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Gap Junctional Conductance Produced by Cx50, but not Cx46, is Regulated by the PI3K Signaling Pathway

A Dissertation Presented

by

Jennifer Marie Martinez

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Abstract of the Dissertation

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Gap junction channels and cell signaling are essential components for proper organ development in multicellular organisms. The ocular lens is one such organ that has been well studied because of its large dependence on intercellular communication. The lens relies heavily on gap junction channels for intercellular communication during growth and differentiation due to the absence of a vascular system.

Gap junctions are comprised of hexameric oligomers of connexin subunits that are inserted into the plasma membrane. Genetic knockouts of lens connexins have helped to identify the function of each connexin. Cx46 knockout mice developed severe nuclear cataracts. Targeted deletion of Cx50 resulted in mild nuclear cataracts and a significant ocular growth defect not seen in the Cx46 deletion mutants. In lenses with a functional replacement of Cx50 with Cx46 knocked in to the Cx50 coding region, transparency is restored, however, the growth defect was not rescued.

These results suggested that Cx46 and Cx50 lack redundancy and demonstrated the possibility that Cx50, specifically, was involved in regulation of lens growth. Previous experiments showed the MAPK signaling pathway differentially regulated Cx50 and Cx46 by altering Cx50 junctional conductance with no effect on Cx46 mediated coupling. This supports the hypothesis that Cx50, but not Cx46, is interacting with growth signaling pathways and that this signaling regulates Cx50 gap junctions specifically in a manner that modulates their activity.

This dissertation addresses the effects of PI3K signaling on gap junctional conductance produced by Cx50 and Cx46. Connexin expressing HeLa cells were incubated with inhibitors of either the PI3K catalytic subunit, p110α, or its downstream effector, Akt. There was a three-fold decrease in Cx50-mediated gap junctional conductance when treated with either inhibitor. Gap junctional conductance of Cx46 transfected cells were unaffected by inhibitor treatment. Cx50 expressing oocytes showed increased junctional coupling when co-expressed with a constitutively active PI3K subunit and had no effect on Cx46 mediated coupling. Single channel currents of Cx50 expressing HeLa cells were measured after inhibitor treatment. Neither inhibitor had any effect on the unitary conductance. These results suggest that PI3K signaling regulates Cx50, specifically, by a mechanism other than altering its unitary conductance.

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List of Figures/Tables/Illustrations

List of Abbreviations

Akt	Protein kinase B (PKB)
Akti	Akt Inhibitor VIII
ATP	Adenosine tri-phosphate
BMP	Bone Morphogenetic Protein
BSA	Bovine serum albumin
Ca^{2+}	Calcium
$CaCl_2$	Calcium chloride
caPI3K	Constitutively active p110α mutant ($p110\alpha^{H1047R}$)
CK1	Casein Kinase 1
cDNA	Complementary deoxyribonucleic acid
cRNA	Complementary ribonucleic acid
C-terminal	Carboxy terminus
Cl	Chloride
Cx	Connexin
Cx26	Connexin26
Cx38	Connexin38
Cx43	Connexin43
Cx46	Connexin46
Cx50	Connexin50
DF	Differentiated fiber cells
DMSO	Dimethyl sulfoxide
E1	Extracellular loop 1
E2	Extracellular loop 2

EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethylene glycol tetra acetic acid
ERK	Extracellular Regulated Kinase
FGF	Fibroblast Growth Factor
FGFR	Fibroblast Growth Factor Receptor
Ga	Gauge
GFP	Green fluorescent protein
G_j	Gap junctional conductance
G_{jss}	Steady state junctional conductance
G_{jmax}	Maximum junctional conductance
G_{jmin}	Minimum junctional conductance
H_2O	Dihydrogen Oxide
HeLa	Immortalized human epithelial cell line
HEPES	4-(2-Hydroxyethyl) piperazine-1-ethanesulfonic acid
HRP	Horseradish Peroxidase
I_j	Junctional 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
LC-MS/MS	Liquid chromatography-tandem mass spectrometry

MB	Modified Barth's Medium
MAPK	Mitogen Activated Protein Kinase
MEK	Mitogen Activated Protein Kinase Kinase
MEK1(E)	Constitutively active Mitogen Activated Protein Kinase
MF	Mature fiber cells
$MgCl_2$	Magnesium chloride
mm	Millimeter
mM	Millimolar
mV	Millivolt
MΩ	Megaohm
nA	Nano-amperes
Na^+	Sodium
NaOH	Sodium hydroxide
nM	Nanomolar
nS	Nanosiemens
p110α	Catalytic subunit of PI3K
$p110\alpha^{H1047R}$	Constitutively active p110α mutant
p-Akt	Phosphorylated Akt (activated)
PBS	Phosphate buffered saline
PCR	Polymerase Chain reaction
PHTS	PTEN hamartoma tumor syndrome
PI3K	Phosphoinositide-3 Kinase
PIK-75	Inhibitor of catalytic subunit of PI3K, p110α
PIP_3	Phosphatidylinositol-3,4,5 triphosphate
PKA	Protein Kinase A

PKC	Protein Kinase C
pS	Picosiemens
PTEN	Phosphatase and tensin homolog
RNA	Ribonucleic acid
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
T-Akt	Total Akt
TBS	Tris buffered saline
UO126	MEK1/2 Inhibitor
V_j	Junctional voltage
V_m	Membrane voltage
μg	Microgram
μM	Micromolar

Chapter I

Introduction

Gap junction formation and function

Gap junctions are an essential component of intercellular communication required by multicellular organisms for proper growth and development (Bennett). Gap junctions are intercellular channels that permit the passage of ions, metabolites, and small signaling molecules between adjacent cells to allow for rapid communication without secretion into the extracellular space (Bennett and Goodenough , Bruzzone, White et al. 1996). Gap junction channels are composed of connexin proteins. Connexins are translated as monomeric proteins in the endoplasmic reticulum, oligomerize into a hexameric complex in the Golgi apparatus, and are transported and inserted into the plasma membrane (Harris , Evans and Martin). These hexameric complexes, called connexons, can dock with connexons of adjacent cells to form an intercellular channel (Figure I-1) (Bruzzone, White et al. 1996, Fleishman, Unger et al. 2004). Connexins contain four transmembrane domains with cytoplasmic amino and carboxy termini (Purnick, Benjamin et al. 2000, Willecke, Eiberger et al. 2002).The extracellular loops (E1 and E2) are believed to be involved in connexon docking, while the cytoplasmic termini and intracellular loop contain sequences for post translational modifications (Figure I-2)(Bukauskas, Elfgang et al. 1995, Bruzzone, White et al. 1996, Foote, Zhou et al. 1998, Willecke, Eiberger et al. 2002).

There are over 20 members in the connexin gene family, each having tissue specific expression (Willecke, Eiberger et al. 2002). Connexon complexes can be formed by a single isoform or multiple isoforms creating either a homomeric or hetermeric connexon respectively. Based on the composition of the connexons, gap junction channels can also be classified as homotypic, the connexons have the same isoforms, or heterotypic (Figure I-3). Studies have shown that the protein composition of gap junctions influence the activity and permeability of the channel

(Veenstra , Goldberg, Valiunas et al. 2004). In addition, alterations in channel properties by mutations or deletions of connexin genes can cause cellular disorganization and possibly lead to disease (White, Goodenough et al. 1998, Willecke, Eiberger et al. 2002, Gerido and White).

Lens structure and development

The lens is one of the largest organs in the body that lacks vascular circulation; therefore, it greatly relies on intercellular channels for development, homeostasis, and maintenance of transparency (Mathias, Kistler et al. 2007, DeRosa, Mese et al. 2009). During the neurulation stages of vertebrate development, specified cells surrounding the neural plate form the lens placode. The placodal cells migrate to form a hollow ball of epithelial cells called a lens vesicle, surrounded by a capsule (Figure I-4). Cells from the posterior pole elongate to fill the lumen and form the primary fiber cells. These cells begin to degrade light scattering organelles and synthesize large amounts of crystallin proteins (Piatigorsky , Donaldson, Kistler et al. 2001). The epithelial cells at the anterior pole proliferate and migrate toward the equator, where they elongate and differentiate into secondary fiber cells. These cells enclose the primary fiber cells from anterior pole to posterior pole. Throughout the lifetime of an organism, this process continues, creating the three cell types (McAvoy, Chamberlain et al. 1999). A single layer of epithelial cells is maintained at the anterior portion of the lens. The epithelial cells migrate at the equatorial region, elongate, and differentiate into differentiating fiber cells (DF) (Harding, Reddan et al. 1971, McAvoy , Beyer, Ebihara et al. 2013). The differentiating fiber cells continue to undergo the process of organelle degradation and crystallin accumulation, migrate to the core to develop into mature fiber cells (MF) (Bassnett).

Important areas of the eye that support the lens are the aqueous and vitreous humors. The aqueous humor fills the space between the cornea and the lens. The vitreous humor fills the space between the lens and the retina. These fluid filled cavities are comprised of water, oxygen, carbon dioxide, ions, carbohydrates, and proteins especially growth factors (Goel, Picciani et al. 2010). Previous studies demonstrated that lens epithelial cells withdraw from the cell cycle to differentiate into secondary fiber cells in response to factors in the vitreous humor (Wang, Stump et al. 2009).

Maintenance of lens homeostasis

Due to the absence of a vascular system, the lens relies heavily on gap junction channels for cell-to-cell communication during growth and differentiation (Goodenough , Mathias, White et al. 2010, Berthoud, Minogue et al. 2014). Gap junctions are essential for direct transport of ions, metabolites, and small signaling molecules throughout the lens (Goodenough, Dick et al. 1980, Donaldson, Kistler et al. 2001). Development and differentiation of the lens requires intracellular responses to signaling molecules that are present in the vitreous humor, such as FGF and BMP (Wang, McAvoy et al. 2010). Therefore, intercellular communication in the lens through gap junction channels is necessary for signaling to propagate to the inner cells at the core that are not directly exposed to the vitreous humor (Dahm, van Marle et al. 2011).

One major function of the lens is to maintain transparency so it can focus light on the retina. Therefore, blood vessels would scatter light, interfering with this ability. It has been hypothesized that the lens has evolved an organized circulatory system through intercellular channels. The transport of ions such as Na^+, K^+ and Cl^- is essential for lens homeostasis by creating a circulating current, with sodium as the primary current-producing ion (Mathias, Rae et al. 1997). The lens

circulation model states that a current, produced by Na^+, enters the lens from both the anterior and posterior poles and exits at the equator (Figure I-6). Sodium is withdrawn from the extracellular matrix across plasma membranes into intracellular spaces of fiber cells by an electrochemical gradient produced by Na^+/K^+ pumps. The outward current is presumably produced by Na^+/K^+ - ATPase activity that is concentrated at the equator of the lens that draws Na^+ from the inner cells, which is then transported out of the lens (Figure I-5) (Candia and Zamudio , Tamiya, Dean et al. 2003, Gao, Sun et al. 2004). The circulation generated by Na^+ current allows Ca^{2+} to flow through the lens as well (Donaldson, Kistler et al. 2001, Gao, Sun et al. 2004).

Exclusive functionality of lens connexins

There are more than 20 connexin isoforms, to which there are only three known to be present in the lens, each with distinct expression patterns: Connexin43 (Cx43) is expressed in the lens epithelium (Beyer, Kistler et al. 1989), Connexin46 (Cx46) is present in the differentiating and mature fiber cell types (Paul, Ebihara et al. 1991), and Connexin50 (Cx50) is expressed in all three cell types (Figure I-6) (White, Bruzzone et al. 1992, Dahm, van Marle et al. 1999, Rong, Wang et al. 2002).

Gap junctions are responsible for the circulation of the ions and small signaling molecules between the aqueous and vitreous humors to all cells of the lens to maintain homeostasis (Goodenough, Dick et al. 1980, Donaldson, Kistler et al. 2001). Gap junctions are especially important in maintaining lens transparency and homeostasis. Studies have shown that Cx46 and Cx50 are essential for proper development and clarity of the lens but presumed to serve separate functions (Martinez-Wittinghan, Sellitto et al. 2003). Cx46 and Cx50 have distinct, non-redundant

functions and diverse protein-protein interactions (White 2003). For example, Cx46 was shown to aggregate in lipid rafts by interacting with caveolin-1 whereas Cx50 does not (Schubert, Schubert et al. 2002).

There are an increasing number of studies that have shown mutations of connexin proteins can lead to physiological diseases. Connexin mutations result in a wide variety of pathologies including skin diseases, deafness, and cataracts (White and Bruzzone , Gong, Cheng et al. 2007, Mhaske, Levit et al. 2013, Levit, Sellitto et al. 2015). At least 20 mutations were identified in both Cx46 and Cx50 that lead to familial cataracts. Figure I-7 shows the topology of the human lens connexins Cx46 (A) and Cx50 (B). The diagram displays the amino acid residues with missense and frame shift mutations commonly found in humans with familial cataracts (Beyer, Ebihara et al. 2013).

Previous studies have shown that deletion or alterations of distinct connexins have various effects on the growth and differentiation of the developing mouse lens. Cx46 knockout lenses develop nuclear cataracts without affecting lens size or eye growth (Gong, Li et al. 1997). However, targeted deletion of Cx50 results in nuclear cataracts and a significant ocular growth defect (White, Goodenough et al. 1998). Figure I-8 displays the functional replacement of the Cx50 gene with Cx46 (Cx50KI46) restores transparency of the lens by expressing a quantity of connexin channels similar to that of wild-type. This supports the idea that Cx46 and Cx50 work similarly in the maintenance of transparency. However, the genetic substitution of the Cx50 coding region with the Cx46 gene did not rescue the ocular growth defect shown in Cx50 knockouts alone (Sellitto, Li et al. 2004). Based on these results, the involvement of Cx50 in ocular growth is a unique function of Cx50 and cannot be compensated by simple genetic replacement

with Cx46. Therefore, the possibility arises that Cx50 may interact with growth signaling pathways in a manner in which Cx46 cannot.

Connexins and cell signaling

An increasing number of studies have shown that activation of signaling pathways has the ability to alter intercellular communication by changing gap junction structure and function (Lampe and Lau , Solan and Lampe). In addition to channel composition, post-translational modifications of connexins resulting from signal transduction have the ability to regulate gap junction activity (Solan and Lampe , Wang, Han et al. 2013). Since the discovery of Cx43 being a phosphoprotein, many studies have focused on the c-terminal tail for consensus sequences to predict possible protein-protein interactions (Figure I-9) (Musil, Beyer et al. 1990). In addition to phosphorylation, connexins can get glycosylated, truncated, and ubiquitinylated in response to signal transduction (Cooper, Solan et al. 2000). Connexin phosphorylation has been thought to regulate gap junctional protein trafficking, gap junction assembly, channel gating, and turnover (Laird, Puranam et al. 1991, Herve, Derangeon et al. 2012)

A well-documented example of a connexin protein shown to interact with signaling pathways that alter its regulation is Cx43 (Buo and Stains). It was shown that PKC had the ability to phosphorylate Cx43 at S368 which decreased gap junctional conductance and permeability by altering selectivity of the channel *in vitro* (Lampe, TenBroek et al. 2000, Solan and Lampe , Ek-Vitorin, King et al. 2006). The increase in Cx43 phosphorylation and decrease in conductance was also seen in lens cells after PKC-gamma activation (Lin, Harris et al. 2007). The inverse effect was demonstrated after Cx43 phosphorylation by Akt, which in turn, increased gap junctional

conductance by stabilizing the complex on the plasma membrane (Dunn, Su et al. 2012). The other lens connexins, Cx46 and Cx50, are also phosphoproteins with multiple consensus sequences for phosphorylation by protein kinases (Shearer, Ens et al. 2008). One study in particular demonstrated PKA phosphorylation does not change the number of Cx50 gap junctions on the plasma membrane, but increased permeability by increasing its open probability (Liu, Ek Vitorin et al. 2011).

