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Genetic alterations to Cx26 causing palmoplantar keratoderma and hearing loss modify Cx43 channel behavior

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Abstract of the Dissertation

Genetic alterations to Cx26 causing palmoplantar keratoderma and hearing loss modify Cx43 channel behavior

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Junctional-mediated intercellular communication in vertebrates is primarily achieved by clusters of connexin proteins which form gap junctions at the plasma membrane. It is believed connexins are crucial to the regulation of physiological homeostasis in various tissues such as the cochlea, and epidermis. Mutations in the *GJB2* gene encoding connexin26 (Cx26) cause either deafness, or deafness associated with skin diseases. The fact that very different disorders can be caused by distinct mutations within the same gene suggests that unique channel activities are influenced by each class of mutation. We have examined the functional characteristics of two human mutations, Cx26-H73R and Cx26-S183F, causing palmoplantar keratoderma (PPK) and deafness. Both mutant proteins failed to form gap junction channels or hemichannels when expressed alone. Co-expression of the mutants with wild-type Cx43 showed a

trans-dominant inhibition of Cx43 gap junction channels, without reductions in Cx43 protein synthesis. In addition, the presence of mutant Cx26 shifted Cx43 channel gating and kinetics towards a more Cx26-like behavior. Immunolocalization of Cx43 in the presence of mutant Cx26 showed a reduction of gap junction plaque formation, and resulted in a dispersal of Cx43 throughout the cell. Co-immunoprecipitation of Cx43 and mutant Cx26 showed the formation of a novel heteromeric structure that could act as a pathological unit in disrupting the stability of the epidermis. This novel formation of heteromeric Cx43 and mutant Cx26 connexons resulted in significantly increased Cx43 hemichannel activity. These findings suggest a mechanism whereby Cx26 mutations causing PPK and deafness trans-dominantly influence multiple functions of wild-type Cx43. They also implicate a role for aberrant hemichannel activity in the pathogenesis of PPK, and further highlight an emerging role for Cx43 in genetic skin diseases. While these mutants cause PPK through an interaction with Cx43, we have identified a novel Cx26 mutation, Cx26-N54H, which causes PPK through an unknown mechanism. Cx26-N54H formed functional gap junctions and hemichannels; furthermore, it did not interfere with the function of Cx43. The range of mechanisms through which Cx26 mutants cause syndromic hearing loss illustrates the functional heterogeneity of this protein.

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List of Abbreviations

35delG — Deletion of guanine at position 30, resulting in a frame-shift

42delE — Deletion of glutamic acid at 42

A40V — Missense mutation of alanine at 40 to valine

A88V — Missense mutation of alanine at 88 to valine

A — Gating Charge

ATP — Adenosine triphosphate

BPS — Bart-Pumphrey Syndrome

BSA — Bovine serum albumin

Ba^{2+} — Barium ion

Ca^{2+} — Calcium ion

cAMP — 3'-5'-cyclic adenosine monophosphate

CL — Cytoplasmic loop

CMTX — X-linked Charcot-Marie Tooth disease

cRNA — Complementary ribonucleic acid

Cx — Connexin

Cx26 — Connexin26

Cx30 — Connexin30

Cx30 — Connexin30.3

Cx31 — Connexin31

Cx32 — Connexin32

Cx38 — Connexin38

Cx40 — Connexin40

Cx43 — Connexin43

Cx46 — Connexin46

Cx50 — Connexin50

D50A — Missense mutation of aspartic acid at 50 to alanine

D66H — Mutation of aspartic acid at 66 into histidine

Δ42E — Deletion of glutamic acid at 42

E1 — first extracellular loop domain of connexin topology

EDTA — Ethylenedinitrilotetraacetic acid

eGFP — Excitatory green fluorescent protein

EGTA — Ethylene glycol tetraacetic acid

EKVP — Erythrokeratodermia variabilis et progressiva

ER — Endoplasmic reticulum

G11R — Missense mutation of glycine at 11 to arginine

G12R — Missense mutation of glycine at 11 to arginine

G45E — Missense mutation of glycine at 45 to glutamic acid

G59A — Missense mutation of glycine at 59 into alanine

G130V — Missense mutation of glycine at 130 into valine

Gj — Junctional conductance

Gjss — Macroscopic steady state junctional conductance

Gjmax — Maximum conductance normalized to utility

Gjmin — Residual conductance at large values of Vj

GFP — Green fluorescent protein

GJA1 — Gap junction alpha 1 gene encoding connexin43

GJB2 — Gap junction beta 2 gene encoding connexin26

GJB6 — Gap junction beta 6 gene encoding connexin30

H73R — Missense mutation of histidine at 73 to arginine

HeLa — Transformed (immortal) cell line derived from human cervical cancer

HEPES — 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

IACUC — Institutional animal care and use committee

Ij — Junctional current

Ijss — Steady state junctional current

Im — Membrane current or hemichannel current

Inx — Innexin

IP_3 — Inositol triphosphate

IRES — Internal ribosome entry site

k — Boltzmann constant

K^+ — Potassium ion

KCl — Potassium chloride

kDa — kiloDalton

KHLS — Keratoderma-hypotrichosis-leukonychia totalis syndrome

KID — Keratitis-ichthyosis-deafness syndrome

µA — microAmpere

MB — Modified Barth's medium

$MgCl_2$ — Magnesium chloride

µM — microMolar

mM — milliMolar

mRNA — Messenger ribonucleic acid

µS — microSiemens

mV — milliVolt

N14K — Missense mutation of asparagine at 14 to lysine

N14Y — Missense mutation of asparagine at 14 to tyrosine

N54K — Mutation of asparagine at 54 into lysine

N54H — Mutation of asparagine at 54 into histidine

NT — amino terminal domain of connexin topology

N-terminus — Amino terminus of protein

ODDD — Oculodentodigital dysplasia

OMIM — Online Mendelian inheritance in man

Panx — Pannexin

PBS — Phosphate buffered saline

PCR — Polymerase chain reaction

PPK — Palmoplantar keratoderma

R75W — Mutation of asparagine at 75 into tryptophan

S17F — Missense mutation of serine at 17 to phenylalanine

S183F — Missense mutation of serine at 183 to phenylalanine

SD — Standard deviation

SDS — Sodium dodecyl sulfate

SE — Standard error

siRNA — Small interference ribonucleic acid

SNHL — Sensorineural hearing loss

TBS — Tris-buffered saline

TM1 — first transmembrane domain of connexin topology

TM2 — second transmembrane domain of connexin topology

TM3 — third transmembrane domain of connexin topology

TM4 — fourth transmembrane domain of connexin topology

V0 — Transjunctional Voltage at which Gjss=(Gjmax-Gjmin)/2

V1 — Voltage of cell 1

V2 — Voltage of cell 2

V_{i-o} — Potential difference between intracellular and extracellular space

Vj — Transjunctional potential

Vm — Membrane voltage

VS — Vohwinkel Syndrome

WT — Wild-type

Zn^{2+} — Zinc ion

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Chapter 1 . Introduction

As organisms evolved into multicellular eukaryotes, the dilemma of maintaining a proper homeostatic balance along with coordinating developmental signals across a large cellular network arose. Multicellular organisms, from plants to animals, were able to independently evolve various mechanisms of intercellular communication (Van Norman, Breakfield, & Benfey, 2011) including the use of cellular junctions. While plants use their plasmodesmata for intercellular communication (Spanswick, 1972; Whaley, Mollenhauer, & Kephart, 1959), animals have developed structures called connexins (Cxs), innexins (Inxs) and pannexins (Panxs) to directly connect cells to mediate intercellular communication (Goodenough, 1974; Panchin et al., 2000; Phelan et al., 1998). Although structurally similar, innexins, pannexins and connexins do not share sequence homology, but do maintain a varying degree of analogous functional properties (Bao et al., 2007; Barbe, Monyer, & Bruzzone, 2006; G. Dahl & Locovei, 2006; G. Dahl & Muller, 2014; Simonsen, Moerman, & Naus, 2014). Mutations in connexin genes are responsible for a wide range of human genetic disorders contributing to their role as crucial mediators of intercellular communication in tissues. Thus far, there have been no reports of pannexin mutations linked to a disease, however, numerous studies have linked them to keratinocyte differentiation, tumor suppression, and ischemia and stroke (Celetti et al., 2010; Lai, Bechberger, & Naus, 2009; Lai et al., 2007; Thompson et al., 2008; Thompson, Zhou, & MacVicar, 2006).

GAP JUNCTION STRUCTURE AND FUNCTION

Junctional-mediated intercellular communication in vertebrates is primarily achieved by clusters of connexin (Cx) proteins found in gap junctions at the plasma membrane (Revel & Karnovsky, 1967; Robertson, 1963). The oligomerization of six connexin protomers results in the formation of a connexon/hemichannel (Makowski, Caspar, Phillips, & Goodenough, 1977). Gap junctions are composed of two connexons which dock on adjacent cells (Goodenough & Paul, 2003). They are assembled either in the endoplasmic reticulum or Golgi apparatus and transported to the plasma membrane through vesicles from the *trans*-Golgi (Musil & Goodenough, 1993; C. A. Scott & Kelsell, 2011); Cx26 has been shown to bypass the Golgi and go directly from the endoplasmic reticulum to the plasma membrane (Diez, Ahmad, & Evans, 1999; George, Kendall, & Evans, 1999; Martin, Blundell, Ahmad, Errington, & Evans, 2001). Once at the membrane, the connexon subunit can either form a gap junction channel by docking with another connexon on an adjacent cell, or nonjunctional channels. Connexins are dynamic proteins with short half-lives (Fallon & Goodenough, 1981), and the insertion of hemichannels in the plasma membrane to form gap junction plaques is followed shortly thereafter by removal of the proteins (Gaietta et al., 2002). The connexons are internalized from the center of the plaque into vesicular structures called annular junctions (Jordan, Chodock, Hand, & Laird, 2001) and subsequently targeted to the lysosome or proteasome (Laing & Beyer, 1995; Musil, Le, VanSlyke, & Roberts, 2000; Qin et al., 2003; VanSlyke, Deschenes, & Musil, 2000).

Gap junctions can be made of homomeric or heteromeric connexons (Nicholson et al., 1987) (Figure 1-1). Homomeric connexons are composed of the same connexin

proteins, while heteromeric structures can be made of different connexins subunits (Figure 1-1). These variations in connexons can build homotypic or heterotypic gap junction channels (Figure 1-1). Homotypic channels are composed of homomeric connexons or heteromeric connexons composed of similar connexins in apposed cells (Figure 1-1). The numerous interactions increase the structural and functional diversity of the channels, and are important for regulation of the selective permeability, or permselectivity, of molecules. However, not all connexins can combine to form hemichannels and connexin compatibility is heavily dependent on sequence homology (Evans & Martin, 2002).

Connexin proteins have four α-helical transmembrane domains (TM1-4), two extracellular loops (E1 and E2), one cytoplasmic loop (CL) and intracellular termini (Bruzzone, White, & Paul, 1996). The structure of connexins is highly conserved, with most differences found in the cytoplasmic domains, particularly the carboxyl termini (Dbouk, Mroue, El-Sabban, & Talhouk, 2009), and they are encoded by 21 genes in humans (Sohl & Willecke, 2004). The extracellular loops control the docking of connexons, as well as the oligomerization of connexins (Bruzzone, White, & Paul, 1994; White, Bruzzone, Wolfram, Paul, & Goodenough, 1994), while the second transmembrane domain and amino terminus are believed to play a role in the voltage gating of some connexins (Barrio et al., 1991; Oh, Rubin, Bennett, Verselis, & Bargiello, 1999). The sequence of the second extracellular loop is critical for compatibility in heterotypic structures (White, Paul, Goodenough, & Bruzzone, 1995). The sequences on the N-terminus and extracellular loops are highly conserved (Harris, 2001), which may lead to heteromeric/heterotypic interactions.

The nomenclature of connexins is divided amongst two conventions, according to their weight in kiloDaltons (Beyer, Paul, & Goodenough, 1987), or based on their gene structure, gene homology and sequence similarity resulting in a classification into five groups (A-E) (Sohl & Willecke, 2003). The molecular mass naming convention is used to describe proteins, while the latter is used to name genes. Categorization of connexins based on structure and homology also helps organize them according to their ability to interact and form heteromeric structures (Evans & Martin, 2002).

The twenty-one connexin isomers have minimal structural differences, yet, have significant differences in their sequences and length, especially at the C-terminus where connexin26 (Cx26) has the shortest C-terminus and connexin50 (Cx50) has the longest. The cytoplasmic components of the structure are also more prone to post-translational modifications such as phosphorylation (Cruciani & Mikalsen, 2002). Cx26 (*GJB2*) and connexin30 (Cx30, *GJB6*) share a higher degree of sequence homology than connexin43 (Cx43, *GJA1*), which plays a role in connexin-connexin interaction. The determination of the Cx26 structure gave stronger evidence for residues involved in connexon-connexon interactions, which may explain connexon specificity.

CONNEXIN26 STRUCTURE

Cx26 is the only connexin whose structure has been resolved (Maeda et al., 2009). This structure of Cx26 revealed many residues mutated in syndromic and non-syndromic hearing loss were involved in inter- or intra-protomer interactions, and thereby interfere with proper folding and/or oligomerization of connexins. Three cysteine residues found in each of the two extracellular loops form disulfide bonds between the loops; changes in

these residues result in a loss of function (G. Dahl, Werner, Levine, & Rabadan-Diehl, 1992; Krutovskikh & Yamasaki, 2000).

The major intra-protomer interactions occur in the extracellular parts of the transmembrane domains. Arginine 32 (TM1) interacts with glutamine 80 (TM2), glutamic acid 147 (TM3), and serine 199 (TM4). Tryptophan 44 (E1) and tryptophan 77 (TM2) stabilize the protomer structure and form a hydrophobic core. Alanine 39 (TM1), alanine 40 (TM1), valine 43 (E1) and isoleucine 74 (TM2) form one hydrophobic core around tryptophan 44, and phenylalanine 154 (TM3), while methionine 195 (TM4) forms the second core with tryptophan 77. In the intracellular part of the transmembrane domain, arginine 143 (TM3) forms hydrogen bonds with asparagine 206 (TM3) and serine 139 (TM3). The inter-protomer interactions occur between glutamic acid 47 (E1), glutamine 48 (E1), asparagine 62 (E1), aspartic acid 66 (E1), tyrosine 65 (E1), arginine 75 (TM2) and serine 72 (E1) from one protomer with aspartic acid 46 (E1), aspartic acid 50 (E1), arginine 184 (E2) threonine 186 (TM4) and glutamic acid 187 (TM4) from the adjacent protomer (Maeda et al., 2009).

The interactions between two hemichannels involve the E1 and E2 domains. In the E1 region, asparagine 54 forms hydrogen bonds with leucine 56 on the opposite protomer, and glutamic acid 57 binds to the same residue on the opposite protomer (Figure 1-2). In the E2 domain, lysine 168, aspartic acid 179, threonine 177 and asparagine 176 form hydrogen bonds and salt bridges with the opposite protomer (Figure 1-2). These interactions between protomers create a tight wall that separates the gap junction pore from the extracellular environment. The residues involved in these interactions are conserved, again emphasizing their significance in the structural and

functional stability of the protein. The interaction of Cx26 with Cx30 is expected based on their close sequence homology and the presence of residues necessary for key interactions between protomers; of the five residues identified as critical for docking, Cx26 and Cx30 share all in common, while Cx43 differs in the E2 region with a threonine in place of a lysine at position 168 and histidine instead of an asparagine at position 176 (Maeda et al., 2009).

The pore lining residues located in E1 and the N-terminus in Cx26 are important for the gating and regulation of the channel (Kronengold, Trexler, Bukauskas, Bargiello, & Verselis, 2003; Oh, Abrams, Verselis, & Bargiello, 2000; Purnick, Benjamin, Verselis, Bargiello, & Dowd, 2000; Purnick, Oh, Abrams, Verselis, & Bargiello, 2000; Srinivas, Kronengold, Bukauskas, Bargiello, & Verselis, 2005; Verselis, Ginter, & Bargiello, 1994). There are nine positively charged residues in TM2 and two in TM3. Together, they create a positively charged environment at the pore entrance which makes it more permeable to negatively charged molecules. The residues lining the pore opening of the connexin also determines permselectivity based on size. Cx26 has a conserved threonine at position 5, whereas Cx43 has an alanine at the same location; the smaller side chain is a possible explanation for the wide pore in Cx43 channels which allows for the passage of large molecules such as siRNAs (Valiunas et al., 2005). The voltage sensitivity of the channel is also derived from residues lining the TM1/E1 boundary (Maeda et al., 2009; Verselis et al., 1994). Lysine 41 is specifically responsible for creating a positively charged opening and along with the N-terminus serves as a voltage sensor. Consistent with previous reports, the Cx26 structure also showed the switch to an opposite polarity with the negatively charged aspartic acid 46 and aspartic acid 50 residues.

The intercellular pore formed by adjoining connexons allows ions (K^+, Ca^{2+}), secondary messengers (cAMP, cGMP, IP_3) and small metabolites (glucose) to flow between the cells (Bevans, Kordel, Rhee, & Harris, 1998; Kanno & Loewenstein, 1964; Lawrence, Beers, & Gilula, 1978; Veenstra, 1996) resulting in electrical or biochemical coupling. The diffusion of these molecules is dependent on the channel type and is limited by pore size and charge (Brink & Dewey, 1980; Flagg-Newton, Simpson, & Loewenstein, 1979). The permeability of the gap junction can vary based on connexin composition. A disruption in ionic or molecular transport between cells has the ability to result in deleterious phenotypes. It is believed connexins are crucial to the regulation of physiological homeostasis in various tissues such as the cochlea and epidermis (Brissette, Kumar, Gilula, Hall, & Dotto, 1994; Caputo & Peluchetti, 1977; Zhao, Kikuchi, Ngezahayo, & White, 2006).

CONNEXINS IN THE EPIDERMIS AND COCHLEA

The skin is the largest organ in the human body and is composed of two tissues: the epidermis and dermis. The epidermis is formed by keratinocytes and is divided into four layers: stratum basale, stratum spinosum, stratum granulosum and stratum corneum (Figure 1-3). The basal layer is composed of proliferating keratinocytes that are attached to the dermis through hemidesmosomes and integrin-based adhesions (Simpson, Patel, & Green, 2011). Terminal differentiation of basal keratinocytes results in an outward migration of these cells towards the spinous and granular layers (Blanpain & Fuchs, 2006, 2009). Keratinocytes in the granular layer begin to flatten and form a water-impermeable cornified envelope. Cells in the cornified layer lack major organelles and are tightly cross-

linked to complete the cutaneous barrier of the skin. Cells in the basal, spinous and granular layers have various intercellular connections, including gap junctions (Kam, Melville, & Pitts, 1986). There are nine connexins, including Cx26, Cx30 and Cx43, expressed in the different layers of the epidermis (Di, Rugg, Leigh, & Kelsell, 2001), in overlapping spatial and temporal patterns during keratinocyte differentiation (Figure 1-3). The most prominently expressed connexin is Cx43; which is expressed throughout the keratinocytes of the epidermal layers (Guo, Acevedo, Parsa, & Bertram, 1992; Risek, Klier, & Gilula, 1992). Cx26 is found mainly in the palmoplantar skin, and the lower layers of the epidermis (Rouan et al., 2001).

Within the inner ear, connexin proteins play an integral role in homeostasis of the cochlea (Zhao et al., 2006). Cochlear supporting cells, such as pillar, Deiters (Zhao, 2000), Hensen, and Claudius cells are electrically and metabolically coupled by gap junctions (Beltramello, Piazza, Bukauskas, Pozzan, & Mammano, 2005; Y. Zhang et al., 2005). Aside from this epithelial network, a second gap junction network also exists between connective tissue, the stria vascularis and spinal limbus (Figure 1-4) (Kikuchi, Kimura, Paul, & Adams, 1995). The epithelial gap junction network has been postulated to play a role in the recirculation of K^+ ion in the cochlea (Kikuchi, Adams, Miyabe, So, & Kobayashi, 2000; Kikuchi, Kimura, Paul, Takasaka, & Adams, 2000).

The predominant connexins expressed in the cochlea are Cx26 and Cx30 (Lautermann et al., 1998). Although these connexins share a high degree of sequence homology, they display different biophysical properties. (E. Dahl et al., 1996). In the inner ear Cx26 is essential for the post-natal development and maturation of the organ of Corti (Y. Wang et al., 2009), and Cx30 mediated intercellular communication is necessary for

epithelial repair (Forge, Jagger, Kelly, & Taylor, 2013). Studies on gap junctional voltage gating in cochlear supporting cell pairs (Zhao & Santos-Sacchi, 2000), co-immunoprecipitation experiments (Forge, Marziano, Casalotti, Becker, & Jagger, 2003), and immunohistological studies on the cochlea have provided evidence for the presence of heterotypic Cx26 and Cx30 gap junctions. The loss of either Cx26 or Cx30 can result in hearing loss due to the inability of larger solutes to pass between cells, as well as an alteration in cochlear ionic homeostasis (Chang, Tang, Ahmad, Zhou, & Lin, 2008; Y. Zhang et al., 2005). The loss of Cx26 has been shown to reduce transport of IP_3 between cells (Beltramello et al., 2005), even in the presence of Cx30, thus demonstrating the differential permeability of Cx26 and Cx30. However, a transgenic mouse model of deafness, where Cx26 was overexpressed in a Cx30 null background showed restoration of hearing sensitivity and prevented hair cell death (Ahmad et al., 2007), suggesting that heteromeric Cx26/Cx30 channels might be unnecessary for normal cochlear function. Instead, it has been proposed that a reduction in gap junction plaques, due to a decrease in Cx30 or Cx26 protein may be responsible for disruption in cochlear function (Schutz et al., 2010). Loss of function mutations in Cx26 can cause autosomal recessive (Kelsell et al., 1997) or autosomal dominant non-syndromic hearing loss (Tekin et al., 2001). The most common recessive Cx26 mutation is a single base deletion (35ΔG), which results in a frameshift at residue 12 and premature termination of the protein (Estivill et al., 1998; D. A. Scott et al., 1998). Although Cx26 function is lost, resulting in deafness, afflicted individuals do not display an epidermal abnormality.

