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Analysis of the Hemichannel properties of Cx26 mutants causing Keratitis-Ichthyosis-Deafness Syndrome

A Thesis Presented

by

Pallavi V Mhaske

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The Graduate School

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We, the thesis committee for the above candidate for the
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This thesis is accepted by the Graduate School

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Abstract of the Thesis

Analysis of the Hemichannel properties of Cx26 mutants causing Keratitis-Ichthyosis-Deafness Syndrome

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Keratitis-Ichthyosis-Deafness (KID) Syndrome has been linked with various mutations of the Cx26 encoding GJB2 (Gap Junction β2) gene. Each mutation was associated with symptoms and severity of varying degree in both adult and neonatal patients. Here, the hemichannel activity of two recently discovered mutations of GJB2 – the A88V and D50A mutants found in patients of KID syndrome were examined using *Xenopus* oocytes. The data recordings show that while the oocytes were able to express both the wild type and mutant Cx26 genes, the hemichannel activity of the mutants was much greater. This increase in hemichannel activity led to the lysis of the oocytes within 24 hours of protein expression. The presence of increased levels of extracellular calcium led to better survival of the A88V and D50A injected oocytes delaying cell death. Although the exact molecular mechanism of disease progression is not known, the analysis suggests that mutant hemichannels may play a role in the patho-physiology of skin abnormalities characteristic of KID syndrome.

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List of Abbreviations

BPS	Bart-Pumphrey Syndrome
Ca^{2+}	Calcium
$CaCl_2$	Calcium chloride
cAMP	Cyclic adenosine monophosphate
cGMP	Cyclic guanine monophosphate
CL	Cytoplasmic Loop
C-terminal	Carboxy terminus
Cx26	Connexin 26
Cx30	Connexin 30
Cx32	Connexin 32
Cx46	Connexin 46
Cx50	Connexin 50
dsDNA	double-stranded DNA
EC	Extracellular loop
Ga	Gauge
GJB2	Gap Junction β2
H_2O	Water
HEK-293	Human Embryonic Kidney 293 cells
HID	Hystrix-like Ichthyosis Deafness syndrome
IACUC	Institutional Animal Care and Use Committee
IP_3	Inositol triphosphate
K+	Potassium
KCl	Potassium chloride

kDa	Kilodalton
KID	Keratitis-Icthyosis-Deafness
MB	Modified Barth's Medium
mm	Millimeter
mM	Millimolar
mRNA	messenger ribonucleic acid
mV	Millivolt
nA	Nanoamperes
Na^+	Sodium
Na_2CO_3	Sodium bicarbonate
NaOH	Sodium hydroxide
nM	Nanomolar
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PPK	Palmoplantar Keratoderma
RNA	Ribonucleic acid
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SNHL	Sensorineural Hearing Loss
TM	Transmembrane domain
VS	Vohwinkel Syndrome
µg	Microgram
µM	Micromolar

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I. INTRODUCTION

Gap Junctions in the regulation of keratinocytes:

The human skin is divided into the dermis and epidermis. The epidermis is made up of skin cells or keratinocytes which are further sub-divided into the stratum basale, the stratum spinosum, the stratum granulosum and the uppermost stratum corneum layers. Maintaining a uniform epidermal thickness requires the growth and differentiation of these keratinocytes to be tightly regulated. Gap junction mediated cell communication has been shown to play a role in this regulation (Buultjens et al., 1988). Gap junctions are responsible for the physiological processes of ionically coupling cells via the regulated exchange of ions (Ca^{2+}, Na^{+}, K^{+}) between neighboring cells (Veenstra et al., 1994), a process vital for the normal functioning and existence of cells.

These gap Junction channels are formed by the alignment of two hemichannels from the plasma membranes of adjacent cells in the extracellular space. A single hemichannel or a connexon is in-turn formed by the oligomerization of six connexin protein subunits. There are at least 20 different connexin genes that are expressed throughout the body in unique patterns in different cell types (Evans and Martin, 2002). This heterogeneous expression allows hemichannels to be formed from mixtures of connexin sub-units, resulting in gap junctions that are either homomeric, i.e of the same connexin or heteromeric, made

of different connexins (Figure 1). The composition of these channels is significant as this determines how a particular gap junction will modulate biochemical coupling, or how the sharing of larger metabolites and nutrients such as cAMP, cGMP and IP_3 occurs between cells (Goldberg et al., 1999; Valiunas et al., 2002). Each connexin has a cytoplasmic amino and carboxyl termini that are thought to mediate channel voltage gating (Bennett et al., 1993; Peracchia, 2004; Purnick et al., 2000) and pH sensitivity (Eckert, 2002; Peracchia, 2004) respectively. In addition to these termini each connexin gene contains four transmembrane domains (TM1-TM4) that are joined by a single cytoplasmic loop (CL) and two extracellular loops, EC1 and EC2. The extracellular loops are hypothesized to play a role in the docking of connexons to form a complete gap junction (Bukauskas et al., 1995; Foote et al., 1998) (Figure 1). Recent studies have shown that hemichannels also may play a role in maintaining homeostasis under different physiological conditions (Goodenough and Paul, 2003; Saez et al., 2005).

Connexin mutations:

It has been seen that the mutation, complete removal, or replacement of specific connexins can lead to physiological defects and disease (Gerido and White, 2004; White and Paul, 1999; Willecke et al., 2002). The X-linked Charcot-Marie Tooth disease (CMTX) was the first connexin related disease discovered in humans. This disease occurred due to mutations in the Cx32 gene (Bergoffen et al., 1993). Subsequently, mutations in either Cx46 or Cx50 were found responsible for autosomal dominant cataracts (Mackay et al., 1999;Shiels et al., 1998). Mutations in genes encoding connexins have been observed in a diverse set of hereditary diseases with pathology of the epidermis and cochlea, two organs where connexins and gap-junctions are vital for normal functioning. The most common connexin related disease is Sensorineural hearing loss (SNHL). This may be classified as syndromic deafness when associated with skin pathology and other ectodermal symptoms or non-syndromic when no associated pathology is found. An example of such a connexin commonly linked to SNHL is the Connexin-26. The gene encoding this connexin-26 protein is the GJB2 (Gap Junction β2) gene that is normally present in all

