

FUNCTIONAL ROLE OF CONNEXIN 46 IN LENS EPITHELIAL CELL
DIFFERENTIATION AND GROWTH

by

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B.S., Kansas State University, 2007

A THESIS

submitted in partial fulfillment of the requirements for the degree

MASTER OF SCIENCE

Graduate Biochemistry Group

KANSAS STATE UNIVERSITY
Manhattan, Kansas

2008

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Abstract

The vertebrate lens relies on gap junction-mediated intercellular communication to maintain cellular homeostasis and lenticular transparency. Differentiation of cuboidal lens epithelial cells into cortical fiber cells involves the degradation of endogenous gap junction protein, connexin 43 (Cx43) and the up-regulation of connexin 46 (Cx46). Cx46 may also be involved in the hypoxia response in other tissues; a function that can possibly be attributed to unique phosphorylation sites at the cytoplasmic C-terminus. In this study, we have developed a mammalian (rabbit) lens epithelial cell (RLEC) culture model that overexpresses Cx46 to ascertain the role of Cx46 in differentiation and oxidative stress response. The cell line N/N1003A was stably transfected with a GFP-Cx46 plasmid construct, and analyzed for differentiation markers including endogenous gap junction protein isoforms (Cx43 and Cx50). Western blot analysis and visual observation determined that the stable overexpression of Cx46 (sCx46OE) induced the degradation of Cx43 and elicited morphological changes indicative of fiber cell elongation. Total RNA from RLEC culture was isolated and analyzed for mRNA levels using RT-PCR. Comparable levels of Cx43 transcript were present in wild type, transient Cx46OE (tCx46OE), and sCx46OE which suggests a post-transcriptional regulation of Cx43 degradation. Treatment of sCx46OE with proteasome inhibitors restored Cx43 protein levels, and scanning confocal microscopy supported our hypothesis that Cx43 is degraded in differentiating lens cells by way of a ubiquitin-mediated proteasomal pathway. It is our conclusion that Cx46 has application in hypoxic conditioning and differentiation in addition to its conventional role as a gap junction protein.

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Acknowledgements

It is my humble privilege to have worked under the guidance of Dr. Dolores Takemoto. I am thankful for her motivation, encouragement, and mentorship. Without her, I would still be fixing toilets in Aggieville. I would like to express gratitude to the entire Kansas State University biochemistry faculty, including committee chairs, Drs. Larry Davis and Michael Kanost, for equipping my station as a scientist. I would also like to personally thank Drs. John Tomich (Biochemistry) and Michael Finnegan (Anthropology) for their advice and guidance.

This thesis would not have been possible without the gracious help of Drs. Vladimir Akoyev, Micheal Barnett, and Dingbo Lin. They took the time to patiently answer my incessant questions and quell my ignorance of laboratory workings. My appreciation is extended to my fellow graduate and undergraduate students who made my academic career not only possible, but bearable.

Most importantly, I would like to thank my wife, Sarah, and my children Phinneas and Georgia for their undying support and love. They endured long nights and days without me, and even longer nights and days with a man that had already given his all at the lab. Thank you for your patience, your understanding, and your laughter. It makes it all worth it.

Thanks and love to my mother, father, siblings and the rest of my family, who made me who I am today.

Finally, I praise God, from whom all things, good and alive, flow.

Dedication

This thesis is dedicated to my wife, Sarah, my children, Phinneas and Georgia, and my future children. You are my greatest experiment of all.

CHAPTER 1 - INTRODUCTION

The Lens

An Atypical Tissue

The lens presents a unique microcosm for the study of cell growth, development, epithelial function, cancer and aging. Morphological simplicity, defined developmental history and easy access by the experimenter make the lens an appealing tissue to investigate. In particular, there are two aspects of the lens that make it highly relevant to the researcher. Firstly, there are no known clinically recognizable cancers of the lens (Mathias and Rae, 2004). The intrinsic architecture of the lens is essentially an asymmetrically folded epithelium with two distinct domains of specialization (Zampighi et al., 2000). This morphology makes lens tissue highly relevant to the study of epithelial derived malignancies, which account for a majority of tumor propagations. Secondly, the lens could be considered an essential paradigm for the characterization of the effects of aging on epithelial function. Human lens tissue can be discerned around the 5th or 6th week of gestation, and remains intact for the remainder of an adult's life (Bhat, 2001). Due to the general ease of manipulation of lens tissue, cellular interactions in embryonic induction can be elucidated.

Lens Cell Differentiation

Throughout embryonic development, the mammalian lens cell undergoes a complex process of morphological and physiological change during differentiation. The embryonic lens vesicle begins as a spherical cyst of epithelial cells derived from placoidal ectoderm (Fig 1.1A). As the lens progresses developmentally, the posterior epithelial cells elongate internally toward the anterior; these become the primary fiber cells (Fig 1.1B-C) (Chow and Lang, 2001).

Throughout the lifetime of an individual, lens growth is marked by the retrograde deposition of new fiber cells over older, existing fiber cells, forming a graduated

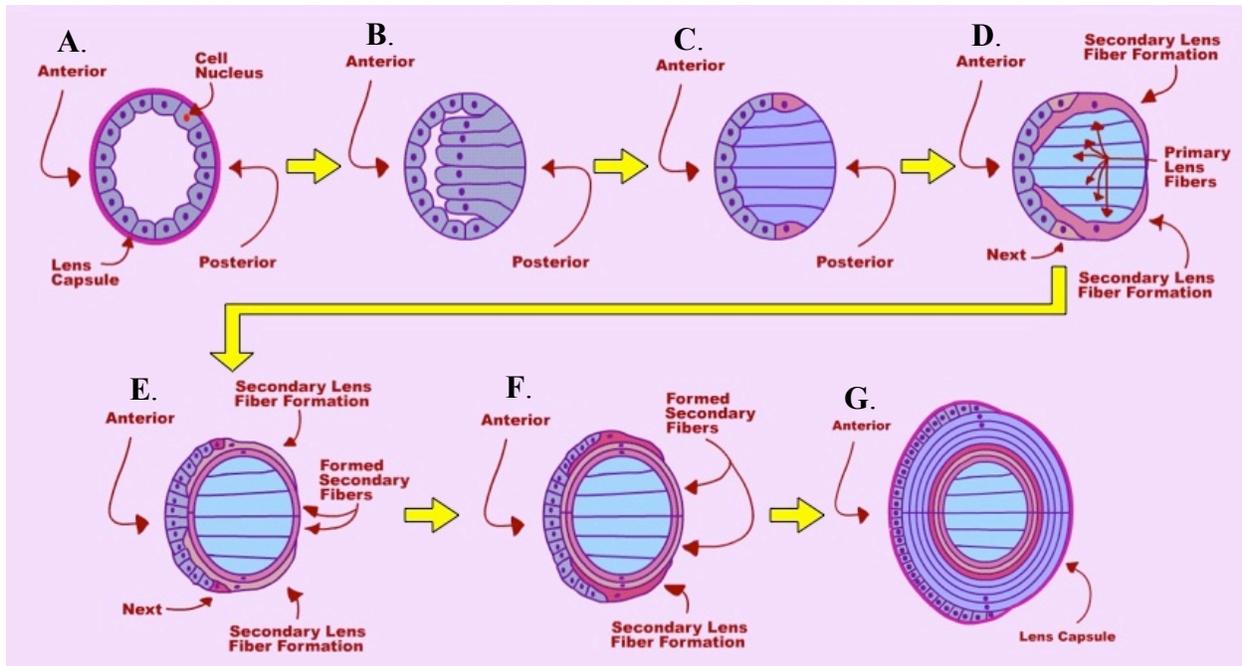


Figure 1.1: Embryonic development and cell differentiation of the vertebrate lens.

A. Embryonic lens begins as a vesicle composed of cuboidal epithelial cells enveloped in a collagenous capsule. **B.** Posterior epithelial cells elongate toward anterior to form primary fiber cells. **C.** Primary cells lose nuclei and organelles; equatorial epithelial cells (at bow region) begin process of differentiation. **D.** Differentiated secondary fiber cells are deposited in retrograde position over (and around) older (primary) fiber cells. **E.** Epithelial cells divide in germinative zone prior to differentiation. **F.** Mature fiber cells elongate to form medial suture with fiber cells. **G.** New secondary fibers form (deposited) throughout life, but are retained at the cortex in an inert state. [Figure modified from http://www.dogstuff.info/lens_development]

arrangement of cells in various stages of differentiation (Fig 1.1D-G) (Donaldson et al., 2001). Terminally differentiating fiber cells, at the cortex, demonstrate redistribution of gap junction proteins (Gu et al., 2003; Jiang et al., 1995) and crystallins (Wang et al., 2004), reorganization of centrosomal markers (Dahm et al., 2007), and the degradation of organelles (Bassnett and Beebe, 1992; De María and Arruti, 2004; Girao, 2005) and DNA (Nagata, 2005).

Differentiation of lens fiber cells depends on several secreted factors, including fibroblast growth factors (FGF), Wingless (Wnt) and transforming growth factor-B (TGF β) proteins, and insulin-like growth factors (IGF) which regulate many differentiation factors and/or cell-cycle regulators during lens formation. Additionally, N-

cadherin and $\alpha 6$ integrin have also been shown to play a role in fiber-cell induction (Beebe et al., 2001; Walker et al., 2002). At the bow region of the lens, the up-regulation of cyclin-dependent kinase inhibitors (p27, p57) and Rb (retinoblastoma) proteins, together with the inhibition of Src kinase (sarcoma tyrosine kinase) activity, induces withdrawal from the cell cycle and initiates the start of fiber cell differentiation (Tholozan and Quinlan, 2007). The characteristic elongation of the primary fiber cells has been attributed to an increase in K^+ permeability and the reconfiguration of the increasing cell volume into an accommodating morphology. The extrusion mechanism of the secondary fiber cells, however, is not fully understood. The observation of up-regulated cytoskeletal protein expression (CP49 and filensin) and the increased association of the plasma membrane with the cytoskeleton suggests that the mechanism of elongation is independent of volume change (Bassnett, 2005). Interestingly, growth factors, cadherins, and integrins all activate RhoGTPases to control actin organization and the subsequent disassembly of actin stress fibers, which is indicative of cell elongation (Weber and Menko, 2006).

Lens differentiation is also characterized by the activation of crystallin synthesis. Composing about 90% of mature fiber cell protein content, these water-soluble structural proteins impart the transparent and refractive characteristics of the lens. The prevailing view of differential crystallin expression holds that most epithelial and fiber cells in vertebrate lenses contain a mixture of alpha-crystallins, represented by the two isoforms, αA - and αB -crystallin, whereas β - and γ -crystallins are solely found in differentiated fiber cells. Wang et al. have reported finding β - and γ -crystallin mRNA in some epithelial cell cultures as well as extremely low protein levels (2004). This, however, does not negate the long-held view that β - and γ -crystallin are up-regulated in the differentiating fiber (DF) cell.

Apoptosis, or programmed cell death, also plays a large role in the process of lens development. While typical apoptotic pathways herald the removal of unwanted cells, the activation of primary apoptotic signaling components, caspase-3 and endonuclease, instead initiate the degradation of nuclei and DNA in DF cells. Suppression or enhancement of developmental apoptosis because of genetic mutations and

manipulations, or environmental conditions, causes formation of abnormal lenses or absence of the ocular lens (Yan et al., 2006). For example, in addition to its function as a structural protein, α A-crystallin acts as an excellent anti-apoptotic regulator in ocular lens and it prevents apoptosis induced by a large number of different stress factors. Knock-out of both α A and α B-crystallin has been shown to lead to cataractogenesis (Boyle et al., 2003). Concordantly, a recent study demonstrates that in the double knock-out mice, enhanced expression and activity of both caspase-3 and -6 may contribute to apoptosis of secondary lens fiber cells, and eventual cataractogenesis (Morozov and Wawrousek, 2006).

The biochemical signaling pathway for organelle degradation, including mitochondria, in DF cells may follow a similar pathway with slight modifications. Organelle degradation by way of hypoxia-induced apoptosis is discussed in the next section.

