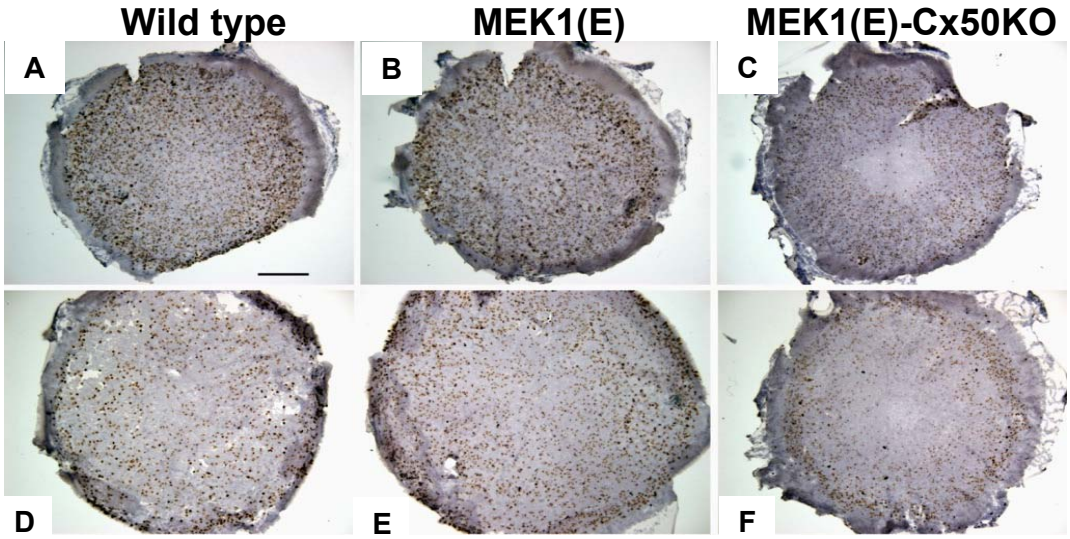

lens glucose [mM]
2.0
1.5
1.0
0.5
0.0
wild type
MEK1(E)
MEK1(E)-Cx50KO
Cx50KO

Figure V-11. MEK1(E) stimulated mitosis is reduced in Cx50 knockouts
Differences in the extent and spatial localization of mitosis in wild type, MEK1(E) and MEK1(E)-Cx50KO in P2 and P5 lenses. BrdU positive labeled cells in equatorial, transitional and central epithelium. At P2 (A and B), wild type and MEK1(E) lens show similar distributions of mitotically active cells. MEK1(E)-Cx50KO (C) lens capsules show a dramatic reduction of BrdU labeling in the central epithelium. By P5 (D, E and F), mitotic activity is reduced in all genotypes. We calculated the density of BrdU positive labeled cells in the lens epithelium from P1 to P6 (G). On postnatal day 2, wild type and MEK1(E) lens capsules display a high density of BrdU incorporation, and by P3 all the genotypes have a significant decrease in mitosis. However, MEK1(E) lenses show sustained activation of mitosis on P5. ($P < 0.05$, mean ±SE, ANOVA) Thus, Cx50 and MAPK signaling may interact on P2 to achieve optimal postnatal growth and cell differentiation.



Scale = 200 microns

G.