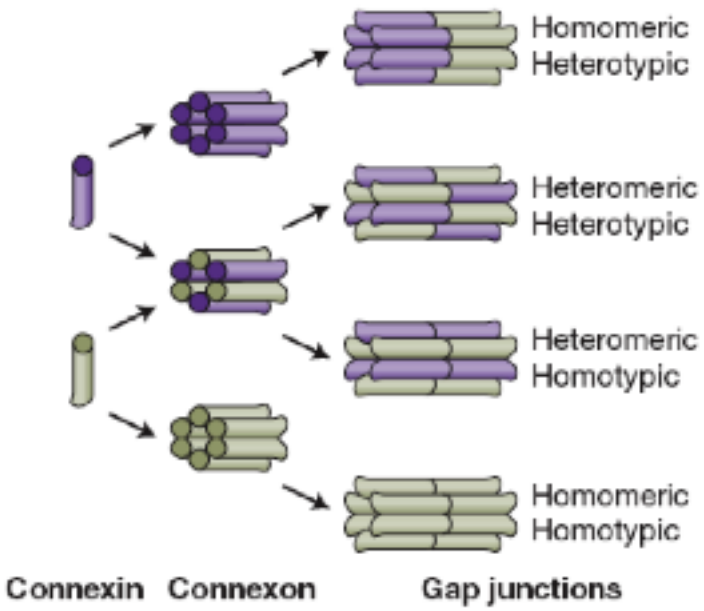


Figure 1. Connexin/Connexon channel formation. The Diagram shows how connexons (hemichannels) form at cell membranes and the terminology used to describe the different types of combinations. Formation of connexin proteins into hemichannels and intercellular channels. Connexons may be homomeric or heteromeric, and gap junction channels may be homotypic or heterotypic. (Lee and White, 2009)

locations in which cochlear gap junctions are present (Kikuchi et al., 1995). Studies have also shown that when normal human skin is exposed to retinoic acid there is an increase in keratinocytes correlating with a relative induction of Cx26. The high prevalence of Cx26 mutations in human population has made genetic testing routine in cases of pediatric hearing impairment (Apps et al., 2007; Smith, 2004; Tranebaerg, 2008). On analysis of the Cx26 topology it was seen that whereas non-syndromic deafness is associated with mutations of Cx26 throughout the coding region, syndromic mutations causing skin disease are predominantly present in the amino terminus and first extracellular domain (Figure 2) (Lee and White, 2009). It has also been seen that Cx26 mutants causing skin disease and deafness are due to single amino acid changes that result in an autosomal dominant mutation. Skin disorders that are known to be associated with dominant Cx26 mutants are Vohwinkel Syndrome (VS), Bart-Pumphrey Syndrome (BPS), Palmoplantar Keratoderma (PPK), Hystrix-like Ichthyosis Deafness syndrome (HID) and Keratitis-Icthyosis-Deafness (KID) syndrome.

Role of Connexin-26 mutants in Keratitis-Ichthyosis-Deafness:

Keratitis-Ichthyosis-Deafness (KID) syndrome is a disease affecting the corneal epithelium, the epidermis of the skin and the cochlea. Burns first documented a patient suffering from this triad of symptoms in 1915 and Skinner coined the acronym 'KID' syndrome in 1981. This is a congenital disease with patients typically displaying skin changes that range from a mild to severe presentation that persist with time in addition to SNHL (Cushing et al., 2008). Additional symptoms include palmoplanter keratoderma, dental abnormalities, dystrophic nails and scarring alopecia (Arndt et al., 2010; Koppelhus et al., 2011). The skin lesions may spread throughout the body leading to a disruption of its normal function as a protective barrier therefore causing an increased susceptibility to muco-cutaneous infections and carcinoma (Grob et al., 1987; Nazzaro et al., 1990; Szymko-Bennett et al., 2002). Keratitis characterized by corneal epithelium thickening causes visual impairment and this coupled with hearing loss leads to complex communication problems. Being a rare disease most cases are sporadic, however an