Growth factor signaling in the lens

In general, organ development is tightly regulated by a multitude of signaling pathways, lens development is no exception. A number of studies have investigated the regulation of development of lens cells by signaling pathways and proven that growth factor signaling plays a crucial role (Lovicu and McAvoy , Robinson , Martinez, Wijesinghe et al. 2009). One example that has been extensively studied in the lens is fibroblast growth factor (FGF) signaling.

FGF signaling is an essential growth factor signaling pathway necessary for proper embryonic development of multicellular organisms. The role of FGF during organ development is being extensively studied, especially its function in the lens. FGF is a factor found in the vitreous humor that has been shown to induce lens epithelial cell migration and differentiation. Both in vitro and in vivo experiment confirm FGF activates both the MAPK and PI3K signaling pathways in the lens (Boswell, Overbeek et al. 2008, Wang, Stump et al. 2009, Goetz and Mohammadi). Fiber cell specific crystallin proteins are synthesized in response to ERK and Akt activation (Wang, McAvoy et al. 2010). These results suggest an important function for both ERK1/2 and PI3K/Akt signaling pathways in lens cell proliferation and fiber cell differentiation (Iyengar, Patkunanathan et al. 2009, Wang, Stump et al. 2009).

The role of FGF in the lens has been further elucidated by loss of function experiments. Robinson et al. 1995 generated transgenic mice with lens cells expressing a truncated FGF receptor that were unable to activate downstream signaling. These mice with mutant FGFR developed a range of phenotypes, from cataracts to severe microphthalmia, proving FGF signaling was required for normal lens formation (Robinson, MacMillan-Crow et al. 1995). Furthermore, proper activation of these pathways has been shown to be necessary for lens epithelial cell proliferation and fiber cell differentiation (Weber and Menko , Wang, McAvoy et al. 2010).

FGF activates the PI3K signaling pathway, and this activation was shown to be required for lens fiber cell differentiation (Le , Weber and Menko). In addition to FGF signaling, ILK activation of PI3K/Akt pathway has recently been shown necessary for complete activation of signaling cascades downstream of the FGF receptor in lens epithelium and fiber cells during development (Teo, McQueen-Miscamble et al. 2014).

One study proved the lens requires a fully functioning Ras protein, an activator of the mitogen-activated protein kinase (MAPK) signaling pathway, for proper lens development (Xie, Overbeek et al. 2006). This is of particular interest because the MAPK signaling pathway is a proliferative signaling pathway. A recent study showed that manipulation of the MAPK signaling pathway differentially regulated Cx50 and Cx46, essentially effecting Cx50 mediated coupling with no effect on Cx46 junctional conductance (Shakespeare, Sellitto et al. 2009).

PI3K signaling pathway

Lipids at the plasma membrane can be phosphorylated as a signaling mechanism, recruiting and activating downstream effector proteins to the membrane. Phosphoinositide 3-kinase (PI3K) catalyzes the production of phosphatidylinositol-3, 4, 5-trisphosphate. The PI3K signaling pathway has been implicated in many human diseases including diabetes and cancer, promoting research for understanding the pathway for possible disease treatments (Cantley).

The PI3K signaling pathway has become of great interest in studying lens development and homeostasis. As previously stated, this proliferative signaling pathway is stimulated by growth receptor activation, leading to lipid phosphorylation at the plasma membrane. This phosphorylation site recruits proteins, such as Akt, to the plasma membrane which in turn become phosphorylated and activated (Figure I-11). Interestingly, one study revealed Cx43 was a substrate for Akt phosphorylation in vitro (Park, Wallick et al. 2007). Later the same research group discovered phosphorylation of Cx43 by Akt is a necessary component for Cx43 gap junctional stability in two kidney cell lines (Park, Wallick et al. 2007, Dunn, Su et al. 2012).

The antagonist to PI3K is the phosphatase and tensin homolog (PTEN). Mutations in the PTEN gene cause PTEN hamartoma tumor syndrome (PHTS) One human pathology for this disorder is cataract formation. Mice with lens specific PTEN deletion formed cataracts, as well as developed other phenotypes such as increased hydrostatic pressure, increased intracellular sodium concentration, and decreased Na^+/K^+ ATPase activity (Sellitto, Li et al. 2013). This study concluded that the lens requires a functioning PTEN to maintain organ homeostasis and transparency (Sellitto, Li et al. 2013).

Here, we investigated the effects on gap junctional conductance produced by Cx50 or Cx46 when inhibiting or activating the PI3K signaling pathway. Based on the effects of MAPK signaling

on these lens connexins, it is postulated that PI3K signaling will also differentially regulate Cx50 and Cx46. These results suggest a mechanism of differential regulation of these two lens connexins by the PI3K signaling pathway. This dissertation addresses the interaction between PI3K and the lens connexins Cx50 and Cx46.

Figure I-1. Gap junction formation. Connexin mRNA is translated in the endoplasmic reticulum, monomeric connexin proteins oligomerize into a hexameric complex in the Golgi apparatus, and are transported and inserted into the plasma membrane. Connexons can then dock with connexons of neighboring cells to form a gap junction channel.

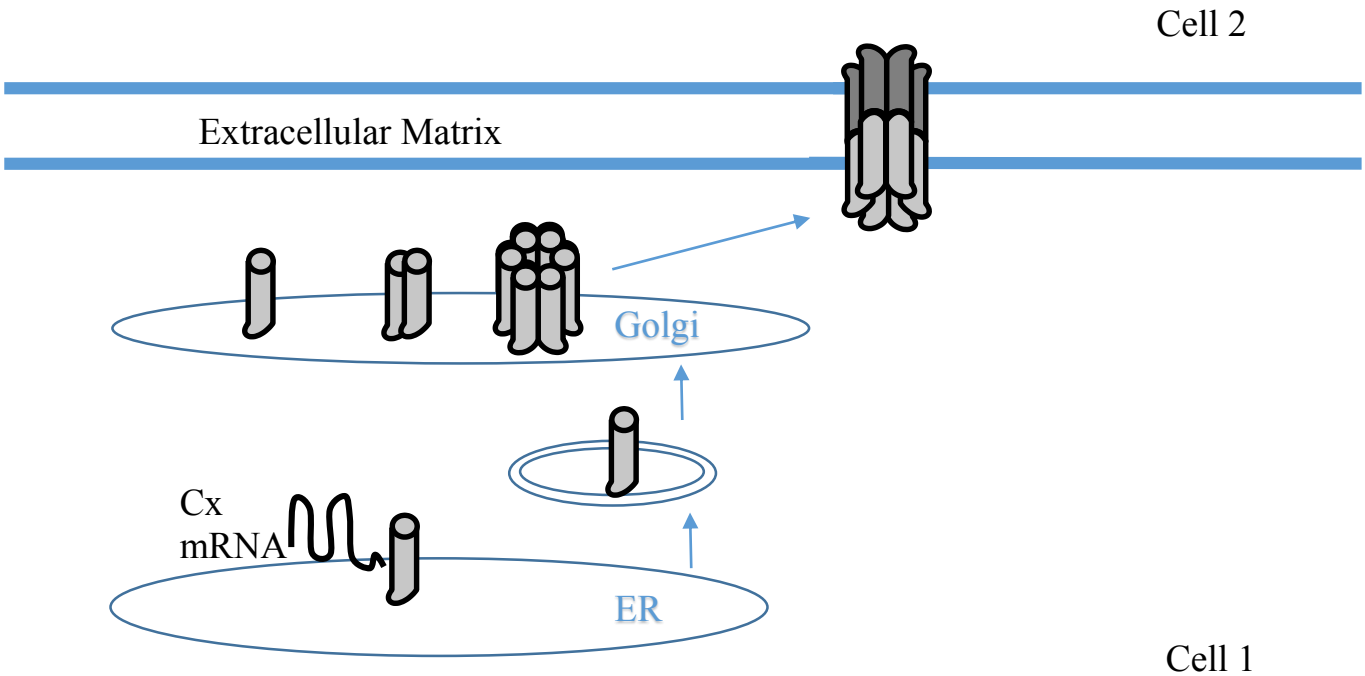

Cell 2
Extracellular Matrix
Golgi
Cx
mRNA
ER
Cell 1

Figure I-2. 2D structure and sequence of Cx50 protein. Diagram of the mouse Cx50 protein amino acid sequence. The protein contains four transmembrane domains, with cytoplasmic N and C termini. Extracellular loops, E1 and E2, are located in the extracellular matrix. (Illustration from A. DeRosa).

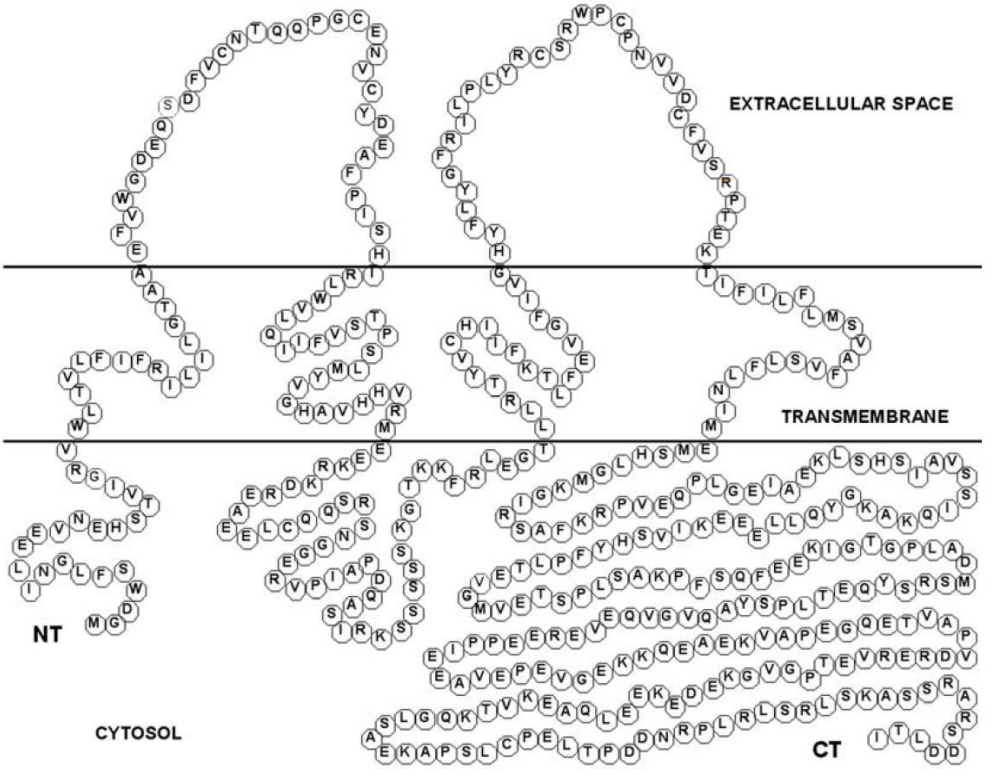

EXTRACELLULAR SPACE
TRANSMEMBRANE
NT
CYTOSOL
CT

Figure I-3. Structure and variations of gap junction channels. Physiological characteristics such as permeability and selectivity of gap junctions vary based on protein composition. Various combinations of isoforms in connexon or gap junction complexes can form numerous types of intercellular channels such as homotypic, heterotypic, or heteromeric gap junctions. (Evans and Martin 2002)

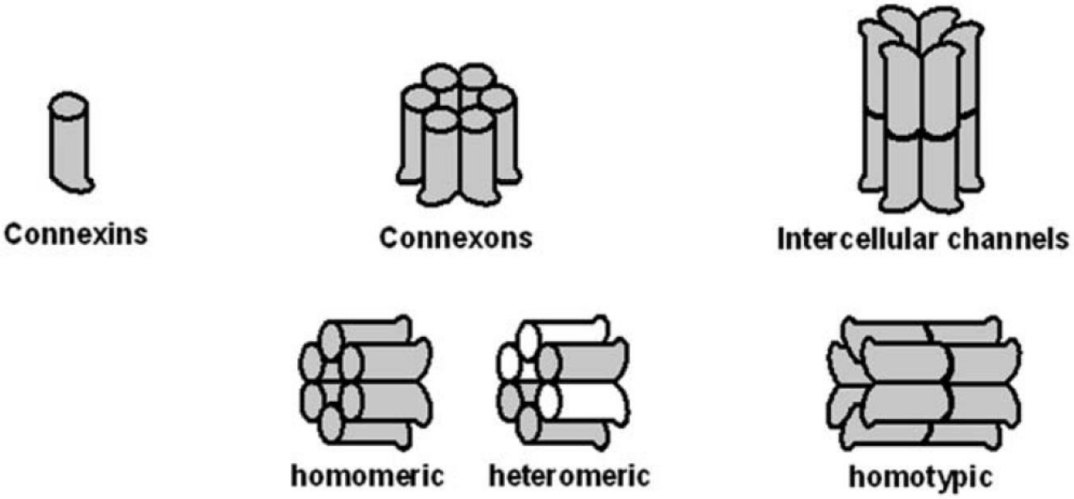

Connexins
Connexons
Intercellular channels
homomeric
heteromeric
homotypic
cytoplasm of cell A
cAMP
3.5 nm
20 nm
cytoplasm of cell B
Ca2+
heteromeric
heterotypic

Figure I-4. Embryonic lens development. Schematic of the developmental stages of the vertebrate lens. Development begins as a hollow all of epithelial cells surrounded by a lens capsule. The posterior cells elongate to fill the lumen. These cells fully differentiate into primary fiber cells by accumulating high levels of crystallin proteins, as well as undergo organelle degradation. The cells of the anterior epithelium proliferate and migrate toward the equator, where they elongate and differentiate into secondary fiber cells. These cells enclose the primary fiber cells from the anterior to the posterior poles. This process continues throughout the lifetime of an organism.