GAP JUNCTION PHYSIOLOGY: CONNEXIN-LINKED SYNDROMIC EPIDERMAL DYSPLASIA

Mutations in *GJB2*, the gene encoding Cx26, can result in many syndromic diseases including palmoplantar keratoderma (PPK, OMIM no. 148350) with hearing loss and keratitis icthyosis deafness (KID) syndrome (OMIM no. 148210) (Bakirtzis et al., 2003; de Zwart-Storm, Rosa, et al., 2011; de Zwart-Storm, van Geel, et al., 2011; Gasparini et al., 1997; Richard, Brown, Ishida-Yamamoto, & Krol, 2004; van Steensel, 2004; Zelante et al., 1997). These mutations are autosomal dominant and present as either a single amino acid substitution or deletion (Laird, 2008). Despite their genetic similarities, the clinical presentations of these two syndromes are distinct. Patients with Cx26 mutations associated with PPK present with hyperkeratosis in the palm and plantar skin (Sharland, Bleach, Goberdhan, & Patton, 1992), as well as sensorineural hearing loss. Mutations associated with KID syndrome have been found in patients with epidermal abnormalities such as hyperkeratotic lesions (Langer, Konrad, & Wolff, 1990), the follicular occlusion triad (dissecting folliculitis, hidradenitis suppurativa, and cystic acne) (Montgomery, White, Martin, Turner, & Holland, 2004), and squamous cell carcinoma (Mazereeuw-Hautier et al., 2007). Studies of mutations causing non-syndromic hearing loss have indicated a loss of function resulting in an alteration in cochlear intercellular communication, however, this mechanism does not affect the epidermis (Bruzzone et al., 2003; White, 2000). This suggested a loss of connexin function in the epidermis did not contribute to the disease, therefore, connexin related syndromic hearing loss likely occurs due to a gain of function mutation in the gene. The mechanisms whereby Cx26 mutations

result in KID syndrome have been studied extensively (Lee & White, 2009); however, a conclusive determination has yet to be reached for mutations associated with PPK.

A recent study of two mutations linked to KID syndrome, Cx26-D50A and Cx26-A88V, showed an increase in Cx26 hemichannel activity, which resulted in accelerated cell death in low extracellular calcium (Ca^{2+}) (Mhaske et al., 2013). Electrophysiological assays were used to study the hemichannel activity of these mutants. Based on these assays, it was determined that Cx26-D50A and Cx26-A88V produced large outward currents that increased with greater depolarization (Figure 1-5). These currents were significantly higher than those measured in cells expressing wild-type Cx26 alone or the H_2O control, and the mean steady-state currents were at least four times higher than wild-type Cx26 and ten times higher than the H_2O control (Figure 1-6). Based on the high outward hemichannel currents, it was concluded these mutant proteins produced aberrant hemichannel activity. Large outward currents are routinely measured in functional studies of Cx26 mutations causing KID syndrome and linked to leaky hemichannels (Gerido, DeRosa, Richard, & White, 2007; Lee, Derosa, & White, 2009; Levit et al., 2015; Montgomery et al., 2004; Stong, Chang, Ahmad, & Lin, 2006). In the study of Cx26-D50A and Cx26-A88V, protein translation remained unchanged in spite of increasing currents, indicating the significant increase in hemichannel current magnitude was not due to an increased expression of mutant protein (Figure 1-7). Although the phenotypes observed with the majority of KID syndrome mutations, along with the presence of increased hemichannel currents appear to be a shared similarity, the mechanism underlying this aberrant increase may be different.

The processes through which Cx26 mutations cause syndromic hearing loss are not limited to irregularities in hemichannel activity. Although KID syndrome generally occurs due to aberrant hemichannel function, mutations associated with PPK are hypothesized to inhibit gap junction channel activity. PPK mutations, Cx26-delE42, Cx26-G59A, Cx26-D66H and Cx26-R75W, have been studied using electrophysiological and permeability assays (Rouan et al., 2001; Thomas, Telford, & Laird, 2004; Yum, Zhang, & Scherer, 2010). In the study of Cx26-delE42, Cx26-D66H and Cx26-R75W, these mutants were unable to form functional gap junction channels when expressed alone, and also inhibited the gap junctional activity of wild-type Cx26 and Cx43. Additionally, co-expression of Cx37 with these mutant proteins showed a reduction in Cx37 channel activity only in the presence of delE42. A dye transfer assay was used to investigate Cx26-G59A and Cx26-D66H. Expression of these mutants alone in mammalian cells showed an inability to form functional channels that were permeable to Lucifer yellow. Co-expression with wild-type Cx43 and Cx32 revealed Cx26-G59A was able to trans-dominantly inhibit both wild-type connexin channels, whereas Cx26-D66H only inhibited Cx43. The differential inhibition of wild-type connexins by Cx26 mutants may explain the phenotypic heterogeneity observed in patients presenting with Cx26 PPK mutations.

Cx26, Cx30 and Cx32 belong to the same category of connexin proteins and their interactions suggest the possibility of heteromeric channel formation. However, the trans-dominant inhibition of Cx43 by Cx26 mutant proteins is perplexing since these proteins belong to different groups of connexins. This dissertation will aim to understand Cx43 channel behavior in the presence of Cx26 mutants associated with PPK, specifically Cx26-H73R, Cx26-S183F and Cx26-N54H. These three mutations occur in the highly

conserved extracellular domains which are essential for proper docking of hemichannels between adjacent cells. All three were inherited as autosomal dominant mutations with patients presenting with sensorineural hearing loss (SNHL) and hyperkeratosis of palmar and plantar epidermis.

HYPOTHESIS

Cx26 plays a role in the causation of skin diseases in patients suffering from syndromic deafness. A loss of Cx26 function does not result in skin defects, only hearing loss. We hypothesize that Cx26 loss of function is not sufficient to cause skin disease; rather, an abnormal dominant gain of function in Cx26 contributes to epidermal pathologies associated with syndromic deafness. Cx26 mutants associated with palmoplantar keratoderma (PPK) have been linked to altered channel activity. Some of these mutants have also been studied in the presence of other wild-type connexins and have been found to have a negative effect on these proteins. The purpose of this dissertation was to further our understanding of how syndromic hearing loss associated mutations in Cx26 lead to epidermal dysplasia and how these genetic mutations can modify the activity of other proteins, specifically, Cx43.

Specific Aim 1: Determine the hemichannel activity of two Cx26 mutants.

Most Cx26 mutations associated with KID syndrome are known to have functional defects in their hemichannel activity, however, the hemichannel activity of Cx26 mutations associated with PPK has not been studied. We used single whole-cell voltage clamp to measure membrane currents and determine the hemichannel activity of these two mutants. Next we investigated the hemichannel activity of Cx43 channels in the presence of Cx26-H73R and Cx26-S183F.

Specific Aim 2: Characterize the channel properties of Cx43 gap junctions in the presence of Cx26 mutant proteins.

Previous reports have shown a lack of gap junction channel activity in Cx26 mutants associated with PPK. These studies have also revealed a dominant inhibition of wild-type Cx26, Cx30, Cx32 or Cx43 by the Cx26 mutant proteins. We screened the gap junction channel activity of Cx26-H73R and Cx26-S183F alone and also their effect on wild-type Cx26, Cx30 and Cx43. These wild-type connexin proteins were chosen because of their overlapping expression with Cx26 in the epidermis.

Our preliminary data indicated Cx43 channel activity was altered by Cx26 mutant proteins. The voltage gating properties, including channel closure kinetics, of Cx43 gap junction channels were examined in cells co-expressing Cx26-H73R or Cx26-S183F proteins. Since these mutants were non-functional, modifications in Cx43 channel behavior could be attributed to a gain of function from the mutant proteins.

Specific Aim 3: Examine Cx43 protein localization, and heteromeric channel formation when co-expressed with Cx26-H73R and Cx26-S183F.

Changes in Cx43 activity in the presence of the mutant proteins could be ascribed to defects in the translation of the protein or its localization within the cell. Hence we analyzed the translation of Cx43 when co-expressed with the mutant proteins. We also assessed its localization in mammalian cells to determine if Cx43 could make intercellular gap junction plaques. Since our preliminary data suggested the possibility of Cx43 channel activity being modified by the mutant proteins, we hypothesized Cx43 and the mutant connexins made heteromeric channels. We used a co-immunoprecipitation assay to conclusively make this determination, and validate our electrophysiological evidence.

Specific Aim 4: Investigate channel activity of a third mutant, Cx26-N54H.

Our initial data indicated that unlike other Cx26 mutations associated with PPK, Cx26-N54H forms functional channels. We performed functional assays to test hemichannel and gap junction channel activity.

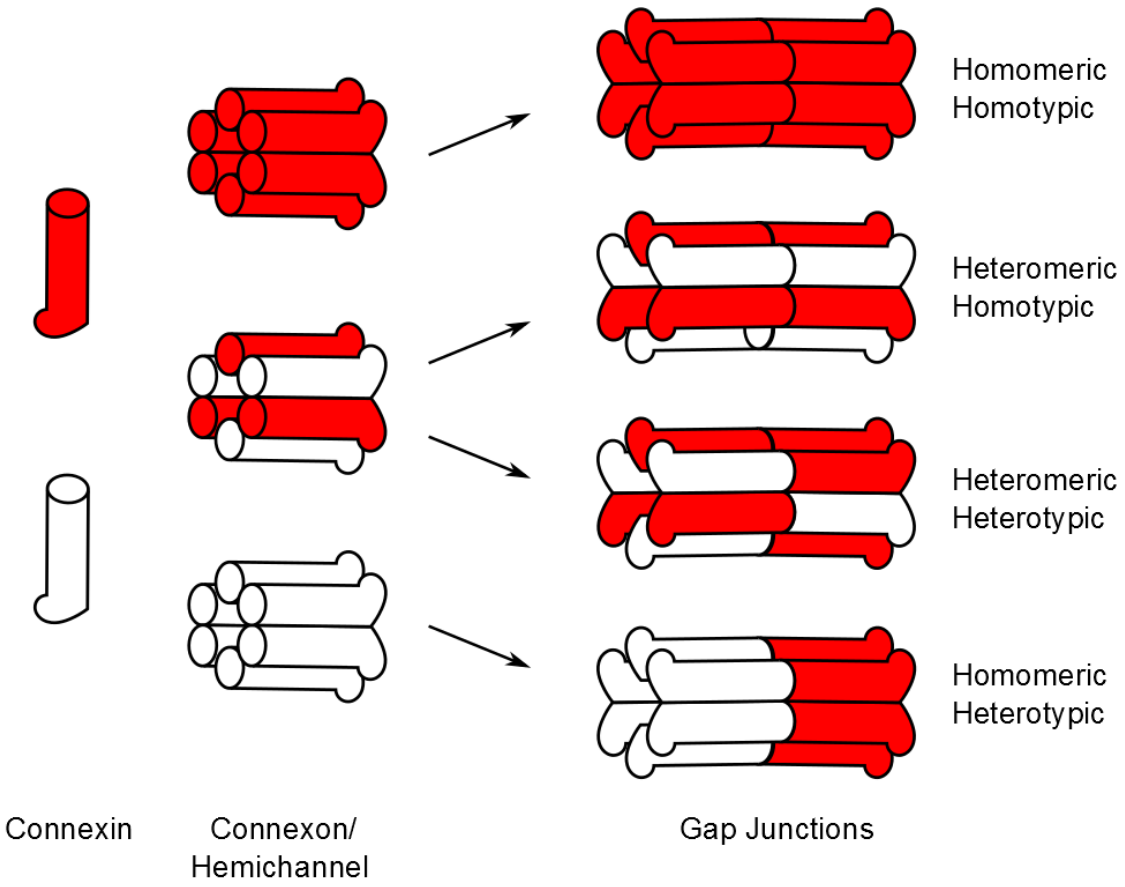

Figure 1-1. Hemichannel and gap junction formation. Connexin proteins oligomerize to form a hexameric structure called a connexon or hemichannel. Connexons can be composed of the same connexin proteins to form a homomeric structure, or more than one connexin, resulting in a heteromeric hemichannel. The homomeric or heteromeric channels can form intercellular channels called gap junctions. Gap junctions can be either homotypic or heterotypic. Homotypic gap junctions are composed of two homomeric connexons, or heteromeric connexons, where hemichannels from the two cells are made of the same types of connexins. Heterotypic gap junctions result from the docking of connexons comprising different connexins. These channels can form from two different heteromeric hemichannels or two different homomeric hemichannels. (Lee & White, 2009)

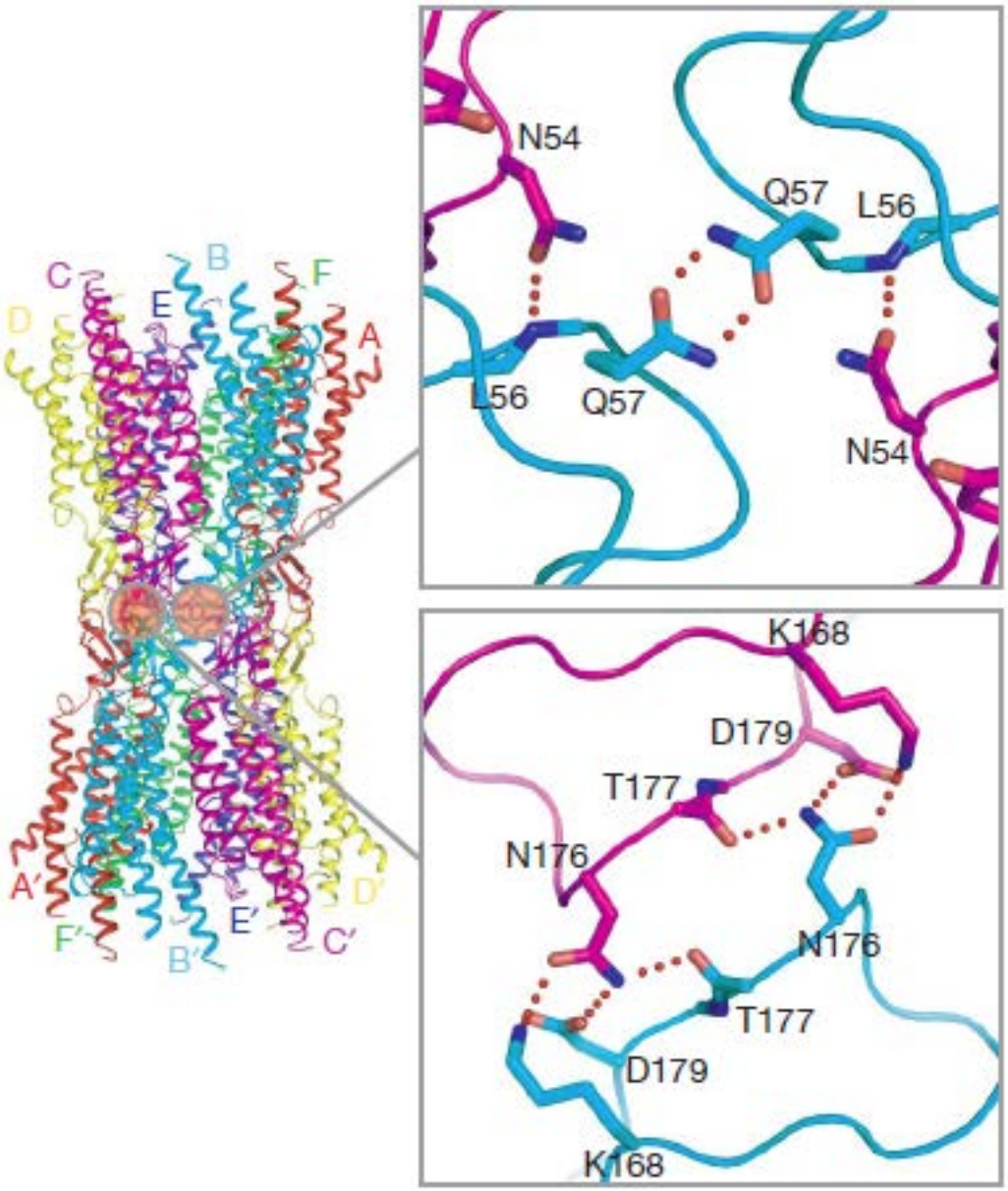

Figure 1-2. Key residues identified for gap junction formation. Docking of two hemichannels to form a gap junction channel requires the interaction of residues on the first and second extracellular loops resulting in hydrogen bonds and salt bridges. In the first extracellular loop, leucine 56 bonds with asparagine 54 and glutamic acid 57 bonds with the same residue on the opposite protomer. In the second extracellular loop, lysine 168, asparagine 176, threonine 77 and aspartic acid 179 play key roles in docking and bind to the opposite protomer. (Maeda et al., 2009)

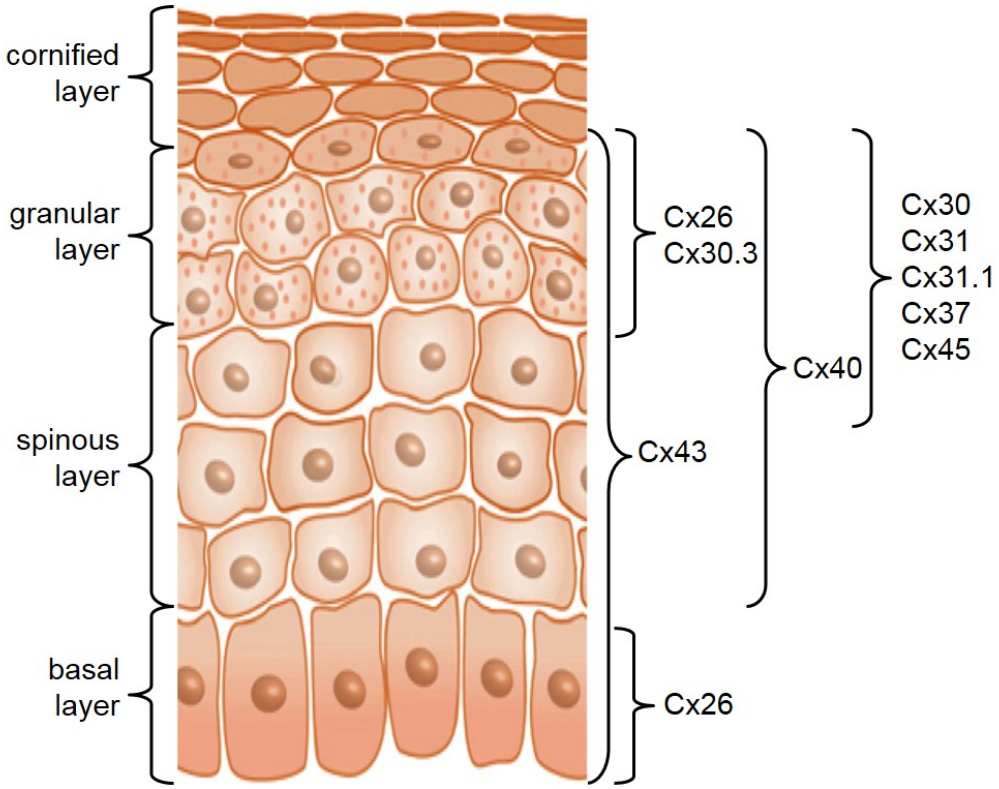

Figure 1-3. Expression pattern of connexins in the epidermis. At least nine connexin isoforms have been shown to be expressed during epidermal morphogenesis with distinct spatial and temporal expression pattern as well as some overlapping distribution. Cx43 is the most broadly expressed, whereas Cx26 is limited to basal keratinocytes in palms and soles, or occasionally cells in the stratum granulosum. (Mese, Richard, & White, 2007)

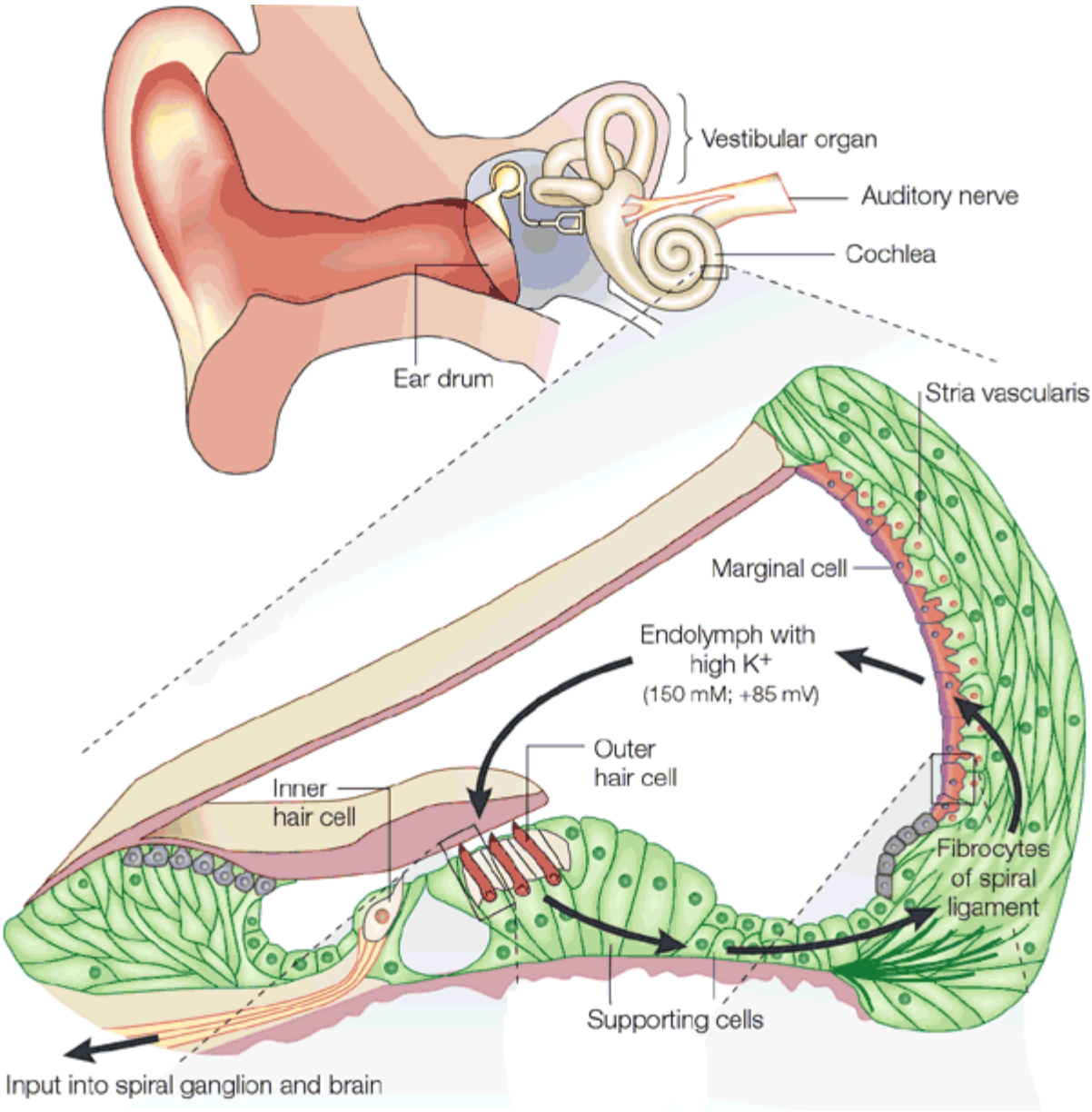

Figure 1-4. Structure of the cochlea. The cochlea contains two gap junction networks: epithelial and connective. The epithelial gap junction network is composed of the supporting cells and the connective gap junction network is made up of the stria vascularis and spiral ligament. The endolymph contains a high concentration of potassium and a +85 mV potential difference relative to the perilymph. Upon a mechanosensory stimulus, K+ moves into the outer hair cells from the endolymph and is released into the extracellular space. The supporting cells take up the K+ and the epithelial gap junction network spatially buffers K+ since high concentrations of the ion are cytotoxic. K+ is transported across the gap junction networks and released back into the endolymph. The recycling of K+ ions maintains sensitivity of hair cells and the high potassium concentration in the endolymph. (Jentsch, 2000)