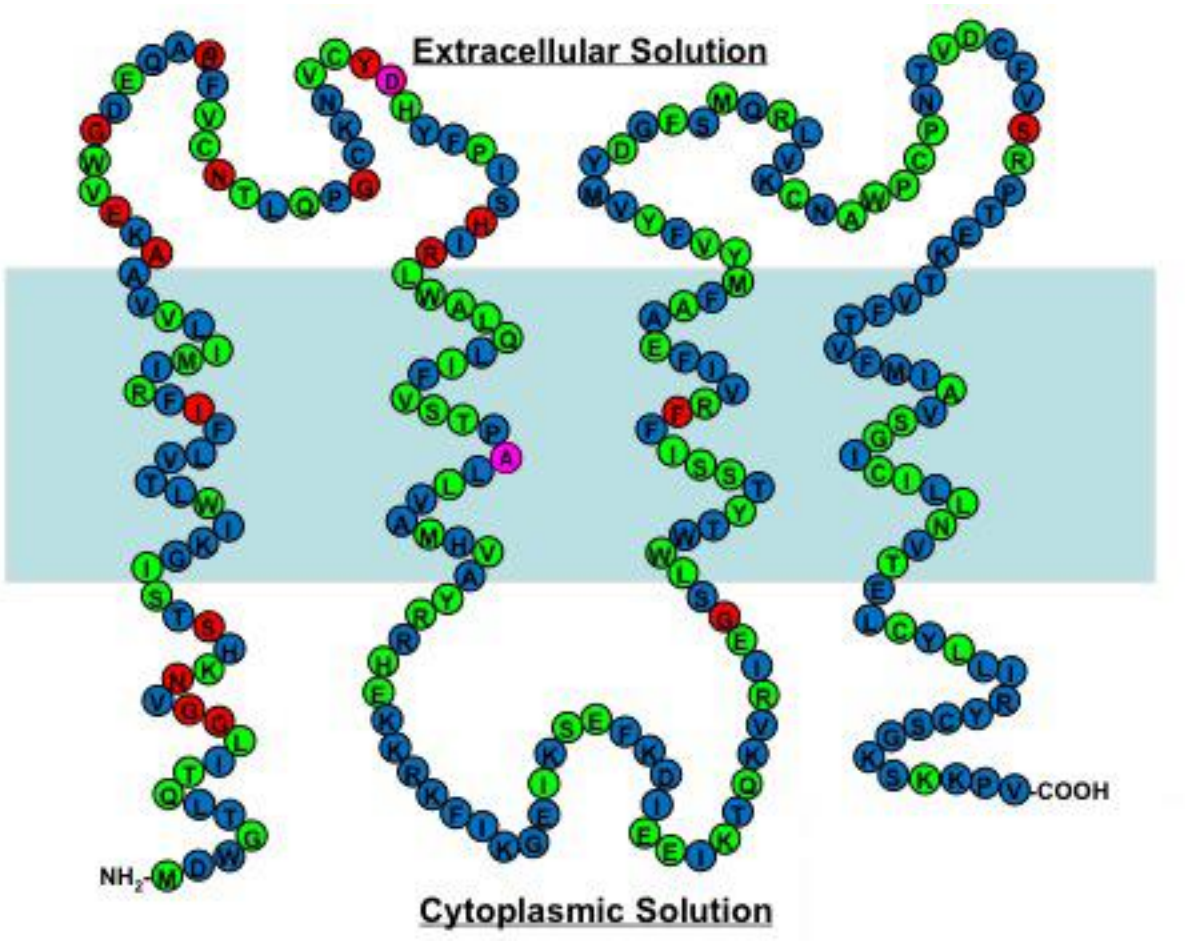


Figure 2. Connexin-26 topology and mutated residues. This illustration displays the location of amino acid residues in Cx26 relative to the membrane. The D50A mutant is seen in the first extracellular loop and the A88V mutation is present in the inter-membrane region indicated with Purple residues. Green amino acids are mutated residues found in non-syndromic deafness and can be seen throughout the protein. Red residues represent amino acids mutated in syndromic deafness, which are generally found within the N-terminus and first extracellular loop. Blue residues are not mutated. (Lee et al., 2009)

autosomal dominant inheritance (in 30% cases)(Jonard et al., 2008; Kelly et al., 2008) has been observed (Arndt et al., 2010).

Dominant mutations in the GJB2 gene, Cx26 cause KID syndrome. Previously, the G12R, N14K, D50N, D50Y and the G45E mutants of Cx26 found in KID syndrome have displayed aberrant hemichannel properties (Gerido et al., 2007; Lee et al., 2009). Recently the A88V, and D50A mutants have been discovered in neonatal and adult patients presenting with KID syndrome.

A88V mutant:

The GJB2 mutation caused by the replacement of Alanine by Valine at position 88 (A88V) has been recently reported by Koppelhus et al., 2011 in a premature neonate diagnosed with KID syndrome with infectious tendency leading to fatality. The neonate presented with thick hyperkeratotic plaques seen on the scalp and extensor side of the extremities seen in KID syndrome (Koppelhus et al., 2011).

D50A mutant:

The D50A mutant caused bilateral severe SNHL with KID syndrome in a neonate. The mutation was a heterozygous missense mutation resulting in a single amino acid change from aspartic acid to alanine at nucleotide position 149 of the cDNA. It was hypothesized that since a hydrophilic aspartic acid was replaced by a hydrophobic alanine this novel mutation may alter protein function. When the genomic DNA of both parents was analyzed no familial mutation was seen confirming the de novo nature of this mutation (Cushing et al., 2008).

Experimental goals

The goal of this research project is to understand how the A88V and D50A mutations of Cx26 affect hemichannel properties. The characterization of these pathological mutations will help us improve our understanding of the in vivo functions of the Cx26 gene. Previously, various mutations that have been shown to cause lethal hemichannel activity contribute to hearing loss and skin disease. In contrast various

mutations of Cx26 that are found in non-syndromic deafness display loss of channel function without causing cell death (Montgomery et al., 2004; Stong et al., 2006).

In order to analyze how these connexins affect hemichannels we need to perform single cell voltage clamp recordings to test how oocytes injected with the different mutants react to pulses of increasing voltage. My specific experimental goals were to:

1) Construct the A88V and D50A mutant versions of Cx26 found in patients of KID syndrome, each of which is a single mutation found in the amino terminus and the first extracellular loop of the Cx26 gene respectively.

2) Confirm that the Cx26 mutant genes found in patients with KID syndrome alter hemichannel activity. It is crucial to demonstrate that this difference in hemichannel activity is due to varying properties of different mutants and not due to the lack or over-expression of mutant protein itself.

Xenopus laevis was chosen as the experimental system to evaluate the functionality of each Cx26 mutant. There are many practical advantages in choosing *Xenopus* as an experimental animal (Dawid and Sargent, 1988; Dawid et al., 1994). Many of the Connexin genes have been characterized in *Xenopus* (Gerido et al., 2007; Lee et al., 2009). Injected mRNAs are translated efficiently and their protein products are processed and transported to appropriate cellular locations in the oocytes therefore it has been commonly used as an expression system.

II. MATERIALS AND METHODS

Expression Construct Generation and Site Directed Mutagenesis:

The forward and reverse primers for A88V and D50A mutations were designed using the wild-type Connexin 26 as a double-stranded DNA (dsDNA) template. In addition Cx26 BamH1 forward (sense) and Cx26 Xba1 (antisense) primers were designed to ensure incorporation of the Cx26 mutated insert in the right orientation into the desired vector.

Cx26 BamH1 forward Primers

5'- ACG TGG ATC CAT GGA TTG GGG CAC GCT GCA GAC G – 3'

Cx26 Xba1 Reverse primers

5' – ACG TTC TAG ATT AAA CTG GCT TTT TTG ACT T – 3'

These primers in conjunction with the primer specific to each mutant were used to create each mutant by the overlap extension method (Horton et al., 1990).

A PCR was run as follows:

95.0 ° C	95.0 ° C	60.0 ° C	72.0 ° C	72.0° C	4.0° C
4 min	30 sec	30 sec	45 sec	2min	∞

Once the Forward and reverse strands for each mutation were amplified they were gel purified using the QIAquick gel extraction kit (Qiagen, Valencia, CA). A second PCR was then run using the same program to allow the amplified forward and reverse strands to anneal.