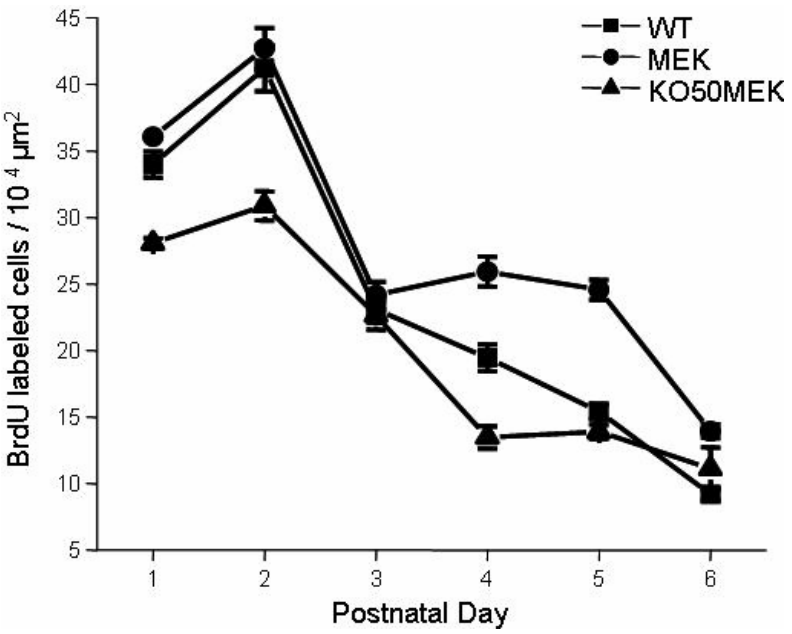
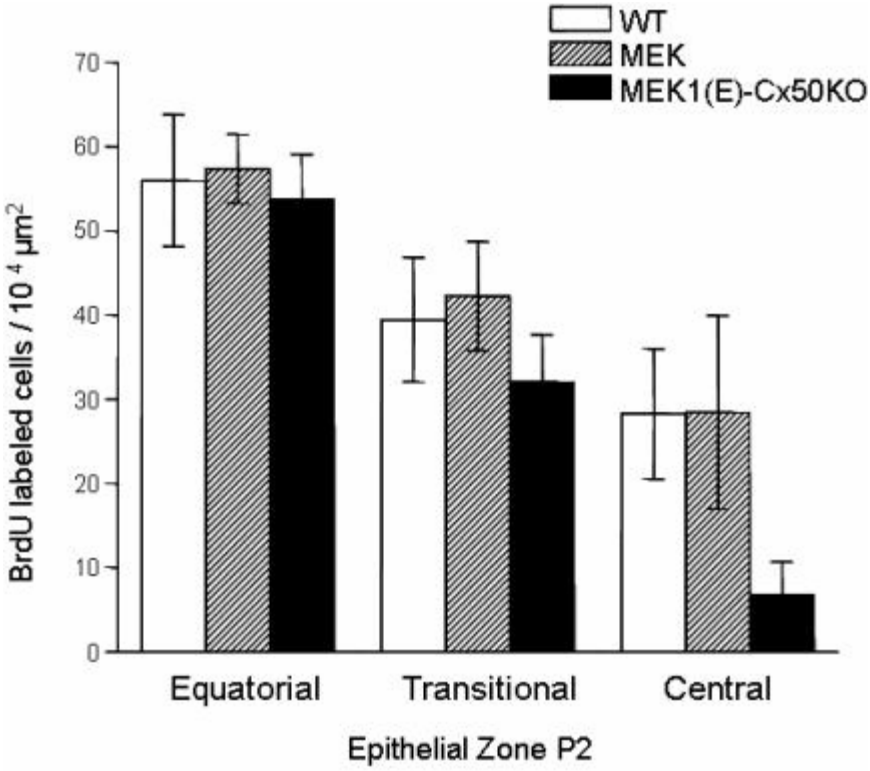
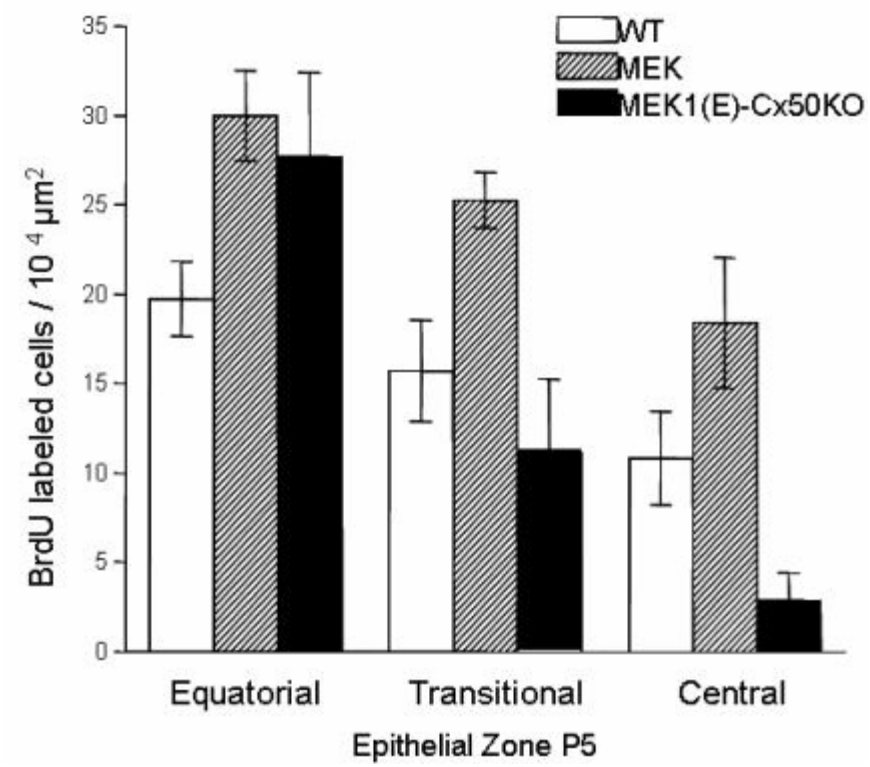


Figure V-12. Quantitation of BrdU incorporation and patterning in the epithelial zone. Mean densities of BrdU labeled cells in the equatorial, transitional and central sub-regions of the epithelial zone for wild type, MEK1(E) and MEK1(E)-Cx50KO were plotted at P2 and P5. On P2, only BrdU labeling in the central epithelium showed a dramatic reduction in the density of BrdU labeled cells, however, on P5 BrdU was reduced in both the central and transitional zones ($P < 0.05$, mean ±SE, ANOVA).