Structure and Function of the Lens

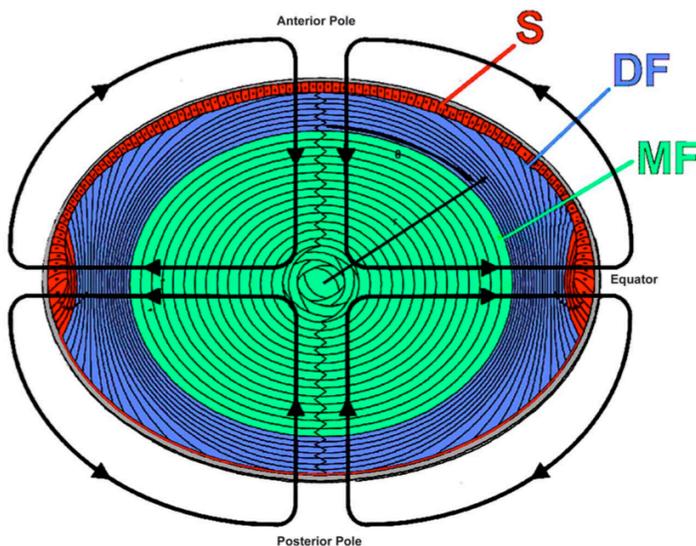


Figure 1.2: Cellular structure and arrangement in the vertebrate lens. S=surface epithelium (red); DF=differentiating fiber cells (blue); MF=mature fiber cells (green); Arrows=microcirculatory pathways. [Mathias and Rae, 2004]

There are three perceivable zones of cells in lens (Fig 1.2): an outer layer of surface (red S) cells, an intermediate layer of differentiating fiber cells (blue DF) that still have organelles and other distinct transport properties, and a core of mature fiber cells (green MF) that have lost their organelles and deposited large amounts of crystallins (Mathias and Rae, 2004). The primary function of the lens is to retain

transparency, which requires a major departure from other somatic tissue; particularly, it is avascular and noninervated. To maintain optimum transparency and refractive index, the lens must reduce obstructive occlusions and minimize metabolic machinery (Goodenough et al., 1996). Consequently, a distinctive, microcirculatory transport system that is coupled to ion flux and fluid movement has been adapted in the vertebrate lens (indicated in illustration by pathway arrows). Osmotic and metabolic homeostases are preserved through a highly developed communication network mediated by gap junctions (Mathias et al., 1997), which will be discussed at length in the next section.

Another consequence of this arrangement is that it leads, inevitably, to the generation of standing gradients of small, diffusible metabolites within the tissue. An example of this effect is the gradient of intracellular pH (pHi) observed within the lens. The lens obtains much of its ATP from anaerobic glycolysis. Lactic acid generated by glycolysis diffuses from the lens only across its outer surface. Consequently, a standing gradient of lactic acid is established, resulting in values for core lens pHi that are significantly lower than those in the outer cortex. Similarly, the distribution of intralenticular oxygen follows a gradient that can be attributed to limited diffusion from the vitreous humor through the mitochondriated epithelium. Mitochondrial respiration, which is responsible for most oxygen consumption in vertebrate tissue, in the surficial layers of the lens would account for the low dissolved oxygen levels in cortical fiber cells (Bassnett and McNulty, 2003). The normal oxygen level measured in the vitreous body of normal rabbit, feline, rat, and human eyes is between 8 and 20 mm Hg (1%–3% O₂) (Shui et al., 2006). This low oxygen (hypoxia) and pHi gradient may provide spatial cues for the coordination of differentiation and organelle degradation. Support for this hypothesis is presented by Bassnett and McNulty, who observed that the organelle-free zone (OFZ) in the lens cortex remained delineated at a fixed distance (850 μm) below the surface of the embryonic chicken lens despite a matched growth rate to whole lens (2003). Hyperoxic treatment of the embryos yielded a significant increase in this distance, which suggests that there is a correlative relationship between organelle degradation and intraocular oxygen. Furthermore, while hyperoxic in vivo experiments

of mature chicks resulted in high mortality, increased water gas levels of oxygen have been shown to increase growth-related cataract formation in salmon (Waagbø et al., 2008). These findings may indicate an elevated occurrence of intralenticular occlusions due to incomplete degradation of organelles by way of hypoxic signaling.

Hypoxia inducible factor-1 (HIF-1), a regulatory transcription factor that manages oxygen homeostasis in metazoan tissue, has been implicated in mitochondrial autophagy and proteasomal degradation in mouse embryo fibroblasts (MEFs) (Zhang et al., 2008). HIF-1 is a heterodimer composed of an O₂-sensing HIF-1 α subunit and a constitutively expressed HIF-1 β subunit, or aryl hydrocarbon receptor nuclear translocator (ARNT). HIF-1 primarily functions as a response element to protect cells from accumulated reactive oxygen species (ROS) or hypoxic insult, which can cause cell injury or death. Under normoxic conditions, HIF-1 α is continuously synthesized and degraded. Conserved proline residues, proline 402 and/or 564, are hydroxylated, allowing the von Hippel-Lindau (VHL) E3 ubiquitin ligase complex to bind with HIF-1 α and target it for proteasomal degradation. Hypoxic conditions decrease essential rate-limiting oxygen levels for prolyl hydroxylation, which decrease the ubiquitination of HIF-1 α . This in turn allows the dimerization of HIF-1 α and HIF-1 β , and subsequent transcription activation of hundreds of genes such as glycolytic enzymes (eg. aldolase), glucose transporters (eg. GLUT 1 and 3), angiogenic molecules such as vascular endothelial growth factor (VEGF) and angiogenin, survival/growth factors such as TGF- β and IGF-II, and proteins involved in tumor invasiveness and metastasis (Semenza, 2007). In the normally hypoxic environment of the inner eye, high levels of HIF1- α are maintained in lens epithelium. When the lens is exposed to high levels of oxygen saturation (ie. post-vitreectomy), the lens increases cell proliferation and has been shown to exhibit heightened occurrence of cataract formation. Inversely, expression of oxygen insensitive HIF1- α or pharmacological inhibition of HIF1- α degradation has been shown to suppress the ability of oxygen to increase cell proliferation (Shui et al., 2003). These findings suggest that low levels of oxygen in the eye maintain high levels of HIF-1 activity, which controls lens cell proliferation and regulates intralenticular homeostasis.

Gap Junctions

Gap Junction Structure and Function

Gap junctions are intercellular membrane channels that permit the low-resistance passage of ions and diffusion of small molecules ($M_r \leq 1$ kDa). These specialized

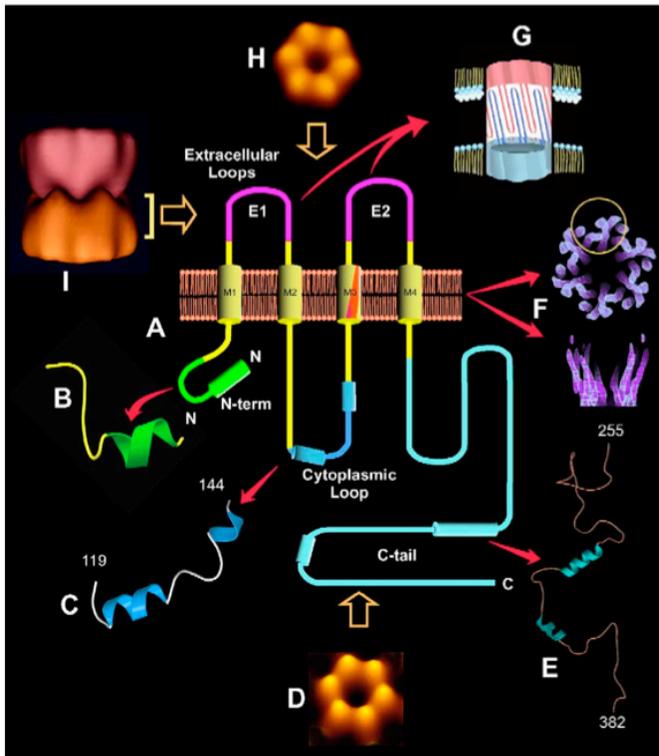


Figure 2.3: Information on the structure and arrangement of gap junctions. A. Topology diagram of a generic connexin monomer. B. N-terminal secondary structure. C. Secondary structure of cytoplasmic loop. D. AFM topographic image of cytoplasmic side of connexon. E. Carboxy terminus of Cx43 (NMR). F. 3-D structure of transmembrane domain seen perpendicular (top) and parallel (bottom) to plasma membrane. G. Barrel extensions at the docking surface of hemichannels. H. AFM topography of extracellular side of connexon. I. 3-D structure of complete gap junction. [Sosinsky and Nicholson, 2005]

channels are formed through the hexameric oligomerization of connexin proteins which, in turn, couple with other hexamers (connexons) on adjacent cell membranes; bridging the cytoplasmic interior across the intercellular space (Fig 1.3) (Benedetti et al., 2000; Donaldson et al., 1995).

As one connexon dimerizes to another, high-density clusters of these gap-junctions form spontaneously at cell-cell interfaces and are referred to as plaques (Gaietta et al., 2002; Musil and Goodenough, 1991; Yamasaki, 1990). These functional plaques

assemble and disassemble approximately every 2-5 hours, which is relatively high compared to the typical membrane protein turnover rate of 24+ hours (Berthoud et al., 2000; Berthoud et al., 2004; Musil et

al., 2000).

Connexons can be assembled from one connexin (called a homomeric

connexon) or more than one connexin (called a heteromeric connexon). Consequently, an intercellular channel can be composed of two identical homomeric connexons (called a homotypic junction) or two connexons of different heteromeric or homomeric composition (called a heterotypic junction) (Fig 1.4). Mixing of connexins within the channel is hypothesized to be possible because of the high conservation of primary sequence in the extracellular and transmembrane domains, however, there is some selectivity as to which connexins partner with others. Heterotypic junctions can have distinct molecular permeabilities from their parental homotypic junctions, which suggests that connexin composition strongly influences the passage of molecules through the pore. This selectivity may pertain to size, charge and shape of the permeant, the effective pore size and affinities between the pore wall and the permeants (Hervé et al., 2007).

Topographic analysis and x-ray diffraction studies of gap junctions have established a universal structural motif consisting of four hydrophobic transmembrane domains (M1-M4), two extra cellular loops (E1 and E2), and the cytoplasmic loop (CL). The carboxy-terminal cytosolic tail represents the most variable portion of the connexin molecule, while the remaining N-domain, capable of self-assembly and hexamer formation, is conserved between members of the connexin family. The C-terminus has been shown to possess an array of regulatory binding sites and influence gating properties due to variable phosphorylation and protein-protein interactions (Goodenough et al., 1996; Sosinsky and Nicholson, 2005).

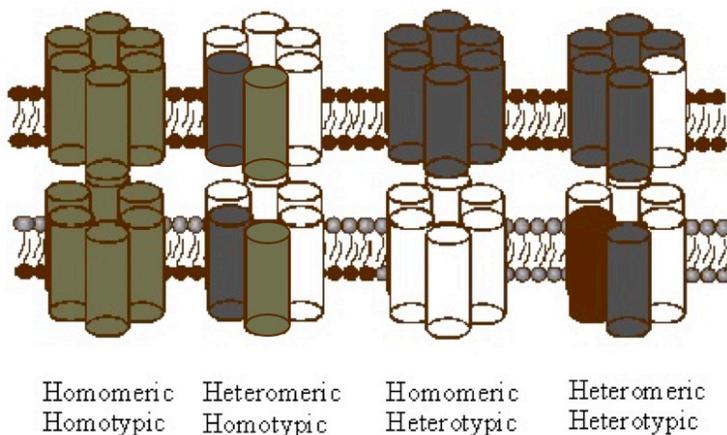


Figure 1.4: Connexin and connexon membrane arrangements. Connexin composition can be classified as homomeric or heteromeric. Connexons can dimerize to form homotypic or heterotypic gap junctions. [Modified from <http://herkules.oulu.fi>]

In humans there are at least 20 connexin genes that exhibit complex and overlapping patterns of expression. Connexin genes are commonly divided into alpha, beta, and an unnamed third group, all of which encode gap junction channels of different molecular weights. Examination of these groups shows an apparent separation of channels that is partially dependant on the molecular weight of each individual gap junction protein. In general, connexin genes within the beta group encode proteins smaller than 32 kDa, whereas the alpha genes encode connexins larger than 37 kDa. The unnamed group is composed of at least seven connexins of intermediate molecular weight (31-47 kDa) that violate this dichotomy (Kardami et al., 2007). Similarly, the connexin transcriptional motif is fairly conserved as most connexin genes have a first exon containing only 5'-untranslated (UTR) sequences and a large second exon containing the complete coding region as well as all remaining untranslated sequences (3'-UTR) (Fig1.5). Only a few connexins (Cx34.7, Cx36) demonstrate an intronic interruption in the coding region (Saez et al., 2003).