The Alanine 88 to Valine mutation was generated using the following forward and reverse primers:

5’ – GTG TCC ACG CCA GTG CTC CTA GTG GCC - 3;

5’ – GGC CAC TAG GAG CAC TGG CGT GGA CAC – 3’

The Isoleucine 30 to Asparagine mutation was generated using the following primer.

5’–ACC GTC CTC TTC AAT TTT CGC ATT ATG–3’;

5’–CAT AAT GCG AAA ATT GAA GAG GAC GGT–3’

The Aspartic acid 50 to Alanine mutation was generated using the following primers.

5’–GAT GAG CAG GCC GCC TTT GTC TGC AAC –3;

5’–STT GCA GAC AAA GGC GGC CTG CTC ATC–3’

Plasmid construction:

The Cx26 A88V, Cx26 D50A gel purified PCR samples were doubly digested with BamH1/Xba1 (New England Biolabs) to release a 690 bp insert and cloned into pBlueScript vector (Stratagene, La Jolla, CA). The generated plasmid sequence was confirmed by restriction digest mapping and sequence

analyses (GeneWiz South Plainfield, NJ). Mutants with correct sequences were sub-cloned into the pCS2+ vector (Turner and Weintraub, 1994).

In Vitro Transcription:

Once the sequences of the mutant Cx26 vectors were confirmed, they were used as templates for in vitro transcription of mRNA. Human Cx26, A88V and D50A were linearized using the *Not*1 restriction site of pCS2+. In vitro transcription to mRNA was carried out using the mMessage mMachine SP6 polymerase kit (Ambion) as described by Moon and Christian (1989).

Oocyte micro-injections:

Xenopus laevis adult females were anesthetized in a solution of Sodium Bicarbonate (0.17mg/ml Sigma-aldrich) and ethyl-m-amino-benzoate methane sulfonic acid (0.15mg/ml Sigma-aldrich) in tap water. After 30 minutes in this solution the ovaries were surgically removed. SBU IACUC approves all procedures. The ovaries were treated with 1X Modified Barths (MB) solution without calcium containing Collagenase B (7.5mg/ml type IV, Roche) and Hyaluronidase (5mg/ml Sigma) for 15 minutes at 37 degrees Celsius with constant rocking. This was done to release the oocytes from ovarian tissue and remove the follicle cells that surround them. The oocytes were then treated with CLP (10 μg/ml Chymostatin, 10 μg/ml Leupeptin, 10 μg/ml Pepstatin in MB) protease inhibitors for 20 min and transferred to 1X MB solution with 2mM Calcium.

In order to eliminate the presence of endogenous connexins healthy oocytes were isolated and injected with 1:1 amounts of antisense *Xenopus* Cx38 oligonucleotide with mRNA encoding the wild-type Cx26 and the A88V and D50A Cx26 mutants. Fifty oocytes were injected per mutant mRNA. Oocytes injected with water only were also collected as a negative control. Injections were done using a Nanoject II Drummond Scientific company, an Olympus micromanipulator. Once injected the oocytes were incubated at room temperature overnight in MB with elevated Ca^{2+} (4mM $CaCl_2$) to allow translation of the mRNA to protein.

Electrophysiology:

9-12 hours after injections single cell voltage clamp electrophysiology readings were conducted. Current and voltage electrodes (1.2 mm diameter, omega dot; Glass company of America, Millvile, NJ) were pulled to a resistance of 1-2MΩ with a horizontal puller (Narishige, Tokyo, Japan) and filled with solution containing 3M KCl, 10mM EGTA, and 10mM HEPES, pH 7.4. Macroscopic recordings of hemichannel currents were recorded from single *Xenopus* oocytes using a GeneClamp 500 amplifier controlled by a PC-compatible computer through a Digidata 1320 interface (Axon instruments, Foster City, CA). pClamp 8.0 software (Axon instruments) was used to program stimulus and data collection paradigms. The hemichannel I-V curves were obtained by initially clamping cells at -40mV and then subjecting them to 5 second depolarizing voltage steps ranging from -30 to +60mV in increments of 10mV.

Preparation of oocyte samples for Western blot analysis and quantification:

Oocytes once injected were left overnight at room temperature and then stored at -80 degrees C the next day. These oocytes were lysed in 1ml lysis buffer (5mM EDTA, 5mM Tris pH 8.0, CLP protease inhibitor). The oocytes were subjected to a series of mechanical passages through needles of diminishing caliber (20,22,26 Ga). The oocyte lysates were then centrifuged at 2000 rpm for 5 minutes at 4 degrees C. The supernatant was then collected, being careful to avoid the layer of yolk proteins and was ultra-centrifuged at 45,000 rpm at 4 degrees C for 30 min using the TLA45 rotor. The pellet was re-suspended in 3X SDS sample buffer (2μl per oocyte). Samples were heated at 55 degrees C for 20 minutes. Ten oocyte equivalents (Volume of solution that is the fractional representation of the protein content of 1 oocyte) of each sample along with standard molecular weight markers (Novex Sharp Marker,Invitrogen and MagicMarker,Invitrogen) were then loaded onto a Sodium Dodecyl Sulfate -Polyacrylamide (15%) gel in SDS-PAGE Running Buffer (192mM Glycine, 25mM Tris, 0.1% Sodium Dodecyl Sulfate Buffer). After electrophoresis, the separated proteins were electro blotted onto Protran nitrocellulose transfer

members (Whatman) in 1X Tranfer buffer (25mM TRIS pH 8.3, 192mM glycine, 10% methanol) at 90 V constant voltage using a Mini-transblot electrophoretic transfer cell with plate electrodes (Bio-Rad) for 1 hour with an ice pack in place as per manufacturer instructions.