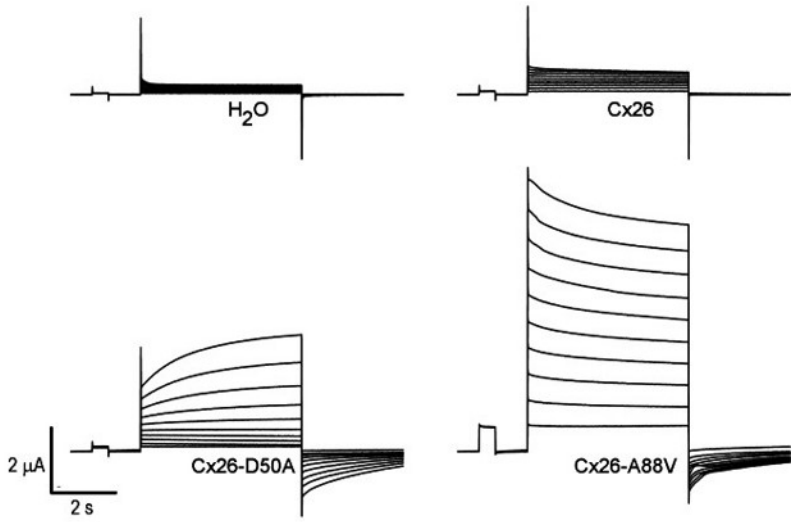

Figure 1-5. Cx26-D50A and Cx26-A88V induce large hemichannel currents in Xenopus oocytes. Single cells were clamped at a holding potential of -40mV and subjected to voltage pulses ranging from -30 to +60 mV in 10 mV steps. (A) H_2O injected cells displayed negligible membrane currents. Wild-type Cx26 (B), Cx26-D50A (C) and Cx26-A88V (D) expressing oocytes exhibited hemichannel currents with both of the KID mutation hemichannels displaying much larger currents than wild-type. (Mhaske et al., 2013)

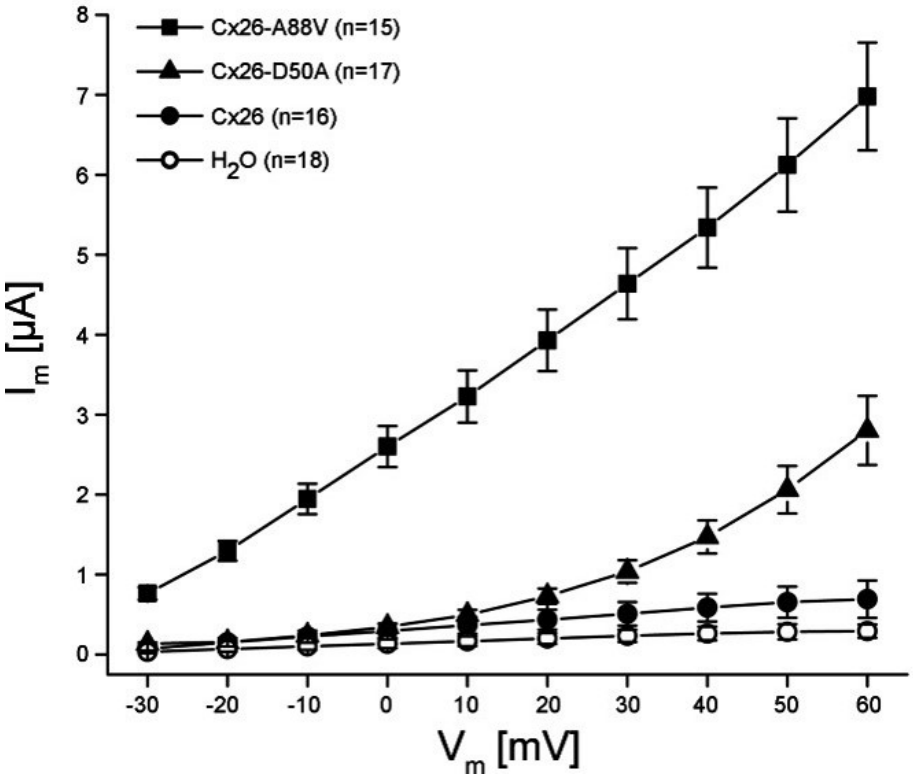

Figure 1-6. Steady-state currents for Cx26 mutants. Steady-state currents from each pulse were plotted as a function of membrane voltage. Steady state currents in H_2O injected control cells (○) were negligible at all membrane voltages. Cx26 currents (●) were similar to those observed in control cells at lower voltages, but increased at higher membrane voltage. Cx26-D50A (▲) or Cx26-A88V (■) expressing cells exhibited significantly increased steady-state currents compared to either control or Cx26 oocytes. Data are the mean ± SE. (Mhaske et al., 2013)

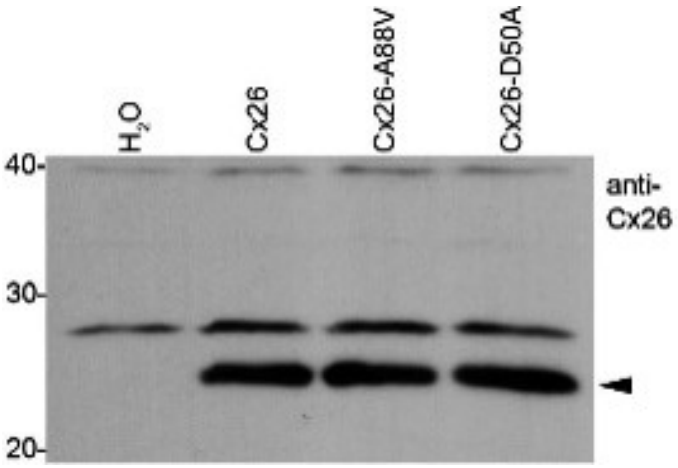

Figure 1-7. Wild-type and mutant connexins are equivalently expressed in Xenopus oocytes. Equal amounts of membrane extracts were first probed with an antibody that recognized Cx26. H_2O injected controls did not express Cx26 as expected. Wild-type Cx26, Cx26-D50A and Cx26-A88V were readily detected in lanes corresponding to each injection condition with similar band intensities. (Mhaske et al., 2013)

Chapter 2 . Materials and Methods

MOLECULAR CLONING

Human wild-type Cx26, Cx30 and Cx43 were cloned into the BamHI (DeRosa et al., 2009) restriction site of the pCS2+ expression vector for functional studies in *Xenopus* laevis oocytes (Turner & Weintraub, 1994). DNA primers with BamHI restriction sites (Integrated DNA Technologies, Inc. Coralville, IA) were used to generate mutant , Cx26-H73R, Cx26-N54H and Cx26-S183F constructs, which were prepared by site directed mutagenesis (Horton, Cai, Ho, & Pease, 1990) using wild-type human Cx26 as a template. DNA constructs were first cloned into pBlueScript II (Agilent Technologies, Santa Clara, CA) and sequenced on both strands prior to subcloning into the pCS2+ vector for *Xenopus* laevis expression, or into the pIRES2-EGFP2 vector (Clontech Laboratories, Mountain View, CA) for mammalian cell transfection.

IN VITRO TRANSCRIPTION, OOCYTE MICROINJECTION, AND PAIRING

Human Cx26, Cx30, Cx43, Cx26-H73R, Cx26-N54H and Cx26-S183F constructs were linearized via the NotI restriction site of pCS2+. The constructs were transcribed using the SP6 mMessage mMachine (Ambion, Austin, TX). Adult *Xenopus* laevis females were anesthetized with ethyl 3-aminobenzoate methanesulfonate. The ovarian lobes were surgically removed and digested for 10 minutes at 37ºC with gentle shaking in a solution containing 75 mg/ml collagenase B, 50 mg/ml hyaluronidase in modified Barth's medium (MB) without Ca^{2+}. The defolliculated oocytes were incubated with a protease inhibitor cocktail (Roche, Indianapolis IN) for 15 minutes. Stage V-VI oocytes were

collected and injected with of 10 ng of antisense *Xenopus* Cx38 oligonucleotide to eliminate coupling caused by endogenous Cx38 intercellular channels (Barrio et al., 1991; Bruzzone, Haefliger, Gimlich, & Paul, 1993). Anti-sense treated oocytes were then injected with wild-type Cx26, Cx26-H73R, and Cx26-S183F cRNA transcripts alone (5 ng/cell) or in combination (5 ng/cell of each cRNA transcript, 10 ng total). Oocytes injected with H_2O were used as a negative control. Wild-type Cx43 RNA was injected at a concentration that would yield average electrical conductance of ~5-10 µS. Other wild-type (Cx26, or Cx30) or mutant Cx26 RNA was injected at comparable levels. For measurement of gap junctional conductance, the vitelline envelopes were removed in a hypertonic solution (200mM aspartic acid, 10mM HEPES, 1mM $MgCl_2$, 10mM EGTA, and 20mM KCl at pH 7.4), and the oocytes were manually paired with the vegetal poles apposed in MB medium containing 2mM $CaCl_2$.

ELECTROPHYSIOLOGICAL RECORDINGS OF HEMICHANNEL CURRENTS

Macroscopic recordings of hemichannel currents were taken from single *Xenopus* oocytes 24 hours after cRNA injection using a GeneClamp 500 amplifier controlled by a PC-compatible computer through a Digidata 1440A interface (Axon Instruments, Foster City, CA). pClamp 8.0 software (Axon Instruments) was used to program stimulus and data collection paradigms. Current and voltage electrodes (1.5mm diameter glass, World Precision Instruments, Sarasota, FL) were pulled to a resistance of 1-2 MΩ with a horizontal puller (Narishige, Tokyo, Japan) and filled with solution containing 3M KCl, 10mM EGTA, and 10mM HEPES, pH 7.4. Hemichannel current-voltage (I-V) curves were

obtained by clamping cells at -40 mV and subjecting them to 5 second depolarizing voltage steps ranging from -30 to +60 mV in 10 mV increments (Mhaske et al., 2013).

ELECTROPHYSIOLOGICAL RECORDINGS OF JUNCTIONAL CONDUCTANCE

Gap junctional coupling between *Xenopus* oocyte pairs was measured using the dual whole-cell voltage clamp technique using two GeneClamp 500 amplifiers. Junctional conductance (Gj) was measured by initially clamping both cells in a pair at -40 mV, or at a transjunctional potential (Vj) of zero. Following this, one cell in the pair was subjected to alternating pulses of ±20 mV, while the current produced by the change in voltage was recorded in the second cell (DeRosa et al., 2009; DeRosa, Xia, Gong, & White, 2007). The current measured in the second cell was equal in magnitude to the junctional current (Ij). The junctional conductance was calculated by dividing the measured current by the voltage difference, Gj = Ij/(V1-V2) (Spray, Harris, & Bennett, 1981). Voltage-gating properties of connexin channels were determined by studying the response of the junctional current to different Vjs. Vjs of opposite polarity were generated by hyperpolarizing or depolarizing one cell of a pair in 20-mV steps (range: ±120 mV) while clamping the second cell at –40 mV. Steady-state currents (Ijss) were measured at the end of the voltage pulse. Steady-state macroscopic conductance (Gjss) was calculated by dividing Ijss by Vj, normalized to the values determined at ±20 mV, and plotted against Vj. The data illustrating the relationship of Gjss as a function of Vj was analyzed and fit to a Boltzmann relation of the form: $\mathrm{Gj}ss = \frac{\mathrm{Gjmax}-\mathrm{Gjmin}}{1+\exp[\mathrm{A}\ (\mathrm{Vj}-\mathrm{V0})]} + \mathrm{Gjmin}$, where Gjmax (normalized to unity) is the maximum conductance, Gjmin is the residual conductance at

large values of Vj, and V_0 is the transjunctional voltage at which Gjss= (Gjmax–Gjmin)/2. A (=nq/kT) is the cooperativity constant, representing the voltage sensitivity in terms of gating charge as the equivalent number (n) of electron charges (q) moving through the membrane, k is the Boltzmann constant, and T is the absolute temperature.

PREPARATION OF OOCYTE SAMPLES FOR WESTERN BLOT ANALYSIS

Oocytes were collected in 1 ml buffer containing 5mM Tris pH 8, 5mM EDTA and protease inhibitors (White, Bruzzone, Goodenough, & Paul, 1992) and lysed by mechanical passage through three different caliber needles (20, 22, 26 Ga). Samples were centrifuged at 1000 g at 4ºC for 5 minutes to remove yolk granules. The supernatant was then centrifuged at 100,000 g at 4ºC for 30 minutes. Membrane pellets were resuspended in SDS sample buffer (2 µl per oocyte). Samples were separated on 12% SDS gels and transferred to nitrocellulose membranes. Blots were blocked for 1 hour in 5% milk in 1X TBS with 0.1% Tween 20 and then probed with a polyclonal Cx26 antibody (Invitrogen, Camarillo CA) or a polyclonal Cx43 antibody (Zymed, Carlsbad CA). They were then incubated with appropriate horseradish peroxidase conjugated secondary antibodies (Jackson Laboratories and GE Healthcare). As a loading control, blots were probed with a monoclonal β-actin antibody (Abcam, Cambridge, MA) followed by incubation with HRP conjugated anti-mouse secondary antibody (GE Healthcare Biosciences, Pittsburgh, PA). Band intensities were quantified using ImageJ software and values from four independent blots were normalized to the mean band intensity of the wild-type sample.

PREPARATION OF OOCYTE SAMPLES FOR CO-IMMUNOPRECIPITATION ANALYSIS

Oocytes were collected and processed as described above. For whole cell lysate analysis, the membrane fraction was resuspended in SDS sample buffer (2 µl per oocyte) and boiled. However, for the co-immunoprecipitation assay, the membrane fraction was resuspended in RIPA lysis buffer (10 mM sodium phosphate, pH 7.0, 150 mM NaCl, 2 mM EDTA, 50 mM sodium fluoride, 1% Nonidet P-40, 1% sodium deoxycholate, 0.1% SDS and 1 mM Na_2VO_4) (Yum et al., 2007). Samples were pre-cleared with protein G agarose beads (Roche, Mannheim, Germany) that had been blocked overnight in 5% BSA-PBS. They were subsequently incubated with a monoclonal Cx26 antibody (Invitrogen, Frederick MD). The Cx26 antibody was selected based on a previous study that had verified its inability to cross-react with Cx43 (Yum et al., 2010). Following this incubation, protein G agarose beads were added to the samples and incubated. Beads were then washed, boiled in SDS sample buffer and run on a gel as described above. Protein bands were detected using a polyclonal Cx26 antibody (Invitrogen, Camarillo CA) or a polyclonal Cx43 antibody (Sigma, St. Louis MO).

TRANSIENT TRANSFECTION AND IMMUNOFLUORESCENCE STAINING

HeLa cells were plated on 22 mm^2 coverslips, grown to ~50% confluence, and then transiently transfected with 2.5 µg DNA (subcloned in pIRES2-EGFP) using TransIT HeLaMONSTER (Mirus Bio LLC, Madison, WI). After 24 hours, the cells were fixed using 1% paraformaldehyde in PBS and subsequently blocked using 5% BSA in 0.1% Tween20. Cells were stained with either a polyclonal Cx26 (Invitrogen) or polyclonal Cx43

antibody (Invitrogen) followed by incubation with Cy3-conjugated Affinipure goat anti-rabbit secondary antibody (Jackson ImmunoResearch Labs Inc., West Grove, PA). Cells were viewed and photographed on a BX51 microscope using a DP72 digital camera (Olympus America).

Chapter 3 . Alteration of Cx43 hemichannel activity by Cx26 mutants associated with palmoplantar keratoderma

ABSTRACT

Syndromic hearing loss associated with palmoplantar keratoderma (PPK) is caused by mutations in connexin26 (Cx26). Unlike Cx26 mutations linked to keratitis icthyosis deafness (KID) syndrome, PPK mutations are non-functional and able to dominantly inhibit wild-type connexins present in the epidermis. Cx26 mutations associated with KID syndrome are known to form hemichannels that have altered Ca^{2+} regulation. Previous work has indicated that while KID syndrome mutations seemingly cause aberrant hemichannel activity, PPK mutants need to interact with other connexins to confer pathogenicity. Although the hemichannel activity of mutations associated with KID syndrome has been well documented, the hemichannel activity of PPK mutant proteins has not been studied, and it is unknown whether they can form hemichannels in cells. We used electrophysiological assays to investigate the hemichannel activity of two Cx26 mutations associated with PPK, Cx26-H73R and Cx26-S183F. We present data showing a lack of hemichannel activity by both mutant proteins. Since PPK mutants need to act upon other connexins to cause disease, we co-expressed the mutants with wild-type Cx26 and Cx43 to show a reduction in wild-type Cx26 hemichannel activity, but interestingly, an enhancement in Cx43 hemichannel function. The alteration in Cx43 hemichannel activity brings into focus an emerging role of Cx43 protein in the causation of epidermal dysplasia. The difference in hemichannel behavior highlights a mechanistic difference between KID syndrome mutations and PPK mutations.

INTRODUCTION

Connexins are tetraspan integral membrane proteins that oligomerize in a hexameric arrangement to form intercellular channels called gap junctions. The oligomerization of the gap junction structure begins with the assembly of the connexon/hemichannel subunit, and subsequent docking of two hemichannels between adjacent cells to form an intercellular pore. These channels are responsible for the transport of ions, secondary messengers and small metabolites between cells (Bruzzone et al., 1996). Many hemichannels are appreciated to form non-junctional membrane channels which can also transport ions and small molecules (DeVries & Schwartz, 1992; Ebihara & Steiner, 1993; Malchow, Qian, & Ripps, 1993). Connexins are divided into five major classes, A-E, of which connexin26 (Cx26) belongs to B or β and connexin43 (Cx43) belongs to A or α (Bennett, Zheng, & Sogin, 1994). Hemichannel composition can vary, and studies indicate only connexins of similar classes can interact to form heteromeric hemichannels (Ahmad, Diez, George, & Evans, 1999; Elfgang et al., 1995; Falk, Buehler, Kumar, & Gilula, 1997).

Connexin proteins are expressed ubiquitously in vertebrate cells. Cx26 and Cx43 are both expressed in the epidermis, and mutations in the genes encoding them can result in various diseases, including keratitis icthyosis deafness (KID) syndrome, sensorineural hearing loss (SNHL) with palmoplantar keratoderma (PPK), erythrokeratodermia variabilis et progressiva (EKVP) and keratoderma-hypotrichosis-leukonychia totalis syndrome (KHLS) (Boyden et al., 2014; Gerido et al., 2007; Laird, 2014; H. Wang et al., 2015; Xu & Nicholson, 2013). Studies of mutations associated with KID syndrome have implicated the formation of pathological Cx26 hemichannels as the mechanism of action

(Mese et al., 2011; Sanchez, Villone, Srinivas, & Verselis, 2013). However, defects in hemichannel activity have not been implicated in SNHL associated with PPK. While Cx26 hemichannels have been well studied, and mutations in them are associated with disruption of normal tissue physiology, formation of functional hemichannels has not been demonstrated in Cx43 (Contreras, Saez, Bukauskas, & Bennett, 2003). Most diseases associated with Cx43 arise due to abnormal gap junctional activity (Laird, 2014), and changes in the functional properties of Cx26 gap junctions are responsible for SNHL associated with PPK.

Studies on three Cx26 mutant proteins linked to SNHL associated with PPK have indicated the possibility of a *trans*-dominant inhibition of Cx43 by the mutants (Rouan et al., 2001). It is understood that these classes of mutations use a different method to disrupt epidermal homeostasis, where Cx43 gap junctional coupling is affected. However, the role of Cx43 hemichannels in SNHL associated with PPK is poorly understood. The emergence of two novel mutations, Cx26-H73R and Cx26-S183F, which are also tied to SNHL associated with PPK, brings to focus the possibility of an interaction between Cx43 and the mutant proteins. Since wild-type Cx26 and Cx43 belong to different classes of connexins, it is hypothesized that they cannot form heteromeric hemichannels (Gemel, Valiunas, Brink, & Beyer, 2004), yet it is unknown whether Cx26 mutant proteins are able to alter Cx43 hemichannel activity.

RESULTS

Cx26 mutants associated with palmoplantar keratoderma do not form functional hemichannels

Studies of Cx26 mutations that cause syndromic hearing loss have found that the majority of mutants associated with KID syndrome were able to form functional hemichannels with aberrant activity (Gerido et al., 2007; Mhaske et al., 2013; Sanchez, Mese, Srinivas, White, & Verselis, 2010; Stong et al., 2006). While KID syndrome mutations were routinely found to form leaky hemichannels, it is yet unknown whether PPK mutations employ a similar mechanism to disrupt epidermal homeostasis. The palmoplantar keratoderma associated with hearing loss mutations Cx26-H73R and Cx26-S183F are single amino acid substitutions found in the first extracellular loop and the highly conserved second extracellular loop of Cx26, respectively. To analyze the hemichannel activity of these mutations, wild-type Cx26, mutant Cx26-H73R and Cx26-S183F were expressed in *Xenopus* oocytes, the cells were subjected to depolarizing voltage pulses and membrane currents were recorded. Control oocytes injected with water showed negligible current flow for voltage steps from -30 to +60 mV (Figure 3-1A). The hemichannel activity of wild-type human Cx26 has been previously characterized by outward currents that increase with depolarization (Figure 3-1B) (Gerido et al., 2007; Gonzalez, Gomez-Hernandez, & Barrio, 2006; Lee et al., 2009; Mhaske et al., 2013; Ripps, Qian, & Zakevicius, 2004; Sanchez et al., 2010; Sanchez et al., 2013). The PPK mutants, Cx26-H73R and Cx26-S183F both showed a large reduction in membrane current when compared to wild-type Cx26 injected cells (Figure 3-1C, 1D). Mean steady-state currents were plotted as a function of membrane potential to quantify differences in

the recorded hemichannel currents. Control cells injected with H_2O had negligible currents at all tested voltages, while wild-type Cx26 injected cells displayed larger outward currents that increased at greater depolarizing voltages (Figure 3-1E). At +60 mV, wild-type Cx26 produced maximum currents that were at least 10-times larger than control cells, as well as both Cx26-H73R and Cx26-S183F mutants (Student's t-test $p<0.05$). The mutant injected cells were similar to H_2O injected negative control cells, thereby suggesting these mutants have lost hemichannel activity.