Like other genes, connexin expression can be regulated at many of the steps in the pathway from DNA to RNA to protein, i.e., transcriptional control, RNA processing control, RNA transport and localization control, translational control, mRNA degradation control, and protein activity control. Transcriptional controls represent the most important and widely observed regulatory activities (Oyamada et al., 2005). In most connexin genes, the basal promoter P1 is located within 300 bp upstream of the trascription initiation site in exon 1. Located within this region, transcriptional binding sites, which can bind cell type-dependant or -independant transcription factors, have been identified. Cell type-dependant factors are exemplified in cardiac-specific factors



Figure 1.5: Structure of connexin genes. A. Most connexin genes contain two exons and one intron, where first exon is entirely 5'-UTR. B. A few connexin genes have split coding regions. Dark regions = non-coding, light grey = coding. [Saez, 2003]

(GATA4 and Nkx2-5) and hepatic nuclear factors (HNF-1). The more ubiquitous cell type-independent binding sites include sequences for TATA box-binding protein, human transcription factors Sp1/Sp3 (GC box recognition), and activator protein-1 (AP-1). Additionally, various cell types subjected to different treatments are known to posttranscriptionally increase the levels of connexin mRNA due to enhanced transcription. For example, cAMP has been shown to induce elevation of Cx43 transcript levels in hepatoma cells (Mehta et al., 1992), and the phorbol ester [12-O-tetradecanoylphorbol-13-acetate (TPA)] induces transcriptional up-regulation of Cx26 in human immortalized MCF-10 mammary epithelial cells (Li et al., 1998). This variance in genetic regulation governs cell coupling and mediates the cell-specific adaptations of gap junctions in tissue such as lens.

Gap junctions respond to a number of stimuli, including voltage (both transjunctional and transmembrane), pH, Ca^{2+} and phosphorylation events. Connexin sensitivity to transjunctional voltage (V_j), or differences in voltages between coupled cells, varies widely in degree, but it's partially this variance that renders functional diversity and permeant selectivity in GJIC (Mathias et al., 1997). V_j is determined by the intrinsic properties of the connexon component hemichannel in plasma membrane. Typically, connexon hemichannels are closed in nonjunctional cell membranes, but form a hydrophilic pathway between cell interiors after docking with another hemichannel (Bukauskas and Verselis, 2004). Recent evidence indicates that there are two distinct voltage-dependent gating mechanisms that can close GJ channels and hemichannels. One mechanism closes channels and hemichannels to a subconductance state and has molecular components of its voltage sensor localized to the N-terminal domain (Saez et al., 2003; Trexler et al., 1996) (fast gating). The other mechanism completely closes gap junction channels and hemichannels, leaving no residual conductance, and has a distinctive feature in that the gating transitions appear slow, often taking tens of milliseconds to complete (slow gating). Molecular components of this form of gating may be at the extracellular end of the hemichannel because of strong sensitivity to extracellular Ca^{2+} and resemblance to gating associated with docking during cell-cell channel formation. The provisional name "loop gating" was assigned because of the

plausible involvement of the extracellular loop domains (Sosinsky and Nicholson, 2005). The gating properties of some asymmetric, heterotypic gap junctions have been associated with voltage conductance independent of their combined predicted hemichannel conductances. This phenomenon, however, will be discussed in the next section.

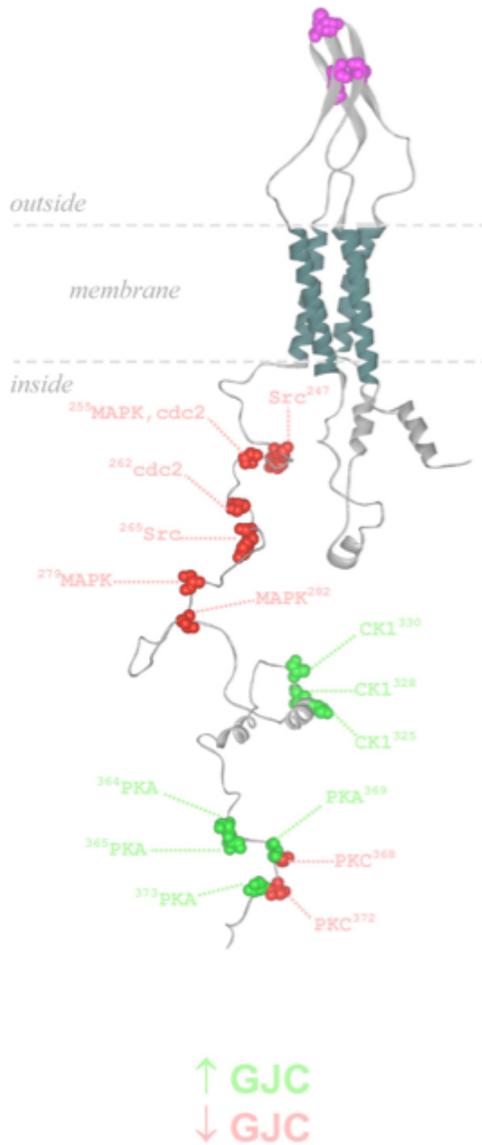


Figure 1.6: Phosphorylation sites on Cx43. Monomeric Cx43 with phosphorylation sites targeted by known kinases are labeled by amino acid number and phosphorylating kinase. [Pahujaa et al., 2007]

Acidification of the cytoplasm, as seen in the lens cortex, leads to closure of the gap junction channels composed of most connexin isoforms, although the sensitivity differs. The predominant hypothesis in literature, as determined by mutation mapping, predicts that the regulatory sites for pH and other chemical gating processes are located at the carboxyl terminus (CT) of the connexin molecule and within the second half of its intracellular loop structure. This pH gating mechanism has been termed a “ball-and-chain” type schematic in which a gating particle (the CT) binds to a separate region of the protein (a “receptor”) to close the channel (Sosinsky and Nicholson, 2005). In lens, the CT of fiber cell connexin proteins is cleaved as they are internalized to the cortex, which alters their physiological sensitivity to acidification (Eckert, 2002).

Similarly, the connexin CT contains gating components in association with phosphorylation control (Table 1.1 and Fig1.6). Two kinase systems have been shown to mediate closure of gap junction

Event activity	Amino acid residue	Kinase
Decrease communication	Y247	Src
	S255	MAPK, CDC2
	S262	CDC2, PKC
	Y265	Src
	S279	MAPK
	S282	MAPK
	S368	PKC
	S372	PKC
Increase communication	S325	CK1
	S328	CK1
	S330	CK1
	S364	PKA
	S365	PKA
	S369	PKA
	S373	PKA

Table 1.1: Cx43 phosphorylation events that affect gap junctional communication.

Established Cx43 phosphorylation sites conserved between humans, rodents, primates, and pigs are shown.

[Modified from Pahuja et al., 2007]

channels, notably v-src (Pahuja et al., 2007) and extracellular signal-regulated kinase (ERK) (Stains and Civitelli, 2005), while PKC has been associated with modulation of channel conductance (King and Lampe, 2005). V-src is known to target Y265 and Y247 on the CT of Cx43 and has been found to bind to the tail through a combination of src homology domain 3 (SH3)-driven interactions and SH2 binding to the phosphorylated tyrosines (Moreno and Lau, 2007). CT phosphorylation sites in Cx43 primary sequence studies have also been linked to regulating the assembly of gap junctions: S328/330 (Casein kinase), S368 (Protein Kinase C), and residues 251 – 257. Disassembly and degradation of connexins have also been shown to be regulated by other sites (e.g., a tyrosine sorting signal, Y286) (Sosinsky and Nicholson, 2005) on the CT by way of kinase activation, including PKC γ

activity (Barnett et al., 2007; Laird, 2005).

Gap Junction Isoforms in Human Lens

There have been three endogenous connexin isoforms identified in the lens: Cx43 ($\alpha 1$), Cx50 ($\alpha 8$), and Cx46 ($\alpha 3$) (Donaldson et al., 2001; Goodenough et al., 1996; Zampighi et al., 2000). Lens epithelium primarily contains Cx43 and, to a lesser extent, Cx50. Differentiating fiber cells and mature fiber cells degrade Cx43 and preferentially express Cx46 and Cx50 (Lin et al., 2004). Patch clamp measurements of *Xenopus* oocyte membrane conductances have revealed that exogenously expressed Cx46 and Cx50 have the ability to form hemichannels with non-junctional conductances and heteromeric connexons that possess unique gating characteristics from homomeric analogs (Srinivas et al., 2005). Additionally, Cx46 was able to form functional heterotypic channels with Cx43 and Cx50; however, Cx50 was unable to pair with Cx43 (White et al., 1994). This may be physiologically relevant because Cx46 hemichannels have also been shown to have mechanosensitive properties that may be important in the equilibration of hydraulic and mechanical stress during visual accommodation and differentiation (Bao et al., 2004).

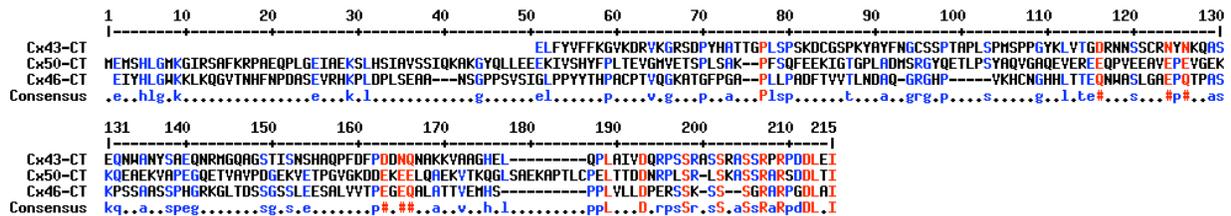


Figure 1.7: C-terminal protein sequence alignment of connexin isoforms in lens. Cx43, Cx50, and Cx46 cytosolic C-terminus alignment. Red letters are high consensus indicators and blue are low consensus indicators.

All three connexin isoforms are essential to the proper development and function of the lens. Cx43 null mice develop oculodentodigital dysplasia (ODDD) which is characterized by microphthalmia, cataract, and optic atrophy (Gerido and White, 2004). While Cx50 knockout mice are characterized by reduced ocular growth and mild fiber

cell opacities, Cx50 deficient cataracts are corrected with the subsequent knock-in of Cx46 (Xia et al., 2006a). Conversely, knockout and mutation studies of Cx46 in mice show a marked increase in cortical cataracts, but no measurable difference in lens growth (Dunia et al., 2006; Gong et al., 1998; Gong et al., 2007). Though gap junction sequences reflect a high degree of conserved sequence similarity, these findings suggest extremely specific roles for individual connexin proteins in maintaining lens clarity and overall lens development.

Thirteen different mutations in Cx46 have been associated with congenital cataract formation, of which five have been reported as autosomal dominant (ADCC) (Li et al., 2004b). Several these mutations are associated with nuclear and “Zonular pulverulent” cataracts, which are opacifications that take on a pulverized (powdery) appearance and affect the lamellar regions around the nucleus. These findings are relevant to functional studies of Cx46 because they elucidate sequence significance that knockout studies and random point mutation cannot. Mutations of Cx46 have been identified in the N-

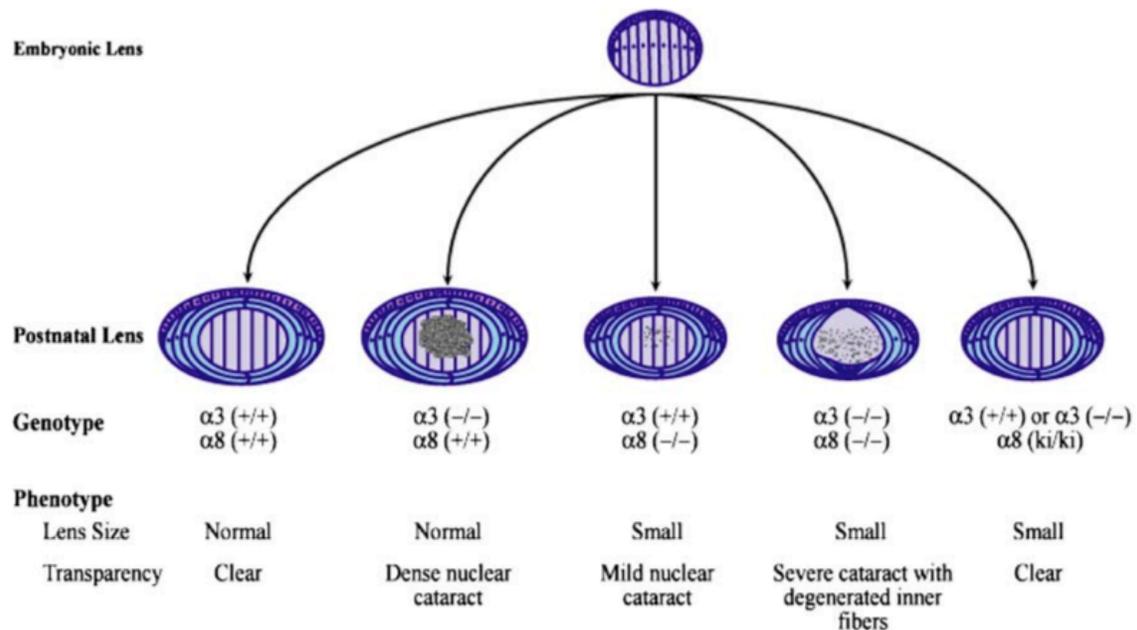


Figure 1.8: Lens phenotypes of different connexin knockouts. $\alpha 3$ and $\alpha 8$ correlate with Cx46 and Cx50 respectively. The wild-type ($\alpha 3^{+/+}\alpha 8^{+/+}$) lens is a normal control. Cx46 knockout ($\alpha 3^{-/-}\alpha 8^{+/+}$) lenses develop nuclear cataracts, and Cx50 knockout ($\alpha 3^{+/+}\alpha 8^{-/-}$) lenses are smaller with mild nuclear cataracts. Double knockout ($\alpha 3^{-/-}\alpha 8^{-/-}$) lenses have severe cataracts with degenerated inner fibers, and Cx46 knockin (ki/ki) with or without endogenous Cx46 ($\alpha 3^{+/+}\alpha 8^{ki/ki}$ or $\alpha 3^{-/-}\alpha 8^{ki/ki}$) lenses are clear but smaller than wild-type lenses. [Gong et al., 2007]

terminal domain (D3Y and L11S) (Addison et al., 2006; Hansen et al., 2006), first transmembrane domain (V28M and F32L) (Devi et al., 2005; Jiang et al., 2003), E1 loop (W45S, P59L, and N63S) (Bennett et al., 2004; Ma et al., 2005; Mackay et al., 1999), second transmembrane domain (R76G, R76H, and T87M) (Burdon et al., 2004; Devi et al., 2005; Guleria et al., 2007), E2 loop (P187L and N188T) (Li et al., 2004b; Rees et al., 2000) and C-terminal domain (S380fs) (Mackay et al., 1999). Additionally, heteromeric channels formed by wild-type and mutant subunits of Cx46 and Cx50 connexins have been shown to inhibit the elongation of primary fiber cells and disrupt the formation of fiber cells in embryonic lens. This “gain-of-function” study conducted by Xia et al. not only reinforces the importance of lens fiber cell connexins in maintaining lens transparency, it suggests that heteromeric connexin proteins may have unique regulatory properties in addition to independent gating characteristics (Xia et al., 2006b).