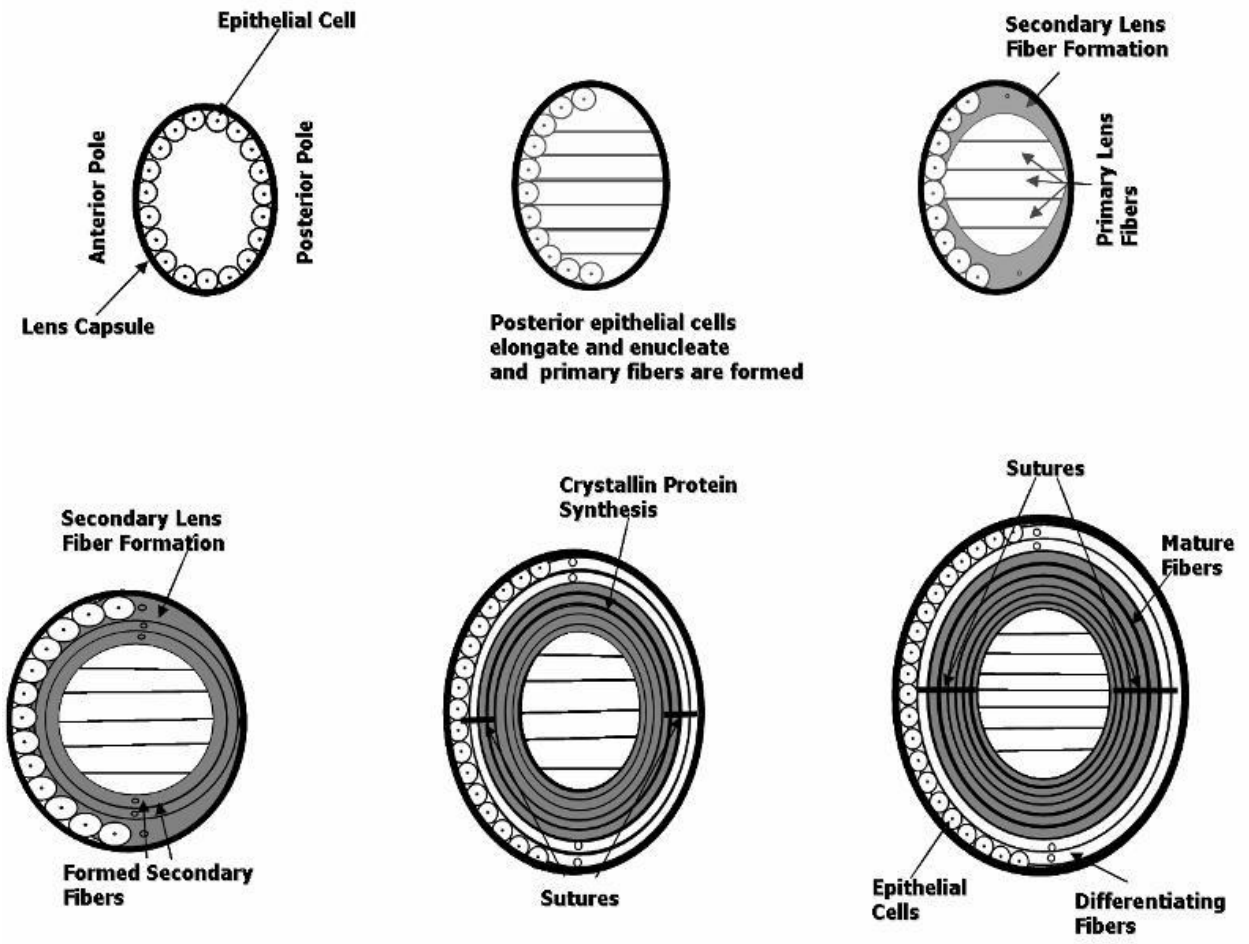

Epithelial Cell
Anterior Pole
Posterior Pole
Lens Capsule
Posterior epithelial cells
elongate and enucleate
and primary fibers are formed
Secondary Lens
Fiber Formation
Primary Lens
Fibers
Secondary Lens
Fiber Formation
Formed Secondary
Fibers
Crystallin Protein
Synthesis
Sutures
Sutures
Mature
Fibers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

Figure I-5. Diagram of an ocular lens and the lens circulation model. (A) Cross-section of a lens displaying the anterior (Calera, Topley et al.) to posterior (bottom) poles. The anterior surface is covered by a single layer of epithelial cells that express Cx43 and Cx50 (light gray). During development these cells migrate toward the equator, elongate, and differentiate into Cx46 and Cx50 expressing differentiating fiber cells (white). The mature fiber cells are found at the core of the lens that express Cx46 and Cx50 (dark gray). In the lens circulation model, the current flows in the direction indicated by the arrows. (B) A schematic of the lens circulation model. In this model, Na+ is the main ion in the circulating current, which enters the lens along the extracellular spaces between cells at the anterior and posterior poles, then moves down its electrochemical gradient across membranes into fiber cells. Gap junctions are thought to be the main ion transporter that returns the ions back to the surface to complete the circuit. The outward current is presumably produced by Na/K-ATPase activity that is concentrated at the equator of the lens that draws Na+ from the inner cells through gap junctions and then transported out of the lens (Mathias, Rae et al. 1997, Shakespeare, Sellitto et al. 2009).

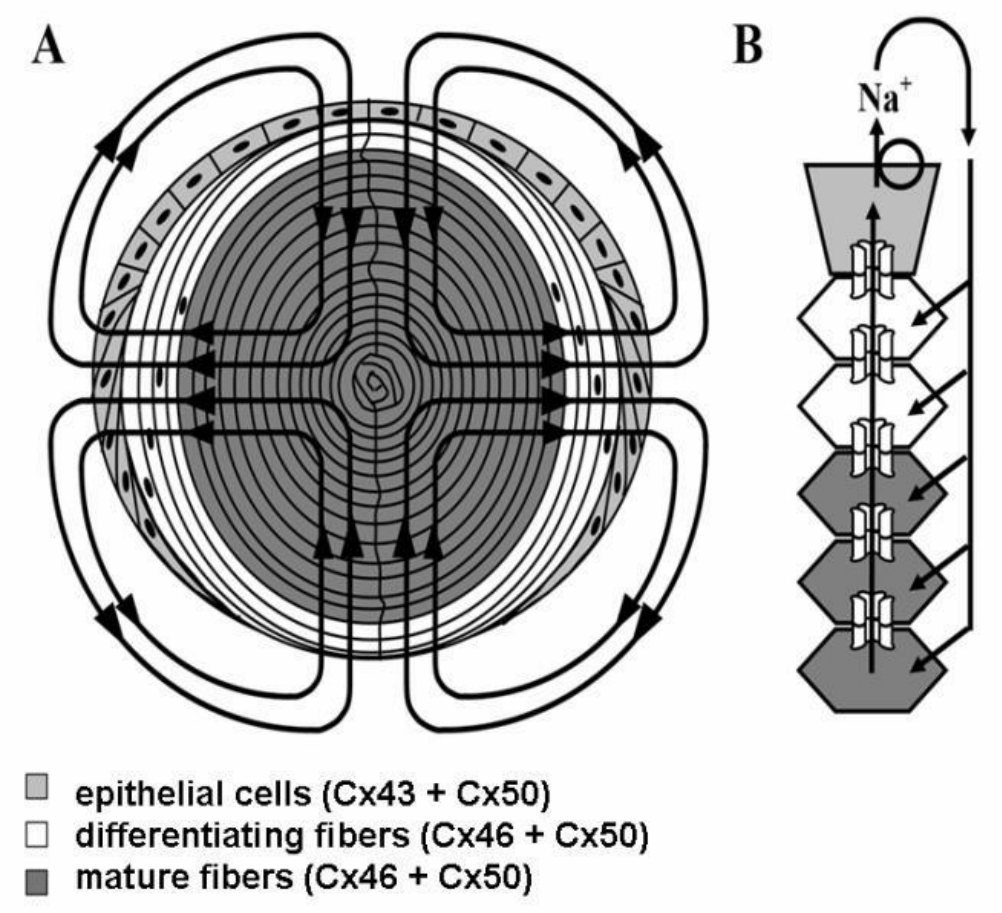

A
B
Na+
epithelial cells (Cx43 + Cx50)
differentiating fibers (Cx46 + Cx50)
mature fibers (Cx46 + Cx50)

Figure I-6. Anatomy of the eye and lens structure. (A) Anatomy of the eye highlighting the lens that is surrounded by the aqueous and vitreous humors. (B) Figure of the lens showing the distribution of connexin isoforms. Cells from the anterior epithelial cell layer express Cx43 and Cx50, differentiating and mature fiber cells express Cx46, and Cx50. (Beyer, Ebihara et al. 2013)

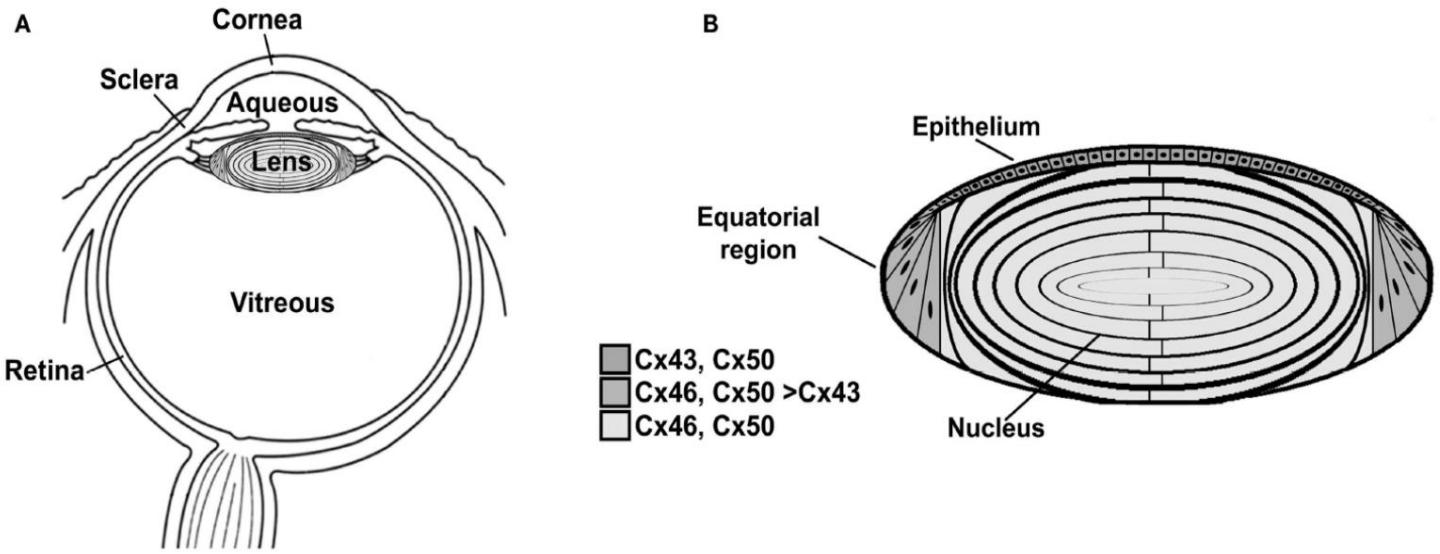

A
Cornea
Sclera
Aqueous
Lens
Vitreous
Retina
B
Epithelium
Equatorial region
Cx43, Cx50
Cx46, Cx50 >Cx43
Cx46, Cx50
Nucleus

Figure I-7. Topology of the human lens connexins. Diagram of Cx46 (A) and Cx50 (B) and amino acid residues as a result of missense and frame shift mutations found in humans with familial cataracts**.** (Beyer, Ebihara et al. 2013)

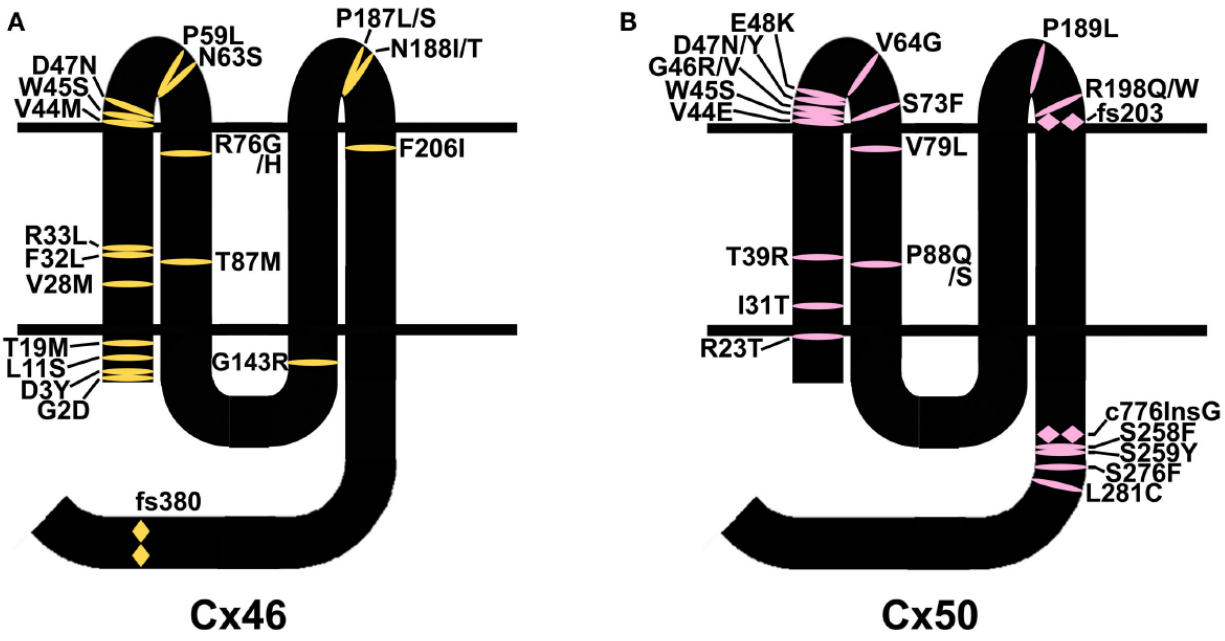

A
P59L
N63S
P187L/S
N188I/T
D47N
W45S
V44M
R76G
/H
F206I
R33L
F32L
V28M
T87M
T19M
L11S
D3Y
G2D
G143R
fs380
Cx46
B
E48K
D47N/Y
G46R/V
W45S
V44E
V64G
S73F
P189L
R198Q/W
fs203
V79L
T39R
P88Q
/S
I31T
R23T
c776InsG
S258F
S259Y
S276F
L281C
Cx50

Figure I-8. Connexin mutants mouse lenses. (A) Wild-type adult lens (B) Cx46 knockout lenses display severe cataract formation. (C) Cx50 knockout lenses develop mild nuclear cataracts with a severe growth defect. (D) Cx50KI46 lenses, replacement of Cx50 with Cx46 rescued lens transparency but not the ocular growth defect. (White)