WT
MEK
MEK1(E)-Cx50KO
BrdU labeled cells / 10 4 µm2
70
60
50
40
30
20
10
0
Equatorial
Transitional
Central
Epithelial Zone P2


WT
MEK
MEK1(E)-Cx50KO
BrdU labeled cells / 10 4 µm2
35
30
25
20
15
10
5
0
Equatorial
Transitional
Central
Epithelial Zone P5

Chapter VI

Concluding Remarks and Future Directions

The importance of lens connexins and their role in the maintenance of lens homeostasis and growth have been well documented {White et al., 1998; Gong et al., 1997; Rong et al., 2002; Sellitto et al., 2004; Chang et al., 2002; Xia et al., 2006; White et al., 2007}; and many studies have sought to elucidate the role of mitogen signaling during lens proliferation and differentiation {Lovicu et al., 2001; Xie et al., 2006; Reneker et al., 2004; Gong et al., 2001; Li et al., 2003}. There are compelling data that suggest an interaction between connexins and mitogens in the lens {Le and Musil et al. 2001; Sellitto et al., 2004; White et al., 2007}. Data presented in these studies revealed that mitogens, such as FGF or ERK may interact with lens connexins to increase gap junctional intercellular communication at the equatorial region of the lens. The later two studies support a role for Cx50 signal propagation in the central epithelium of the lens and necessity of specific connexin isoforms for proper growth of the lens. Based on these studies, we designed experiments to further test the above hypotheses.

In this dissertation, MEK1(E) and Cx46 or Cx50 were tested to determine if their junctional conductance could be influenced by MAPK signaling *in vitro*. Co-expression of Cx50 and MEK1(E) resulted in an increase in Cx50-mediated junctional conductance. Interestingly, we found that Cx46 did not increase junctional conductance. In both cases, we reported no change connexin or MAPK protein synthesis. We then focused the study to explore the implications of connexin functionality *in vivo*, whereby, we crossbred several recently characterized mouse models, Cx50KO, Cx46KO or Cx50KI46 with a transgenic MEK1(E) mouse. Through the use of transgenic, knock out and knock in

animal models, we provide new insight on the physiological importance of connexin and mitogen signaling.

Analysis of MEK1(E)-Cx50KO mice, revealed that deletion of Cx50 in MEK1(E) lenses delayed the onset and progression of cataractogenesis in the lens for ~3 weeks in comparison to MEK1(E) and MEK1(E)-Cx46KO lenses, which a have a cataract as early as P7 and eventually rupture. MEK1(E)-Cx50KO lenses did not rupture and have improved cortical clarity. The deletion of Cx50 eliminated macrophthalmia caused by MEK1(E) and reduced postnatal mitosis in the central epithelium of the lens. Lastly, we found that the deletion of Cx50 eliminated the enlarged vacuoles in the equator of MEK1(E) lenses. Analyses of lens glucose showed that deletion of Cx50 reduced glucose concentrations to a value similar to wild type.

We were able to show an increase in junctional conductance in Xenopus *laevis* oocytes that co-expressed Cx50 and MEK1(E) or FGFr, without increasing connexin protein synthesis. Oocytes provide a rapid and flexible method for screening the electrophysiological properties of connexins. However, co-expression of connexins and mitogen activators in this system can complicate experimentation. For instance, expression of mitogens in oocytes can activate endogenously silenced pathways (Mos/MAPK) {Mood et al., 2003} and limit cell viability due to germinal vesicle breakdown. Therefore, the use of mammalian cell lines may provide further results that may explain the underlying mechanisms associated with our results.

We could use lens epithelial cells from MEK1(E) and MEK1(E)-Cx50KO lenses to measure unitary conductance and that may provide further insight about Cx50 mediated coupling and MAPK stimulation. Because FGF and MEK1(E) increased the

magnitude of coupling in Cx50, but not Cx46 expressing oocytes, we can speculate that Cx50 may have regulatory sites that respond to MAPK signaling. Thus, it would be useful to investigate the phosphorylation states of Cx46 and Cx50 in the presence of growth factors. Phosphorylation has been shown to be important for gap junction assembly and turnover and connexin phosphorylation can be altered by the activation of intercellular signaling pathways {Warn-Cramer and Lau 2004}. The literature, primarily explores Cx43 and phosphorylation in response to MAPK signaling {Cruciani et al., 2002; Cushing et al., 2005}, and little is known about the phosphorylation states of Cx50 when expressed with tyrosine receptor kinases. Because phosphorylation sites have been mapped in the C-termini of lens connexins, and junctional coupling and protein stability have been shown to be regulated by phosphorylation {Berthoud et al., 1997; Yin et al., 2000}, it would be interesting to express truncated Cx50 isoforms which have a cleaved C- termini with FGF or MEK1(E) to examine their response to MAPK stimulation. Other experiments to examine the trafficking of Cx50 to the cell membrane in the presence of MAPK stimulation could also be useful.

In conclusion, this dissertation demonstrated that Cx50 and MAPK signaling might function together to maintain lens homeostasis and growth and is consistent with the previous hypothesis for a role for Cx50 necessity for growth.

Chapter VIII

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