Amino acid change	Location	Cataract type	Phenotype description	Family origin	Reference
D3Y	NT cytoplasmic loop	Zonular pulverulent	Progressive zonular pulverulent cataract	Hispanic	Addison, 2006
L11S	NT cytoplasmic loop	Ant-egg	Lamellar cataract with dense ant-egg like structures imbedded in the lens, primarily confined to the perinuclear layers and to lesser degree in the fetal nucleus	Danish	Hansen, 2006
V28M	First transmembrane domain	Variable	Variable cataract types like total, anterior capsular cataract with posterior cortical opacities in different individuals	Indian	Devi, 2005
F32L	First transmembrane domain	Nuclear pulverulent	Punctate opacities in the central zone of the lens limited to the embryonal nucleus	Chinese	Jiang, 2003
W45S	First extracellular loop	Nuclear	Progressive nuclear cataract	Chinese	Ma, 2005
P59L	First extracellular loop	Nuclear punctate	Coarse punctate opacities located in the central or nuclear region of the lens	American	Bennett, 2004
N63S	First extracellular loop	Zonular pulverulent	Coarse and granular opacities in the central zone of the lens. Fine dust-like opacities predominated in the peripheral zone of the lens	Caucasian	Mackay, 1999
R76G	Boundary of first extracellular loop and second transmembrane domain	Total	Total lens opacification	Indian	Devi, 2005
R76H	Boundary of first extracellular loop and second transmembrane domain	Nuclear pulverulent	Faint lamellar nuclear opacity surrounding pulverulent nuclear opacities, some with fine gold dots or haze and some with needle-like peripheral riders	Australian	Burdon, 2005
T87M	Second transmembrane domain	Pearl box	A bunch of white spots seen in the embryonal nucleus. The central white spots distributed in a radial manner. The space between the surface opacity and central white spots is optically empty. Surface opacity gives the appearance of a box while central white spots as of pearls in it.	Indian	Guleria, 2007
P187L	Second extracellular loop	Zonular pulverulent	Central dust-like opacity affecting the embryonal, fetal and infantile nucleus of the lens surrounded by snowflake-like opacities in the anterior and posterior cortical region of the lens	Caucasian	Rees, 2000
N188T	Second extracellular loop	Nuclear pulverulent	Progressive, central pulverulent opacity affecting the embryonal, fetal, and infantile nucleus of the lens	Chinese	Li, 2004
S380fs	CT cytoplasmic loop	Zonular pulverulent	Coarse and granular opacities in the central zone of the lens. Fine dust-like opacities predominated in the peripheral zone of the lens	Caucasian	Mackay, 1999

Table 1.2: Mutation spectrum of the Cx46 gene (GJA3) and associated phenotypes.

[Modified from Guleria et al., 2007]

Gap Junction Role in Cell Differentiation

Gap junction proteins Cx50 and Cx43 have both been studied and implicated for their apparent role in regulating various developmental processes, including lens development (Gong et al., 2007). However, very few publications have suggested a possible function for Cx46 in cellular differentiation independent of its inherent role in gap junction-mediated intercellular communication (GJIC). Cheng et al. describe lens development abnormalities in Zebrafish after disruption of Cx48.5 expression, an orthologue of human and rat Cx46 (Cheng et al., 2004b). In contrast, several studies have implied that the functional gap junction activity of Cx56, the chick orthologue of Cx46, is not required for lens fiber cell differentiation (Banks et al., 2007; Gu et al., 2003; Le and Musil, 1998; Reza and Yasuda, 2004). While Cx46 is primarily found in human lens fiber cells, it is also expressed in several tissues of the cancer-prone Min mouse model (Cruciani et al., 2004) and Schwann cells after peripheral nerve injury (Chandross et al., 1996). Several studies have also documented Cx46 in heart, liver, and retina tissues, though this unique connexin is retained as a non-functional monomer in the trans-Golgi compartment (Koval et al., 1997).

Both Cx43 and Cx50 have been implicated in the promotion of cell growth and proliferation. Neural progenitor cell (NPC) proliferation has been shown to be Cx43-dependant when induced with fibroblast growth factor-2 (FGF-2) (Cheng et al., 2004a). Cell surface receptors for FGF-2 initiate an intracellular signaling cascade involving p42/p44 mitogen-activated protein (MAP) kinases, also known as extracellular signal regulated kinases (ERK1 and ERK2), and has been demonstrated to phosphorylate Cx43 at S279 and S282 (Abdelmohsen et al., 2007; Warn-Cramer et al., 1996). Similarly, phosphorylation of Cx43 at S262, a PKC site that becomes phosphorylated in response to growth factor stimulation, has been reported to control DNA synthesis in cardiomyocytes independent of cell-cell communication (Doble et al., 2004). This control of growth activity is also seen in Cx45.6, the chick ortholog of Cx50, where overexpression of Cx45.6 is observed to induce differentiation in epithelial cells in spite

of mutational impairment of gap junction assembly (Banks et al., 2007).

As stated previously, the differentiation of epithelial cells to fiber cells initiates the degradation of Cx43. Gap junction turnover has been shown to be facilitated by lysosomal and proteasomal degradation (Berthoud et al., 2004). Immunofluorescent colocalization studies have discovered the presence of annular gap junctions, or cytosolic connexon dimers, which suggests that endocytosis is highly instrumental in gap junction degradation. Moreover, in GFP (green fluorescent protein)-connexin chimeras, internalization of gap junctional plaques occurs in the center of the plaque while newly formed channels are incorporated at the periphery of gap junctional plaques. After being internalized, gap junctions may fuse with lysosomes and be degraded by lysosomal enzymes (Gaietta et al., 2002). This has been evidenced in SKHep1 cells where a decrease in the rate of Cx43 degradation is observed after site-directed mutagenesis of the tyrosine at position 286 to alanine. Stable transfection of SKHep1 cells with this Cx43 mutant rendered gap-junctional plaques insensitive to treatment with lysosomal inhibitors and increased half-life from 2 to 6 hours. Tyr286 in Cx43 is part of a putative tyrosine-based sorting signal, YXX ϕ (ϕ = hydrophobic residue) corresponding to YKLV on the C-terminus, which suggests that Cx43 can be targeted by another protein for degradation through the lysosomal pathway (Thomas et al., 2003).

There is also significant evidence for the involvement of the proteasomal pathway in connexin/gap junction degradation. Increased interaction of Cx43 and ZO-1 has been associated with proteasome-regulated endocytosis in ventricular muscle tissue under pathological conditions (Barker et al., 2002; Girao and Pereira, 2007). Similarly, an increase in Cx43 levels and a decrease in Cx43 degradation have been observed after treatment with proteasomal inhibitors and lysosomal inhibitors in cardiomyocytes (cell type, BWEM) (Laing and Beyer, 1995; Laing et al., 1997). Lysosomal inhibitors, however, had no effect on Cx43 degradation levels in mouse sarcoma and fibroblast cells, but proteasomal inhibitors decreased these levels (Musil et al., 2000).

Furthermore, the tyrosine at position 286 in Cx43, which may be part of a tyrosine-based sorting signal for internalization, has also been hypothesized as being part of a PY (proline rich targeting) motif similar to the degradation motif in the amiloride-sensitive

epithelial sodium channel (ENaC) membrane protein. In ENaC, this PY motif is part of the sequence pPPxYxxL. Nedd4, a ubiquitin-protein ligase (E3 family), binds ENaC first and ubiquitinylates it, which leaves the PY motif to interact with adaptor proteins allowing endocytosis. The similarity between the ENaC PY motif and CT sequence in Cx43 (282-SPPGYKLV-289) raises the possibility that a similar possibility may be involved in Cx43 degradation (Berthoud et al., 2004; Lu et al., 2007).

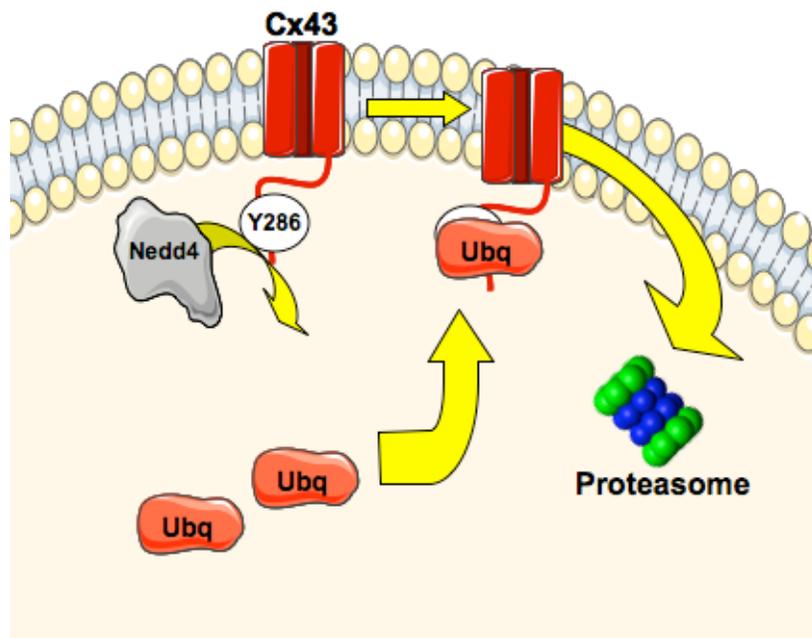


Figure 1.9:
Hypothesized Cx43 degradation in lens fiber cells. Nedd4 binds to a tyrosine sorting motif on Cx43-CT. Nedd4 recruits ubiquitin to Cx43 which targets it for degradation via the 26S proteasome.

Goals of Study

In this study, rabbit lens epithelial cell culture was used as an experimental model to demonstrate the role of connexin proteins in the differentiation process of the lens. The overexpression of exogenous Cx46 was facilitated to elucidate whether Cx46 has a function in fiber cell differentiation in addition to its utility as a gap junction protein. We have examined morphological changes, protein expression as determined by Western blot and RT-PCR, and protein colocalization by confocal immunofluorescence to determine the role of Cx46 in lens fiber cell differentiation.

CHAPTER 2 - Materials and Methods

Reagents

Dulbecco's modified Eagle's medium (low glucose), trypsin–EDTA, gentamicin and penicillin/streptomycin, were purchased from Invitrogen Corporation (Carlsbad, CA). Dithiothreitol (DTT), sodium fluoride (NaF), bovine serum albumin (BSA) were purchased from Fisher Scientific (Hampton, NH). Fetal bovine serum was purchased from Atlanta Biologicals (Norcross, GA). Phenylmethanesulfonyl fluoride (PMSF), protease inhibitor cocktail and ATX Ponceau S red staining solution were from Sigma-Aldrich (St. Louis, MO). Super-Signal West Femto Substrate kit with secondary anti-mouse or anti-rabbit IgG conjugated with horseradish peroxidase were purchased from Pierce (Rockford, IL). All electrophoresis reagents and protein molecular weight markers for electrophoresis were purchased from Bio-Rad Laboratories (Hercules, CA). Protein Assay dye (Bio-Rad Protein Assay) and Coomassie Brilliant Blue R-250 stain were purchased from Bio-Rad Laboratories (Hercules, CA). Whatman Optitran BA-S-85 nitrocellulose 0.45 μ m membrane was purchased from MidSci (St. Louis, MO). Protease Inhibitor Set II (ALLN, Epoxomicin, clasto-Lactacystin β -Lactone) was purchased from Calbiochem (EMD Biosciences, #539165).