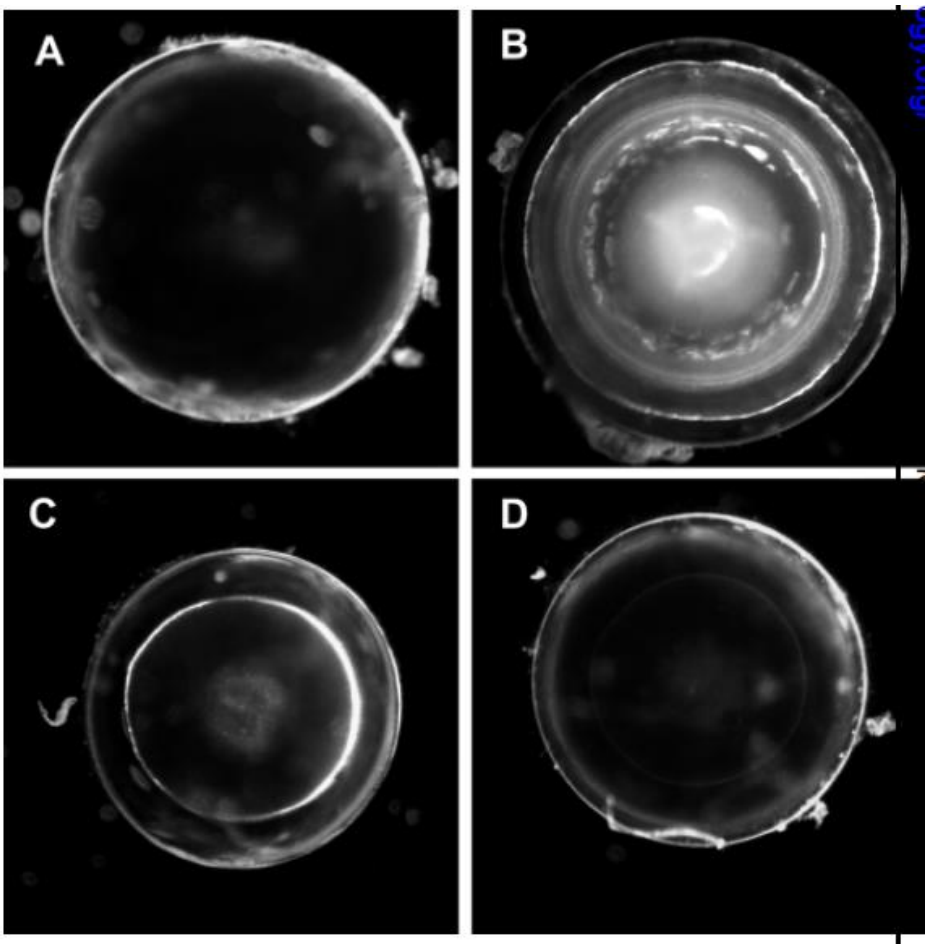

A
B
C
D

Figure I-9. Predicted Cx43 domains. Diagram of the C-terminal tail of Cx43 displaying the predicted protein interactions based on consensus sequences and known protein-protein interactions. (Herve, Derangeon et al. 2012)

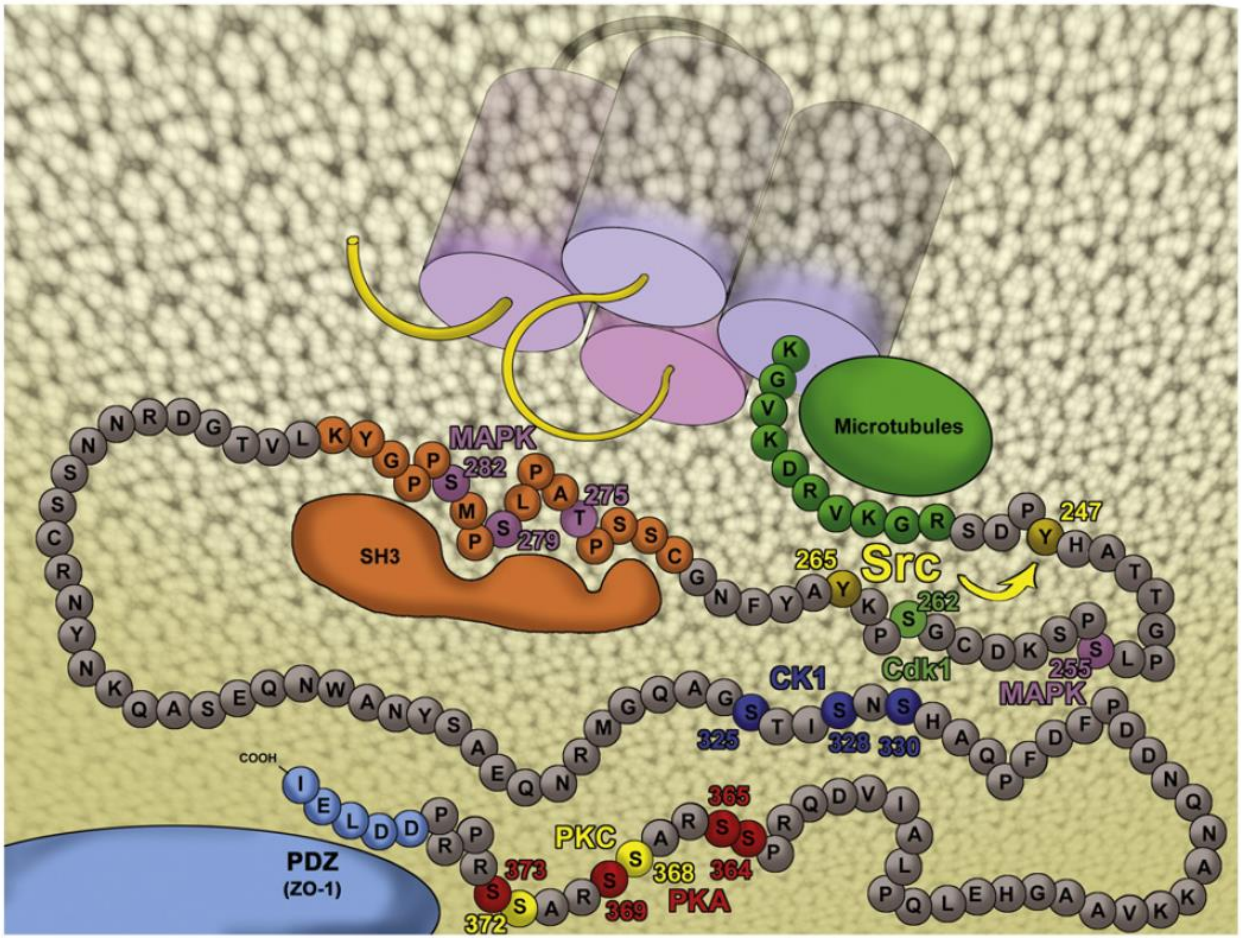
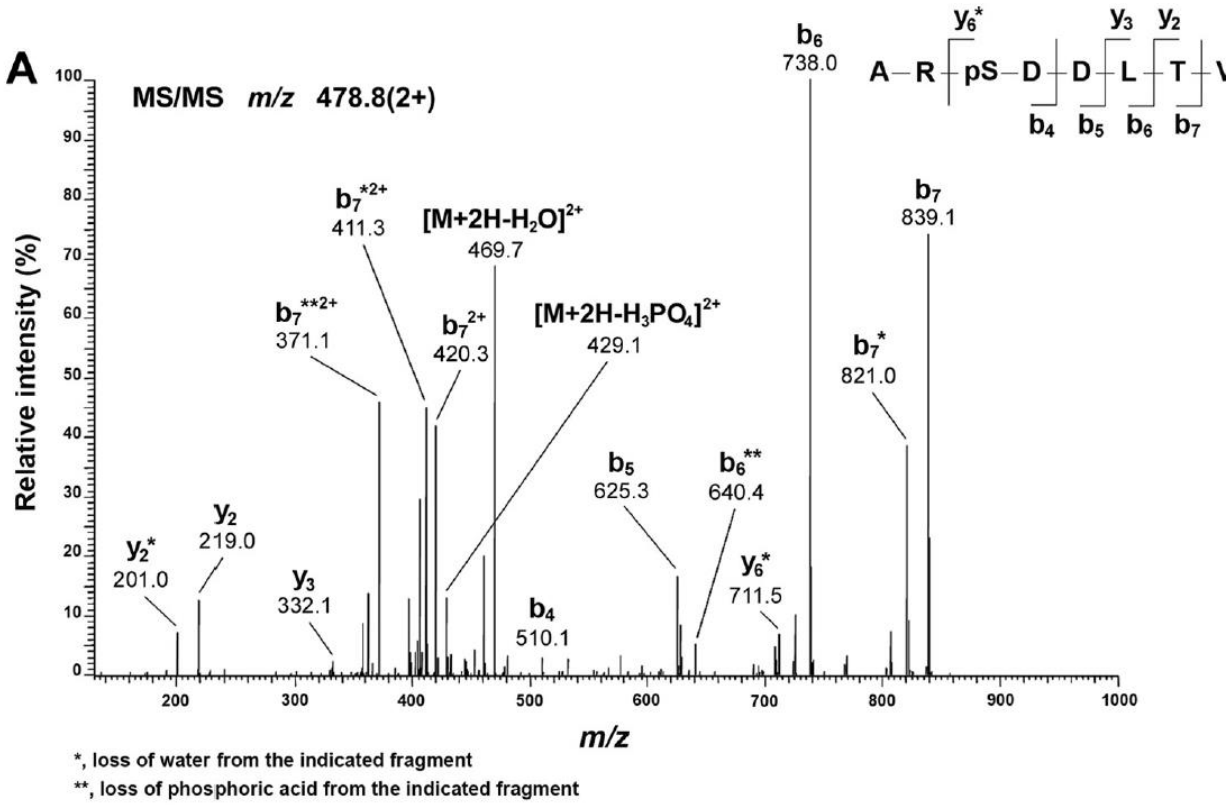

Microtubules
MAPK
282
275
279
SH3
Src
265
247
262
CK1
Cdk1
MAPK
255
325
328
330
COOH
PDZ
(ZO-1)
PKC
365
373
368
364
372
369
PKA

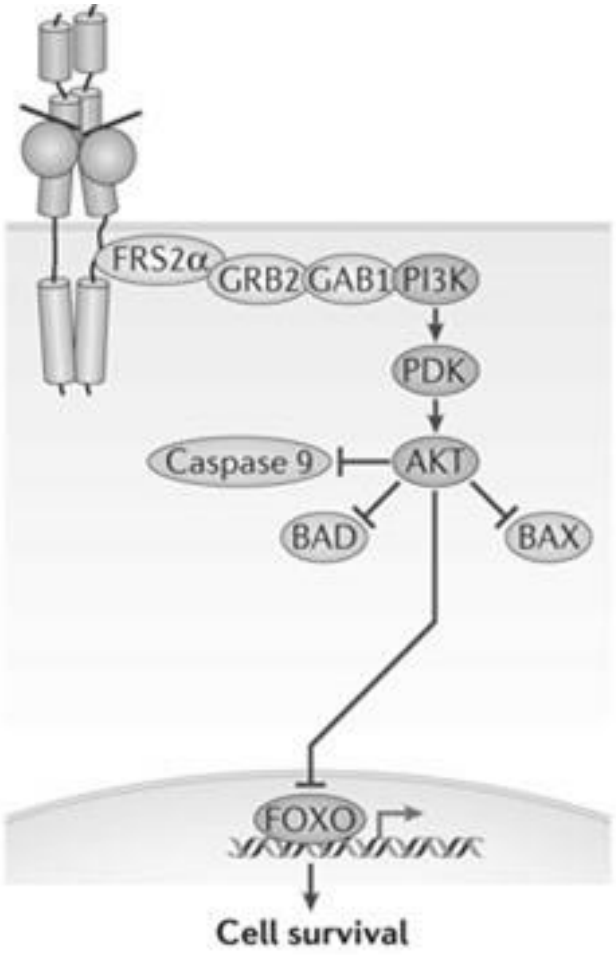
Figure I-10. Lens Cx50 phosphorylation site. Cx50 extracted from embryonic chick lenses were purified for LC-MS/MS. (A) Representative MS/MS spectrum. (B) Species comparison of highly conserved amino acid sequences surrounding the phosphorylation site (Ser-395).



B

	369	395	400
Chicken	GPSAPAELAT-DVRSLSRLSKASSRAR**S**DDLTV-COOH		
Human	TPSLCPELTTDDARPLSRLSKASSRAR**S**DDLTV-COOH		
Rat	APTLCPELTTDDNRPLSRLSKASSRAR**S**DDLTI-COOH		
Mouse	APSLCPELTTDDNRPLSRLSKASSRAR**S**DDLTI-COOH		
Ovine	APSLCAELPGEDTRPLSRLSKASSRAR**S**DDLTV-COOH		
Bovine	APSLCAELPGDDTRPLSRLSKTSSRAR**S**DDLTV-COOH		

Figure I-11. PI3K signaling pathway. Activated FGFR initiates downstream signaling through the PI3K–AKT pathway. The activation of this pathway by FGFs vary depending on cell type. In the lens, it is thought that the PI3K–AKT pathway promotes cell survival by protein-protein interactions such as inhibiting pro-apoptotic proteins BAD, BAX, and caspase-9 (Goetz and Mohammadi).


FRS2α
GRB2
GAB1
PI3K
PDK
Caspase 9
AKT
BAD
BAX
FOXO
Cell survival

Chapter II

Hypothesis and Specific Aims

Specific Aims

Gap junction channels are formed by oligomers of connexin (Cx) proteins. When these intercellular channels are open and active, they connect the cytoplasm of adjacent cells to allow for the passage of ions and small molecules. These channels are essential for proper development and homeostasis of many organs including the lens. The connexins in the lens are: Cx43, found in the epithelial monolayer; Cx46, found in the differentiating fiber and mature fiber cells; and lastly Cx50, which is found in all three cell types. Previous experiments have shown that deletion of Cx50 in the lens of mice results in cataract formation and reduced lens growth. It also has been shown that Cx46 replacement of Cx50 restores normal transparency of the lens but does not correct the ocular growth defect. This proves that Cx50 has a function that cannot be compensated for by simply inserting Cx46 in the Cx50 coding region. This posed the hypothesis that Cx50, specifically, interacts with growth signaling pathways in ways that Cx46 cannot. The purpose of this dissertation is to investigate a growth signaling pathway, the PI3K signaling pathway, and its interaction with Cx46 and Cx50.

Specific Aim 1: To determine if manipulation of the PI3K signaling pathway affects the gap junctional coupling produced by Connexin50 or Connexin46.

a) Gap junctional conductance can be measured in vitro by electrophysiology recording techniques. Dual whole-cell patch clamp measurements will be taken of HeLa cells transfected with Cx50 or Cx46 DNA after incubation with inhibitors of the PI3K signaling pathway.

b) By using the *Xenopus* oocyte expression system, dual whole-cell voltage clamp experiments will be used to record the effects of co-expression of either Cx50 or Cx46

with a constitutively active PI3K, p110α^{H1047R} (caPI3K) in oocyte pairs. These were the very first experiments using caPI3K in the *Xenopus* oocyte expression system.