Wild-type Cx26 hemichannel activity is reduced by co-expression with Cx26 mutants

Since Cx26 PPK mutants are known to cause disease through an inhibition of wild-type connexin proteins, we tested the hemichannel activity of wild-type Cx26 in the presence of Cx26-H73R and Cx26-S183F. This analysis suggested a reduction in the activity of wild-type Cx26 hemichannels. Mean steady-state currents were plotted as a function of membrane potential to quantify differences in the recorded hemichannel currents. Control cells injected with H_2O had negligible currents at all tested voltages and cells injected with wild-type Cx26 had larger outward currents that increased at greater depolarizing voltages (Figure 3-2). Unlike wild-type Cx26 alone, cells co-expressing wild-type and mutant Cx26-H73R proteins displayed a significant decrease in hemichannel activity (Student's t-test $p<0.05$). Although Cx26-S183F behaved similarly to Cx26-H73R, it did not have as potent effect on wild-type Cx26. Co-expression of Cx26-S183F with wild-type Cx26 resulted in a slight reduction in wild-type hemichannel activity, which was not statistically significant (Student's t-test $p>0.05$).

Cx26 mutants linked to palmoplantar keratoderma enhance Cx43 hemichannel activity

Cx26 PPK mutants demonstrated a lack of functional activity when expressed alone and a reduction in wild-type Cx26 hemichannels when co-expressed. Changes in wild-type Cx26 hemichannel function led us to study the activity of Cx43 hemichannels in the presence of Cx26 PPK mutants. *Xenopus* oocytes were injected with H_2O, Cx43, Cx43 and Cx26-H73R, and Cx43 and Cx26-S183F cRNAs as described above. Cells were subjected to depolarizing voltage pulses from -30 to +60 mV and membrane currents recorded. Control oocytes injected with water exhibited negligible current flow for all tested voltages (Figure 3-3A). As previously reported (White et al., 1999), oocytes expressing Cx43 alone exhibited little hemichannel activity (Figure 3-3B). Although Cx43, Cx26-H73R and Cx26-S183F were all unable to form hemichannels alone, study of heteromeric channels of Cx43 with either Cx26-H73R or Cx26-S183F showed current flow significantly higher than control or Cx43 expressing oocytes (Figure 3-3C, 3D). Investigation of the mean steady-state currents suggested a gain of Cx26-like activity by Cx43 hemichannels. Quantification of the data indicated a marked increase in hemichannel activity in cells co-expressing Cx26-H73R and Cx43 at increasing voltage pulses, clearly demonstrating acquired hemichannel function by Cx43 (Figure 3-3E). We observed a similar, albeit less consistent, trend in cells co-expressing Cx26-S183F and Cx43. Of the 55 oocytes recorded, 33% (n=18) showed a clear difference in hemichannel activity from control or Cx43 alone expressing cells, whereas, the remaining cells failed to produce membrane currents greater than the water injected negative control. Cx43, and the Cx26 mutants Cx26-H73R and Cx26-S183F were able to form functional hemichannels when co-expressed as heteromeric connexons. As with many KID

syndrome associated mutations, it is possible that this aberrant formation of functional hemichannels contributes to the disruption of epidermal homeostasis resulting in skin disease.

DISCUSSION

Cx26 mutations associated with syndromic deafness have been studied in the context of aberrant hemichannel formation and non-functional gap junctions. In this study, we have examined the role played by two Cx26 mutations associated with syndromic hearing loss in the disruption of epidermal homeostasis. Our data indicated innate functional defects in the Cx26 mutations, which caused an effect on other epidermal connexin proteins, namely wild-type Cx43. The mutations we examined, Cx26-H73R and Cx26-S183F, were unable to form functional hemichannels when expressed alone. However, co-expression of mutant proteins with Cx43 resulted in Cx43 exhibiting aberrant hemichannel activity. We believe this acquired behavior is a gain of function that distinguishes Cx26 mutations associated with PPK from other Cx26 syndromic mutations, such as those linked to KID syndrome. The ability of both mutants to modify Cx43 hemichannel behavior brings into focus an emerging role of Cx43 in the causation of some epidermal disorders.

Cx43 has been studied extensively for its function in the heart (Delmar & Makita, 2012; Veeraraghavan, Poelzing, & Gourdie, 2014), brain (Marquez-Rosado, Solan, Dunn, Norris, & Lampe, 2012; Meier & Rosenkranz, 2014), cancer (McLachlan, Shao, & Laird, 2007) and epidermis (Laird, 2014). Within the skin, Cx43 is well known to be abundantly expressed in all layers of the epidermis (Butterweck, Elfgang, Willecke, & Traub, 1994; Kamibayashi, Oyamada, Oyamada, & Mori, 1993) and also found in the dermis (Choudhry, Pitts, & Hodgins, 1997; Risek et al., 1992; Salomon et al., 1994). Genetic mutations in *GJA1*, the gene encoding Cx43, can result in diseases such as oculodentodigital dysplasia (ODDD) (Paznekas et al., 2003), erythrokeratodermia

variabilis et progressiva (EKVP) (Boyden et al., 2014), ODDD with palmoplantar keratosis (Kogame et al., 2014) and keratoderma-hypotrichosis-leukonychia totalis syndrome (KHLS) (H. Wang et al., 2015). Interestingly, these disorders share some clinical features with syndromic Cx26 mutations such as hyperkeratosis and leukonychia. ODDD (OMIM no. 164200) manifests itself with numerous traits such as neuropathies, a range of facial and digit abnormalities and occasionally skin disease. EKVP (OMIM no. 133200) first linked to Cx30.3 (Macari et al., 2000) and Cx31 (Richard, Smith, et al., 1998) and more recently to Cx43 (Boyden et al., 2014), results in hyperkeratosis and transient figurate patches of erythema. It is usually inherited in an autosomal dominant manner and has shown phenotypic heterogeneity, even in individuals with the same mutations. KHLS is categorized as a subtype of palmoplantar keratoderma-congenital alopecia syndrome (OMIM no. 104100), with an autosomal dominant inheritance pattern. It is characterized by severe skin hyperkeratosis, congenital alopecia and leukonychia. Recently, Cx43 has been linked to EKVP and KHLS, both rare epidermal disorders, from patients who were diagnosed with either disease and then through exome sequencing, determined to have Cx43 mutations (Cx43-E227D, Cx43-A44V and Cx43-G8V), thereby suggesting a potential role of this protein in skin disease (Boyden et al., 2014; H. Wang et al., 2015).

The similarities between the clinical features observed in patients with Cx26-H73R and Cx26-S183F, and Cx43-G138S (Kogame et al., 2014), Cx43-E227D, Cx43-A44V and Cx43-G8V mutations are striking. The presence of hyperkeratotic lesions on frictional surfaces (palms and soles) is a common feature among these patients, and points to possible mechanistic similarities through which palmoplantar keratoderma arises. Additionally, KHLS shares many clinical correlations with the Cx26 associated Bart-

Pumphrey syndrome (OMIM no. 149200) including hyperkeratosis and leukonychia (Bart & Pumphrey, 1967). Although Cx26-H73R and Cx26-S183F share many phenotypic similarities, functional studies of Cx43 mutations linked to ectodermal disorders need to be completed before any definitive correlation between their mechanisms can be determined.

One such functional study of the Cx43-G8V mutation resulting in KHLS indicated a possible hemichannel gain of function that may be associated with the disease (H. Wang et al., 2015). This study found that Cx43-G8V resulted in the formation of functional hemichannels, which allowed an influx of Ca^{2+} into cells, thereby decreasing cellular viability, but presumably also resulted in the loss of essential metabolites like ATP. The formation of aberrant hemichannels by a connexin mutation was frequently observed in Cx26 mutations associated with KID syndrome, and has been shown in heteromeric connexons in our study as well. A comparative study of two Cx26 KID syndrome mutations, A40V and G45E, showed that while both displayed increased hemichannel currents, they had a different mechanism of Ca^{2+} regulation and permeability (Sanchez et al., 2010). Wild-type Cx26 and Cx26-G45E hemichannels were more sensitive to higher extracellular Ca^{2+} than Cx26-A40V and Cx26-A40V had impaired Ca^{2+} regulation. However, activation of the hemichannels resulted in a robust activation of Ca^{2+}-activated chloride channels. Although an increase in Ca^{2+} resulted in a decrease in currents, hyperpolarization of the channels resulted in a large transient inward current in cells expressing Cx26-G45E, but not wild-type Cx26 or Cx26-A40V. Substitution of Ca^{2+} with Ba^{2+}, eliminated the inward current, demonstrating the current was Ca^{2+} dependent. These results showed two different mechanisms through which KID syndrome may

manifest; Cx26-A40V drastically impaired regulation by extracellular Ca^{2+}, thereby producing “leaky” hemichannels, while Cx26-G45E hemichannels showed an increase in Ca^{2+} permeability.

Another recent study of Cx43 hemichannels utilized a co-expression assay of Cx43 with Cx26 KID syndrome mutations to demonstrate an increase in Cx43 hemichannel activity in the presence of the non-hemichannel forming KID mutation Cx26-S17F (Garcia et al., 2015). Cx26-S17F has been shown to result in KID syndrome, however, unlike all other tested KID mutants, it is unique in its lack of hemichannel formation, rendering it a non-functional mutant (Lee et al., 2009; Richard et al., 2002). Consistent with our findings, this Cx26 syndromic mutation, while not being able to form hemichannels or gap junctions alone, was able to increase Cx43 hemichannel activity when co-expressed, providing further evidence that Cx43 activity in the skin can be modified by association with Cx26 mutations. Our data expands the spectrum of mutations with the ability to do this.

While Cx43 has been associated with many physiological functions in mammals, it has not been widely implicated in the context of cutaneous disorders. The emergence of Cx43 mutations linked directly to skin disease has brought into focus the importance of this protein in the maintenance of epidermal homeostasis. Aside from Cx43 mutations being involved in the causation of skin disease, by analyzing the effect of other proteins on Cx43, we can further broaden the function of Cx43 in the skin. Cx43 has now been determined to interact with Cx26 mutations, as well as desmoplakin mutations (Patel, Dubash, Kreitzer, & Green, 2014) which interfere with microtubules essential for Cx43 biogenesis. Taken together, these findings highlight a growing importance of Cx43 in

epidermal disorders, resulting not only from mutations in *GJA1*, but also from the *GJB2* gene.

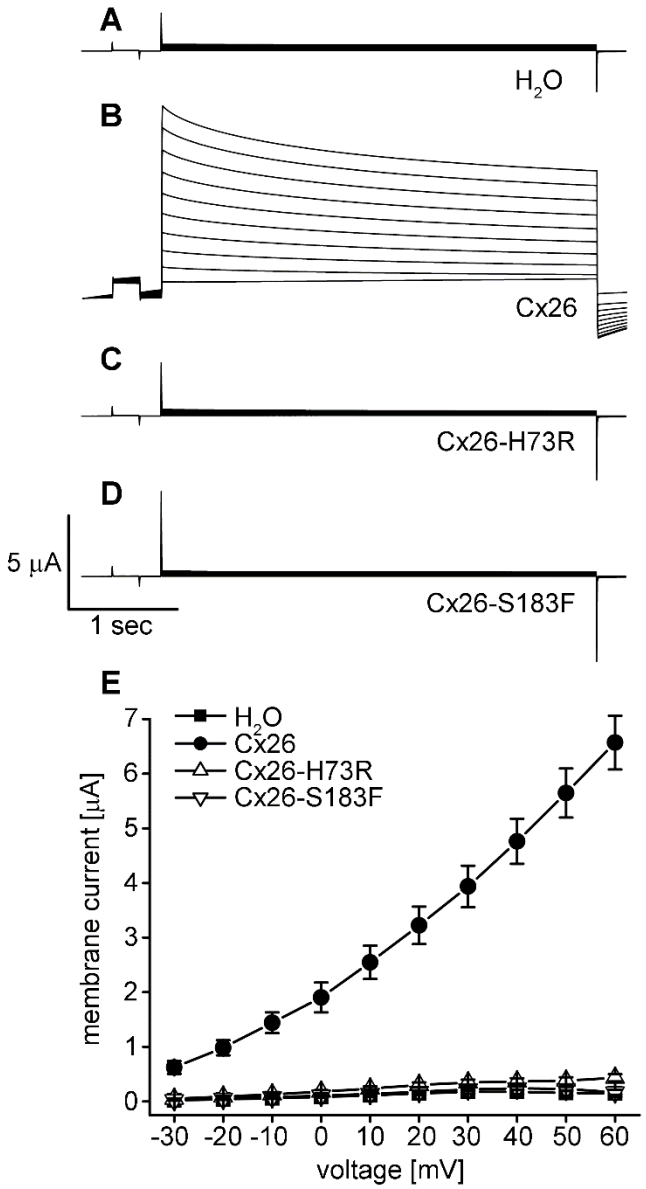

Figure 3-1. Cx26 mutants associated with PPK display a loss of hemichannel activity. Cells were placed in a Ca2+ free solution and subjected to voltage steps from -30 to +60 mV. (A) Oocytes injected with H_2O exhibited minimal current at all voltage steps, while (B) wild-type Cx26 cells displayed current that increased at each voltage step. (C, D) Mutants Cx26-H73R and Cx26-S183F showed negligible hemichannel current at all voltage steps. (E) Steady-state membrane current from each step was plotted as a function of membrane voltage. Steady-state currents for H_2O injected control cells (■) were negligible at all membrane voltages (n=12). Wild-type Cx26 (●) steady-state currents increased at each membrane voltage pulse (n=8). Both mutant Cx26-H73R (△) and Cx26-S183F (▽) injected cells failed to show increasing steady-state currents, rather their currents were negligible and resembled control cells (n=9 and n=9, respectively). Data are the means ± SE.

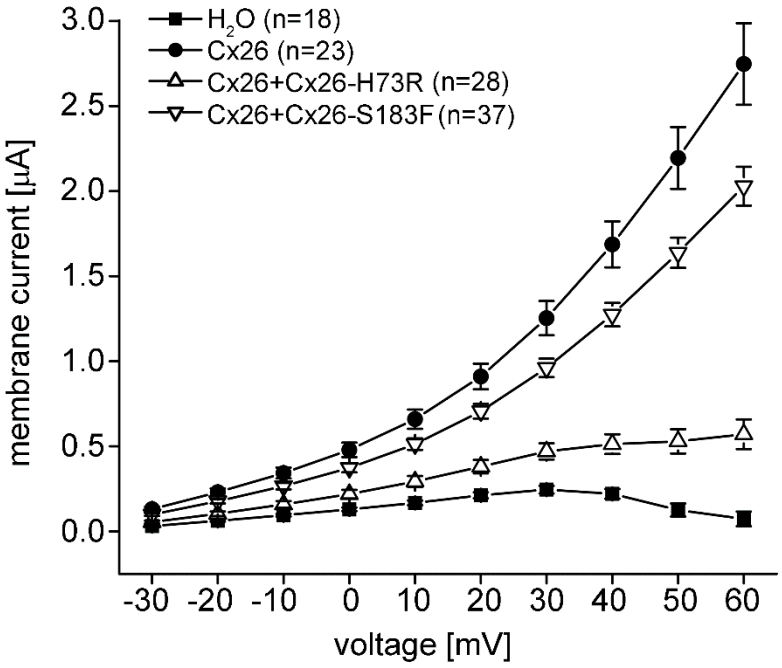

Figure 3-2. Cx26 hemichannel activity is reduced in the presence of Cx26-H73R and Cx26-S183F. Cells were placed in a Ca2+ free solution and subjected to voltage steps from -30 to +60 mV. Steady-state membrane current from each step was plotted as a function of membrane voltage. Steady-state currents for H_2O injected control cells (■) were negligible at all membrane voltages. Wild-type Cx26 (●) steady-state currents increased at each membrane voltage pulse. Heteromeric channels consisting of wild-type Cx26 and mutant Cx26-H73R (△) or Cx26-S183F (▽) showed a decrease in steady-state currents, indicating reduction of wild-type Cx26 hemichannel activity in the presence of Cx26 mutants. Data are the means ± SE.

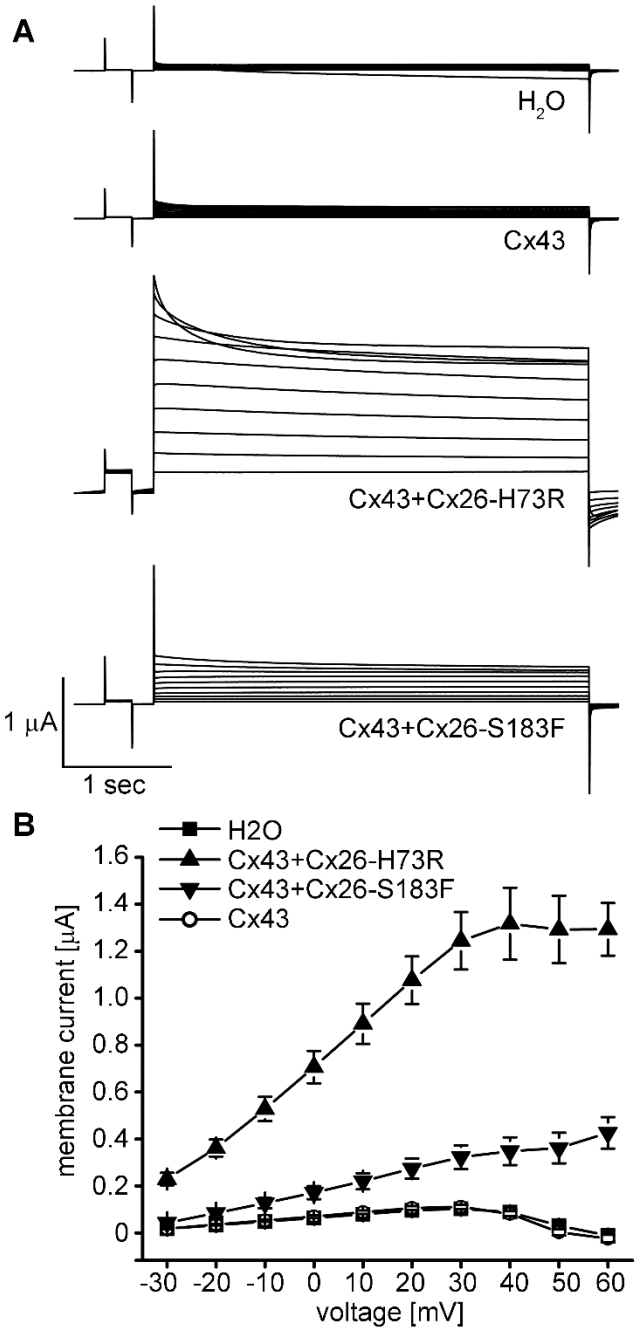

Figure 3-3. Cx43 acquires hemichannel activity in the presence of Cx26-H73R and Cx26-S183F. Cells were placed in a Ca2+ free solution and subjected to voltage steps from -30 to +60 mV. (A) Oocytes injected with H_2O exhibited negligible current at all voltage steps. (B) Wild-type Cx43 cells displayed little hemichannel activity. (C, D) In the presence of Cx26-H73R and Cx26-S183F, Cx43 showed higher hemichannel activity than when expressed alone. (E) Steady-state membrane current from each step was plotted as a function of membrane voltage. Steady-state currents for H_2O injected control cells (■) were negligible at all membrane voltages (n=21). Wild-type Cx43 (○) steady-state currents were similar to the negative control at each membrane voltage pulse (n=21).

Heteromeric channels consisting of Cx43 and mutant Cx26-H73R (▲) or Cx26-S183F (▼) showed increasing steady-state currents, indicating an enhancement of Cx43 hemichannel activity in the presence of Cx26 mutants (n=10 and n=18/55, respectively). Data are the means ± SE.

Table 3-1. Hemichannel activity of wild-type Cx26 and Cx43 co-expressed with Cx26 PPK mutants

Oocyte injection	Alone	+ Cx26	+ Cx43
Cx26-H73R	No	↓↓	↑↑
Cx26-S183F	No	↓	↑

Cx26-H73R and Cx26-S183F were expressed in oocytes alone or in combination with Cx26 or Cx43. Cx26-H73R exhibited a more pronounced effect on wild-type connexin channels than Cx26-S183F.

Chapter 4 . Modification of Cx43 gap junction channel properties by Cx26 mutant proteins

ABSTRACT

Syndromic hearing loss resulting from Cx26 mutations can cause keratitis icthyosis deafness (KID) syndrome or sensorineural hearing loss (SNHL) with palmoplantar keratoderma (PPK). Studies on Cx26 mutations associated with PPK have found these mutant proteins to be non-functional as opposed to those causing KID syndrome which result in aberrant hemichannel activity. Previous reports have found Cx26 PPK mutant proteins to dominantly inhibit wild-type connexins, yet, they have not tested the channel properties of the downregulated proteins. We investigated the functional properties of two mutations, Cx26-H73R and Cx26-S183F. These mutations were then co-expressed with wild-type Cx26, Cx30 or Cx43 and found to dominantly inhibit Cx43 and reduce its conductance; Cx26-S183F also inhibited wild-type Cx26. The biophysical properties of Cx43 channels was assessed in cells co-expressing mutant Cx26 proteins. It was revealed through electrophysiological analysis that Cx43 channels adopt a Cx26-like asymmetric current decay. Mixed Cx43 and mutant Cx26 channels also had a change in closure kinetics from homotypic Cx43 channels. An analysis of heterotypic interactions between mutant Cx26 and wild-type Cx26 or Cx43 did not show the formation of heterotypic channels. Our data suggests null Cx26 PPK mutations can alter the channel activity of Cx43 by conferring the biophysical properties of Cx26 on Cx43, suggesting an interaction between Cx43 and mutant Cx26 proteins.

INTRODUCTION

Gap junctions are formed by the oligomerization of six connexin proteins to form a hemichannel/connexon, which docks to a second connexon on an adjacent cell. Connexins are tetraspan proteins found ubiquitously in vertebrates. There are 21 variants of connexins found in humans that likely evolved from gene duplication events from an ancestral gene (Bennett et al., 1994). These variants can combine with different oligomers to form heteromeric hemichannels and heterotypic gap junctions. The composition of hemichannels and gap junctions is a determinant of channel permeability (Bevans et al., 1998), and suggestive of the non-redundant role of connexin proteins (White, 2003). Mutations in the gene encoding Cx26, *GJB2*, can result in syndromic or non-syndromic hearing loss.

Non-syndromic hearing loss occurs due to a loss of Cx26 function (Petit, 2006), in contrast to syndromic hearing loss. Syndromic hearing loss is a rare disorder that can be further classified into several groups based on clinical features and is hypothesized to result from a gain of function mutation. Sensorineural hearing loss (SNHL) associated with palmoplantar keratoderma (PPK) is one subset of diseases arising from mutation in *GJB2*. Recently, mutations in the first and second extracellular loops were identified in patients exhibiting clinical features of SNHL associated with PPK (de Zwart-Storm, Hamm, et al., 2008; de Zwart-Storm, van Geel, et al., 2008). These mutations, Cx26-H73R and Cx26-S183F, both resulted in demonstrable hyperkeratotic lesions on the patients' palms and plantar skin. Analyses of both mutants *in vitro* suggested abnormal protein trafficking and subsequent aggregation in the endoplasmic reticulum (ER).