Antisera

For confocal and Western blot immunolabeling we used polyclonal rabbit anti-rat Connexin 46 from US Biologicals (C7858-07A); polyclonal Anti-Ubiquitin rabbit pAb from Calbiochem (662099); mouse monoclonal anti-N-terminal-Cx43 IgG as from Fred Hutchinson Cancer Research Center (cat no. Cx43NT1) Seattle, WA; mouse anti- α -tubulin loading control (cat no. 32-2500) as purchased from Zymed-Invitrogen (San Francisco, CA); mouse monoclonal anti-Cx43 (against cytoplasmic C-terminus, cat no. MAB3068), and rabbit polyclonal anti-mouse Cx43 (against cytoplasmic C-terminus, cat no. AB1727), were purchased from Chemicon (Temecula, CA); mouse anti- β -actin loading control IgG (cat no. ab8226) was purchased from Abcam (Cambridge, MA); monoclonal mouse anti-Cx50 (amino acids 290 – 440) from Zymed Laboratories (South

San Francisco, CA); Alexa Fluor 466 and 568 goat anti-mouse or anti-rabbit IgG (H + L) conjugated secondary antibodies and ProLong Gold antifade reagent (cat no. P36934) were purchased from Molecular Probes (Invitrogen, Eugene, OR); Rabbit Anti- α A (SPA-221) and Anti- α B (SPA-223) crystallin polyclonal antibodies were obtained from Stressgen (BC, Canada); Mouse Anti- γ crystallin polyclonal were a kind gift from Larry Takemoto (Kansas State University, Kansas).

Cell Culture

The N/N 1003A rabbit lens epithelial cell culturing was performed as described previously (Wagner and Takemoto, 2001), using low glucose Dulbecco's Modified Eagle Media (DMEM) supplemented with 10% heat inactivated fetal calf serum (Atlanta Biologicals, Norcross, GA), 50 μ g/ml gentamicin (Invitrogen), 0.05 unit/ml penicillin, 50 μ g/ml streptomycin. The cells were grown at pH 7.2, 37 °C in an atmosphere of 5% CO₂ and 95% air for normoxic conditions (21% oxygen). Cells in culture were made hypoxic (5% oxygen) in a O₂/CO₂ dual controlled chamber (Bio-Spherix, ProOx Model, C21, Redfield, NY, USA) inserted into a temperature controlled incubator set at 37° C. The pH was monitored by use of a pH-sensitive dye included in the cell media. All experiments were conducted with cells near 80-90% confluency (6.0×10^6 cells/flask) except where stated differently. Lens tissues or cells were homogenized on ice in lysis buffer (50 mM TrisCl, pH 7.5, 1 mM EDTA, 1 mM EGTA, 2% Triton-100), with protease inhibitor cocktail (Sigma, St. Louis, MO, USA), and phosphatase inhibitor cocktail (Calbiochem, San Diego, CA, USA). After homogenization with a Dounce homogenizer, lysates were sonicated on ice and used immediately for SDS PAGE and subsequent immunoblotting.

DNA Constructs

The GFP-Cx46 plasmid was cloned in a Clontech pEGFP-N3 vector (BD Biosciences; C#6080-1). The holo Cx46 plasmid was derived from the rodent (*Rattus norvegicus*) sequence (GA# X57970). In addition, the EGFP (empty vector) was used as a control in stable transfection experiments.

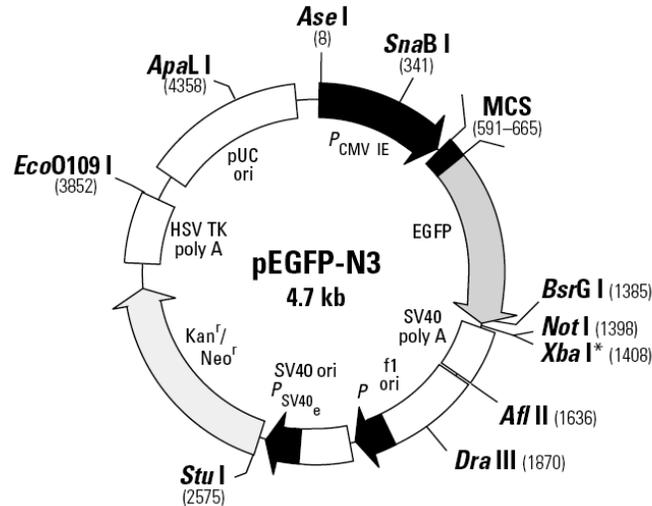


Figure 2.1: EGFP vector

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1 atgggagcact ggagcttcoct gggggcgctg ctggagaatg cgcaggagca ctctacagtc
61 atcggcaagg tgtggctgac cgtcctgttc atcttccgca ttctgggtgct gggggcgcca
121 gccgaggagg tgtgggtga tgagcagtcg gacttcaact gcaacacaca gcagccaggc
181 tgtgagaacg tctgctacga ccgtgccttc cccatctcgc acattcgctt ctgggcgctg
241 caaatcattc tcgtgtccac gccacccttc atctatctgg gccacgtgct gcacatcgtg
301 cgcattggag agaagaagaa agagcgggag gaggagctgc tgaggagaga caaccctcag
361 cacggccgtg gtcgggaacc aatgcgtaca gggagcccgc gggaccgcc actgcgcgat
421 gaccgtggca aggtgcgnat cgcaggcgcg ctgctgcgga cctacgtctt caacatcctc
481 ttcaagacgc tcttcgaagt ggggttcctc gcgggccagt actttctata tggctccag
541 ctgcagccgc tttaccgctg cgaccgctgg ccctgcccc aacacgtgga ctgcttcctc
601 tccaggccca cggagaagac catctttggt atcttcatgt tggctgtggc ctgtgcgtca
661 ctggtgctca acatgctaga gatttaccac ctgggctgga agaagctcaa gcaggaggtt
721 accaaccact tcaaccaga tgccctcaga gtcaggcaca agcccttggg cccctatcc
781 gaggccgcca actctggccc tcccagcgtc tccattgggt tgccacctta ttacacacac
841 cctgcctgtc ccacagtaca gggaaaggcc acagggttct ctggggcccc actgctacca
901 gcagacttca cagtgtgac cctaaacgat gcgcaaggca gaggccacc ggtcaagcac
961 tgcaatggcc accacctgac gacagagcag aactgggcca gcctaggggc agagccgcag
1021 actccagcca gcaagccctc ttcagcagcc tccagccctc atggccgcaa ggggtcact
1081 gacagcagtg gcagcagctt agaggagagt gccttgggtg tgacaccaga gggagagcag
1141 gctttggcga ccacagtgga gatgcactcc ccaccgttgg tctcctcctgga cccagaaagg
1201 tccagcaagt ccagcagcgg acgagccaga ccaggtgact tggccatcta g

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Figure 2.2: GFP vector Cx46 insert cDNA sequence. Rat Cx46 CDS obtained from www.ncbi.nlm.nih.gov genebank (X57970).

Western Blotting

Western blot analyses were carried out as previously described (Akoyev and Takemoto, 2007). N/N 1003A cells (6.0×10^6 cells/flask) were harvested in cold 1X phosphate buffered saline (PBS). A 12.5% SDS-PAGE gel containing 25 μ g of total cell protein was transferred to a nitrocellulose membrane (OPTI TRAN, Midwest Scientific, St. Louis, MO). The membrane was incubated for 12 h in primary antibody in 3% powdered milk in water at 4 °C. The membranes were then washed for 10 min, 3 times, in PBS. Mouse anti- α -tubulin and mouse anti- β -actin were used as loading control primary antibodies for all samples. Western blots were visualized with SuperSignal West Femto Substrate kit supplied with secondary anti-mouse or anti-rabbit IgG, conjugated with horseradish peroxidase (Pierce, Rockford, IL, cat no. 34095) followed by exposure to X-ray film (Midwest Scientific, St. Louis, MO). For quantitative analyses, X-ray films were scanned and western blot bands were digitized using UN-SCAN-IT software (Silk Scientific, Inc, Orem, Utah). Digitizing procedure consisted of counting of total pixel numbers in every designated band. These total pixel numbers were used as a measure of amount of proteins in every band.

Transfection

Transfection of N/N 1003A rabbit lens epithelial cells took place when the cells reached approximately 60% confluency. The cells were transfected using Lipofectamine 2000 (Invitrogen). Four μ g of the plasmid DNA and 0.1 mg of Lipofectamine in 250 μ l of serum and antibiotic free media were used for each transfection. The cells were incubated with the DNA plasmid and Lipofectamine for 24 h at 37 °C. After incubation, media containing twice the amount of fetal calf serum (20%) was added for 24 h. Following this incubation, the media was replaced with media containing 10% fetal calf serum. The transfected cells were selected with 1 mg/ml G418 (Research Products International) for 6 weeks and grown in half that concentration of G418 thereafter.

RT-PCR

Total RNA from N/N1003A lens epithelial cells was isolated using RNeasy Mini Kit (Qiagen) and 1.0 µg of total RNA was transcribed to first-strand cDNA using the OneStep RT-PCR System kit (Qiagen) according to the manufacturer's directions. The primer sequences for Cx43 were 5'-GAT GAG CAG TCT GCC TTT CGT-3' for the forward primer and 5'-CGT TGA CAC CAT CAG TTT GG-3' for the reverse primer. The temperature profile for Cx43 was 32 cycles of 30 s at 94 °C, 30 s at 50 °C, and 30 s at 72 °C. Positive controls were performed using β-actin cDNA. The primer sequences for β-actin were 5'-GAA ATC GTG CGT GAC ATT AAG-3' for the forward primer and 5'-CTA GAA GCA TTT GCG GTG GAC GAT GGA GGG GC-3' for the reverse primer. The PCR products were analyzed by 1.2% agarose gel electrophoresis. In negative controls, no DNA template was added to the reaction mixture.

Microscopy

N/N 1003A cells were grown in 6-well tissue culture plates at 5.0×10^2 cells per well (approximately 80% confluent) on 25 mm round glass coverslips. The cells were fixed with 2.5% paraformaldehyde, quenched with 50 mM Glycine, washed with PBS, permeabilized with 0.05% Triton-X100 in PBS for 30 min, washed, and blocked with 3% BSA for at least 1 h at room temperature. To identify target proteins, cells were simultaneously treated with mouse or rabbit primary antibodies directed against different target proteins in 3% BSA, overnight, at 4 °C with constant gentle shaking. After incubation with primary antibodies coverslips with cells were washed with blocking solution and stained with secondary Alexa Fluor-488 (green color) and/or Alexa Fluor-568 (red color) anti-mouse and anti-rabbit antibodies (Molecular Probes, Eugene, OR) for 2 h at room temperature. The coverslips were then washed in cold PBS, 2 times, and mounted onto slides using ProLong Gold antifade reagent. Live cells were viewed on a laser scanning confocal microscope, model Nikon C1 scanning confocal microscope with a 2-laser units system: air-cooled argon laser (454 nm) and He-Ne laser (543 nm). Optical sections (images) of immunolabeled cells were taken using Nikon Plan Fluor 40x/0.60 ELWD DIC oil objective, standardized pinhole (30 µm) and

gain settings. For qualitative analyses, 20 images per slide were saved at resolution 2048 × 2048 pixels (127.3 × 127.3 μm).

Statistical Analysis

All analyses represent at least triplicate experiments and are represented as Mean±SEM. The Student's t-test was used for all statistical analysis in this paper. A value of $p < 0.05$ was considered statistically significant.

CHAPTER 3 - Results

Cx46 overexpression system in N/N 1003A cell line

We introduced a GFP-Cx46 plasmid into RLECs that would functionally increase the expression (overexpression) of Cx46 levels in order to ascertain whether this increase had any notable effect in RLECs. Stable transfectants of the N/N1003A cell line expressing the GFP-Cx46 and EGFP plasmids were selected using 1 mg/mL G418 antibiotic. Confocal laser scanning microscopy (CLSM) was used to visualize successful transfection of the GFP plasmid through autofluorescence at 454nm (green) (Fig 3.1). The EGFP plasmid appeared diffuse and nonspecific throughout the micrograph (Fig 3.1A&C). Overexpressing transfectants with GFP-Cx46 plasmid (sCx46OE) localized on the membrane periphery in distinguishable plaques, which indicated that the vector construct successfully reports exogenous Cx46 localization (Fig 3.1B&D).