Specific Aim 2: Analyze possible mechanisms of Cx50 regulation by PI3K signaling.

The PI3K signaling pathway regulates Cx50 gap junctional coupling. When the pathway is activated, Cx50 conductance increases, also inhibition of the pathway leads to a decrease in conductance. There are a few mechanisms that could be responsible for this direct correlation. This section addresses two possibilities: 1) The PI3K signaling pathway regulates Cx50 gene expression and protein synthesis, or 2) PI3K signaling modifies Cx50 gap junctions directly by some mechanism that alters the amount of conductance produced by each channel.

a) **To determine if the PI3K signaling pathway regulates Cx50 protein expression.** Western blot analysis of recorded oocyte pairs co-expressing Cx50 with the constitutively active PI3K will determine whether the effect seen is due to differences in connexin protein expression. An increase in Cx50 protein might account for the increase in gap junctional conductance.

b) **Characterize the effects of PI3K signaling inhibition on Cx50 unitary conductance.**

Single channel current measurements of Cx50 expressing cells while under inhibitor treatment can show if there is a difference in conductance of individual channels due to inhibition of PI3K signaling. Measuring unitary conductance in PIK-75 and Akti treatment conditions will allow detection of microscopic changes of a single channel that may have caused the overall decrease in gap junctional conductance.

Chapter III

Materials and Methods

Inhibitor treatment and protein analysis of HeLa cells

To selectively block PI3K signaling, cells were incubated in either 50 nM PIK-75 or 10 uM Akt inhibitor VIII (Akti). HeLa cells stably expressing Cx50 (Berthoud, Minogue et al. 2003) were grown to 75% confluence then treated with PIK-75, Akti, or DMSO for 24 hours prior to sample prep. Cells were scraped from plate in SDS sample buffer, heated for 5 minutes at 95°C, and separated on 9% SDS-PAGE gel, and transferred to nitrocellulose membranes. Protein blots were probed with mouse monoclonal phospho (p)-Akt or rabbit total (t)-Akt antibodies (Cell Signaling Technology, Danvers, MA.) at 1:1000 dilution, followed by incubation with ECL-mouse secondary antibody or horseradish peroxidase (HRP)-conjugated goat anti-rabbit secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA).

Cx46 transient transfection of HeLa cells

HeLa cells were plated on glass coverslips, grown to 50% confluence and transiently transfected with 4–5 μg of Connexin 46 DNA subcloned into a pIRES2-eGFP vector (Clontech) using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). Gap junctional conductance of Cx46 expressing cells was measured after overnight incubation. Cx50 stably transfected HeLa cells (Berthoud, Minogue et al. 2003), were used for Cx50 cell experiments.

Dual whole-cell patch clamp electrophysiology

Junctional conductance of cell pairs was measured using dual whole-cell patch clamp with Axopatch 1D patch-clamp amplifiers (Axon Instruments) at room temperature. Cells were bathed in a solution containing 137.7 mM NaCl, 5.4 mM KCl, 2.3 mM NaOH, 1 mM MgCl2, 5 mM HEPES, 10 mM glucose, pH 7.4. Patch electrodes with resistances of 3–5 MΩ were filled with

internal solution containing 120 mM aspartic acid, 120 M KOH, 10 mM EGTA, 3 mM NaATP, and 5 mM HEPES, pH 7.2. Macroscopic and single-channel recordings were acquired using pClamp, sampled at 1–2 kHz and filtered at 0.2– 0.5 kHz. Analysis of recordings was performed with pClamp and Origin software (MicroCal Software, Northampton, MA). Each cell of a pair was initially held at a common holding potential of 0 mV. To evaluate junctional coupling, 200-ms hyperpolarizing pulses from the holding potential of 0 to ±20 mV were applied to one cell to establish a transjunctional voltage gradient (V_j), and junctional current was measured in the second cell (held at 0 mV). To selectively block PI3K signaling, cells were incubated in either 50 nM PIK-75 or 10 uM Akt inhibitor VIII (Akti) for 24 hours prior to recordings. For unitary conductance measurements, alternating bipolar V_j pulses ranging from ±10 mV to ±110 mV were applied to low conductance cell pairs that had only one or two active channels. Single channel currents for the three treatment conditions were plotted against voltage and fit by linear regression to determine the slope which was equal to the unitary conductance. The correlation coefficients of all three linear fits were $r^2 \geq 0.99$.

In vitro transcription, oocyte microinjection, and pairing

Cx50, Cx46, and constitutively active p110α-H1047R (caPI3K) (Lu, Jiang et al. 2013) coding sequences were subcloned into pCS2+, linearized with NotI, and transcribed using the SP6 mMessage mMachine (Ambion, Austin, TX). *Xenopus laevis* oocytes were removed from adult females (Nasco, Fort Atkinson, WI), defolliculated by collagenase B and hyaluronidase digestion, and stage V-VI cells were selected and cultured in modified Barth's (MB) medium. Endogenous connexins were suppressed by injection of an antisense oligonucleotide to *Xenopus* Cx38 (10 ng/cell) (Ebihara) using a Nanoject II injector (Drummond, Broomall, PA). Twenty-four to forty-

eight hours prior to recordings, oligo injected cells were subsequently injected with either Cx50, Cx46 cRNA (5 ng/cell), or H_2O as a negative control. Vitelline envelopes were removed, and oocytes were manually paired with vegetal poles apposed in MB medium. Paired cells were injected with caPI3K cRNA 4 hours before electrophysiology recordings. Gap junctional conductance measurements were taken 16-24 hours after pairing.

Dual whole-cell voltage clamp

Gap junctional conductance was measured by dual voltage clamp (Spray, Harris et al. 1981). Current and voltage electrodes (1.2 mm diameter, omega dot; Glass Company of America, Millville, NJ) were pulled to a resistance of 1–2 MΩ with a vertical puller (Narishige, Tokyo, Japan) and filled with 3 M KCl, 10 mM EGTA, and 10 mM HEPES, pH 7.4. Voltage clamp recordings were performed using two GeneClamp 500 amplifiers controlled by pClamp software using a Digidata 1320A interface. (Axon Instruments, Foster City, CA). Both cells of a pair were clamped at -40 mV to eliminate any transjunctional potential. One cell was then subjected to alternating pulses of ±20 mV, while the current produced by the voltage change was measured in the second cell. The current delivered to the second cell was equal in magnitude to the junctional current (I_j), and gap junctional conductance (G_j) was calculated by dividing the measured current by the voltage difference between first cell (V1) and the second cell (V2), $G_j = I_j/(V1-V2)$.

Preparation of oocyte samples and protein analysis

Oocytes were collected in 1 ml of ice-cold lysis buffer containing 5 mM Tris, pH 8.0, 5 mM EDTA, and protease inhibitors and homogenized using a series of mechanical passages through syringe needles of diminishing diameter (20, 22, and 26 Ga). Extracts were first

centrifuged at 2000 x *g* at 4°C for 5 min, then the supernatants were centrifuged at 45,000 x *g* at 4°C for 30 min. Membrane pellets were resuspended in SDS sample buffer (2 µl/oocyte), separated on 10% SDS-PAGE gels, and transferred to nitrocellulose membranes. Protein blots were probed with antibodies specific for the carboxy-terminal tail of Cx46 (rabbit) (Paul, Ebihara et al. 1991 Bruzzone et al. 1992), or the cytoplasmic loop of Cx50 (goat; Santa Cruz Biotechnology, Santa Cruz, CA) at a 1:1000 dilution followed by incubation with horseradish peroxidase-conjugated rabbit anti-goat, or goat anti-rabbit secondary antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA). In addition, blots were probed with mouse monoclonal phospho (p)-Akt or rabbit total (t)-Akt antibodies (Cell Signaling Technology, Danvers, MA.) at 1:1000 dilution, followed by incubation with ECL-mouse secondary antibody or horseradish peroxidase (HRP)-conjugated goat anti-rabbit secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA). Densitometry quantifications were performed using ImageJ (NIH). Band densities of three independent experiments were analyzed and the normalized mean values plotted.

Chapter IV

Gap junctional conductance analysis after manipulation of the PI3K signaling pathway

Abstract

Gap junction channels have the ability to modulate their activity in response to cell signaling pathways. Manipulation of signaling pathways can specifically affect gap junctional conductance (Lovicu and McAvoy , Boswell, Le et al. 2009, Shakespeare, Sellitto et al. 2009). To investigate whether inhibiting PI3K signaling affected gap junctional conductance, HeLa cells stably expressing Cx50 (Berthoud, Minogue et al. 2003), were treated with PIK-75 to inhibit the p110α catalytic subunit of PI3K an inhibitor of Akt (Akti), or the vehicle (DMSO. To confirm efficacy of these inhibitors on stably transfected Cx50 HeLa cells, western blot analysis showed decreased levels of phosphorylated (activated) Akt. The following set of experiments measure the gap junctional conductance produced by Cx50 and Cx46 in response to PI3K signaling. Measurements of gap junctional conductance were recorded between HeLa cell pairs expressing Cx50 treated with an inhibitor of the p110α catalytic subunit of PI3K (PIK-75). Patch clamp measurements of Cx50 expressing cells showed a three-fold decrease in gap junctional conductance following treatment with PIK-75. To identify if this decrease was caused by a reduction in PIP_3 or inhibition of downstream signaling, Akti was used to inhibit the effector of p110α, Akt. Cells demonstrated the same three-fold decrease in gap junctional conductance in response to Akti, confirming the resulting reduction is due to the inhibition of the PI3K signaling pathway. Alternatively, cells expressing Cx46 were unaffected by treatment with either inhibitor.

Next, using the *Xenopus* oocyte expression system, gap junctional conductance was measured by dual whole-cell voltage clamp. These were the very first experiments using caPI3K in the *Xenopus* oocyte expression system. First, a western blot analysis of oocytes injected with caPI3K cRNA to confirm activity of the constitutively active p110α. Oocytes were injected with connexin or the constitutively active p110α, or both cRNA. Cx50 gap junctional conductance

significantly increased when co-expressed with constitutively active p110α compared to Cx50 pairs alone**.** Cx46-mediated coupling was unaffected by the presence of the constitutively active p110α

Results

PIK-75 and Akti decreases Akt phosphorylation in Cx50 expressing HeLa cells

Initial experiments tested the effectiveness of the inhibitors PIK-75 and Akti to prevent PI3K signaling in HeLa cells stably expressing Cx50. After 24 hour treatment with either inhibitor, proteins were separated on SDS-PAGE gels, transferred to nitrocellulose membranes and probed for total Akt (t-Akt) and phosphorylated Akt (p-Akt). Cx50 expressing HeLa cells treated with either inhibitor showed no significant difference in t-Akt levels as compared to DMSO alone (Figure IV-1). Cx50 HeLa cells treated with either PIK-75 or Akti significantly decreased activated Akt levels (p-Akt) when compared to the vehicle treated sample. When compared to the DMSO control, PIK-75 and Akti reduced p-Akt by 91% and 95%, respectively. Therefore, both inhibitors decreased activation of Akt in stably transfected Cx50 HeLa cells, proving to be effective inhibitors for the proposed patch clamp experiments.

Treatment with PIK-75 decreases Cx50 gap junctional conductance

To determine the effects of inhibiting p110α on Cx50 gap junctional conductance, Cx50 HeLa cell pairs were patch clamped with or without PIK-75 treatment. Control Cx50 HeLa cells had high conductance measurements with an average G_j of 22.2 nS. Incubation of Cx50 expressing cells with DMSO (inhibitor vehicle) alone had no effect on coupling. PIK-75 treatment

significantly decreased Cx50 gap junctional conductance by 3-fold to a mean G_j of 6.8 nS ($p < 0.05$, Student's *t* test). Referring back to the PI3K signaling pathway, inhibition of p110α by PIK-75 directly results in a decrease in PIP_3 which, then, indirectly decreases the level of Akt that is recruited to the plasma membrane to be activated. The decrease in gap junctional conductance observed could be a result of reduced interaction between Cx50 gap junctions and PIP_3 in the plasma membrane or an indirect result of inhibited downstream signaling.

Cx50 gap junctional conductance is reduced by Akti treatment

To test if the decrease in conductance was caused by inhibiting PIP_3 directly, or inhibition of the signaling cascade, Cx50 HeLa cells were treated with Akt Inhibitor-VIII (Akti) to block downstream signaling. This allosteric inhibitor specifically blocks Akt phosphorylation, therefore inhibiting activation. Akti treatment showed a similar 3-fold decrease in Cx50-mediated conductance with a mean G_j of 8 nS (Figure IV-2). This 3-fold reduction observed after Akti treatment proved that it is inhibition of the PI3K signaling cascade that decreases Cx50 gap junctional conductance, not just the absence of PIP_3 in the plasma membrane.

Cx46-mediated coupling is unaffected by PIK-75 treatment

Whether or not the effects of inhibiting PI3K signaling on Cx50 gap junctional conductance is specific to Cx50, similar patch clamp experiments were performed on cells expressing Cx46. HeLa cells were transiently transfected with Cx46 DNA three days prior to patch clamp recordings. This DNA plasmid encoded a GFP reporter gene to identify paired cells expressing Cx46 via fluorescence. Incubation of Cx46 expressing cells with PIK-75 for 24 hours had no significant effect on gap junctional conductance with a mean G_j of 10.5 nS, compared to 7.3 nS with vehicle alone, exhibiting a p value > 0.05 (Figure IV-3). Therefore, the inhibition of p110α by PIK-75

specifically decreased Cx50-mediated coupling, but had not effect Cx46 gap junctional conductance.

Injection with caPI3K efficiently up-regulates Akt activation

Prior to testing the effect of constitutive activation of PI3K signaling on Cx50 gap junctional conductance by dual whole-cell voltage clamp, we needed to ensure efficacy of injecting caPI3K cRNA to activating Akt. CaPI3K, (Lu, Jiang et al. 2013), is a constitutively active p110α mutant, $p110\alpha^{H1047R}$. *Xenopus* oocytes were injected with caPI3K at multiple time points. Control cells were injected with H_2O. Western blot analysis confirmed activity of caPI3K by the presence of p-Akt as early as 4 hours post-injection (Figure IV-4). Paired cells were injected with caPI3K cRNA 4 hours prior to voltage clamp recordings for future experiments.

Constitutive activation of PI3K signaling increases Cx50-mediated conductance in *Xenopus* oocytes

To test the effect of constitutive activation of PI3K signaling on Cx50 gap junctional conductance, the Xenopus oocyte expression system was used with dual whole-cell voltage clamp recordings. These experiments were performed by injecting either Cx50 cRNA (5 ng/cell) or H_2O as a negative control into oocytes. Following injection, vitelline envelopes were removed and oocytes were manually paired with vegetal poles apposed in MB medium. Gap junctional conductance measurements were taken 24 hours after pairing. A subset of paired cells were subsequently injected with caPI3K cRNA 4 hours prior to voltage clamp recordings. As expected, control oocyte pairs injected with H_2O or caPI3K cRNA alone had nominal junctional conductance (Figure IV-5). Cell pairs injected with Cx50 cRNA had a mean G_j of 3.0 μS. When cells

expressing Cx50 were subsequently injected with caPI3K, gap junctional conductance significantly increased approximately 4-fold to a mean G_j of 11.8 μS ($p< 0.05$). These data suggest a direct relationship between activation of PI3K signaling and an increase in gap junctional conductance produced by Cx50.