Ponceau S (Sigma) solution (0.1% Ponceau S in 5% acetic acid) was applied to the nitrocellulose filters after the transfer. Ponceau S solution is a reversible staining solution designed for rapid (15 minute) staining of protein bands on nitrocellulose and is easily reversed with 3X 5 minute washes with 1X TBS-Tween buffer (20mM/Tris base, pH 7.6, 137mM NaCl, 1N HCl and 0.1% Tween-20). This confirms the transfer was successful. Nitrocellulose blot were then blocked for 1 hour shaking at room temperature with the TBS Blotto (5% milk in a 1X TBS- 0.1% Tween solution). The blot was then probed overnight with a polyclonal Rabbit anti-Cx26 primary antibody (1:500 dilution; Invitrogen C# 71-0500) at 4 degrees shaking. The next day the nitrocellulose was then washed with 0.1% TBS-Tween for 3X 15 minutes. The nitrocellulose was then incubated in 5% Milk in TBS Tween containing the Goat anti-Rabbit alkaline phosphatase conjugate secondary antibody (1:5000 dilution; Jackson Laboratories) for 1 hour shaking at room temperature. The washes were repeated as above and a Luminol Peroxide mixture in a 1:1 ratio was added to the nitrocellulose blot for one minute. (Immunocruz Western Blotting Luminol Reagent; SantaCruz). The Cx26 antigens were visualized by exposure to X-ray film (HyBlot CL Autobiography Film, Denville Scientific). Once exposed, the film was processed using the Kodak 1D Image analysis software (Eastman Kodak, Rochester, N.Y., USA).

III. RESULTS

The A88V and D50A mutants of Cx26 induce increased hemichannel currents in single Xenopus oocytes*:*

The A88V and D50A mutations in the GJB2 gene are both involved in KID syndrome. To characterize the functional properties of these mutations single oocytes were individually subjected to depolarizing voltage pulses. The corresponding membrane currents were recorded over a range of -30mV to +60mV. Water injected negative controls displayed insignificant amounts of current. The wild-type Cx26 injected oocytes showed currents of greater magnitude than the water injected controls confirming previous reports of low hemichannel activity (Gerido et al., 2007; Gonzalez et al., 2006; Ripps et al., 2004). The D50A mutant displays hemichannel activity of higher magnitude than the wild-type Cx26; and the A88V mutant displays current of the greatest magnitude amongst the wild type and two mutant connexins (Figure 3).

In order to quantitate these results the mean steady state current values were plotted as a function of membrane potential (Figure 4). The water injected control oocytes displayed small currents at all voltages. The Cx26 injected oocytes showed small currents at smaller voltages, with an increase in currents at higher voltages. This was in accordance with previously published literature. On comparison

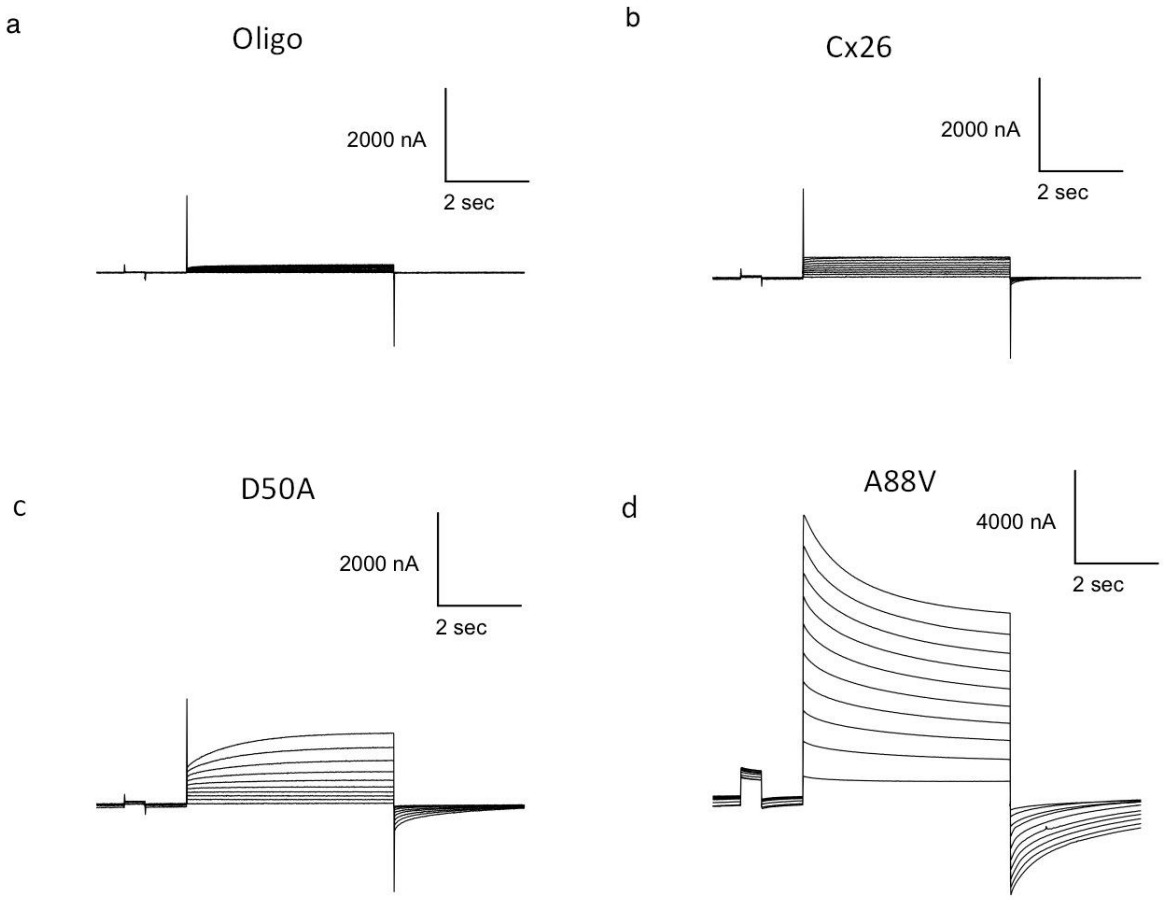
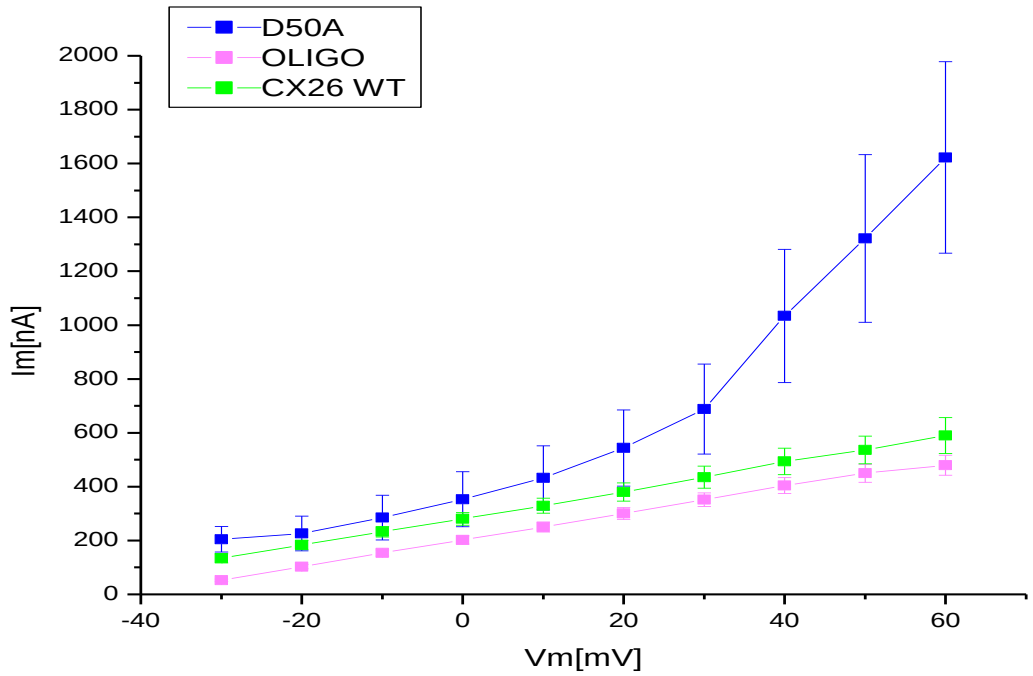