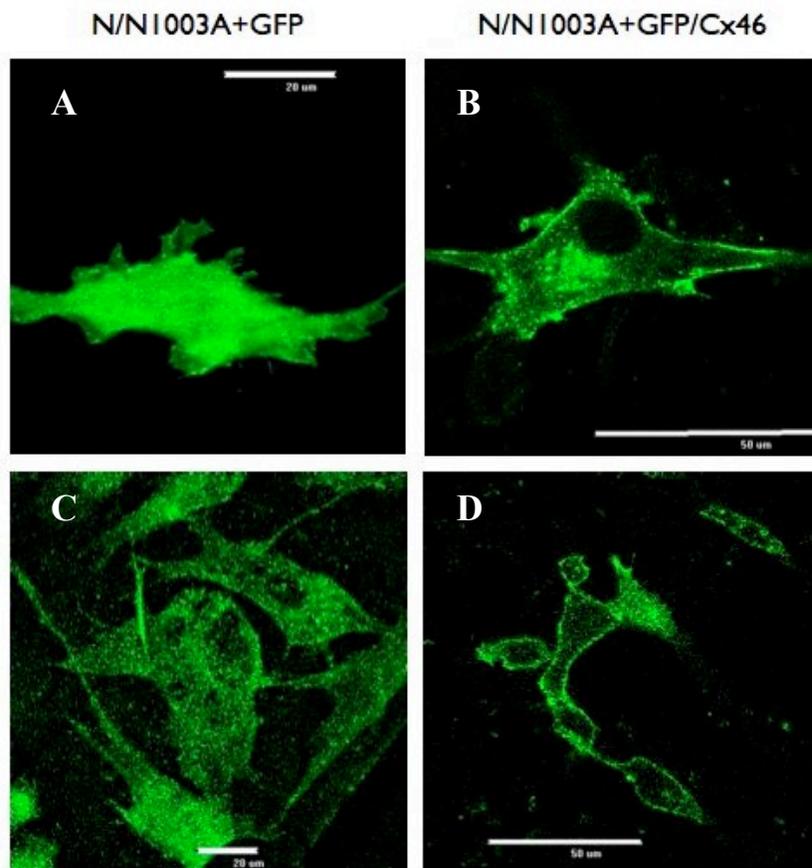


Figure 3.1: CLSM micrograph of EGFP and GFP/Cx46 transfected cells. A&C.Empty vector cells with diffuse GFP fluorescent report. **B&D.**Stable Cx46 transfected cells with punctate plaques and peripheral localization of GFP construct.

Western blot analysis of wild type (WT) and sCx46OE revealed a five-fold increase in Cx46 in transfected cells compared to WT (Fig 3.2 A&B). This dramatic increase also confirmed successful transfection of RLECs with the GFP-Cx46 plasmid.

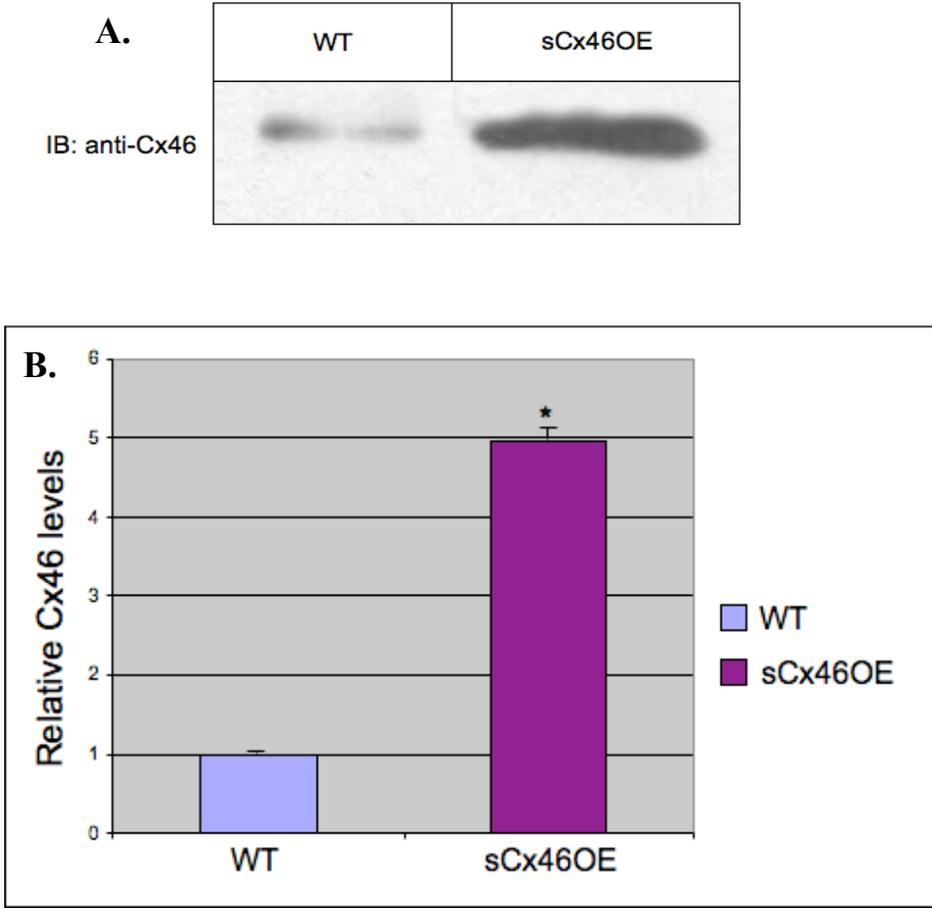


Figure 3.2: Cx46 levels in WT and sCx46OE N/N1003A cells. A. Western blot of N/N1003A WCH probed with Cx46 antibody. B. Blots were scanned and digitized. sCx46OE was measured against WT levels of Cx46. Error by SEM indicated by bars. * $p < 0.05$ by student's t test compared to WT-RLECs.

Overexpression of Cx46 in N/N 1003A cell line induced morphological changes

After passing the EGFP (empty vector) and GFP-Cx46 stably transfected N/N 1003A cells to 90% confluency, comparable morphological changes were evident. The empty vector GFP-N/N 1003A cells (Fig 3.3A) exhibited a more rounded shape and randomized organization, which was identical to control cells. The GFP-Cx46 stable N/N 1003A cells (Fig 3.3B), however, became more striated and elongated. Their cellular organization appeared more unidirectional and concerted. This apparent elongation of Cx46 overexpressing epithelial cells was indicative of fiber cell morphology, and may reflect induction of the differentiation process. This was not observed after overexpression of Cx50 (Fig 3.3C).

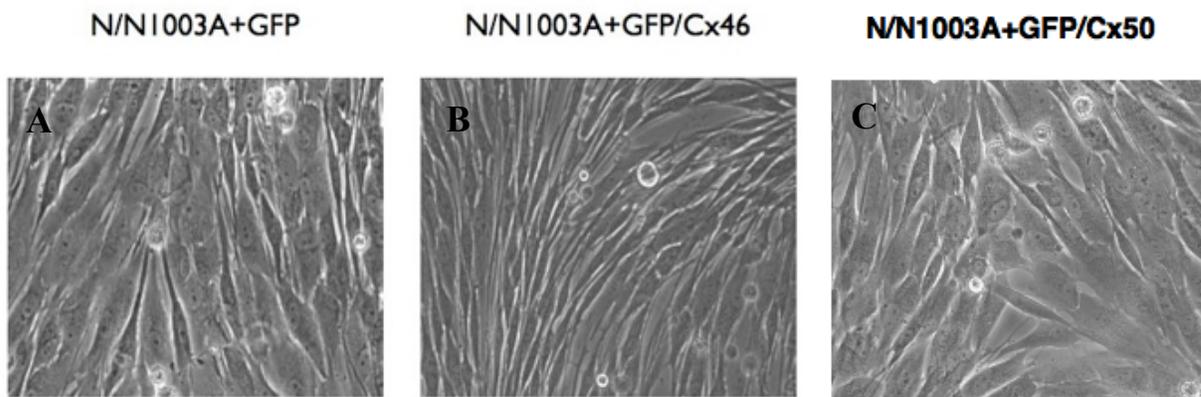


Figure 3.3: Morphology of empty vector and overexpressing RLECs. A.RLECs transfected with empty GFP vector. B.RLECs transfected with GFP-Cx46. Note shape change compared to EV and GFP-Cx50 expressing cells. C.RLECs transfected with GFP-Cx50 have no notable shape change compared to EV.

Overexpression of Cx46 caused a decrease in Cx43; Cx50 was not affected

To address whether overexpression of Cx46 affected other connexin isoforms in lens epithelial cells, Cx43 and Cx50 levels in both control N/N 1003A and stable GFP-Cx46 and EGFP transfected cells were determined by Western blot. Both Cx43 and Cx50, which are normally expressed in N/N 1003A control cells, were found in all three samples (Fig 3.4A). However, a detectable level of Cx43 was significantly reduced in the the Cx46 overexpressing N/N cells (Fig 3.4B). This change in Cx43 levels represented a $55\pm 2\%$ decrease compared to control N/N 1003A cells. The levels of Cx50 were not significantly altered by the overexpression of Cx46. Blots were scanned and analyzed for pixel intensity. Quantitative analysis was expressed as the ratio of sample intensity to loading control intensity.

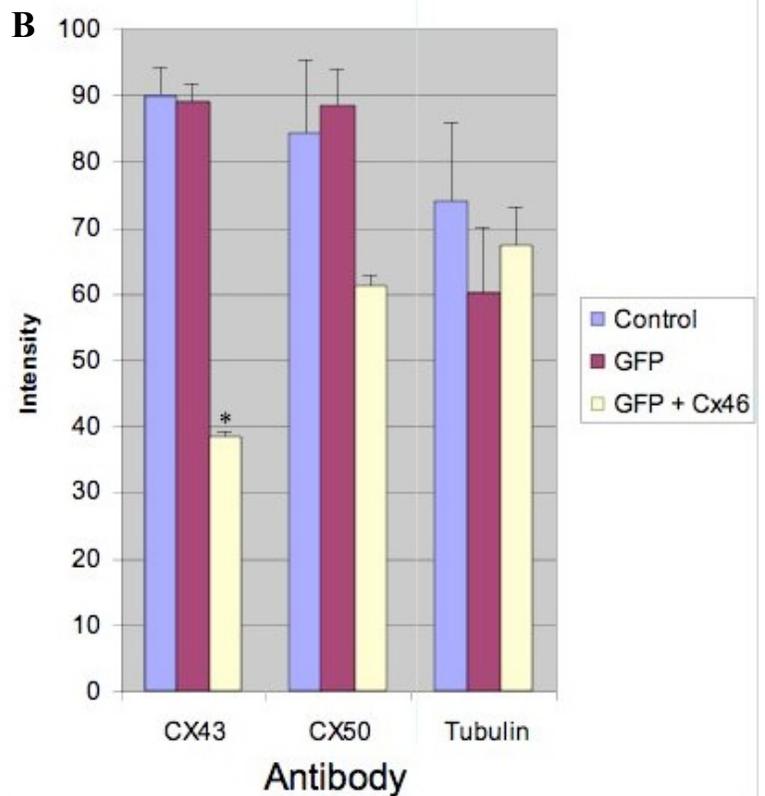
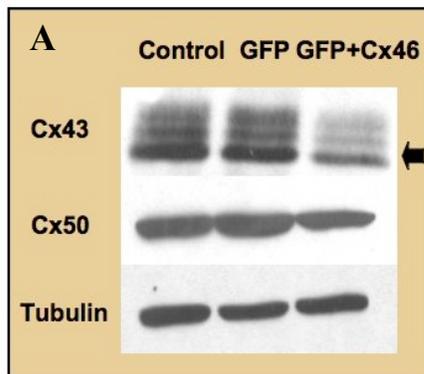


Figure 3.4: Cx43, Cx50, and tubulin protein levels in WT and experimental RLECs. A. Western blot of WCH probing for Cx43, Cx50, and tubulin protein levels. Arrow indicates decreased Cx43 levels in sCx46OE cells compared to WT and EV. B. Blots were scanned and digitized. * $p < 0.05$ by student's t test compared to WT in each treatment.

RT-PCR Analysis of Cx43 transcript in t/sCx46OE cells; Cx43 transcript was not altered.

In order to determine whether Cx43 degradation in Cx46 overexpressing N/N 1003A cells was pre- or post-transcriptionally initiated, Cx43 transcripts were measured in 1 ug of total RNA by RT-PCR (Fig 3.5A). N/N 1003A cells were transiently transfected with the GFP-Cx46 plasmid and harvested in 24, 48, and 72 hour time periods after transfection. RT-PCR confirmed that Cx43 transcript levels at all three time periods were normal (not significantly reduced) compared to control and stably transfected Cx46 overexpressing N/N 1003A cells (Fig 3.5B). These results indicate a post-transcriptional modification of Cx43 levels in the Cx46 overexpressing N/N 1003A cell line.