Studies of Cx32 mutations causing X-linked Charcot-Marie-Tooth disease (CMTX) found mutants proteins that either did not form functional gap junctions (Bruzzone, White, Scherer, Fischbeck, & Paul, 1994) or made channels with altered voltage gating (Abrams, Freidin, Verselis, Bennett, & Bargiello, 2001; Rabadan-Diehl, Dahl, & Werner, 1994; Ressot, Gomes, Dautigny, Pham-Dinh, & Bruzzone, 1998). Analysis of null mutations of Cx32 demonstrated a dominant inhibition of wild-type Cx26, but not Cx40 (Bruzzone, White, Scherer, et al., 1994), suggesting an interaction of the mutant protein with Cx26. Electrophysiological assays of other Cx32 mutations revealed the formation of functional channels with altered biophysical properties from wild-type channels. Examination of four Cx32 mutants demonstrated a distinct difference in voltage gating from wild-type channels (Ressot et al., 1998). The mutant homotypic channels had a pronounced sensitivity to voltage and faster closure kinetics than wild-type Cx32. These mutants were paired with wild-type Cx32 to form heterotypic channels, and showed considerable asymmetry in their current decays indicating alterations to the channel's gating properties. Altogether, it was found Cx32 mutants were able to modify the channel properties of wild-type Cx32.

Previous work analyzing the gating properties of wild-type Cx50 and Cx46 when co-expressed with a Cx50 mutation, Cx50-S50P, showed the mutant was able to affect the wild-type channels (DeRosa et al., 2007). This study first determined the functionality of Cx50-S50P, concluding it was unable to form functional gap junctions, however, it did not hinder wild-type Cx46 or Cx50. Additional examination on the functional heteromeric channels formed by the mutant and wild-type connexins revealed alterations in channel activity. Channels containing both wild-type Cx50 and Cx50-S50P did not show drastic

changes with respect to current decay, however, their channel closure kinetics showed a significant decrease. Despite the heteromeric channels mixed with Cx46 and Cx50-S50P being active, they appeared to be more voltage sensitive than homotypic Cx46 channels. The channel closure kinetics of the heteromeric channels were also faster than homotypic channels, yet slower than heteromeric channels of wild-type Cx46 and Cx50. Together, this indicated the mutant protein exerted a dominant effect on the wild-type proteins, thus altering their behavior.

Functional studies on Cx26 mutations associated with PPK suggest loss of functional gap junctions by the mutant proteins and subsequent inhibition of Cx43 as the probable cause of the disease (Rouan et al., 2001). The effect of mutant Cx26 proteins on wild-type connexins has been shown in the context of non-syndromic hearing loss, where Cx26 mutants were able to inhibit Cx30 (Marziano, Casalotti, Portelli, Becker, & Forge, 2003). Taken together, it is likely Cx26 mutants associated with PPK may interact not only with Cx43, but also Cx30. Although these studies established groundwork on the functionality of Cx26 mutant proteins, they did not investigate alterations to wild-type connexin channel behavior.

RESULTS

Cx26-S183F does not form functional gap junction channels and dominantly inhibits wild-type epidermal connexins

While the hemichannel activity of PPK mutations had not been fully investigated, many studies have demonstrated the ability of some mutant connexins to dominantly interfere with the function of wild-type proteins (DeRosa et al., 2009; Gong, Shao, Langlois, Bai, & Laird, 2007; Marziano et al., 2003; Mesnil et al., 1995; Pal et al., 1999; Richard, White, et al., 1998; Rouan et al., 2001; Tong et al., 2011; Tong et al., 2013), resulting in diverse pathologies. To further our understanding of the role Cx26-S183F plays in the pathophysiology of PPK, this mutant was expressed in *Xenopus* oocytes in various combinations with other epithelial connexins and gap junctional conductance, G_j, was measured. Wild-type Cx26, Cx30 and Cx43 were selected for study with Cx26-S183F because of their expression in human epidermis (Guo et al., 1992; Lamartine et al., 2000; Lucke et al., 1999; Macari et al., 2000; Richard, Smith, et al., 1998; Salomon et al., 1994). Control oocytes injected with anti-sense oligonucleotide and water showed negligible conductance (Figure 4-1A). In contrast, cells injected with wild-type Cx26 cRNA had conductance of 8.6 µS, which was significantly higher than the control (Student's t-test $p<0.05$) (Figure 4-1A). While the presence of conductance in homotypic wild-type Cx26 channels is indicative of functional gap junctions, the conductance (G_j = 0.03 µS) measured in oocytes injected with Cx26-S183F cRNA was the same as the water injected negative control cells, demonstrating a loss of functionality (Figure 4-1A). To study the effect of the mutant Cx26-S183F on wild-type Cx26, the cRNA of both connexins were injected into oocytes. The resulting measurement showed a mean conductance of 3.2

μS, which was significantly less than wild-type Cx26 alone (Student's t-test $p<0.05$) (Figure 4-1A) and demonstrated a functional mechanism of dominant inhibition of the wild-type protein by Cx26-S183F.

Since the mutant Cx26-S183F was able to inhibit wild-type Cx26, wild-type Cx30 was selected next to screen for additional inhibitory effects. When wild-type Cx30 cRNA was injected alone in oocytes, a mean conductance of 12 μS was measured (Figure 4-1B). As an extra control, wild-type Cx26 and Cx30 cRNA were co-injected in oocytes to determine the effect of the wild-type proteins on the functionality of the gap junctions, and a mean conductance of 10 μS was ascertained (Figure 4-1B). The wild-type heteromeric channels formed by the combination of wild-type Cx26 and Cx30 were evaluated against the channels formed by the introduction of Cx26-S183F, and it presented a mean conductance of 11 μS (Figure 4-1B). Thus, the addition of the mutant Cx26-S183F to wild-type Cx30 protein did not hinder its functionality.

Based on the presence of a significant skin disease in the proposita and her daughter with the Cx26-S183F mutation (de Zwart-Storm, van Geel, et al., 2008), wild-type Cx43 was examined because of its prevalence in the majority of human epidermal layers (Butterweck et al., 1994; Di et al., 2001; Goliger & Paul, 1994; Risek et al., 1992; Salomon et al., 1994; Wiszniewski, Limat, Saurat, Meda, & Salomon, 2000). As with the previously studied connexin proteins, wild-type Cx43 was first expressed alone in oocytes with a mean conductance of 5.8 μS, which was significantly higher than the background (Student's t-test $p<0.05$) (Figure 4-1C). To assess the impact of wild-type Cx26 protein on wild-type Cx43, both were simultaneously injected in oocytes producing a conductance of 5.6 μS (Figure 4-1C), indicating no antagonistic effect of the proteins upon each other.

However, the mutant Cx26-S183F protein was able to significantly inhibit wild-type Cx43 when both were present in oocytes (G_j = 0.62 µS, Student's t-test $p<0.05$) (Figure 4-1C). While mutant Cx26-S183F protein was unable to functionally impede wild-type Cx30 gap junctions, it displayed a potent effect on both wild-type Cx26 and Cx43. Based on these functional data, it is possible to conclude that Cx26-S183F may result in PPK through an interaction between the mutant and wild-type proteins.

Cx26-H73R does not form functional gap junction channels, and can dominantly inhibit conductance of Cx43, but not Cx26, or Cx30

Similar to Cx26-S183F, mutant Cx26-H73R was identified in a patient and two of her children with palmoplantar keratoderma with hearing loss (de Zwart-Storm, Hamm, et al., 2008). Our functional analyses of Cx26-H73R were again performed using *Xenopus* oocytes to express epidermal connexins in a variety of combinations, followed by a measurement of G_j. Wild-type Cx26, Cx30 and Cx43 were once more selected for co-expression as described above. Control oocytes injected with anti-sense oligonucleotide and water showed negligible conductance (G_j = 0.12 µS), while in contrast, cells with homotypic wild-type Cx26 channels had an average G_j of 9.3 µS. Cell pairs expressing mutant Cx26-H73R RNA had conductance levels similar to water injected cells (G_j = 0.14 µS) (Figure 4-2A). Similar to Cx26-S183F, Cx26-H73R was unable to form functional gap junction channels. While both Cx26 mutants exhibited comparable loss of functional activity when expressed alone, Cx26-H73R did not display a dominant inhibitory effect on wild-type Cx26; cells co-injected with wild-type Cx26 and mutant Cx26-H73R displayed G_j of 9.9 µS (Student's t-test $p>0.05$) (Figure 4-2A).

While Cx26-H73R did have a discernible impact on wild-type Cx26 channels, we continued to study this mutant's potential effect on wild-type Cx30 and Cx43. Oocyte pairs expressing homotypic wild-type Cx30 channels had conductance higher than water injected cells (G_j = 20 μS, Student's t-test $p<0.05$) (Figure 4-2B). Cell pairs expressing a mixture of wild-type Cx26 and Cx30 also expressed a mean conductance of 20 μS (Figure 4-2B). Co-expression of both wild-type Cx30 and mutant Cx26-H73R yielded conductance of 23 μS, which was similar to the wild-type controls (Student's t-test $p>0.05$) (Figure 4-2B). The co-expression of the wild-type Cx30 and mutant Cx26-H73R failed to functionally suppress wild-type Cx30 channel activity, similar to the result with Cx26-S183F.

We also studied the effect of mutant Cx26-H73R on wild-type Cx43. Oocytes expressing only Cx43 formed homotypic channels with a mean conductance of 4.6 □S, and cells with both wild-type Cx26 and Cx43 also formed functional channels (G_j = 6.8 μS) (Figure 4-2C). In order to determine if Cx26-H73R was able to negatively impact Cx43 functionality, both proteins were co-expressed in the same cells. Application of a voltage stimulus to these cell pairs yielded significantly lower conductance than pairs expressing Cx43 alone (G_j = 1.1 μS, Student's t-test $p<0.05$) (Figure 4-2C), thereby demonstrating a trans-dominant inhibition of Cx43 by mutant Cx26-H73R.

Voltage gating properties of Cx43 are altered in the presence of Cx26 PPK mutants

If wild-type Cx43 is physically interacting with either Cx26-H73R or Cx26-S183F in gap junctions, then this interaction may modify the voltage gating properties of Cx43. To

examine this possibility, voltage gating of wild-type Cx43 channels in the presence and absence of mutant Cx26 was examined by subjecting oocyte pairs to a series of hyperpolarizing and depolarizing transjunctional potentials [V_j] while recording the junctional current [I_j]. I_js of homotypic wild-type Cx26 channels displayed asymmetric decay at higher voltages as has been noted by previous studies (Figure 4-3A) (Barrio et al., 1991; Bruzzone et al., 2003; Lee et al., 2009; Oh et al., 1999; Ressot et al., 1998; Rubin, Verselis, Bennett, & Bargiello, 1992a; Suchyna et al., 1999) while the I_j of homotypic Cx43 channels decreased much more symmetrically in a voltage-dependent manner with a rapid decrease at higher voltages (Figure 4-3C) (Bukauskas, Bukauskiene, Bennett, & Verselis, 2001; Hoang, Qian, & Ripps, 2010; Valiunas et al., 2004; Valiunas, Gemel, Brink, & Beyer, 2001; H. Z. Wang, Brink, & Christ, 2006; White et al., 1994). Gap junction channels in cell pairs expressing both Cx43 and Cx26-H73R (Figure 4-3E) or Cx26-S183F (Figure 4-3F) behaved differently from homotypic Cx43 channels. Despite the inability of the mutants to form homotypic channels on their own, they shifted the behavior of Cx43 to a more asymmetrical response to voltage, reminiscent of Cx26.

The steady-state voltage gating behavior of these channels was quantified by plotting V_j against G_j and fitting the data to the Boltzmann equation. Analysis of the Boltzmann fit of the homotypic Cx26 and Cx43 channels showed an asymmetric and symmetric decay, respectively (Figure 4-3B and 4-3D). However, the channels containing Cx43 and either Cx26-H73R (Figure 4-3F) or Cx26-S183F (Figure 4-3H) had voltage gating that differed from homotypic Cx43 channels. Since Cx26-H73R and Cx26-S183F cannot form functional homomeric channels, changes in Cx43 gating could be caused by co-assembly of the mutant Cx26 with Cx43 in heteromeric channels. Channels containing

Cx43 and mutant Cx26 displayed gating properties that resembled Cx43, with an acquired asymmetric decay at higher potentials. An examination of the Boltzmann parameters (Table 4-1) was consistent with the observable differences between the mixed and homotypic channels; the mixed channels had adopted properties of Cx26, while retaining elements of Cx43 gating. Similar to homotypic Cx43 channels, the channels containing both Cx43 and mutant Cx26 had at least a 27% change in minimum conductance [G_{min}] values from the negative to positive V_js. The symmetry of the homotypic Cx43 conductance was evident in the potential values at which G_j was halfway to G_{min} [V_o] which were similar at positive and negative V_js (85 mV and -87 mV, respectively). The heteromeric channels resembled homotypic Cx26 channels with asymmetrical values for V_os of +63 mV at a positive V_j and -90 mV at a negative V_j for Cx43 and Cx26-H73R channels, and +59 mV at positive V_j and -82 mV at negative V_j for heteromeric Cx43 and Cx26-S183F channels. The loss of symmetry, or gain of asymmetry, recorded in cells expressing both Cx43 and mutant Cx26 suggests an interaction between these proteins, which may also change other biophysical properties of Cx43 channels resulting in an acquisition of Cx26-like behavior.

Examination of the junctional current data led us to believe that the channel closure kinetics of channels containing Cx43 and mutant Cx26 might also be different. We determined the time constant tau (τ) by plotting the current decay against time and fitting to a mono-exponential function for transjunctional potentials of ±120 mV. Representative current decays measuring τ at +120 mV showed wild-type Cx43 channels expressed alone in cells closed slower (Figure 4-4A) than channels containing both Cx43 and the Cx26 mutants (Figure 4-4B and 4-4C). Mixed channels containing Cx43 and Cx26-H73R

closed 73% faster than wild-type Cx43 channels, with mean channel closure times of 0.90 seconds versus 0.24 seconds (Figure 4-4D). A similar trend was observed with mixed channels containing Cx43 and Cx26-S183F where they closed 53% faster than wild-type channels, with mean closures times of 0.90 seconds against 0.42 seconds (Figure 4-4D). Study of channel kinetics at +120 mV showed that the mixed channels had faster channel closure times than wild-type Cx43 channels alone (Figure 4-4D). The opposite was seen at a transjunctional potential of -120 mV. At the negative pulse, mixed channels had slower channel closure times than wild-type channels (Figure 4-4E). The gain of Cx26-like biophysical properties observed in the steady state data described above was detected again with the channel kinetics of mixed Cx26 mutant and Cx43 channels in comparison with wild-type Cx43 channels alone. The difference observed between wild-type Cx43 channels alone and mixed mutant Cx26 and Cx43 channels appears to be from an acquisition of Cx26-like behavior by Cx43, strengthening the argument for Cx43 interaction with mutant Cx26.

Cx26-H73R and Cx26-S183F fail to form heterotypic channels with wild-type connexins

Cx26-H73R and Cx26-S183F are located on the first and second extracellular loops respectively, domains necessary for docking between connexons on contacting cells. We wanted to confirm that these mutations were not changing the heterotypic interaction of Cx26 with Cx43. *Xenopus* oocytes were paired to allow formation of heterotypic channels consisting of homomeric wild-type Cx26 hemichannels and homomeric mutant hemichannels. Control cells injected with H_2O displayed negligible conductance, whereas cell pairs expressing wild-type Cx26 demonstrated electrical

coupling with a mean conductance of 8.4 μS (Student's t-test $p<0.05$) (Figure 4-5). Cells were paired such that apposing cells expressed either wild-type or mutant Cx26. These pairs failed to exhibit junctional coupling and their mean conductance levels were not above background control (Student's t-test $p>0.05$) (Figure 4-5). A failure to exhibit heterotypic channel formation by the mutant and wild-type proteins suggested the mutant connexins did not form functional homomeric hemichannels, or they were unable to reach the plasma membrane.

The heterotypic channel interaction of Cx43 with the mutant proteins was tested to confirm the asymmetric I_js observed in co-expressing cells above were not due to changes in docking. Oocytes were paired with H_2O control and Cx43 cells to confirm elimination of *Xenopus* Cx38 expression because of the high affinity with which Cx43 and Cx38 form heterotypic channels (Swenson, Jordan, Beyer, & Paul, 1989) (Figure 4-6). Cells forming homotypic Cx43 channels displayed high mean conductance of 9 μS over the control pairs (Student's t-test $p<0.05$) (Figure 4-6). Next, cells expressing Cx43 were paired with cells expressing mutant Cx26 proteins. These pairs failed to form functional heterotypic channels based on negligible conductance similar to the control pairs (Student's t-test $p>0.05$) (Figure 4-6). Based on this observation, we concluded the asymmetric I_js observed in co-expressing cells described above were unrelated to heterotypic interactions.

Since these Cx26 mutants exhibited a lack of gap junctional activity when expressed alone, our interest in studying the effect of these mutants on the voltage gating properties of Cx43 was piqued. These mutants, when combined with Cx43, were able to visibly alter the current decay of Cx43 channels and shifted the equilibrium properties of

the channel towards a Cx26-like behavior. Based on the results of the voltage gating and kinetics studies, there is an indication that Cx26-H73R and Cx26-S183F are not only able to exert dominant inhibitory effects on Cx43 conductance levels, but they can functionally interact with Cx43 such that its gating behavior is altered.

DISCUSSION

The ability of PPK associated connexin mutant proteins to interfere with wild-type connexin proteins has been studied in various electrophysiological and permeability assays (Rouan et al., 2001; Thomas et al., 2004). These studies established a clear link between Cx26 mutations and wild-type Cx26 and Cx43, where the mutant proteins can dominantly inhibit the wild-type proteins. This study examined the functional properties of Cx26, Cx30 and Cx43 gap junctions in the presence of two Cx26 PPK mutants. These wild-type connexins were selected based on their overlapping expression with Cx26 in the epidermis. Cx30 was also chosen due to its prominent role in the causation of Clouston syndrome, a (OMIM no. 129500) (Kibar et al., 1996). Clouston syndrome is marked by fine brittle hair, alopecia, thick or absent nails, palmoplantar hyperkeratosis and hyperpigmentation of the skin (Clouston, 1929). Mutations in Cx30 associated with Clouston syndrome include Cx30-G11R and Cx30-A88V (Lamartine et al., 2000). These mutations share functional similarities with KID syndrome with regard to their increased hemichannel activity (Essenfelder et al., 2004).

Based on the expression of multiple connexins in the same cell and tissue, and high sequence homology, it is likely different connexins will interact to form heterotypic and heteromeric gap junctions. The formation of heterotypic channels was initially seen in a co-expression study of Cx26 and Cx32 (Barrio et al., 1991). This report showed the formation of heterotypic channels of Cx26/Cx32 resulted in asymmetric channels and a change in the channel's behavior. Synthesis of heteromeric channels was observed in vascular smooth muscle cells expressing Cx40 and Cx43 resulting in voltage gating behavior that was distinct from heterotypic channels (He, Jiang, Taffet, & Burt, 1999). It

is possible to observe connexin-connexin interaction based on changes in channel properties from homotypic channels.

In the present study, while both mutants were unable to form functional gap junction channels alone, they demonstrated significant inhibition of wild-type Cx26 and Cx43 channels; neither Cx26-H73R nor Cx26-S183F demonstrated any negative effect on Cx30. The inhibition of wild-type Cx43 did not completely eliminate its channel activity, which allowed for a more comprehensive analysis of its functional properties. This assessment suggested a physical association between wild-type Cx43 and the mutant proteins. Examination of the voltage gating activity of Cx43 channels when co-expressed with Cx26-H73R or Cx26-S183F showed a shift towards a more voltage sensitive current decay. Although Cx43 channels display a symmetric current decay, the addition of the Cx26 PPK mutants, and subsequent formation of heteromeric channels, shifted the current decay towards a Cx26-like asymmetry. The asymmetry observed in these decays are a result of a dependence on the differences in potential between the cytoplasmic and extracellular environments (V_{i-o}) (White et al., 1994). These heteromeric channels still retained some elements of Cx43 channels, but were undoubtedly no longer homomeric. Changes in closure kinetics also showed a reversal in behavior between positive and negative transjunctional potentials, as well as faster channel closure. Unlike PPK mutants, most Cx26 mutants associated with KID syndrome display functional hemichannels, which cause abnormal epidermal pathologies through aberrant hemichannel activity.

Collectively, the biophysical data described above suggests a difference in the mechanism through which Cx26 mutations cause PPK or KID syndrome. While Cx26-

H73R and Cx26-S183F act by eliminating their own gap junctional activity, they also alter the channel activity of Cx43. Co-expression of these mutations with Cx43 results in the wild-type channel adopting a more Cx26-like channel property. This indicates Cx26 mutants associated with PPK need to physically interact with other connexins to cause pathogenicity. Conversely, mutations associated with KID syndrome act by forming functional hemichannels that alter the passage of Ca^{2+} between the cell and extracellular space.

Analyses of KID syndrome mutations have showed some are able to form functional gap junction channels (Cx26-N14K, Cx26-A40V, Cx26-G45E), however, their voltage gating is markedly different from wild-type Cx26 (Gerido et al., 2007; Lee et al., 2009; Sanchez et al., 2010). The three KID syndrome mutations known to make gap junctions display electrical coupling at levels comparable to wild-type Cx26, but their voltage gating varies from the wild-type channels, and also from one another: Cx26-N14K showed a significant loss in voltage sensitivity (Lee et al., 2009), Cx26-G45E had an alteration in gating, with the current decay becoming more symmetrical and voltage sensitive (Gerido et al., 2007), and Cx26-A40V had a slight loss of asymmetry, but retained the voltage dependent behavior of wild-type Cx26 (Sanchez et al., 2010). Unlike these mutations, the majority of PPK mutations do not form functional gap junction channels and their pathogenicity lies in their ability to interact with other connexin proteins. One KID syndrome mutation, Cx26-S17F, behaves functionally similarly to PPK mutants by not forming functional hemichannels or gap junction channels ((Lee et al., 2009), and likely causes disease by interacting with Cx43 (Garcia et al., 2015).