Figure 3. Hemichannel currents recorded from *Xenopus* oocytes. Oocytes were held at a fixed potential of -40mV and membrane currents were recorded at pulses from -30 to +60 mV in 10mV steps were recorded. (a) Oligo injected oocytes displayed negligible amounts of currents at all potentials. (b) Cx26 injected oocytes had currents of a higher magnitude than oligo injected oocytes, but a much lower magnitude than the (c) D50A injected and (d) A88V injected oocytes.

a


D50A
OLIGO
CX26 WT
Im[nA]
Vm[mV]
2000
1800
1600
1400
1200
1000
800
600
400
200
0
-40
-20
0
20
40
60

b

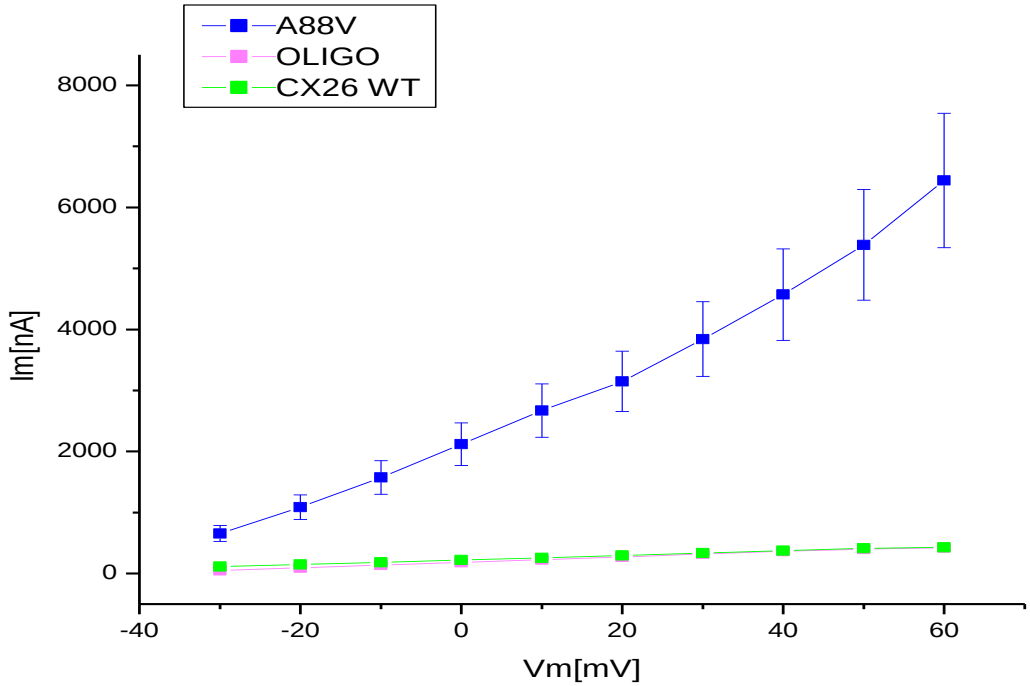

A88V
OLIGO
CX26 WT
Im[nA]
Vm[mV]
8000
6000
4000
2000
0
-40
-20
0
20
40
60

Figure 4. Hemichannel activity from Cx26 mutations involved in KID Syndrome. Membrane currents from ooctyes expressing the mutations D50A and A88V were compared to those from cells injected with Cx26 wild-type RNA or oligo. (a) D50N (n=55), (b) A88V (n=55) both had currents that were similar to Cx26 injected cells at lower voltages but showed a gradual increase larger than Cx26 at increasingly positive potentials. Oligo injected controls displayed lack of current at all voltages.

of the hemichannel activity of A88V and D50A mutants to the wild type injected oocytes it was seen that both displayed high amounts of current. Whereas the A88V mutant recorded high amounts of current at both low and high voltage pulses, the D50A mutant showed a greater increase in current activity at higher voltages.