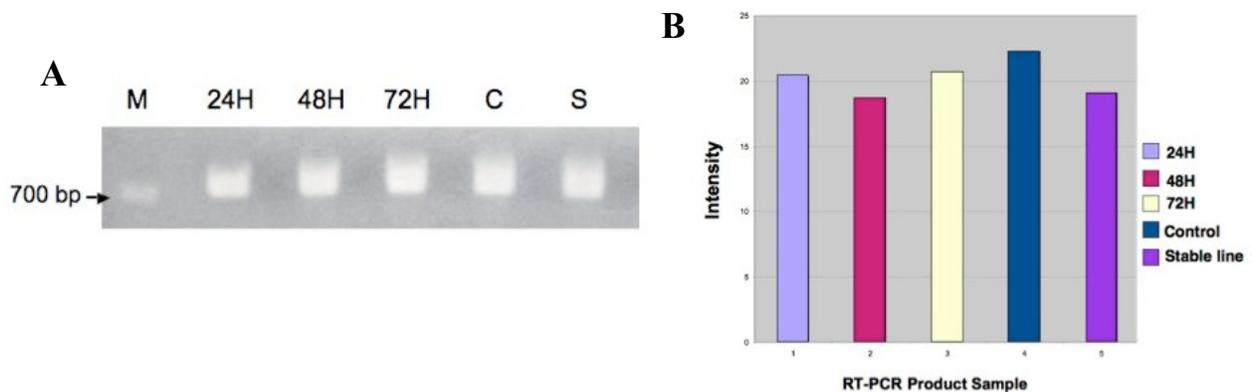


Figure 3.5: Analysis of Cx43 message transcript in RLECs. A. RT-PCR confirms that Cx43 cDNA levels are uniform in all transiently transfected samples (24H, 48H, 72H), WT (C), and stably transfected cells (S). B. Gel was scanned and digitized. All levels of Cx43 transcript were relatively equal.

Proteasome Inhibitors prevented loss of Cx43 levels in sCx46OE cells

To verify post-transcriptional modification of Cx43 levels in Cx46 overexpressing N/N 1003A cells, cells were treated with proteasome inhibitors for 4 hours before harvesting. Cx43 levels in cell lysates were then determined by Western blot (Fig 3.6A). Control and EGFP cells exhibited relatively normalized levels of Cx43 in both treated and untreated samples. N/N 1003A cells overexpressing Cx46 predictably showed a decrease in Cx43, which in turn was normalized by the proteasome inhibitors. These data show a comparable, if not inordinate, return of normalized Cx43 levels to Cx46 overexpressed N/N 1003A cells (Fig 3.6B).

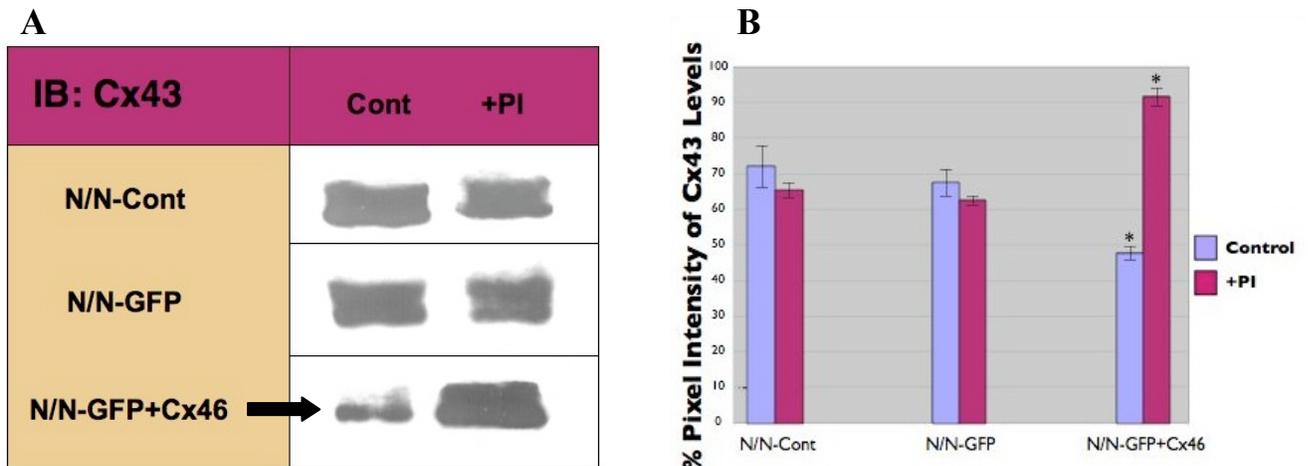


Figure 3.6: Proteasome inhibitors restore Cx43 levels in sCx46OE cells. A. Western blot analysis determined the Cx43 protein levels in WT, EV, and Cx46OE cells with and without treatment of proteasome inhibitors. Arrow indicates decreased Cx43 levels in nontreated sCx46OE cells compared to treated sCx46OE cells. B. Blots were scanned and digitized. $p < *0.05$ student's t test compared to untreated WT (control).

Ubiquitination of Cx43 was increased in sCx46OE cells

Proteasomal degradation may involve direct proteolysis or ubiquitin-mediated degradation of the targeted protein (Breusing and Grune, 2008). To determine if Cx43 degradation is initiated by ubiquitin modification, scanning confocal microscopy was used to visualize colocalized, immunolabeled Cx43 and ubiquitin. Control RLECs showed limited merge (yellow) between Cx43 (red) and ubiquitin (green) (Fig 3.7A-C). However, Cx46OE RLECs demonstrated pronounced colocalization of Cx43 and ubiquitin (Fig 3.7D-F). This data suggests that Cx43 degradation by proteasome is mediated by an active ubiquitin-conjugating system.

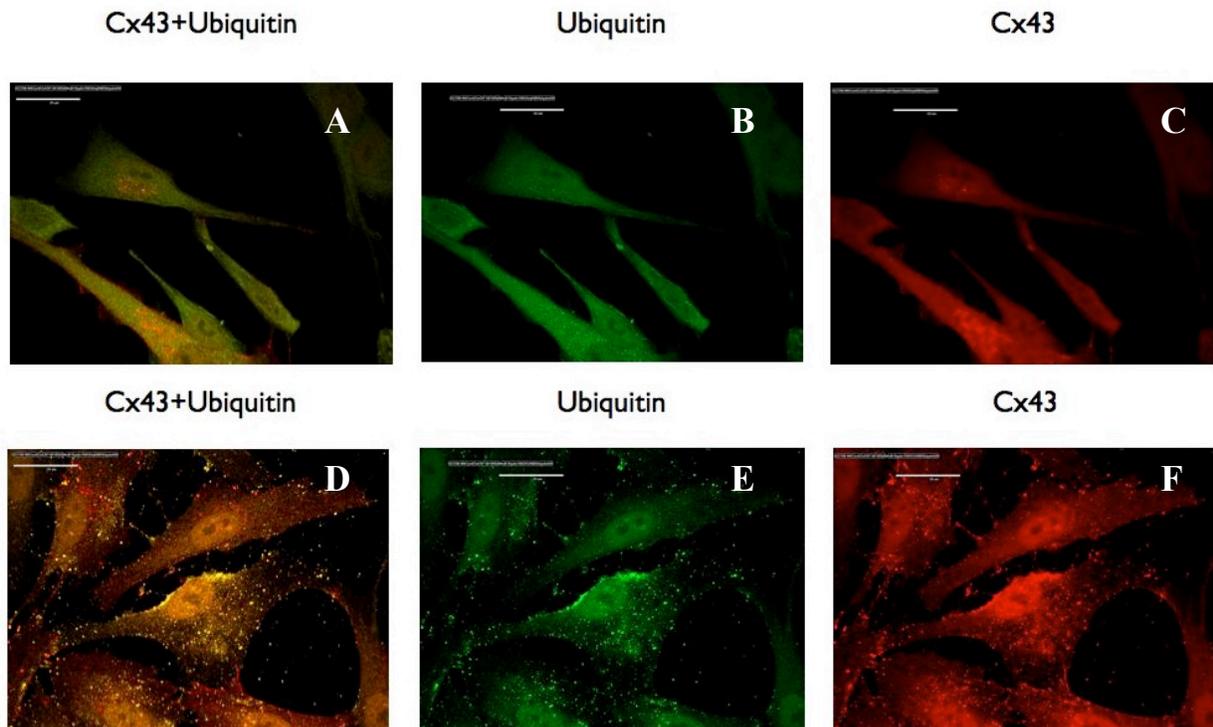


Figure 3.7: Confocal micrograph of Cx43 and ubiquitin colocalization in RLECs.

A. Cx43 and ubiquitin merge in WT cells. **B.** Ubiquitin locality in WT. **C.** Cx43 in WT. **D.** Cx43 and ubiquitin merge in sCx46OE cells. There is a notable increase in colocalized domains (plaques). **E.** Ubiquitin in sCx46OE cells. **F.** Cx43 in sCx46OE cells.

Hypoxia increases expression of Cx46 and decreases Cx43 in WT cells

There are aspects of differentiation such as crystallin expression and organelle degradation that have been speculated to be governed by the oxygen gradient in lens (Bassnett and McNulty, 2003). Since Cx46 expression and subsequent Cx43 degradation may also correspond with this hypoxic response we subjected WT RLECs to a time graduated hypoxic treatment. N/N1003A cells were placed in an incubator under 5% oxygen for up to 72 hours. Cx43 showed a notable decrease in 24-72 hours compared to zero hour (normoxic) conditions (Fig 3.8). Conversely, there appeared to be a spike in Cx46 expression after 24 hours, which correlated with a similar increase in HIF-1 α protein. These data may indicate that Cx46 is up-regulated as part of the hypoxia response pathway. Additionally, Cx43 may be degraded under low oxygen conditions as part of this survival mechanism.

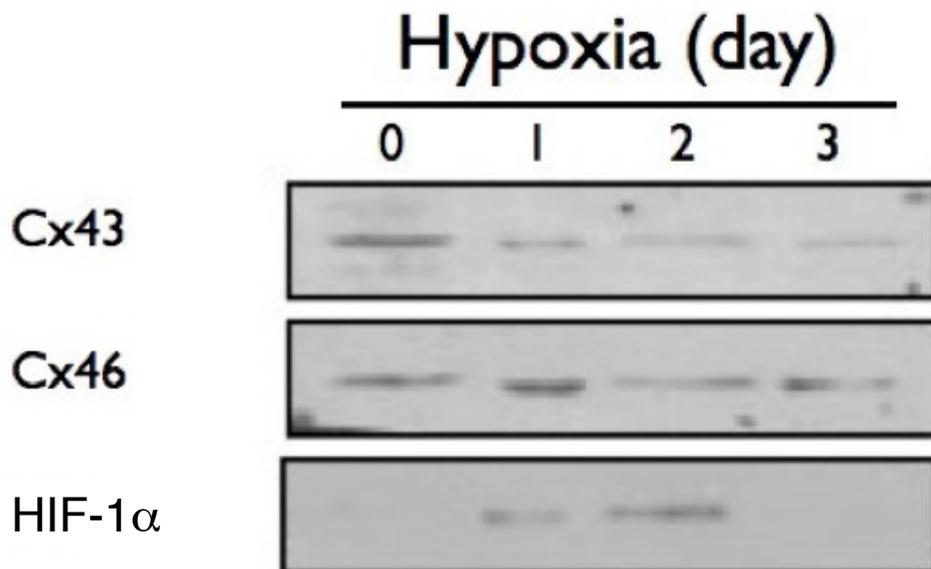


Figure 3.8: WT-RLEC connexin expression under hypoxia. Cx43 protein levels decrease after 24h in 5% oxygen. Cx46 levels increase after 24h, but subside after 48h. HIF-1 α increases after 24 and 48 hrs., but decreases after 72h.

CHAPTER 4 - Discussion

The developing lens serves as a highly accessible paradigm for the universal study of cell growth, epithelial function, and aging. However, the molecular basis for the differentiation process, a focal point of the developmental model, is not completely understood. In the present study, we have utilized a well-characterized, long-term lens epithelial cell culture to further investigate the function of gap-junction proteins in lens fiber cell propagation. The overexpression of Cx46 in the N/N1003A cell line appeared to induce the differentiation process as seen by the degradation of Cx43 and the morphological changes apparent in cell culture. This degradation of Cx43 is probably due to a ubiquitin-proteasomal pathway (UPP) initiated by the upregulation of Cx46. This activity suggests an additional role of Cx46 extraneous to its function as a gap junction protein.