Gap junctional conductance of Cx46 oocyte pairs is not altered by CaPI3K

To determine if the increase in gap junctional conductance by constitutive activation of PI3K signaling is specific to Cx50, voltage clamp recordings were taken of oocytes co-expressing Cx46 and caPI3K. Twenty-four hours prior to voltage clamp recordings, oligo injected cells were subsequently injected with either Cx46 cRNA (5 ng/cell) or H_2O as a negative control. Oocytes were manually paired with vegetal poles apposed in MB medium. Gap junctional conductance measurements were taken 16 hours after pairing. Paired cells were injected with caPI3K cRNA 4 hours prior to recordings. Once again, control cell pairs injected with either H_2O or caPI3K cRNA alone had negligible conductance (Figure IV-6). Unlike Cx50, there was no significant difference in gap junctional conductance between cells co-expressing Cx46 and caPI3K or Cx46 alone, mean G_j values of 18.4 μS and 12.7 μS respectively ($p> 0.05$). These experiments demonstrated that constitutive activation of the PI3K signaling increased junctional conductance mediated by Cx50, but not Cx46.

Discussion

Gap junction channels have the ability to modify their physiological properties as a response to cell signaling. These electrophysiology experiments characterized a direct relationship between Cx50 gap junctional conductance and the PI3K signaling pathway. To confirm efficacy of PIK-75 and Aki on inhibiting Akt activation in stably transfected Cx50 HeLa cells, western blot analysis exhibited decreased levels of phosphorylated (activated) Akt. HeLa cells expressing Cx50 incubated with PIK-75 or Akti showed a three-fold decrease in Cx50 gap junctional conductance. This decrease proved that inhibition of PI3K signaling, either by inhibiting p110α or Akt, leads to a reduction in gap junctional conductance produced by Cx50 *in vitro*. In addition, this reduction was shown to be specific for Cx50 because gap junctional conductance of Cx46 expressing HeLa cells remained unchanged when treated with PIK-75. Therefore, inhibition of the PI3K signaling pathway differentially reduces Cx50 gap junctional conductance without effecting Cx46-mediated coupling.

This chapter introduced the constitutively active $p110\alpha^{H1047R}$ to the *Xenopus* oocyte expression system. Its activity was confirmed by increased levels of p-Akt after caPI3K cRNA injection. Constitutive activation of the PI3K signaling pathway provides additional evidence of differential regulation of Cx50 and Cx46 by the PI3K signaling pathway. Cx50 expressing *Xenopus* oocytes demonstrated a 4-fold increase in gap junctional conductance when co-expressed with caPI3K. In contrast, an effect was absent in oocytes injected with Cx46 cRNA co-expressing the constitutively active PI3K catalytic subunit, producing conductance levels comparable to oocyte pairs expressing Cx46 alone.

There are at least three possible mechanisms of regulation PI3K signaling be influence Cx50 gap junctional conductance. PI3K signaling activation could upregulate Cx50 protein expression, leading to an increase in conductance. Another possible mechanism is a direct modification of Cx50 gap junctions by PI3K signaling that alters the conductance of a single channel. These two possibilities are tested in the next chapter.

Figure IV-1: Western blot of Cx50 stably transfected HeLa cells probed for p-Akt and t-Akt to show PIK-75 and Akti inhibited Akt activation without changing the levels of total Akt. Both PIK-75 and Akti inhibitors decreased Akt phosphorylation with no effect on total Akt. DMSO is used as a vehicle for the inhibitor, which has no effect on either p-Akt or t-Akt.

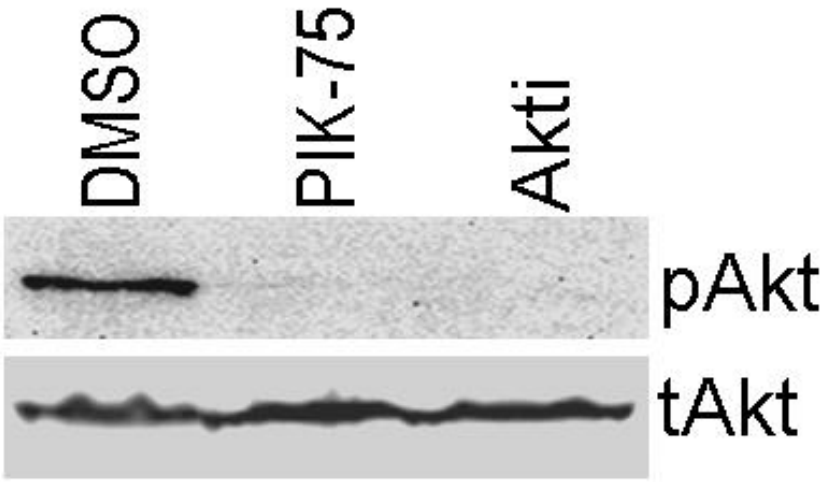

DMSO
PIK-75
Akti
pAkt
tAkt

Figure IV-2. Inhibition of PI3K signaling decreases Cx50 gap junctional conductance. Patch clamp measurements of Cx50 expressing HeLa cell were treated with an inhibitor of the p110α catalytic subunit of PI3K (PIK-75), an inhibitor of Akt (Akti), vehicle (DMSO), or untreated (control). Compared to untreated control, cell pairs expressing Cx50 showed a three-fold decrease in gap junctional conductance after treatment with either PIK-75, or Akti. Treatment with vehicle (DMSO) had no effect.

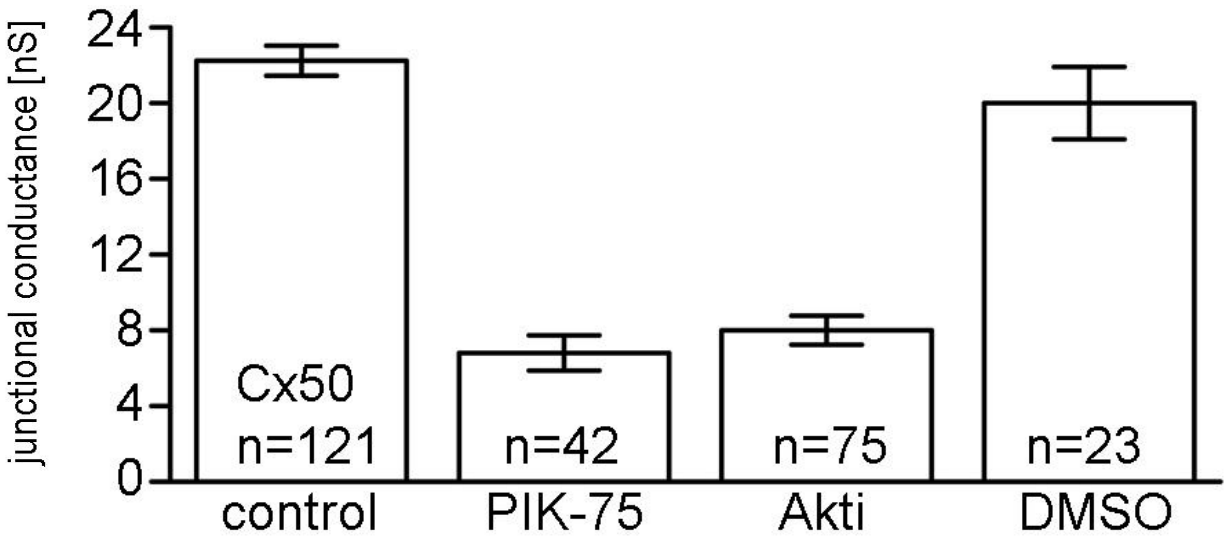

junctional conductance [nS]
24
20
16
12
8
4
0
Cx50
n=121
n=42
n=75
n=23
control
PIK-75
Akti
DMSO

Figure IV-3. **Cx46-mediated coupling is unaffected by PIK-75.** Patch clamp measurements of Cx46 expressing HeLa cell were treated with an inhibitor of the p110α catalytic subunit of PI3K (PIK-75) or vehicle control (DMSO). Compared to untreated control HeLa cells transiently transfected with Cx46, cells incubated with PIK-75 showed no significant difference in gap junctional coupling. Data are the mean ± SE.

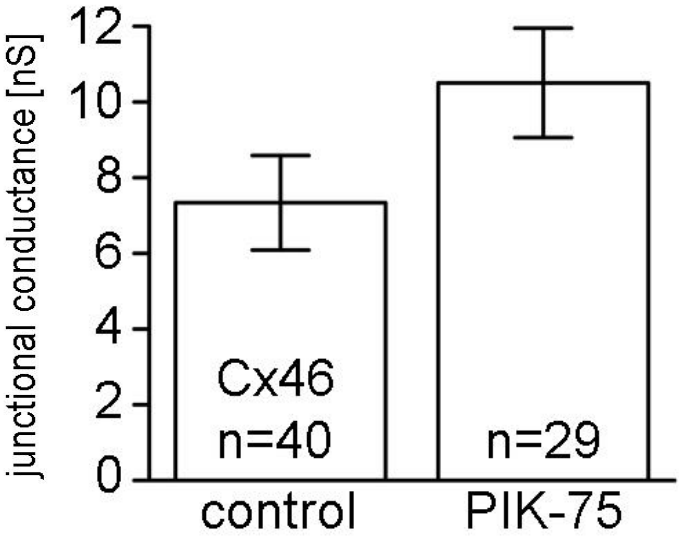

junctional conductance [nS]
12
10
8
6
4
2
0
Cx46
n=40
n=29
control
PIK-75

Figure IV-4. Injection with caPI3K efficiently up-regulates Akt activation. *Xenopus* oocytes were injected with caPI3K at multiple time points. Western blot analysis confirmed activity of caPI3K by the presence of p-Akt 4, 14, and 24 hours post-injection.

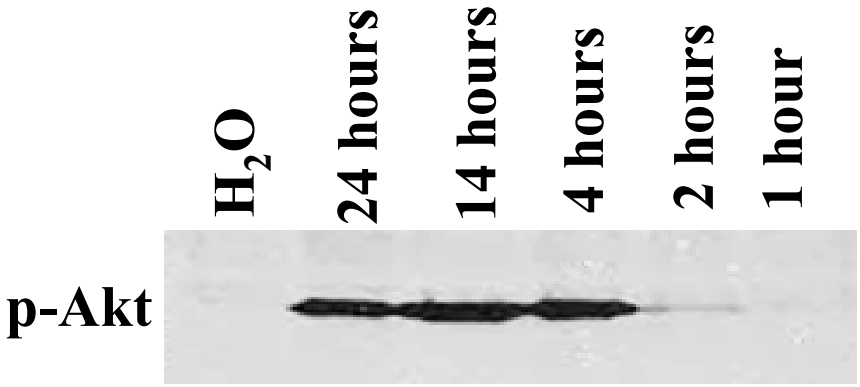

H2O
24 hours
14 hours
4 hours
2 hours
1 hour
p-Akt

Figure IV-5. CaPI3K increases junctional conductance produced by Cx50. PI3K signaling was stimulated by co-expression of the constitutively active catalytic subunit $p110\alpha^{H1047R}$ (caPI3K) in paired *Xenopus* oocytes expressing Cx50. Cells injected with H_2O or caPI3K alone had nominal junctional conductance. Pairs co-expressing Cx50 and caPI3K showed a 4-fold increase in gap junctional conductance compared to cells expressing Cx50 alone. Data are the mean ± SE

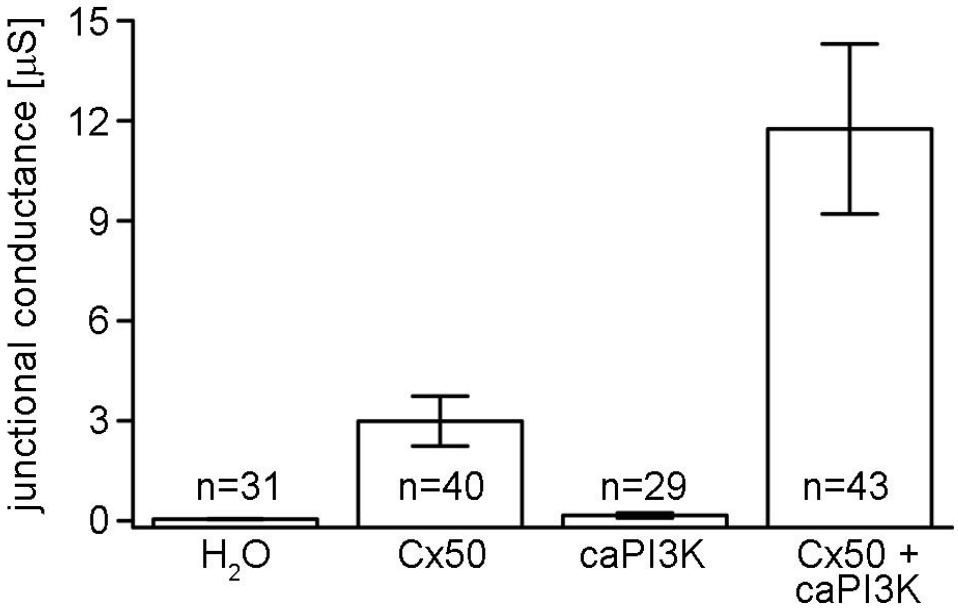
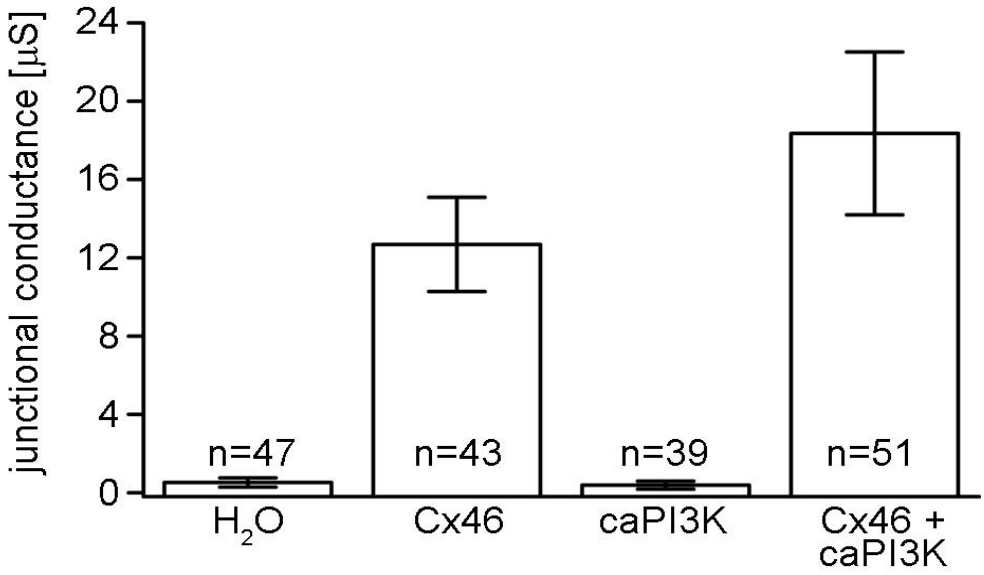

junctional conductance [µS]
15
12
9
6
3
0
n=31
n=40
n=29
n=43
H2O
Cx50
caPI3K
Cx50 +
caPI3K