A88V and D50A induce cell death that may be rescued by increased levels of extracellular Calcium:

Previous studies have shown elevated extracellular calcium levels suppress hemichannel activity. If the *Xenopus* oocytes expressing Cx26 mutants die due to increased hemichannel activity then an increase in calcium levels in the extracellular media should prevent cell death. To identify if the Cx26 injected oocytes do respond to changes in extracellular calcium levels, the oocytes were incubated in 0mM, 2mM and 4mM of extracellular Calcium after injection. It was observed that water injected negative controls and Cx26 wild type injected oocytes remained viable in all levels on extracellular Calcium. The A88V and the D50A Cx26 mutant injected oocytes showed cell death in 0 mM Calcium. Whereas the disruption of normal pigmentation was observed in oocytes injected with both mutants, the blebbing of ooplasm into the media was observed in A88V injected oocytes. Both changes led to cell death and lysis within 24 hours. In contrast the oocytes present in media containing 4 mM extracellular Calcium remained viable (Figure 5). This data suggests that an increased level of extracellular calcium suppresses A88V and D50A hemichannels maintaining viability of oocytes.

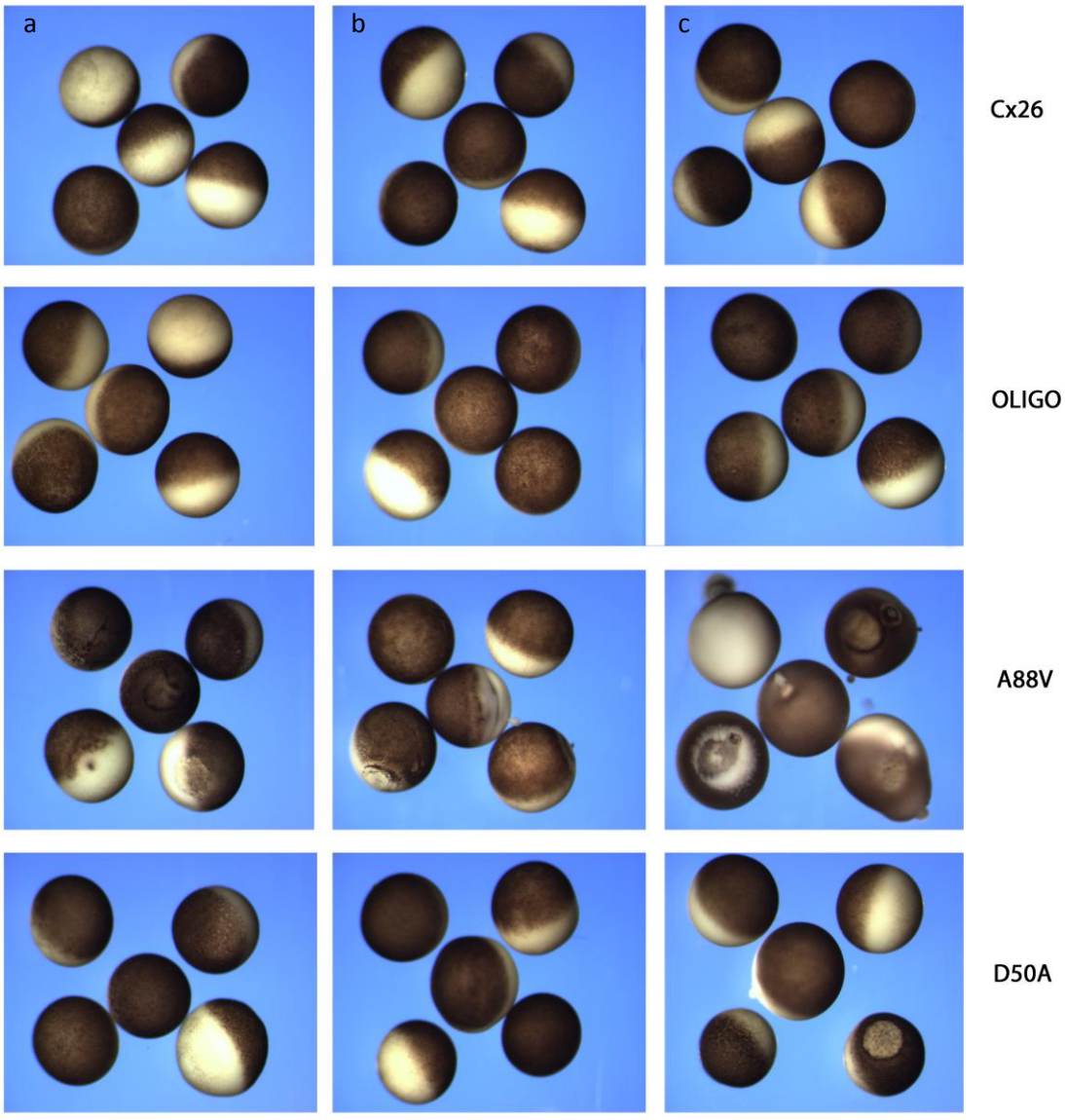

a
b
c
Cx26
OLIGO
A88V
D50A
4 mM Ca2+
2 mM Ca2+
0 mM Ca2+

Figure 5. *Xenopus* oocytes injected with Connexin-26 mutants. (a) Occytes injected with Cx26, Oligo, A88V or D50A were incubated for 24 hours in MB solution with 4mM Ca^{2+}. Occytes injected with Cx26, oligo and D50A were unaffeted and viable. A88V injected occytes display depigmentation. (b) Occytes injected with Cx26, oligo and D50A in MB with 2mM Ca^{2+} were viable. Delayed cell death was observed in A88V injected oocytes. (c) Cx26 and Oligo injected occytes remained viable in MB, D50A injected cells displayed de-pigmentation and blebbing. A88V injected occytes showed accelerated cell death and loss of cell integrity with blebbing.

IV. DISCUSSION

In KID Syndrome, typically all mutations are present in the amino terminal tail and the first extracellular domain (EC-1) of Cx26. It was previously shown that KID mutations G45E, A40V, G12R, N14K and D50N led to increased hemichannel activity and cell death. The recently discovered heterozygous missense mutations, A88V and D50A that were analyzed in this project showed greatly increased levels of hemichannel activity when compared to those displayed by wild-type Cx26 and water injected controls. This increased hemichannel activity resulted in cell death.