The extraconventional role of gap junctions in lens cell differentiation has been contrastingly characterized as both essential and nonexistent. Le and Musil (Le and Musil, 1998) have shown that the inhibition of intercellular couplings with the gap junction blocker 18-glycyrrhetic acid has no effect on lens epithelial-fiber differentiation in primary lens cells. However, this study does not address the necessity of connexin protein expression in differentiation; it only concludes that gap junction mediated intercellular communication is not needed for the induction of the differentiation process. There have also been reports implicating the expression of Cx45.6, the chick ortholog of mammalian Cx50, as being involved in lens epithelial-fiber cell differentiation (Banks et al., 2007). Banks et al. demonstrated differential induction occurs in Cx45.6 overexpressing chick lens epithelial culture, even after loss-of-function mutations (D47A and P88S), which suggests that this role in mediating cell differentiation is independent of Cx45.6's function as a gap junction. It was speculated by Banks et al. that mediation of differentiation was realized through unique binding sites on the cytosolic C-terminus of Cx45.6. However, experiments in the same study did not find promotion of differentiation upon overexpression of Cx56, the mammalian Cx46 ortholog, in chick primary lens culture. Though these findings may seem contradictory to the conclusions

drawn in the present study, several comparative factors should be taken into account. Firstly, Banks et al. used a primary lens culture derived from chick lens, which expresses gap junction proteins homologous to the mammalian proteins. While the orthologous sequences are fairly conserved, there is room for possible discrepancies in connexin behavior that span the interspecies gap. Secondly, and more probable, the comparative study utilized differentiation markers that were not used in the present study. MIP (or Aquaporin0) and filensin were used as primary differentiation markers, and visual confirmation of lens cell differentiation was quantified by lentoid body formation in culture. The present study, instead, relied upon the investigation of endogenous gap junction expression and morphological changes also associated with differentiation in mammalian lens. The N/N1003A cell line does not spontaneously form lentoid bodies, which may indicate that the two comparative models are driven to completely different stages of differentiation and are indeed independently observed phenomena.

While Cx46 is a primary component of cortical fiber cell homeostasis in the lens, its protein expression in the lens is atypically unique. Transcript of Cx46 has been found in virtually all mouse tissues (Anderson et al., 2005), however, actual protein expression of Cx46 has only been reported in lens, cancer tissue (Cruciani et al., 2004; Koval et al., 1997), ischemic and damaged tissue (Chandross et al., 1996; Kamphuis et al., 2007), and germ tissue (Paul et al., 1991). The localization of Cx46 in these atypical tissues is concomitant with its specialized function in the lens. A low sensitivity to pH (Eckert, 2002) and its ability to preferentially modulate gating properties upon formation of heterotypic channels enables the proliferation of intercellular communication despite hypoxic and acidic conditions at the interior of the lens (Hopperstad et al., 2000). A corollary increase in expression of HIF1- α with Cx46 in epithelial cells under hypoxic conditions may indicate that Cx46 bolsters cell survival in adverse microenvironments. This would also explain variable promoter usage and alternative splicing in Cx46 mRNA transcripts. Cx46 mRNA has been found to exist in multiple tissues with two different 5' UTRs (Anderson et al., 2005). Type A-Cx46 mRNA (Gja3A) was found in all mouse tissues except bladder and heart, however, type B-Cx46 mRNA (Gja3B) was primarily

located to lens, uterus, and embryo. Additionally, Cx56, has been found to exist *in vivo* within two kinetic pools which exhibit differing half-lives (Berthoud et al., 1999). Since these two pools correlate with binary phosphorylated forms of Cx56, it is possible that Cx46 depends on phosphorylation to regulate protein stability and post-translational response. Differing regulatory promoters and translational efficiencies of Cx46 transcripts and post-translational modifications may be developmental adaptations to both chronically and acutely hypoxic tissue conditions. That is to say, naturally hypoxic tissues, such as the lens and malignant tumors, would have need to express Cx46 under normal, steady-state conditions, whereas highly vascularized tissue, such as heart, may need to quickly express a responsive form of Cx46 to avoid damage. Needless to say, the exact role of Cx46 in this association remains to be determined.

Both Cx50 and Cx43 have been identified to possess functions that transcend their inherent roles as gap junctions. Cx50 is necessary for normal eye growth (White et al., 1998) and its ortholog may induce differentiation in chick lens models in both wild-type regardless of its ability to form functional homotypic channels (Banks et al., 2007). The more ubiquitous Cx43 has been shown to directly interact with ASK1, an apoptosis pathway kinase, which subsequently rescues C6 cells from hydrogen peroxide-induced apoptosis (Giardina et al., 2007). Additionally, Cx43 has been implicated in protection of ischemic preconditioning without functional gap junction activity (Li et al., 2004a; Lin et al., 2008). When sequence homologies between intrinsic connexin isoforms are taken into account, these findings support the possibility that Cx46 also possesses a nonconventional role in signal transduction conferred by its ability to interact with other proteins.

The transition of epithelial cell to fiber cell is evidenced by several biomolecular rearrangements including the reorganization of centrosomal markers (Dahm et al., 2007), actin cytoskeleton (Weber and Menko, 2006), and filensin (Ong et al., 2003). Furthermore, zonula occludens-1 (ZO-1), a scaffolding/tight junction protein, has been shown to affect gap junction function, including Cx50 and Cx46 (Nielsen et al., 2001) by way of direct interaction. Additional findings have suggested that gap junctional association with structural and regulatory proteins may affect an array of cellular

properties, including differentiation (Hervé et al., 2007). Both Cx46 and Cx50 exhibit differential physiological and gating properties that may be attributed, in part, to their unique C-terminal sequence. It is likely that these novel sequences contain binding domains that recruit regulatory factors that initiate the differentiation process. This hypothetical mechanism would, indeed, explain the role of Cx46 in fiber cell differentiation; it would not, however, fully account for the induced degradation of Cx43.

We have confirmed, with immunofluorescent confocal imagery and treatment with proteasomal inhibitors, the degradation of Cx43 via a ubiquitin-proteasomal pathway (UPP) upon the induction of differentiation. It has been shown that UPP components are upregulated and redistributed throughout the differentiating cell (Girao, 2005). Furthermore, site-directed mutagenesis of various C-terminal sites in lens connexins show decreased degradation when compared to wild-type. A Cx50 phosphorylation site mutant, Cx50P88S, which is associated with formation of cataracts accumulates in transfected cells and shows decreased degradation (Berthoud et al., 2003). Similarly, a decrease in Cx43 degradation is observed after mutation of a residue, not known to be phosphorylated, on a tyrosine-based sorting signal (YXXN) at position 286 (Thomas et al., 2003). It could be inferred that differentiation of epithelial cells and the subsequent redistribution of ubiquitin pathway machinery initiates the phosphorylation or signal processing of a Cx43 C-terminal site which in turn targets the protein for proteasomal degradation. In association with this hypothetical model, we also submit the observation that differentiating epithelial cells (overexpressing Cx46) which were treated with proteasomal inhibitors showed a dramatic increase of Cx43 levels compared to wild-type and empty vector cells treated in the same manner (Fig 3.6A). This observation may be explained by a disruption of steady state connexin levels (connexin synthesis minus connexin degradation) upon differentiation. Whereas changes in Cx43 synthesis are normally masked by increased proteasomal degradation in differentiating cells, they become evident when the proteasome is inhibited. This explanation, however, is contradicted by the lack of significant difference in Cx43 mRNA levels in stable Cx46OE cells as determined by RT-PCR. More probably, the differing levels of PI-treated wild-type Cx43 and experimental Cx43 reflect differing degradation pathways. Both

lysosomal and proteasomal degradation pathways have been observed in the stabilization of Cx43 (Berthoud et al., 2004; Laing and Beyer, 1995; Severs et al., 1989). However, the response of Cx43-expressing cells in differing tissue to lysosomal or proteasomal inhibitors is greatly varied (Musil et al., 2000). To assume that cuboidal epithelium in the lens has different stabilization mechanisms than fiber cells is not unfathomable. Further investigation of the RLEC-Cx46OE differentiation model may serve to clarify these assumptions in the future.

The overexpression of Cx46 in RLECs appeared to invoke an augmentation of ubiquitin colocalization with Cx43 (Fig 3.7D), which confirms that the UPP is involved with the turnover of Cx43 in differentiating cells. The ubiquitinylation of Cx43 has been speculated to be controlled through a MAPK pathway, which recruits E3 ubiquitin ligase, Nedd4, to a PY tyrosine-based sorting motif (pPPXYxx ϕ) on the C-terminus. This process is regulated by phosphorylation modification of nearby phosphorylation sites (Berthoud et al., 2004). The PY motif found in Cx43 (SPPGYKLV) has an analogous region in Cx46, however this region does not have a proximal serine or lysine phosphorylation site (LPPYYTHP). Mutational studies have targeted Y286 and V289 as key sites in the regulation of Cx43 stability, which control degradation through the proteasomal or lysosomal pathway respectively (Thomas et al., 2003). Ubiquitin is covalently conjugated to proteins at the ϵ -NH₂ group of a lysine residue on the substrate protein (Leithe and Rivedal, 2007). Cx43 has many of these residues, however, there is a strategic site within the PY consensus domain at residue 287 that contains such a lysine. Conversely, the PY domain on Cx46 does not have a similar lysine residue. It is our hypothesis that Cx46 induces ubiquitinylation and degradation of Cx43 by binding Nedd4 and transporting it to junctional plaques on the plasma membrane that contain Cx43. Nedd4 is subsequently transferred to the PY motif on Cx43 via a phosphorylation event, which in turn recruits the ubiquitin tag for degradation by the 26S proteasome.

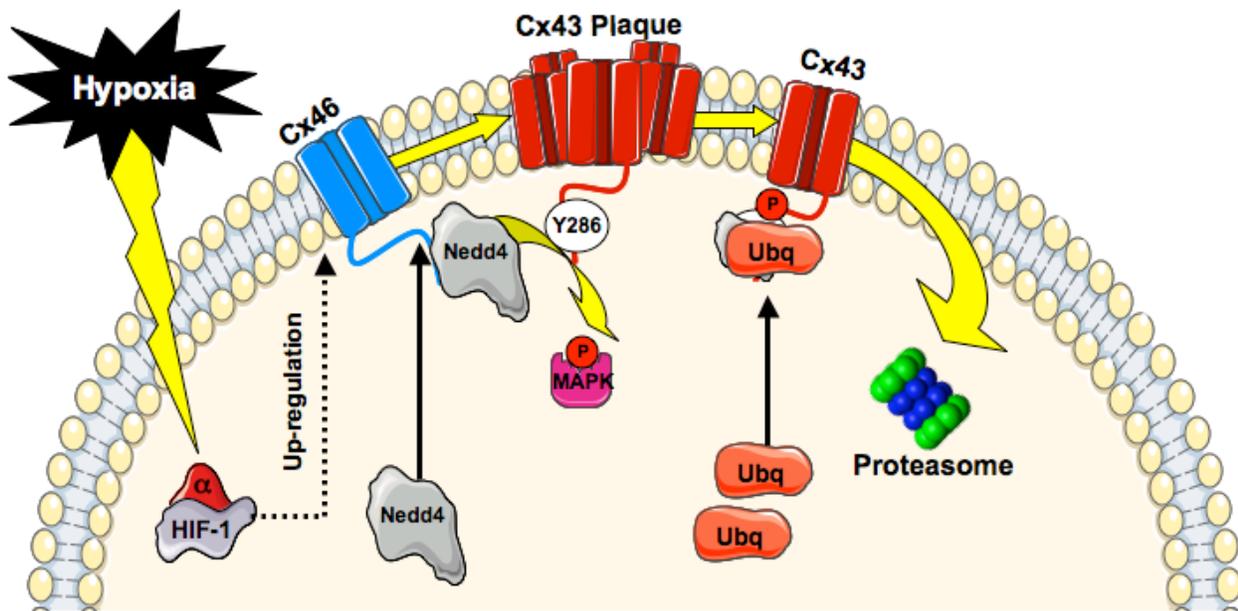


Figure 4.1: Cx46 induces the degradation of Cx43. Hypoxia activates HIF-1 α which up-regulates the expression of Cx46. Cx46 recruits Nedd4 at the C-terminus (CT), but is not ubiquitinated. Cx46 colocalizes with Cx43 plaques and transfers Nedd4 to Cx43 during a phosphorylation event. Nedd4, a ubiquitin ligase, tags Cx43 with ubiquitin and targets the protein for degradation by the 26S proteasome.

The future direction of this work involves investigating the role of Cx46 in tumorigenesis and hypoxic response. Breast tumors, which require a hypoxic state for enhanced metastasis, have been observed to preferentially express Cx46. The projected hypothesis links Cx46 expression to cancer cell survival under hypoxic conditions. This work will also include further elucidation of the Cx43 turnover machinery, which will test the Cx43 degradation model disseminated in this thesis. An innovative investigation into the role of Cx46 in breast cancer propagation will have a substantial impact on pharmaceutical development in the area of cancer treatment.

Conclusions

1. Overexpression of Cx46 (sCx46OE) in rabbit lens epithelial cells (RLECs) induces differentiation as indicated by morphological resemblance to fiber cells and the degradation of Cx43.
2. Degradation of Cx43 in sCx46OE cells does not cause a decrease in message transcription, but does initiate the ubiquitinylation of Cx43.
3. Hypoxia propagates the degradation of Cx43 and the concomitant up-regulation of Cx46 in WT-RLECs.

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