Figure IV-6. Cx46-mediated coupling is unaffected by caPI3k. Up-regulation of PI3K signaling by the constitutively active catalytic subunit $p110\alpha^{H1047R}$ (caPI3K) in paired *Xenopus* oocytes expressing Cx46. *Xenopus* oocyte pairs injected with H_2O or caPI3K alone had nominal junctional conductance. Pairs co-expressing Cx46 and caPI3K showed no significant difference in coupling compared to pairs with Cx46 alone. Data are the mean ± SE.


junctional conductance [µS]
24
20
16
12
8
4
0
n=47
n=43
n=39
n=51
H2O
Cx46
caPI3K
Cx46 +
caPI3K

Chapter V

Investigating possible mechanisms of regulation of Cx50 conductance by PI3K signaling

Abstract

There are at least three possible mechanisms that PI3K signaling can specifically regulate Cx50 gap junctions. Since there was an increase in conductance mediated by Cx50 when co-expressed with caPI3K, the first theory tested is up-regulation of Cx50 protein synthesis in these cells. As a result, it would be desirable to determine if there are changes in connexin protein expression in response to caPI3K. Cx46 injected oocytes were used as a control for connexin protein expression since gap junctional conductance produced by Cx46 is unaffected by co-expression with caPI3K. Western blots of oocyte samples injected with caPI3K and Cx46 were probed with antibodies for Cx46, total Akt, and phospho-Akt. Three western blots for each sample set were used for densitometry calculations. Total Akt levels were equal in all samples. The presence of p-Akt in the cells injected with caPI3K cRNA confirms activity of the constitutively active p110α. In addition, those samples had equivalent p-Akt levels. Lastly, Cx46 protein levels were comparable in cells injected with Cx46 cRNA and cells co-injected with Cx46 and caPI3K.

To analyze Cx50 protein expression after injection with caPI3K, western blots of oocyte samples injected with H_2O, caPI3K, Cx50, or both caPI3K and Cx50 were probed with antibodies for Cx50, total Akt, and phospho-Akt. Immunoblots confirmed connexin expression in oocytes injected with Cx50 cRNA. Once again, total Akt levels were equal in all samples. Most importantly, the blot showed that Cx50 protein expression was unaffected by co-injection of caPI3K. This was validated by band densitometry analysis, confirming that there were no significant differences in connexin protein levels when co-expressed with caPI3K. These results demonstrated the increase in gap junctional conductance seen in the electrophysiology experiments was not due to an increase in Cx50 protein expression in response to caPI3K.

Another mechanism that could result in the altered gap junctional conductance after manipulation of the PI3K signaling pathway is modification of a gap junction channel that changes its unitary conductance. Single channel currents of Cx50 expressing HeLa cells were measured after PIK-75 and Akti treatment. Neither inhibitor had any effect on the unitary conductance. These data suggest that the macroscopic effects in Cx50 gap junctional conductance during PI3K signaling inhibition were not due to a modifications of single channel conductance.

Results

Constitutive activation of PI3K signaling does not alter Cx46 protein expression

Cx46 cRNA injected oocytes were used as a control sample for detection of connexin protein, activated Akt (p-Akt), and total Akt (t-Akt). If co-expression of caPI3K does alter Cx46 protein expression, then the data received from Cx50 oocytes would most likely be impractical for the purposes of supporting expression regulation as a possible mechanism. Therefore, western blots of oocyte samples injected with H_2O, Cx46, caPI3K, or both Cx46 and caPI3K, were probed with antibodies for Cx46, t-Akt, and phospho-Akt. Three western blots for each sample were used for densitometry calculations. Average band densities of total Akt levels were equal in all samples (Figure V-1C). The amount of p-Akt in cells injected with Cx46 and caPI3K were not significantly different than the level of p-Akt in oocytes injected with caPI3K alone (Figure V-1D). Immunoblots and band densitometry analysis confirmed there were no significant differences in connexin protein levels when co-expressed with caPI3K (Figure V-1A and V-1B). These results prove multiple points that are important before testing the effects on protein synthesis in Cx50 expressing cells: 1) injection of caPI3K does activate Akt in oocytes by the up-regulation of p-Akt, 2) Total-Akt levels are unaffected by expression of either the connexin or caPI3K, and 3)

caPI3K does not have a global effect of up-regulating connexin protein because levels of Cx46 were unaffected by caPI3K.

Constitutive activation of PI3K signaling does not alter Cx50 protein expression

The increased conductance mediated by Cx50 when co-expressed with caPI3K could have been a result of increased protein expression. Therefore, western blots of oocyte samples injected with caPI3K and Cx50 were probed with antibodies for phospho-Akt, total Akt, and Cx50 protein. Band densitometry analysis was performed on three western blots. Once again, average band densities of total Akt levels were equal in all samples (Figure V-2C). Immunoblots confirmed caPI3K activity by up-regulation of Akt activation in cells injected with $p110a^{H1047R}$ cRNA. The level of p-Akt remained unchanged in cells co-injected with connexin and caPI3K (Figure V-2A and V-2D). Surprisingly, band densitometry analysis confirmed there were no significant differences in Cx50 protein levels when co-expressed with caPI3K (Figure V-2A and V-2B). These results verified activation of Akt by caPI3K. More importantly, these data revealed there are no modifications of Cx50 protein expression in response to constitutive activation of the PI3K signaling pathway.

Treatment with PI3K signaling inhibitors does not reduce Cx50 unitary conductance

One mechanism that could result in the altered gap junctional conductance after manipulation of the PI3K signaling pathway is a direct modification of a gap junction channel that changes its unitary conductance. Cx50 signal channel currents were recorded in poorly coupled cell pairs to ensure the measurements are in cell pairs that had only one or two active channels. Gap junctional currents were measured by bipolar V_j pulses of a range of voltages from ±10 mV to ±110 mV. Cells were incubated with inhibitors for 24 hours prior to recordings. Figure V-3A

shows representative traces of single channel currents in response to ±70 mV pulses in three treatment conditions; 1) DMSO (vehicle control), 2) PIK-75, and 3) Akti. The traces detected similar channel activity in all three treatment conditions with no clear, discernible qualitative differences. Single channel currents of control (n=71), PIK-75 (n=109), and Akti (n=25) treated cells were plotted against transjunctional voltage and fit by linear regression to determine unitary conductance (Figures V-3B - Figures V-3D). The slope representing unitary conductance of Cx50 had values of 224 pS for untreated control cells, 237 pS for PIK-75 treated cells, and 239 pS after treatment with Akti. The correlation coefficients of all three linear fits were r2 = 0.99. These data suggest that PI3K signaling inhibition does not affect Cx50 single channel conductance.

Discussion

This chapter addressed two possible mechanisms that PI3K signaling could differentially regulate Cx50 gap junction channels. The increase in Cx50 gap junctional conductance demonstrated in response to constitutive activation of PI3K signaling could have resulted from an increase in Cx50 protein expression. Immunoblots and band densitometry analysis confirmed that Cx50 protein expression was unaffected by co-injection with caPI3K. An alternative method of regulation could be modification of individual Cx50 gap junctions that alters conductance produced by individual channels. However, when single channel currents of Cx50 expressing HeLa cells were measured after PIK-75 and Akti treatment, neither inhibitor had any effect on Cx50 unitary conductance. These data suggest that the macroscopic effects in Cx50 gap junctional conductance during PI3K signaling modulation were not due to modifications of individual channel conductance.

Additional experiments testing the protein expression of Cx50 in the presence of PI3K inhibitors would confirm PI3K signaling does not affect Cx50 protein expression. A western blot of Cx50 expressing HeLa cells Cx50 to see if inhibition after 24 hours decreases Cx50 protein synthesis. An experiment to provide additional detail to the effect the inhibitors have on Cx50 gap junctional conductance would be a time course to determine how long Cx50 cells have to be treated with the inhibitors to induce a change in gap junctional conductance.

Figure V-1. Western blot of oocytes injected with Cx46 and caPI3K cRNA. Immunoblots and band density quantification confirmed that expression of Cx46 does not change when co-expressed with caPI3K. (A) Representative western blot confirms expression of total-Akt in all samples, p-Akt, and Cx46 in respective oocyte samples. (B-D) Band densitometry analysis of three western blots confirmed equal levels of t-Akt in all samples, up-regulation of activated Akt (p-Akt) in oocytes injected with caPI3K, and equal levels of Cx46 between cells injected with Cx46 and caPI3K or Cx46 cRNA alone.

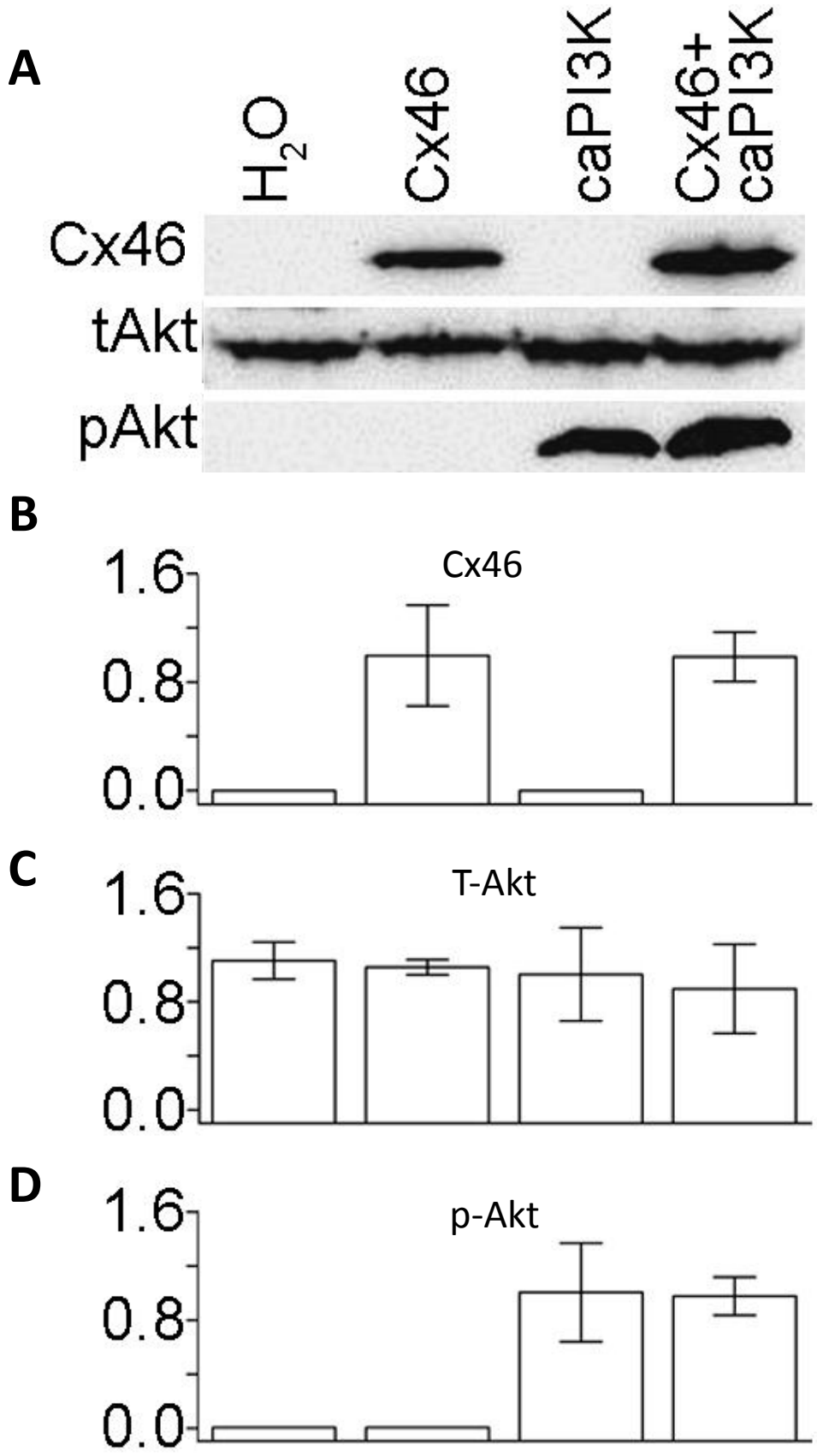

A
H2O
Cx46
caPI3K
Cx46+ caPI3K
Cx46
tAkt
pAkt
B
Cx46
1.6
0.8
0.0
C
T-Akt
1.6
0.8
0.0
D
p-Akt
1.6
0.8
0.0

Figure V-2. Western blot of oocytes injected with Cx50 and caPI3K cRNA. (A) Representative immunoblot confirmed the expression of Cx50 and the activity of caPI3K by the presence of elevated levels of activated Akt (p-Akt). Total Akt expression is used as a control. (B-D) Band densitometry analysis of three western blots. Band densities of t-Akt were similar in all samples. P-Akt levels were also comparable in oocytes injected with caPI3K alone or both caPI3K and Cx46 cRNA. Densitometry measurements show there is no significant difference in Cx50 protein when co-expressed with caPI3K.