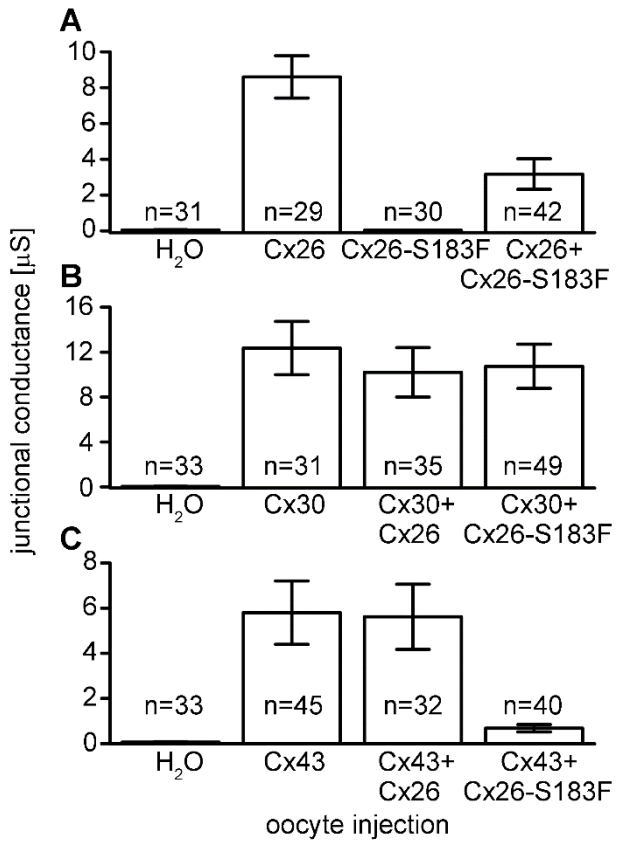

Figure 4-1. Cx26-S183F dominantly inhibits wild-type Cx26 and Cx43, but not wild-type Cx30. (A) Cells injected with H_2O displayed negligible conductance, while cells injected with wild-type Cx26 cRNA formed channels with a conductance value of 8.6 µS. Oocytes expressing mutant Cx26-S183F had minimal conductance which was not above background levels, thereby demonstrating a loss of functionality of the protein. A combination of wild-type and mutant Cx26 cRNA resulted in a significant decrease in channel conductance (Gj = 3.2 µS, Student's t-test $p<0.05$) from wild-type Cx26 alone, revealing a cis-dominant inhibition of wild-type Cx26 function by the mutant. (B) Oocytes were injected with wild-type Cx26, Cx30 and mutant Cx26-S183F, alone or in combination. Wild-type Cx30 was able to form functional channels when expressed alone or in combination with the wild-type or mutant Cx26 cRNA, indicating the mutant is unable to disrupt its functionality. (C) Wild-type Cx26, Cx43 and mutant Cx26-S183F were expressed in cells alone or in combination. When expressed alone, or with wild-type Cx26, wild-type Cx43 is able to form functional gap junctional channels based on conductance that is significantly higher than background (Gj = 5.8 µS and 5.6 µS, respectively, Student's t-test $p<0.05$). However, a combination of wild-type Cx43 and Cx26-S183F cRNA resulted in a trans-dominant inhibition of the wild-type protein's function (Gj = 0.68 µS, Student's t-test $p<0.05$). Data are the means ± SE.

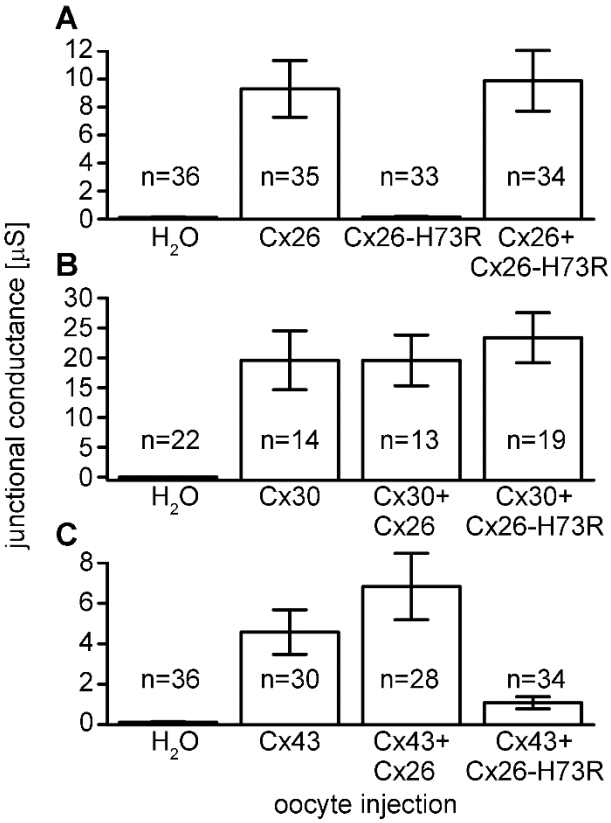

Figure 4-2. Cx26-H73R does not form functional gap junctions and trans-dominantly inhibits Cx43, while not interfering with wild-type Cx26 and Cx30 junctional activity. (A) Cells injected with H_2O displayed negligible conductance, while cells injected with wild-type Cx26 cRNA formed channels with a conductance value of 9.3 µS. Oocytes expressing mutant Cx26-H73R had negligible conductance similar to negative control cells, thereby demonstrating a loss of functionality of the protein. A combination of wild-type and mutant Cx26 cRNA did not alter channel conductance (Gj = 9.9 µS, Student's t-test $p>0.05$) from wild-type Cx26 alone, revealing mutant Cx26 does not affect Cx26-H73R. (B) Oocytes were injected with wild-type Cx26, Cx30 and mutant Cx26-H73R, alone or in combination. Wild-type Cx30 was able to form functional channels when expressed alone or in combination with the wild-type or mutant Cx26 cRNA, indicating the mutant is unable to disrupt its functionality. (C) Wild-type Cx26, Cx43 and mutant Cx26-H73R were expressed in cells alone or in combination. When expressed alone, or with wild-type Cx26, wild-type Cx43 is able to form functional gap junctional channels based on conductance that is significantly higher than background (Gj = 4.6 µS and 6.8 µS, respectively, Student's t-test $p<0.05$). However, a combination of wild-type Cx43 and Cx26-H73R resulted in a trans-dominant inhibition of Cx43 (Gj = 1.1 µS, Student's t-test $p<0.05$). Data are the means ± SE.

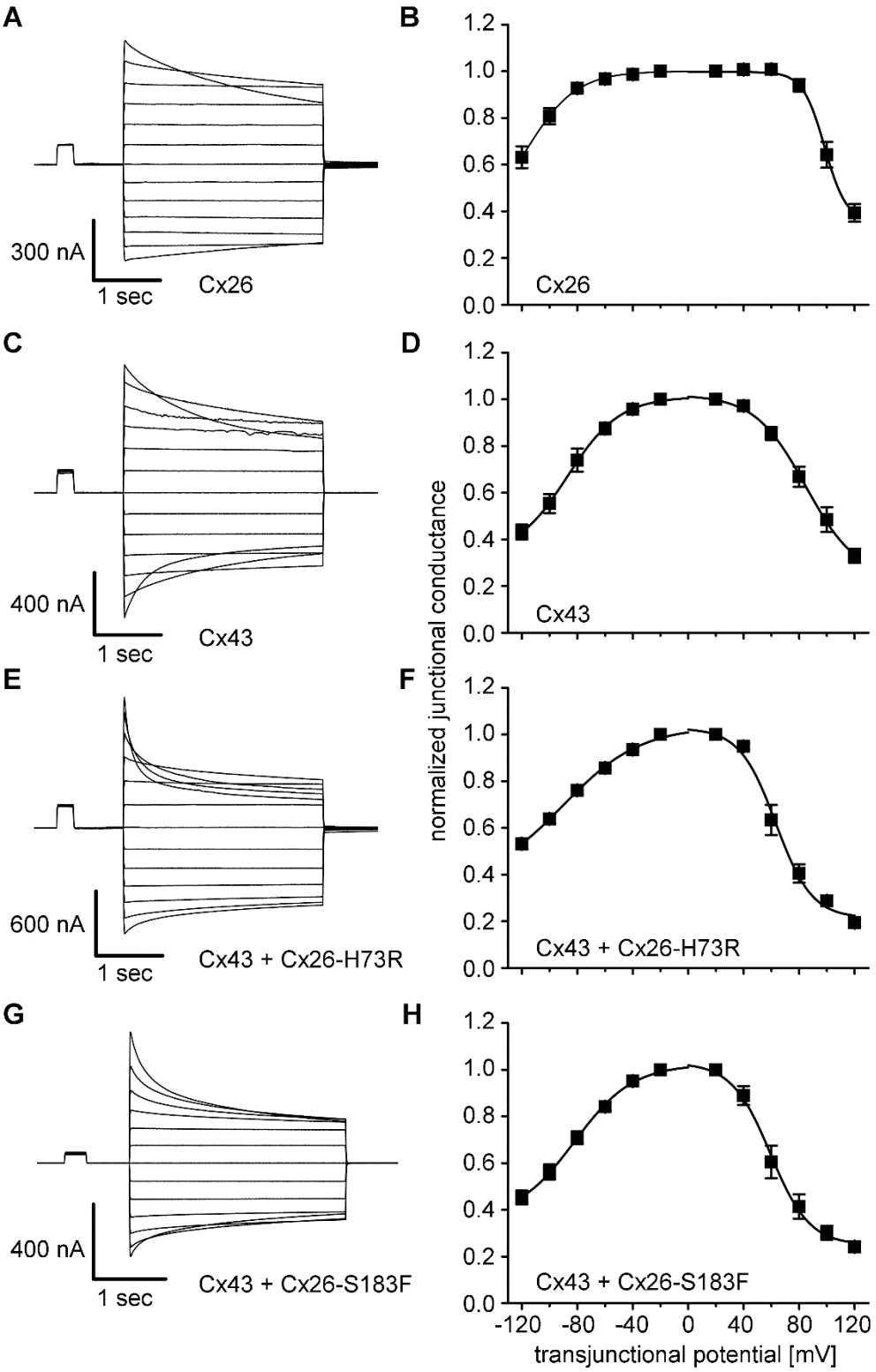

Figure 4-3. PPK mutants associated with deafness can alter the voltage gating of Cx43. (A) Analysis of the voltage gating properties of wild-type Cx26 showed an asymmetry in current decay at higher voltages, (B) which was also observed in the Boltzmann fit at higher negative and positive potentials (n=7). (C) Cx43 showed a typical rapid

symmetrical decrease in a voltage-dependent manner at higher voltages and (D) quantified by a Boltzmann fit of the parameters to show a reasonably symmetrical decay at higher potentials (n=6). (E, G) In the presence of non-functional Cx26 mutants, the gating of Cx43 was modified towards a more asymmetrical response to a voltage stimulus. (F, H) Quantification of the steady-state voltage gating of these mixed channels containing Cx26-H73R (n=4) or Cx26-S183F (n=7) showed a clear shift in Cx43 gating properties towards an asymmetrical behavior at higher potentials, comparable to wild-type Cx26.

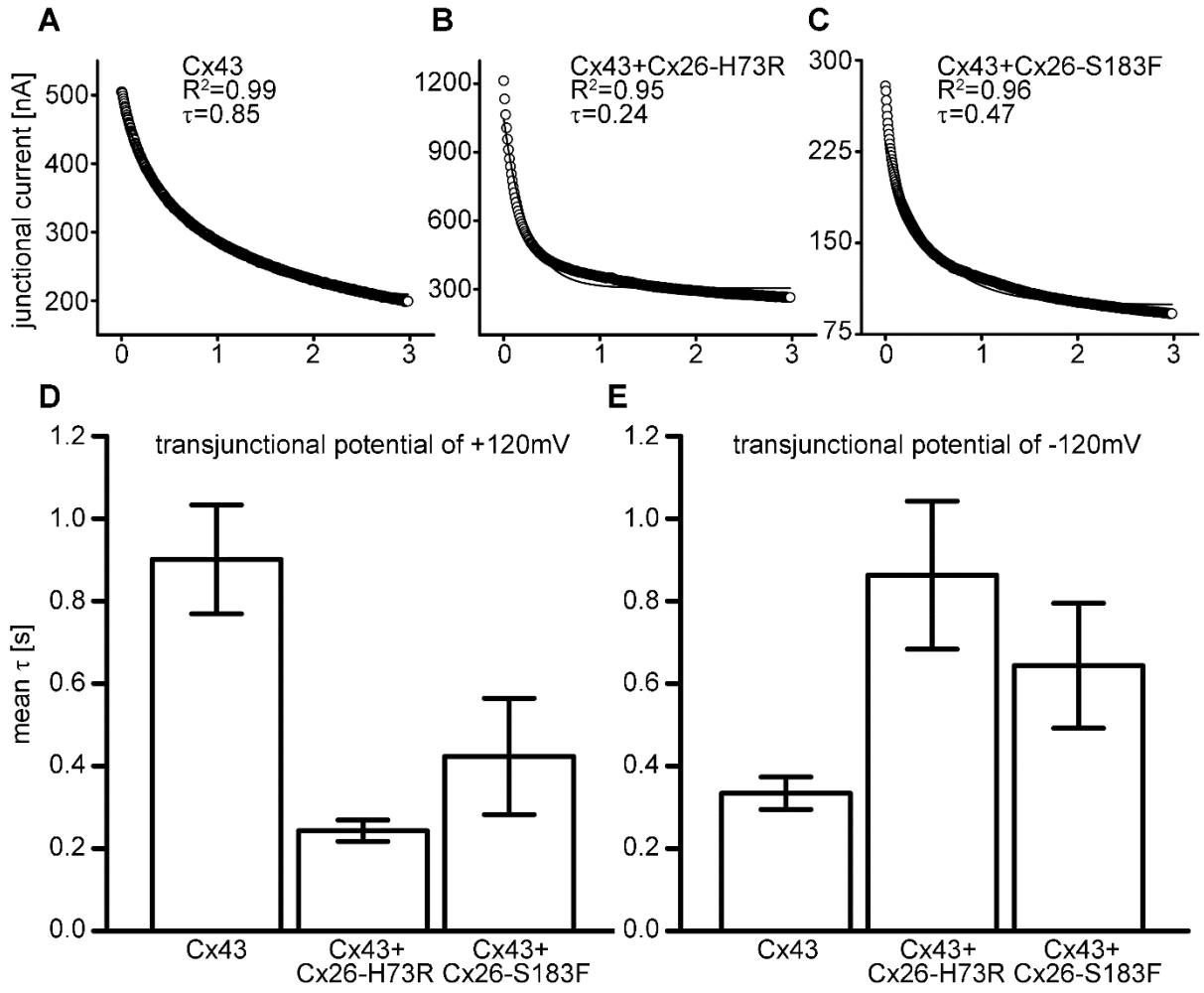

Figure 4-4. The channel closure kinetics of Cx43 can be modified by Cx26 mutants associated with PPK. Junctional currents were fit by a single exponential decay to determine the time constant (τ). Representative decays at a Vj of +120 mV showed (A) slower closure kinetics in channels expressing Cx43 alone, while the presence of (B) mutant Cx26-H73R or (C) Cx26-S183F resulted in a pronounced increase in Cx43 channel closure kinetics. (D) At +120 mV, Cx43 channels closed more slowly than when in the presence of mutant Cx26 based on mean τ values of 0.90 ± 0.26 for Cx43 alone (n=4), 0.24 ± 0.05 for Cx43+Cx26-H73R (n=4) and 0.42 ± 0.31 (n=5) for Cx43+Cx26-S183F. (E) The opposite was observed at -120 mV, where Cx43 channels alone closed faster than those formed in the presence of mutant Cx26. Mean τ values of 0.33 ± 0.07 for Cx43 alone (n=4), 0.86 ± 0.36 for Cx43+Cx26-H73R (n=4) and 0.64 ± 0.34 (n=5) for Cx43+Cx26-S183F suggested a reversal in Cx43 channel closure kinetics in the presence of mutant Cx26. Data are the means ± SE.

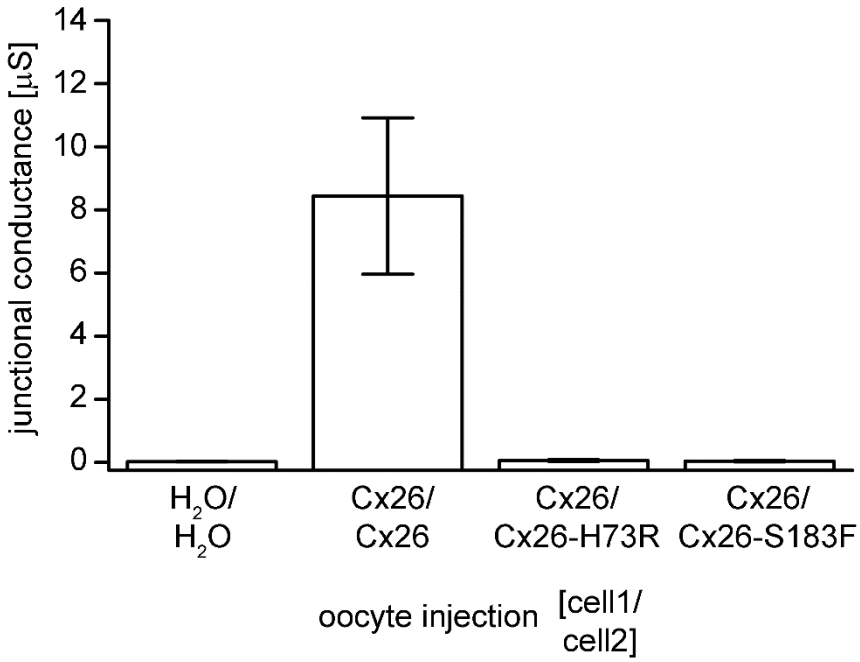

Figure 4-5. Cx26 mutants do not form heterotypic channels with wild-type Cx26. Cells injected with water (n=24) displayed negligible conductance, while cells injected with Cx26 and paired to form homotypic channels had measureable conductance (8.4 μS, n=24). Cells injected with Cx26 paired with cells expressing Cx26-H73R (n=17) or Cx26-S183F (n=17) exhibited negligible conductance similar to control pairs (Student's t-test $p>0.05$) demonstrating an absence of heterotypic channel formation. Data are the means ± SE.

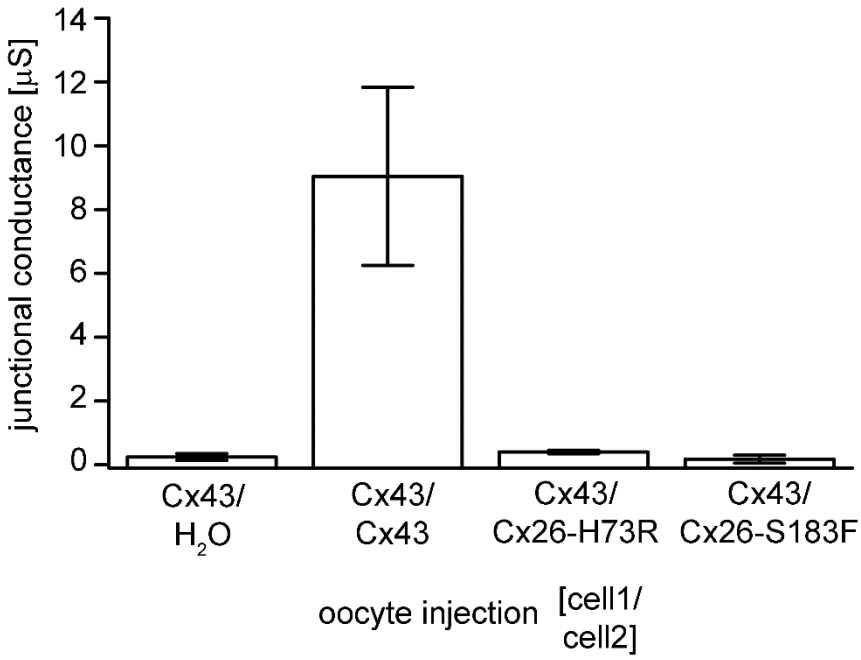

Figure 4-6. Cx26 mutants associated with PPK do not form heterotypic channels with Cx43. Cells injected with water and paired with Cx43 (n=16) displayed negligible conductance, while cells injected with Cx43 and paired to form homotypic channels had conductance of 9.0 μS (n=20). Cells injected with Cx43 paired with cells expressing Cx26-H73R (n=17) or Cx26-S183F (n=19) exhibited negligible conductance similar to control pairs (Student's t-test $p>0.05$) demonstrating an absence of heterotypic channel formation. Data are the means ± SE.

Table 4-1. Boltzmann parameters of gap junction channels

Oocyte injection	V_j	G_{min}	V_0 [mV]	A
Cx26	+	0.35 ± 0.01	98 ± 0.4	0.13
Cx26	–	0.31 ± 0.14	-118 ± 7.2	0.05
Cx43	+	0.24 ± 0.05	85 ± 2.7	0.05
Cx43	–	0.33 ± 0.03	-87 ± 1.8	0.05
Cx26-H73R+Cx43	+	0.21 ± 0.04	63 ± 3.1	0.08
Cx26-H73R+Cx43	–	0.36 ± 0.09	-90 ± 7.4	0.04
Cx26-S183F+Cx43	+	0.25 ± 0.03	59 ± 2.3	0.07
Cx26-S183F+Cx43	–	0.36 ± 0.04	-82 ± 2.8	0.05

G_{min} represents the minimum conductance value, V_0 indicates the voltage measured midway through the G_j decline, and A denotes the cooperativity constant, reflecting the number of charges moving through the transjunctional field. Signs + and – for V_j indicate transjunctional membrane potential polarity.

Table 4-2. Gap junctional properties of connexin channels in the presence of Cx26 mutants

Oocyte injection	Homotypic channel	+ Cx26 (heteromeric channel)	+ Cx43 (heteromeric channel)	+ Cx26 (heterotypic channel)	+ Cx43 (heterotypic channel)
Cx26	Yes				
Cx43	Yes	Yes			
Cx26-H73R	No	No effect	↓	No	No
Cx26-S183F	No	↓	↓	No	No

Cx26-H73R and Cx26-S183F were expressed in oocytes alone or in combination with Cx26 or Cx43.

Chapter 5 . Formation of novel Cx43 and mutant Cx26 heteromeric connexons

ABSTRACT

Multiple connexins can be expressed in the same cell and tissue. That connexin expression can overlap unsurprisingly results in mixing and formation of heteromeric connexons. Yet heteromeric channel synthesis is highly dependent on sequence homology and subsequent compatibility. Nine connexin isoforms are expressed in the epidermis, with Cx26 expression found in the basal layer, and granular layer of palm and plantar skin. Cx26 mutations associated with palmoplantar keratoderma (PPK) alter the channel activity of simultaneously expressed connexin proteins such as Cx43. Electrophysiological assays have shown a decline in mutant Cx26 gap junction channel activity and provided compelling evidence for an interaction between Cx26 mutants associated with PPK and Cx43. We present data demonstrating the absence of electrical coupling between *Xenopus* oocytes expressing Cx26 PPK mutants Cx26-H73R and Cx26-S183F alone and Cx43 is not due to changes in protein levels, but rather a mis-localization of the mutant and wild-type proteins in the cytoplasm. Additionally, we show impairment of Cx43 gap junction channels occurred due to an interaction of the wild-type protein with Cx26 mutant proteins, resulting in the formation of a novel heteromeric channel.