The A88V mutation resulted in KID syndrome in a neonate with profound SNHL and an increased susceptibility to skin infections which ultimately led to fatality within three months of birth (Koppelhus et al., 2010). The functional data obtained is consistent with the dominant role of this mutant, where very high hemichannel activity resulted in death of *Xenopus* oocytes. Previously, the G45E mutation associated with severe KID syndrome also caused death in a neonate similar to the A88V mutation. This mutation was found in caucasian neonates as well as in patients of Asian and African origin (Gilliam and Williams, 2002; Griffith et al., 2006; Janecke et al., 2005; Jonard et al., 2008). Experiments conducted in *Xenopus* oocytes injected with G45E showed rapid death of oocytes. Functional analysis of the hemichannel activity displayed by this mutant was very high when compared to

wildtype Cx26 (Gerido et al., 2007), similar to that displayed by A88V; co-relating with the severe SNHL associated with both mutants. In accordance with these results when A88V injected oocytes were placed in medium with elevated levels of Ca^{2+} they showed delayed cell death, similar to oocytes injected with G45E (Gerido et al., 2007). These results suggest that these two mutants may have some common molecular mechanism by which they might lead to similar symptoms, functional characteristics and prognosis of KID syndrome.

Previously the Cx30 gene was shown to have an A88V mutation that was associated with Clouston syndrome or HED (Hidrotic Ectodermal Dysplasia). Similar to the A88V Cx26 mutation, the HED-associated A88V Cx30 mutants induced cell death in *Xenopus* oocytes, which could have resulted from the presence of functional hemichannels. This was supported by the detection of large voltage-activated currents in single oocytes expressing mutant proteins that were not seen in cells injected with wild-type Cx30 (Essenfelder et al., 2004). These currents could be suppressed by elevation of extracellular calcium, and under these conditions, A88V formed functional intercellular channels with altered voltage gating properties.

The D50A mutation that was found in a neonate presenting with KID syndrome was also a missense mutant with no familial occurrence reported till date similar to A88V. The hemichannel activity displayed by this mutant although of greater magnitude than the wild-type Cx26 mutant and the negetive control, was of lesser magnitude than the A88V mutant. These results co-relate with the better clinical prognosis seen in the neonate suffering with KID syndrome of a milder phenotype as observed by Cushing et al., 2008.

Another previously discovered missense mutation in the GJB2 gene in which the Aspartic acid was mutated to Asparagine (D50N) instead of Alanine (D50A) at the nuleotide position 50 of the cDNA has been commonly associated in patients suffering form KID syndrome (Mazereeuw-Hautier et al., 2007; Richard et al., 2002; van Steensel et al., 2002). This mutation was seen in caucasian siblings with

unaffected parents (Mazereeuw-Hautier et al., 2007). This mutation has also been seen in adolescent patients (Neoh et al., 2009) implying a better prognosis than the lethal A88V and G45E mutants, similar to that seen in the patient with D50A (Cushing et al., 2008). On analysis of the hemichannel activity of the D50N mutation, large currents were produced that increase with cell depolarization similar in pattern to those shown by the D50A mutant (Figure 3) (Lee et al., 2009).

In conclusion, the data obtained from previously characterized Cx26 mutants causing SNHL is in accordance with that from the A88V and D50A mutants, which also result in an increase in hemichannel activity. This increase in hemichannel activity amongst many Cx26 mutations suggests common etiology at a molecular level that might play a role in disturbing the intra and extra cellular milieu of keratinocytes that may lead to defects in growth and differentiation.

Future Directions:

It is vital to test that the increase in hemichannel activity seen when oocytes are injected with the A88V and D50A Cx26 mutants is as a result of the mutations themselves and not merely a variation in the amount of protein being expressed by the *Xenopus* oocytes. Conversely, it would also be interesting to observe if the variation in hemichannel activity amongst mutants is due to differences in the way mutant proteins are expressed as well. Western Blots to test for levels of protein expression should be performed.

Additionaly, the formation of gap-junctions in paired oocytes injected with the A88V and D50A mutants of Cx26 must also be tested. It has been shown that not all disease causing mutations in Cx26 result in a complete loss of gap-junction functionality (Lee et al., 2009). In contrast, when oocytes injected with D50N were paired, they showed a marked decrease in gap junctional conductance when compared to oocyte pairs injected with wild type Cx26 (Lee et al., 2009). It would be interesting to observe gap junctional conductance displayed by oocytes injected with D50A to see if similar results are obtained as this would suggest that a lack of functional gap junctions may lead to various symptoms seen in KID syndrome. The voltage-gating properties of the A88V and D50A mutants should also be tested to

see how they compare to wild-type Cx26 expressing oocytes. These experiments will bring us a step closer in characterizing the A88V and D50A mutants.

It was seen that the addition of calcium preserved the viability of oocytes injected with the A88V and D50A mutants. This suggests that whole cell membrane currents will be greatly reduced in conditions with elevated calcium. Hemichannel activity of A88V and D50A injected oocytes should be recorded in media containing 0mM, 2mM and 4mM Ca^{2+} to see if elevated levels of calcium supress hemichannel activity. Since altered calcium regulation has been observed in some epidermal diseases (Bikle et al., 2004; Fairley, 1991; Hennings et al., 1980; Tu et al., 2004) and calcium plays a role in normal epidermal differentiation and cell to cell adhesion and the terminal differentiation of keratinocytes, its effects must be analysed. The identification of novel hemichannel blocking agents to supress hemichannel activity and therefore supress cell death may lead to a better prognosis of KID syndrome may be used treatment strategy.

Previous HEK-293 cell transfection assays with Cx26-G45E resulted in apoptosis and cell death within 24 hours. This was due to leaky gap junction hemichannel as shown by Stong et al., 2006. It would be interesting to perform a similar cell transfection assay using the A88V mutant to see if similar results are obtained.

Finally, understanding the molecular mechanisms and signaling pathways that play a role between the Cx26 mutations and increased epidermal thickening leading to skin pathology in KID syndrome is key to developing treatment strategies to improve the prognosis of this disease. Additional characterization of disease causing mutations and the generation of a transgenic animal model will further help elucidate the pathophysiology of diseases with disrupted epidermal regulation.

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