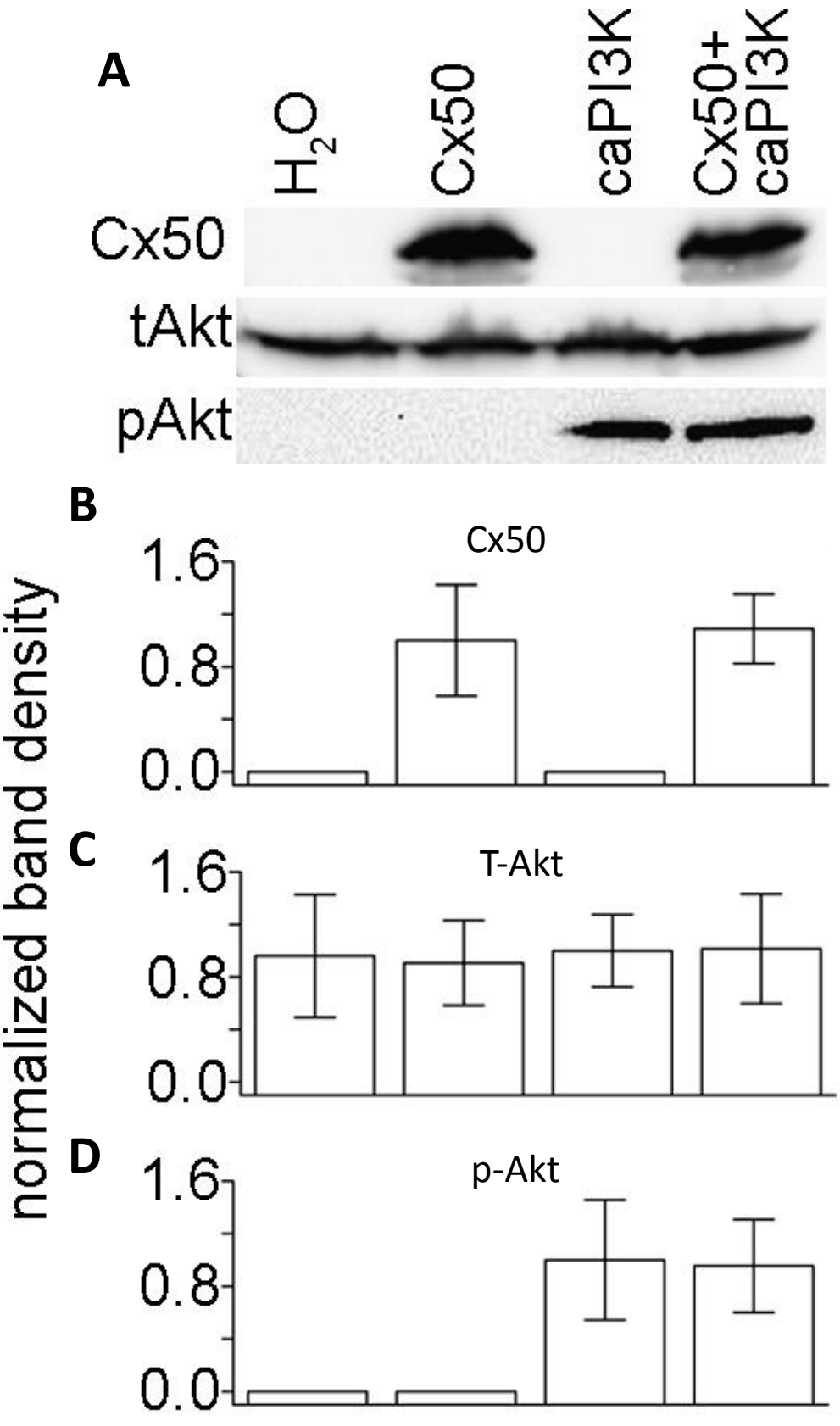
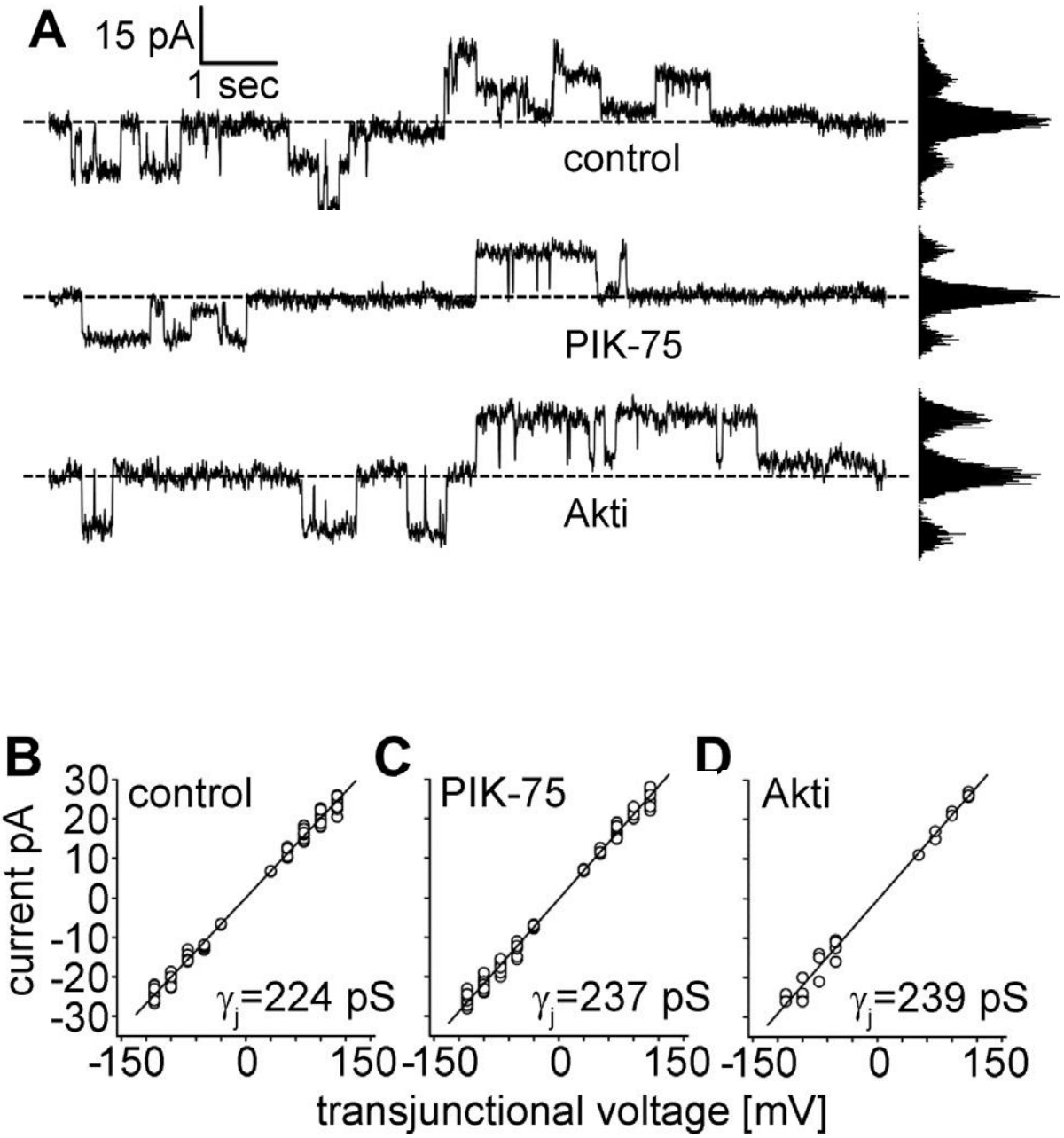

A
H2O
Cx50
caPI3K
Cx50+ caPI3K
Cx50
tAkt
pAkt
B
Cx50
C
T-Akt
D
p-Akt
normalized band density
1.6
0.8
0.0

Figure V-3. Neither PIK-75 nor Akti did not reduce Cx50 unitary conductance HeLa cells stably expressing Cx50 were treated with either PIK-75 or Akti for 24 hours. Junctional currents were measured by bipolar V_j pulses of a range of voltages from±10 mV to ±110 mV in cell pairs that had only one or two active channels. (A) Representative traces of single channel currents in response to ±70 mV pulses in cells treated with DMSO (control), PIK-75, or Akti. (B–D) Single channel currents were plotted against voltage and values were fitted by linear regression for all three conditions. Unitary conductance (the slope) did not decrease in PIK-75 or Akti treated cells, as compared to control; with values of 237 pS, 239 pS, and 224 pS, respectively. The correlation coefficients of all three linear fits were r2 = 0.99.


A
15 pA
1 sec
control
PIK-75
Akti
B
control
C
PIK-75
D
Akti
current pA
30
20
10
0
-10
-20
-30
γj=224 pS
γj=237 pS
γj=239 pS
-150
0
150
transjunctional voltage [mV]

Chapter VI

Concluding Remarks

The *Xenopus* oocyte expression system was used to demonstrate that Cx50 junctional coupling increased when co-expressed with a constitutively active PI3K subunit, $p110\alpha^{H1047R}$ (caPI3K). Cx46- mediated coupling was unaffected by constitutive activation of PI3K signaling. Also, HeLa cells expressing Cx50 were incubated with PIK-75 and Akt Inhibitor-VIII to specifically block p110α and Akt, respectively. There was a three-fold decrease in Cx50-mediated gap junctional conductance when treated with either inhibitor. Once again, this effect was not seen in Cx46 transfected HeLa cells. These results suggest a mechanism of differential regulation of these two lens connexins by the PI3K signaling pathway. Therefore, two mechanisms possible mechanisms were tested. First, since an increase in PI3K signaling increased Cx50 gap junctional conductance in oocytes, a protein analysis was performed to determine if PI3K signaling up-regulates Cx50 protein expression. Western blot and band densitometry analysis confirmed Cx50 protein expression was unaffected by caPI3K.

This dissertation introduced caPI3K to the *Xenopus* oocyte expression system. This is a positive foundation for the use of other constitutively active kinases in the PI3K signaling pathway to be used in voltage clamp experiments. One such protein that would be beneficial for providing more evidence that PI3K signaling pathway regulates Cx50 conductance is the constitutively active Akt mutant. Future experiments measuring Cx50 gap junctional conductance in response to a constitutively active Akt would contribute to the pool of support provided by this dissertation.

In addition, immunocytochemistry experiments of Cx50 HeLa cells to determine gap junction localization in the presence of PIK-75 or Akti would provide additional insight into the possible mechanism of regulation. It is possible that the inhibitors decrease gap junctional conductance by altering the transport of Cx50, either by increasing the rate of turnover or decreasing transport and insertion into the plasma membrane. Alterations in gap junction

localization is a more relevant phenotype of cells expressing mutant connexins, such as Cx50 mutants. Cx50D47N, Cx50P88S, Cx50P88Q are Cx50 mutants that have abnormal localization patterns due to defects in transport or degradation (Berthoud, Minogue et al. 2003, Arora, Minogue et al. 2008).

One mechanism of gap junction regulation not discussed previously in this dissertation is channel activation. At any given moment, a subset of gap junction channels are in the active pool. A channel in the active state has the potential to allow current to flow in response to stimuli. The open probability does not change in a non-active channel, however, the channel does not respond to stimuli and remains closed until a modification occurs. Fluorescence microscopy and electrophysiology of cells transfected with Cx43-EGFP determined that as small as 10-20% of all channels on the plasma membrane were actively communicating (Bukauskas, Bukauskiene et al.). Cell signaling molecules may have the ability to control the amount of channels in the active pool. One research group concluded that an increase in Cx43 conductance in response to a compound, 4-phenylbutyrate, was not because of a change in connexin protein expression but an increase in the number of channels in the active state (Wang, Rosati et al.). Therefore, we predict that PI3K signaling is modulating Cx50 channels so that more are in the active state without effecting open probability. This is different than the effect of Cx50 phosphorylation by PKA. In that study, it was demonstrated that PKA phosphorylation does not change the number of Cx50 gap junctions, but increased single channel permeability by altering its open probability (Liu, Ek Vitorin et al. 2011).

In vivo experiments could characterize the physiological relevance of Cx50 regulation by the PI3K signaling pathway. The PTEN KO lens generated in Sellitto et al. 2013 could be used as a constitutively active PI3K model that may determine if the interaction between PI3K signaling and Cx50 conductance has a role in lens development (Sellitto, Li et al. 2013).

PI3K signaling leads to Akt activation and downstream signaling resulting in cell survival. Research has demonstrated that Akt phosphorylates Cx43, therefore, Akt has the potential to phosphorylate Cx50 directly. Using the GPS 3.0 webserver for predicting kinase-specific phosphorylation sites, with a medium threshold over 28 residues were potential sites of Akt phosphorylation (Xue, Ren et al. 2008). Two predicted phosphorylation sites with high threshold corresponded to phosphorylation sites identified in mass spectrometry screens of bovine lenses, Ser-139 and Ser-431 (Wang and Schey). Ser-139 is a residue found on the cytoplasmic loop of Cx50 and Ser-431 is on the c-terminal tail. Neither site was identified in Cx46, however, Ser-119 and Ser-425 were identified as potential Akt phosphorylation sites at the same threshold (Wang and Schey). Kinetic experiments with purified Cx50, Akt, and P^{32}-ATP would prove if Akt has the ability to directly phosphorylate Cx50. Furthermore, to test if the phosphorylation has physiological relevance, site-directed mutagenesis of the either serine would prove *in vivo* the potential functions of these residues.

The purpose of this phosphorylation and its involvement in lens development is unknown. One may speculate as to the physiological relevance of differential regulation of connexins in developing tissues by signaling pathways. As previously stated, the lens circulation model proposes that the current produced by Na^+ enters the lens from the anterior and posterior poles and exits at the equator. Since the highest levels of FGF signaling is at the equator of the lens, it is possible that this activation upregulates the conductance of Cx50 gap junctions to assist in driving the lens circulation outward from the equator. This may explain why there is a lack of Cx46 response to FGF signaling since Cx46 is expressed in the differentiated and mature fiber cells of the core and may not necessarily be a proponent for directing the current. One study showed that the transport of glutathione to the lens core is solely based on Cx46 and not Cx50 channels.

Interestingly, they showed that the transport of glutathione to the core does not depend on the lens microcirculation (Slavi, Rubinos et al.). This supports the idea that Cx50 channels are more heavily involved in the circulation of current through the lens based on the model.

Furthermore, additional experiments will help elucidate the purpose of differential regulation of these connexins in the lens by determining differences in selective permeability. As stated previously, the protein composition of gap junctions can influence the permeability of the channel (Evans and Martin , Kanaporis, Mese et al. 2008). Also, it is known that the conductance of lens epithelial cells is primarily produced by Cx50 (Boswell, Le et al. 2009). Therefore, it is possible that the regulation of the Cx50 conductance by signaling pathways is truly a mechanism of altering the transport of growth signaling molecules that can permeate the channel. Recent studies are exploring specific signaling molecules that can be selectively transported through connexins, such as the movement of cAMP through Cx43, Cx40, or Cx26 gap junctions (Kanaporis, Mese et al. 2008). Metabolites shown to permeate connexin gap junctions include ATP, cAMP, cGMP, IP_3, Ca^{2+}, and amino acids.

Selectivity studies of ATP in particular have identified a connection between connexins and cell proliferation. For example, Pearson et al. has been studying the effect of ATP transport through Cx43 channels in retinal epithelial cells. Interestingly, they showed that Ca^{2+}-induced release of ATP through Cx43 channels was a necessary mechanism to increase cell division and stimulate epithelial cell proliferation (Pearson, Dale et al.). In addition to Cx43, Cx45 expressed in transit-amplifying precursor cells of the subventricular zone regulates cellular proliferation by modulating the intercellular ATP signaling that controls Ca^{2+} storage and ERK signal activation (Khodosevich, Zuccotti et al.).

By answering these questions, we can better understand the development of the lens, provide another example of differential regulation of connexins by signaling pathways, and more specifically, to determine if the up-regulation of conductance by the PI3K signaling pathway is to regulate the passage of specific signaling molecules through Cx50 or Cx46 gap junction channels.

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