INTRODUCTION

Gap junctions are composed of two hemichannels docked on apposed cells. These hemichannels are comprised of six connexin proteins, which mix to form homomeric or heteromeric channels. Homomeric channels are made up of the same connexins, whereas heteromeric channels contain different connexins (Stauffer, 1995). The formation of heteromeric hemichannels changes the channel's properties, including its gating and permselectivity. Hemichannels and gap junctions of similar classes, such as Cx26 and Cx30 have been demonstrated to mix (Yum et al., 2007), whereas, those belonging to different groups, Cx26 and Cx43, do not form heteromeric channels (Gemel et al., 2004; Yeager & Nicholson, 1996).

The expression of multiple connexins in the same cell is well known: Cx46 and Cx50 in the lens fiber, Cx43 and Cx45 in cardiac myocytes and osteoclasts, and Cx26 and Cx32 in hepatocytes and exocrine pancreas (Kanter, Laing, Beyer, Green, & Saffitz, 1993; Meda et al., 1993; Paul, Ebihara, Takemoto, Swenson, & Goodenough, 1991; Steinberg et al., 1994), and due to their structural and sequence homologies, it is reasonable to expect connexin-connexin interactions. Co-oligomerization of connexins has been demonstrated in many tissues, including the liver (Bevans et al., 1998), heart (He et al., 1999; Valiunas et al., 2001), cochlea (Lautermann et al., 1998; Yum et al., 2007), and lens (Jiang & Goodenough, 1996). These biochemical studies consistently showed heteromeric channel formation by connexins of the same group. Functional studies have shown formation of heteromeric channels by two compatible connexins can alter the channel's permeability and gating (Brink et al., 1997; White et al., 1995). Under "normal" physiological conditions, these channels are composed of wild-type connexin

proteins. However, the presentation of a connexin-related disease can be a result of a disruption in “normal” physiology due to an interaction of mutant connexin proteins with wild-type connexins.

An early report of mutations in Cx32 associated with X-linked Charcot-Marie-Tooth disease (CMTX) found that these mutations though unable to form functional channels alone, were able to inhibit wild-type Cx26 gap junction channels (Bruzzone, White, Scherer, et al., 1994). The authors suggested the possibility of the mutant and wild-type proteins co-oligomerizing to form heteromeric channels that would be non-functional or unable to dock correctly. An analysis of Cx26/Cx32 permeability to cAMP and cGMP provided biochemical evidence of an interaction between Cx26 and Cx32 (Bevans et al., 1998). This investigation concluded that the permeability of connexin channels can be varied based on their composition, while Cx32 is permeable to both cAMP and cGMP, heteromeric channels of Cx26 and Cx32 were impermeable to cAMP. Such permselectivity of connexin channels can provide one possible explanation for the pathogenicity of connexin point mutations. Subsequently, other reports on permselectivity have shown a similar behavior in Cx26 and Cx30 channels (Beltramello et al., 2005; Y. Zhang et al., 2005).

Cx26 and Cx30 are both found in the cochlea and epidermis (E. Dahl et al., 1996; Di et al., 2001; Goliger & Paul, 1994; Xia et al., 2001). Mutations in Cx26 associated with syndromic hearing loss, such as Cx26-G59A and Cx26-R75W, have been shown to have a dominant negative effect on wild-type Cx30 channels (Forge et al., 2003; Marziano et al., 2003). These mutant proteins were found to co-localize in intercellular plaques between cell pairs. Further study found both proteins were able to co-immunoprecipitate

with Cx30, providing compelling evidence for the formation of heteromeric channels (Yum et al., 2010). Taken together research on Cx26/Cx32 and Cx26/Cx30 demonstrated the ability of connexins belonging to the same subgroup to form heteromers. However, other studies have illustrated the inability of connexins belonging to different classes to interact and form heteromeric channels (Gemel et al., 2004; Lagree et al., 2003). Both studies found connexins belonging to the β subgroup (Cx26 and Cx32) were incompatible with Cx43, which is in the α subgroup. Cx26 mutations associated with PPK are able to trans-dominantly inhibit Cx43 through an unexplored mechanism that probably forms aberrant heteromeric channels.

RESULTS

Wild-type and mutant connexin proteins are expressed in *Xenopus* oocytes

One possible reason for a loss of channel functionality in Cx26 PPK mutants could be differences in levels of protein translation. The syntheses of epidermal connexins, both wild-type and mutant, were therefore examined by Western blot. Oocytes injected with H_2O, wild-type Cx26 and Cx43, or mutant Cx26-H73R cRNA alone, or in combination, were studied using polyclonal Cx26 and monoclonal Cx43 antibodies. Immunoblotting for Cx26 protein revealed bands at ~26 kDa in lanes corresponding to oocytes injected with either wild-type Cx26 or mutant Cx26-H73R cRNA, and failed to detect protein in the H_2O injected samples (Figure 5-1A). The antibody for Cx43 also did not identify protein in the H_2O injected oocytes; however, it did show bands at ~43 kDa of equal intensity in all oocytes injected with Cx43 cRNA, whether alone or in combination with wild-type Cx26 or mutant Cx26-H73R (Figure 5-1B). As a qualitative control, β-actin bands detected at ~42 kDa were found to be of similar intensity, thereby confirming equal loading of protein in all lanes (Figure 5-1C).

The expression levels were quantified using band densitometry on replicate Western blots (n=4). The mean band intensity values were normalized to the wild-type mean value. The plot of these values did not show significant differences between the expression of wild-type Cx26 alone, mutant Cx26-H73R alone, and the wild-type and mutant in combination with wild-type Cx43 (Student's t-test $p<0.05$) (Figure 5-1D). The plot for the normalized intensity of Cx43 protein, when expressed alone, or with wild-type Cx26 or mutant Cx26-H73R, did not show significant differences between the samples (Student's t-test $p<0.05$) (Figure 5-1E).

An immunoblot analysis of the protein expression in oocytes injected with H_2O, wild-type Cx26 and Cx43, or mutant Cx26-S183F cRNA alone, or in combination showed the presence of Cx26 protein at 26 kDa in corresponding lanes (Figure 5-1F). Immunoblotting for Cx43 also revealed bands at ~43 kDa in lanes containing Cx43, Cx43 and wild-type Cx26, and Cx43 and Cx26-S183F injected cells (Figure 5-1G). The presentation of protein bands in the correct lanes once again indicated correct translation of the cRNA. Cx26 protein bands differed in the expression of the wild-type and mutant Cx26 proteins. Cx43 proteins were found in equivalent levels, thereby attributing the loss of functional activity in the presence of mutant Cx26-S183F to a mechanism not related to the translational efficiency of Cx43. The blot was re-probed for β-actin (~42 kDa) to ensure equal loading of samples (Figure 5-1H).

As described above, the expression levels were quantified using band densitometry on replicate blots (n=4). Cx26 protein expression levels were equal among the samples containing wild-type Cx26, however, mutant Cx26-S183F expression showed a ~75% reduction in expression compared to wild-type Cx26, despite injection of equivalent levels of cRNA (Figure 5-1I). This reduction in mutant protein expression was consistently found among all Western blots, indicating either reduced translational efficiency, or reduced protein stability for the mutant Cx26-S183F. Examination of Cx43 protein band intensity showed equivalent expression in all corresponding lanes (Figure 5-1J). Although Cx26-S183F protein expression was lower than wild-type Cx26 or mutant Cx26-H73R, the potency with which the mutant was able to affect Cx43 in our functional studies remained high.

Cx26-H73R and Cx26-S183F have irregular localization in transfected cells

The functional evidence indicating a failure of the PPK mutants to form hemichannels and gap junctions alone, yet still displaying sufficient translation, suggested a possible reduction in protein trafficking to the plasma membrane. A change in the connexin proteins' movement towards the plasma membrane would explain the inability of the mutants to form functional channels alone. Expression of wild-type Cx26 in communication deficient HeLa cells established the presence of protein plaques found between cells as expected (Figure 5-2A) (Mese, Valiunas, Brink, & White, 2008). Gap junction plaques were once again observed between cells pairs of stably expressed Cx43 cells transfected with wild-type Cx26, stained for either Cx26 (Figure 5-2B) or Cx43 (Figure 5-2C), demonstrating the presence of either protein does not affect the formation of junctional plaques between adjacent cells. However, cells transfected with Cx26-H73R (Figure 5-2D) or Cx26-S183F (Figure 5-2G) exhibited a reduction in plaque formation between cell pairs; with the mutant proteins dispersed throughout the cell. When the mutant proteins were expressed in the stably expressing Cx43 cells, once again, a reduction of Cx26 gap junctional plaques was apparent (Figure 5-2E and 5-2H). Staining these cells for Cx43 also showed a reduction in Cx43 plaque formation. The Cx43 protein in cells co-expressing Cx26-H73R (Figure 5-2F) or Cx26-S183F (Figure 5-2I) was also found to be dispersed throughout the cell, indicating a disruption in Cx43 protein trafficking due to the presence of the mutant proteins. The mis-localization of Cx43 protein in the presence of Cx26 mutants is consistent with less plaque formation and a decrease in junctional communication, and could result from heteromeric connexon formation between the mutant and wild-type proteins.

Cx26 PPK mutants associate with Cx43

The biophysical data showed that Cx26 PPK mutants were unable to form functional channels alone, but could affect the ability of Cx43 to make channels, as well as alter their gating and kinetics. The changes in Cx43 behavior when co-expressed with mutant Cx26 channels suggested the formation of heteromeric channels due to the acquisition of function. To investigate this possibility, co-immunoprecipitation was performed on cells co-expressing Cx43 and mutant Cx26 to elucidate the mechanism through which Cx43 behavior was altered. *Xenopus* oocytes injected with H_2O, wild-type Cx26, Cx43, Cx26-H73R, Cx26-S183F and mixtures of Cx43 and Cx26 mutants were subjected to co-immunoprecipitation. An immunoblot analysis was performed on cell lysates to confirm the presence of Cx26 and Cx43 proteins in corresponding samples. The cell lysates showed Cx26 protein present in all lanes containing either wild-type or mutant Cx26 (Figure 5-3A and 5-3E). Re-probing for Cx43 revealed similar expression of Cx43 protein in lanes containing Cx43 alone or co-expressed with either wild-type or mutant Cx26 (Figure 5-3B and 5-3F).

Since the biophysical data suggested the formation of heteromeric channels, we tested for co-immunoprecipitation of Cx43 and mutant Cx26. Immunoblotting for Cx26 in the pull-down showed the presence of protein in all samples that had been injected with either wild-type or mutant Cx26 (Figure 5-3C and 5-3G). However, H_2O and Cx43 only samples did not contain Cx26 proteins, thereby indicating wild-type and mutant Cx26 proteins could be immunoprecipitated specifically. Similar to Figure 5-1F and 5-3E, Cx26-S183F expression levels were lower than wild-type Cx26 or Cx26-H73R. In order to determine if the Cx26 mutants interacted with Cx43, an antibody against Cx43 was used

to detect Cx43 protein on the same blot. We observed that wild-type Cx26 inefficiently co-immunoprecipitated with Cx43, consistent with previous reports (Gemel et al., 2004; Yum et al., 2010), however both Cx26 mutants were found to co-immunoprecipitate Cx43 (Figure 5-3D and 5-3H). The levels of protein expression in the samples co-expressing Cx43 and either Cx26 mutant were notably higher than in those co-expressing wild-type Cx26 and Cx43, suggesting the mutant proteins were able to more efficiently co-immunoprecipitate with Cx43. The co-immunoprecipitation of the Cx26 mutant proteins with Cx43 corroborates the channel gating and kinetics data which suggest an interaction between mutant Cx26 and Cx43 in heteromeric connexons resulting in an acquisition of Cx26-like behavior, and provides a potential mechanism whereby mutant Cx26 is able to modify Cx43 activity.

DISCUSSION

Cx43 is the most ubiquitously expressed connexin protein in animal tissues (Dermietzel et al., 1989; Dupont, el Aoumari, Roustiau-Severe, Briand, & Gros, 1988; Goliger & Paul, 1994; Meda, 1996; Risek, Guthrie, Kumar, & Gilula, 1990) and is frequently found co-expressed with other connexins. However, co-expression of Cx43 does not always result in the formation of heteromeric channels; Cx43 only forms heteromeric channels with connexins it shares sequence homology, and therefore compatibility (Koval, 2006). Cx43 has been demonstrated to be unable to interact with Cx26 by several studies using either affinity chromatography or co-immunoprecipitation (Gemel et al., 2004; Yum et al., 2010). They also showed the lack of interaction was due to incompatibility and not differences in the proteins' biosynthetic trafficking. It is hypothesized the N-terminus and first and third transmembrane domain contain residues that are critical for oligomerization (Lagree et al., 2003; Martinez et al., 2011; Maza, Das Sarma, & Koval, 2005). The majority of research has focused on heteromeric channel formation between homologous connexins. We have described a unique interaction between Cx43 and mutant Cx26 proteins.

The trans-dominant inhibition of Cx43 by Cx26 PPK mutant proteins suggested the possibility of an interaction between the mutant and wild-type proteins. Cx26-H73R and Cx26-S183F were shown to modify Cx43 channel activity which provided compelling evidence for a heteromeric interaction. The presence of a Cx43 and mutant Cx26 heteromeric connexon was validated by a co-immunoprecipitation where Cx43 was pulled down by the mutated proteins. Our data showed Cx43 was precipitated more efficiently by the mutant proteins than by wild-type Cx26. The formation of a Cx43 and mutant Cx26

heteromeric channel revealed a structure that could act as a pathological unit, resulting in the disruption of the epidermis. The importance of Cx43 in the epidermis has become increasingly implicit from the skin abnormalities that arise due to mutations in *GJA1*, the gene encoding Cx43 (Boyden et al., 2014; Vreeburg et al., 2007; H. Wang et al., 2015). The role played by Cx43 alongside Cx26 mutations in causing syndromic hearing loss associated with PPK is a major mechanistic difference with KID syndrome.

KID syndrome arises from Cx26 mutations which create functional hemichannels resulting in an aberrant transport of Ca^{2+} between the cell and extracellular space (Sanchez et al., 2010). These mutant channels may or may not form functional gap junctions (Lee et al., 2009). As our data suggests, protein translation of the mutant and wild-type proteins remains unaltered, however, protein localization is affected. This impairment in trafficking to the plasma membrane is a similarity shared by both KID syndrome and PPK mutants (Richard et al., 2002). Unlike KID syndrome, Cx26 mutations linked to PPK do not form functional channels (hemichannels or gap junctions). However, they are able to exert an effect on Cx43, suggesting a possible interaction between the proteins. Cx26 mutations associated with PPK have been demonstrated to mix with Cx30 and form heteromeric channels (Yum et al., 2010), however, their association with Cx43 has not been studied. Similarly, Cx43 has not been studied well in the context of KID syndrome, and only recently has a possible link between Cx26 aberrant hemichannel activity and Cx43 been explored (Garcia et al., 2015). Interestingly, while Cx26 mutations associated with KID syndrome form functional hemichannels, those associated with PPK do not, and instead require an interaction with wild-type connexin proteins to cause disease.

Conversely, a study of Cx43 mutations associated with ODDD, Cx43-G21R and Cx43-G138R, described an interaction of Cx43 with the mutated proteins, yet an inability of Cx32 to bind to the Cx43 mutants (Gong et al., 2007). These mutations were able to co-immunoprecipitate with wild-type Cx43, demonstrating a physical interaction which provides evidence for a possible mechanism causing ODDD, where the overall channel structure is altered by this pathological heteromeric structure. Therefore, it is possible for mutant connexin proteins to interact with their wild-type counterparts, wild-type connexins of the same subgroup, or as our data has determined, with wild-type connexins of a different subgroup. The formation of an aberrant heteromeric structure by co-expression of Cx43 with Cx26-H73R or Cx26-S183F expands the role of Cx43 in epidermal pathologies.

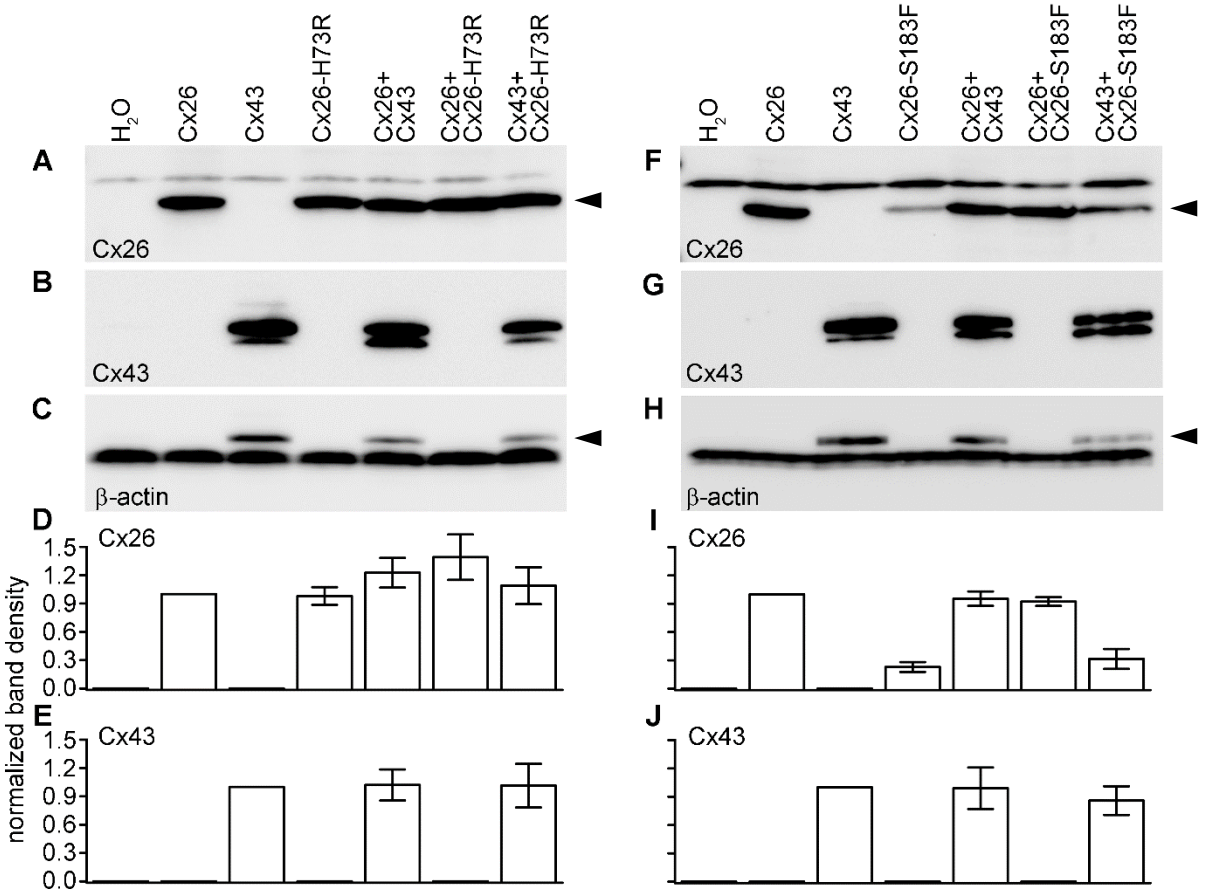

Figure 5-1. Wild-type and mutant connexin proteins were expressed in Xenopus oocytes. (A) Immunoblot analysis of oocytes expressing wild-type Cx26 and mutant Cx26-H73R showed equal expression of Cx26 proteins in all lanes, while (F) analysis of oocytes expressing wild-type Cx26 and Cx26-S183F qualitatively revealed lower expression of the mutant protein as compared to wild-type Cx26. (B, G) Cx43 levels were the same in each condition. (C, H) The blot was re-probed for β-actin to confirm equal loading qualitatively. (D, E) Band densitometry (n=4) confirmed quantitatively that mean protein expression was also equal in all conditions tested, with the exception of wild-type and mutant Cx26 where it was slightly higher. (I, J) Band densitometry (n=4) quantitatively confirmed equal protein expression of all wild-type Cx26 and Cx43, however, it showed a ~75% reduction in Cx26-S183F protein expression.

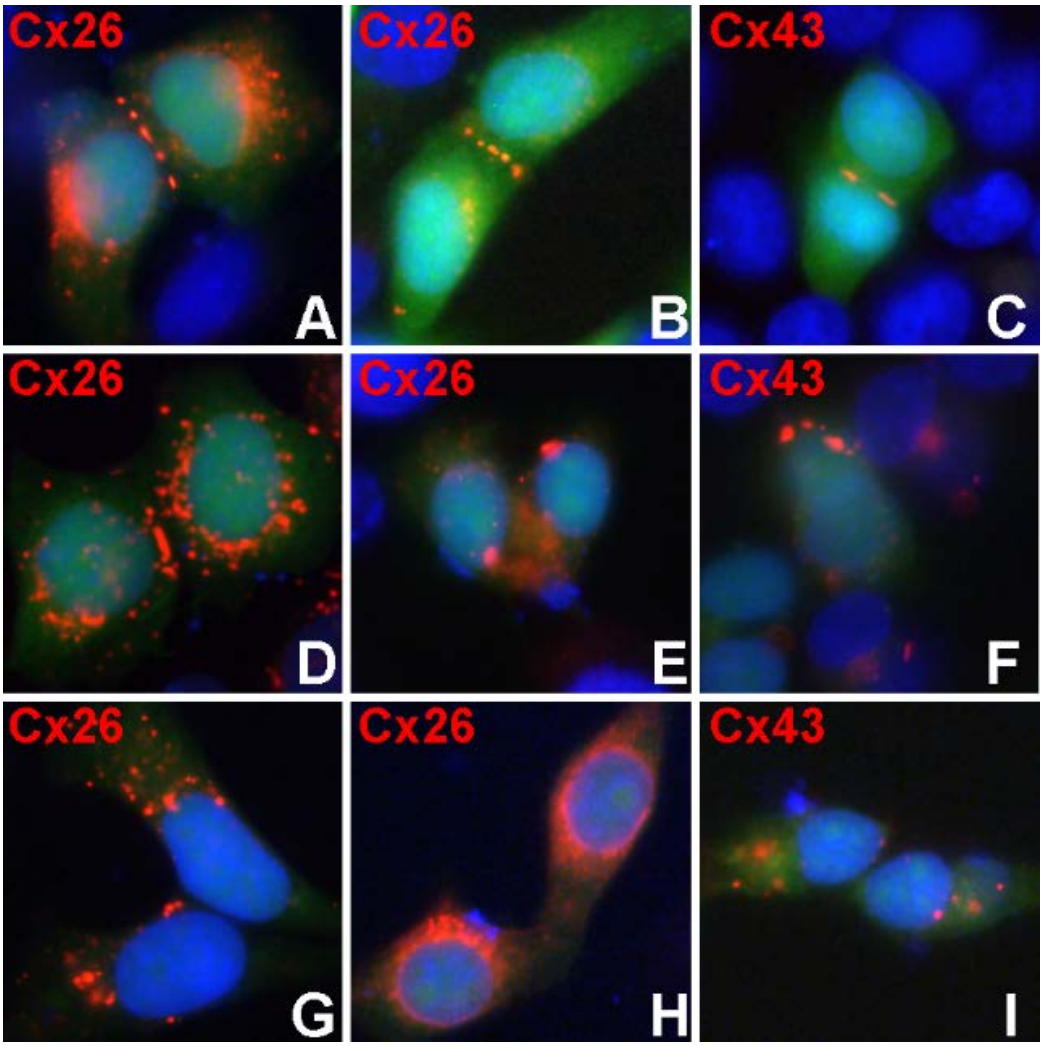

Figure 5-2. Cx26 mutants associated with PPK have irregular localization in mammalian cells, and alter Cx43 membrane trafficking. Two types of HeLa cells were used. Parental cells lacking endogenous connexins (A, D & G), or cells stably transfected with Cx43 (B, C, E, F, H & I). Cells were transiently transfected with either (A, B, C) wild-type Cx26-pIRES2/EGFP, (D, E, F) Cx26-H73R-pIRES2/EGFP or (G, H, I) Cx26-S183F-pIRES2/EGFP. HeLa cells expressing Cx26 proteins alone showed (A) junctional plaque formation between cells transfected with wild-type Cx26, and a loss of junctional plaques in cells transfected with (D) Cx26-H73R and (G) Cx26-S183F alone. In the presence of Cx43, (B) wild-type Cx26 was able to form gap junctional plaques between cells, and (C) Cx43 plaques were observed in cells co-expressing wild-type Cx26 and Cx43. Co-expression of (E, H) mutant Cx26 with Cx43 showed a mis-localization of the mutant proteins within the cell, similar to the observation made when they were expressed alone. However, (F, I) detection of Cx43 in the presence of mutant Cx26 revealed a dispersion of Cx43 protein within the cell, indicating alteration of gap junctional plaque formation.

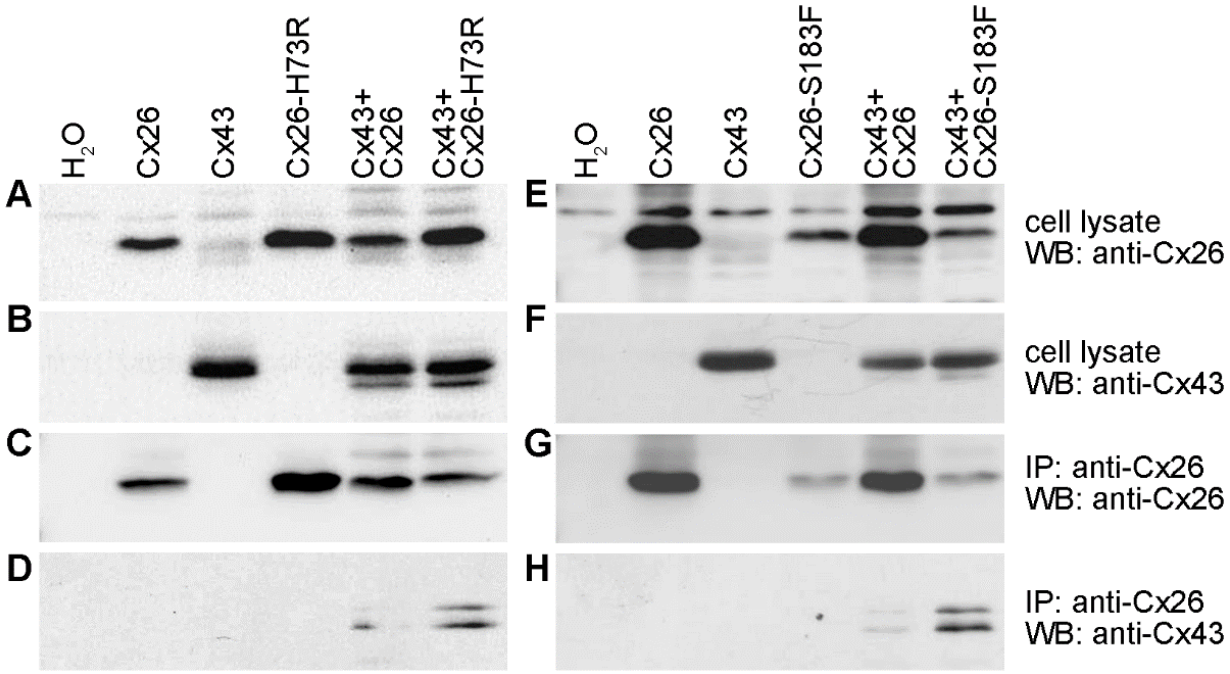

Figure 5-3. Cx26 mutants associated with PPK, biochemically interact with wild-type Cx43. (A, E) Immunoblot analysis of cell lysates showed qualitatively similar expression of wild-type Cx26 and Cx26-H73R, and lower protein expression of Cx26-S183F. (B, F) Detection of Cx43 in the cell lysates revealed equal protein expression in corresponding lanes. (C, G) Immunoprecipitation of Cx26 and subsequent detection of Cx26 protein showed the presence of the protein in all samples containing either wild-type or mutant Cx26. (D, H) Co-immunoprecipitation and Western blot analysis of Cx43 revealed faint bands in samples expressing wild-type Cx26 and Cx43, and notably stronger bands in samples expressing mutant Cx26 and Cx43. The marked difference in band intensity suggested enhanced formation of heteromeric channels containing Cx43 and mutant Cx26.

Chapter 6 . Characterization of Cx26-N54H

ABSTRACT

Cx26 mutations associated with syndromic hearing loss and specifically, palmoplantar keratoderma (PPK) do not form functional channels. The Cx26-N54H mutation is found on the first extracellular domain of Cx26. It occurs on an asparagine residue known to be important for connexon-connexon interaction. We investigated the functional properties of Cx26-N54H and found it formed active hemichannels and gap junction channels. Despite having similarities in the clinical presentation of the disease, Cx26-N54H behaved differently from previously identified Cx26 PPK mutants, and is the first functional Cx26 PPK mutant to be identified.

INTRODUCTION

Gap junctions are formed by the oligomerization of six connexin subunits and their subsequent localization on apposing cellular membranes. These intercellular channels facilitate the passage of ions, secondary messengers and small metabolites (Harris, 2001). An aberration in transport is implicated in the causation of diseases such as syndromic hearing loss (Levit, Mese, Basaly, & White, 2012). Syndromic hearing loss associated with Cx26 mutations is presented clinical under two broad categories: keratitis icthyosis deafness (KID) syndrome or associated with palmoplantar keratoderma (PPK) (Laird, 2008). While Cx26 mutations linked to KID syndrome result in the formation of abnormal hemichannels (Sanchez et al., 2013), mutations causing PPK fail to make functional channels and inhibit other wild-type connexins (Rouan et al., 2001).

The resolution of the Cx26 protein structure confirmed the importance of the first and second extracellular loops in the docking of hemichannels on adjacent cells (Maeda et al., 2009; Rubin, Verselis, Bennett, & Bargiello, 1992b; White et al., 1995). The structure highlighted the importance of a few residues in forming hydrogen bonds and salt bridges, including asparagine 54 (N54). N54 was revealed to form bonds with leucine 56 on the opposite connexon. A recent report of a mutation of asparagine to histidine on residue 54 (N54H) presented a patient exhibiting sensorineural hearing loss (SNHL), with palmoplantar keratoderma and hyperkeratosis of the knuckle pads (Akiyama et al., 2007). Analysis of the patient's skin showed the presence of gap junction channels and an upregulation of Cx26 in the palmar epidermis, a characteristic of hyperkeratosis (Labarthe, Bosco, Saurat, Meda, & Salomon, 1998), and expression of Cx43 similar to control palmar skin.

Interestingly, a second mutation in N54 has also been implicated in syndromic hearing loss with PPK (Richard et al., 2004). This mutation results in the substitution of asparagine with lysine (N54K). Unlike Cx26-N54H, Cx26-N54K was found in several members of the same family. Similar to the epidermis with Cx26-N54H, electron microscopic evaluation of skin obtained from a patient with Cx26-N54K illustrated the presence of gap junctions between keratinocytes. However, immunohistochemical analyses of Cx26-N54K revealed weak protein expression in the palmar skin, but normal expression in the sweat glands and eccrine sweat ducts. Mutant Cx26 was not found localized in the cytoplasm, indicative of normal trafficking, decreased synthesis, or rapid degradation. Additional immunohistochemical study found Cx43 in patient skin was similar to the control, but Cx30 was considerably upregulated as compared to the control epidermis. This suggested Cx26-N54K expression was reduced in the epidermis, but led to an “overcompensatory” increase in the expression of Cx30 while not affecting Cx43.

Both mutations expressed Cx26 channels in palmar epidermis, as evidenced by immunohistochemical assays and did not affect Cx43 expression. Studies of other Cx26 PPK mutants have also shown expression of mutant Cx26 proteins *in vitro* (de Zwart-Storm, van Geel, et al., 2011) and *in vivo* (Rouan et al., 2001). Cx26-N54H and Cx26-N54K present as interesting mutations because of the residue’s role in connexon-connexon binding. Asparagine is an acidic amino acid and both Cx26-N54H and Cx26-N54K mutations change the residue to a basic amino acid. The mutation of asparagine to histidine might add more complexity due to the introduction of large aromatic side chain at position 54 resulting in a conformational change of the first extracellular loop. These

mutations likely change the electron charge at the residue, leading to alterations in voltage gating and function.

RESULTS

Cx26-N54H forms functional hemichannels

The first extracellular domain is highly conserved and mutations in it are linked to different pathologies. Mutations associated with KID syndrome form functional hemichannels that often display irregular activity (Gerido et al., 2007). Skin biopsy from a patient with the Cx26-N54H mutation showed the presence of gap junctions between keratinoctyes (Akiyama et al., 2007). We tested the activity of Cx26-N54H hemichannels by injecting *Xenopus* oocytes with cRNA. Cells were subjected to depolarizing voltage pulses and membrane currents were recorded. Control oocytes injected with water showed negligible current for voltage steps from -30 to +60 mV (Figure 6-1). The hemichannel activity of wild-type human Cx26 was characterized by outward currents that increased with depolarization (data not shown), and mean steady state currents were eleven times greater than control at +60 mV (Figure 6-1). Cells expressing Cx26-N54H expressed functional hemichannels that had outward currents similar to wild-type Cx26 (data not shown). The mean steady state currents for mutant channels were eight times higher than control at +60 mV (Figure 6-1). The mutant currents were slightly lower than wild-type Cx26 indicating a possible reduction in hemichannel activity (Student's t-test $p<0.05$). The presence of functional hemichannels led us to investigate the formation of gap junction channels.

Cx26-N54H forms functional gap junctions and does not inhibit wild-type connexins

Cx26 mutations associated with PPK have been characterized by non-functional gap junctions that also inhibit wild-type connexins (Rouan et al., 2001; Thomas et al., 2004). Since Cx26-N54H expressing cells demonstrated the formation of functional hemichannels, we next tested this mutant's gap junction channel activity alone and when co-expressed with wild-type Cx26 or Cx43. Cell pairs injected with water had negligible conductance (n=49) (Figure 6-2A), whereas pairs expressing wild-type Cx26 homotypic channels had a mean conductance of 2.8 μS (n=44, Student's t-test $p<0.05$) (Figure 6-2A). Oocytes expressing Cx26-N54H exhibited mean conductance of 1.9 μS (n=39) (Figure 6-2A), which was similar to wild-type Cx26 (Student's t-test $p>0.05$), indicating the formation of functional homotypic gap junctions. Co-expression of wild-type and mutant Cx26 also formed functional channels (G_j = 5.3 μS, n=21) (Figure 6-2A). Since Cx26-N54H did not alter wild-type Cx26 functionality, we tested its effect on Cx43.

Xenopus oocyte pairs were injected with water and tested for conductance which was again negligible (n=33) (Figure 6-2B). Cells injected with Cx43 displayed a mean conductance of 1.7 μS (n=23) (Figure 6-2B) which was significantly higher than control (Student's t-test $p<0.05$) and signified the formation of functional homotypic channels. Cells co-expressing Cx43 and wild-type Cx26 exhibited mean conductance of 4.5 μS (n=24) (Figure 6-2B), which was higher than the water injected control pairs. Finally, Cx43 and Cx26-N54H co-expressing cells were paired and found to be electrically coupled (G_j = 5.5 μS, n=24) (Figure 6-2). This assay demonstrated the formation of functional gap

junctions by Cx26-N54H mutant proteins and this mutant's inability to reduce wild-type connexin activity.

Wild-type and mutant connexin proteins are expressed in *Xenopus* oocytes

Although the functional assay demonstrated the presence of gap junction channels in the cells expressing wild-type and mutant connexins, a Western blot was completed for confirmation. *Xenopus* oocytes were injected with water, wild-type Cx26, Cx43, Cx26-N54H, and co-expressed in combinations with wild-type Cx26, Cx43 and Cx26-N54H. Immunoblot analysis showed Cx26 protein expression was similar in all samples injected with either wild-type or mutant Cx26 (Figure 6-3A). Re-probing the blot for Cx43 also revealed similar protein expression in all samples containing Cx43 (Figure 6-3B). This reflected what was suggested by the functional data, that connexin protein translation was unaffected by the mutant protein. The expression of β-tubulin was tested to validate equal loading of protein (Figure 6-3C).

Examination of Cx26-N54H revealed no functional difference between the mutant protein and wild-type Cx26. Asparagine 54 (N54) has been implicated as a residue necessary for connexon-connexon docking on adjacent cells. It would be interesting to see if a residue change, from an acidic asparagine to a basic histidine, which also has an aromatic side chain, would change the voltage gating and permeability of the channel. Study of this mutant's ability to form heterotypic channels with wild-type connexins would also help in its understanding. To date, Cx26-N54H is the only Cx26 mutation associated with PPK to exhibit hemichannel and gap junction functionality.

DISCUSSION

The majority of Cx26 mutations associated with PPK have exhibited a loss of channel activity and an inhibition of wild-type connexins. Cx26-N54H, while clinically presenting with symptoms similar to other PPK mutations, appears to have a different mechanism of action. In the data described above, Cx26-N54H was able to form functional hemichannels and gap junctions. Additionally, it did not exert any inhibitory action on wild-type Cx26 or Cx43. This mutation appears on a residue deemed necessary for connexon-connexon interaction, yet mutating it does not seem to interfere with this interaction. Since a genetic analysis confirmed Cx26-N54H was not a polymorphism, it is likely this mutation causes syndromic hearing loss by altering the voltage gating or permeability properties of the channel. Despite forming functional hemichannels, Cx26-N54H did not display abnormally high hemichannel currents similar to KID syndrome associated mutants. Until now, it had been proposed that Cx26 mutations associated with PPK always resulted in a dominant inhibition of wild-type Cx26. The formation of functional channels and lack of inhibition expands the spectrum mechanism of action for Cx26 PPK mutants.

Asparagine 54 has been implicated in another patient with Cx26 associated with PPK, however this mutation, Cx26-N54K seemingly resulted in a decrease in protein expression (Richard et al., 2004), in contrast to Cx26-N54H. The potential for two different mechanisms to cause the same disease by mutations on the same residue has been observed in KID syndrome with mutations Cx26-N14K and Cx26-N14Y (Arita et al., 2006; Lee & White, 2009). Electrophysiological assay of Cx26-N14K showed formation of functional hemichannels with large outward currents at +60 mV and surprisingly also gap

junctions with mean conductance values similar to wild-type Cx26 (Lee & White, 2009). Despite a similarity in conductance, analysis of voltage gating revealed a stark difference from wild-type Cx26 in that the mutant had a complete loss in voltage dependent gating. In contrast, Cx26-N14Y displayed a decrease in dye transfer indicative of a reduction in channel formation (Arita et al., 2006). The differences in mechanism for both mutations is a possible explanation for the phenotypic heterogeneity observed between the two patients.

A different mechanism resulting in KID syndrome has also been studied with the Cx26-S17F mutation (Lee & White, 2009; Richard et al., 2002). This mutation results in a severe form of KID syndrome, resulting in lethal carcinoma in one case (Mazereeuw-Hautier et al., 2007; Richard et al., 2002). Cx26-S17F does not exhibit functional hemichannels or gap junctions, behavior that is atypical of other KID syndrome mutations. Recently, it was reported that Cx26-S17F was able to increase Cx43 hemichannel activity (Garcia et al., 2015), indicating an interaction between a null mutation and Cx43. These mutants linked to KID syndrome based on clinical presentations have acute mechanistic variations, similar to our observation of Cx26-N54H which is clinically similar to PPK but has a different mechanism.

While all mutations associated with PPK have been demonstrated to be non-functional, Cx26-N54H is the first functional mutation identified. Cx26-N54H forms functional hemichannels and gap junctions, and does not inhibit wild-type connexins. It is likely that the change in residues to a bulkier basic amino acid is able to change the mutant protein's voltage gating and/or permeability. Despite the difference in mechanisms, like KID syndrome mutations, Cx26-N54H can still be classified as a PPK

mutation based on clinical diagnosis. However, the functional assay of Cx26-N54H expands the mechanisms causing syndromic hearing loss associated with PPK. Although an interaction between wild-type connexin proteins and Cx26 mutant proteins associated with PPK is a likely cause for the disease, further analyses of the biophysical properties of Cx26-N54H have to be completed to determine its mode of pathogenicity. As it is capable of forming functional channels, this mutant likely changes the permeability of the channel, thereby altering cellular homeostasis.

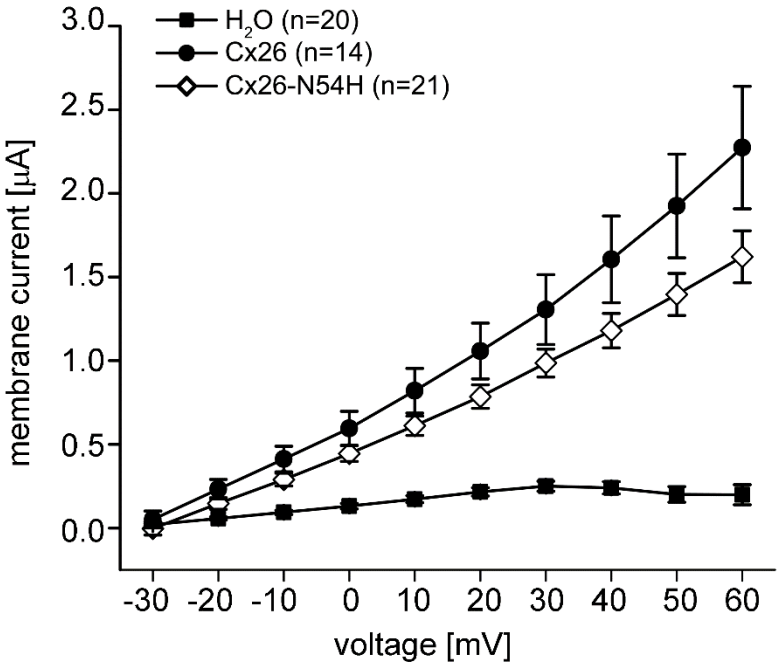

Figure 6-1. Cx26-N54H forms functional hemichannels. Cells were placed in a Ca2+ free solution and subjected to voltage steps from -30 to +60 mV. Steady-state currents for H_2O injected control cells (■) were negligible at all membrane voltages. Wild-type Cx26 (●) steady-state currents were similar to those observed in control cells at lower voltages, but increased at higher membrane voltage. Cx26-N54H (◇) steady-state currents were similar to wild-type Cx26 at lower voltages, but showed a reduction from wild-type at higher voltages. Data are the means ± SE.

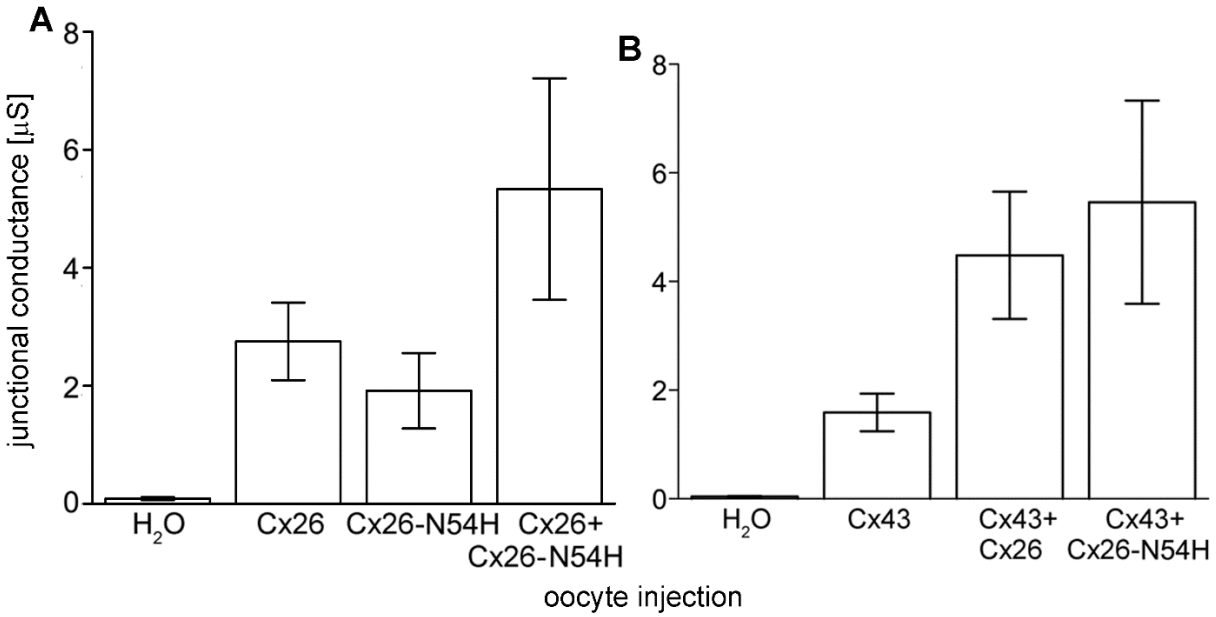

Figure 6-2. Cx26-N54H can form functional gap junctional channels and does not interfere with wild-type connexins. (A) Cells injected with H_2O displayed negligible conductance, while cells injected with wild-type Cx26 cRNA formed channels with a conductance value of 2.8 µS. Oocytes expressing mutant Cx26-N54H formed functional gap junction channels (Gj =1.9). A combination of wild-type and mutant Cx26 cRNA resulted in functional channels (Gj = 5.3 µS, Student's t-test $p>0.05$) from wild-type Cx26 alone. (B) Wild-type Cx26, Cx43 and mutant Cx26-N54H were expressed in cells alone or in combination. When expressed alone, or with wild-type Cx26, wild-type Cx43 is able to form functional gap junctional channels based on conductance that is significantly higher than background (Gj = 1.6 µS and 4.8 µS, respectively, Student's t-test $p<0.05$). A combination of wild-type Cx43 and Cx26-N54H cRNA showed electrical coupling indicating the mutant had no effect on wild-type protein's function (Gj = 5.5 µS, Student's t-test $p>0.05$). Data are the means ± SE.

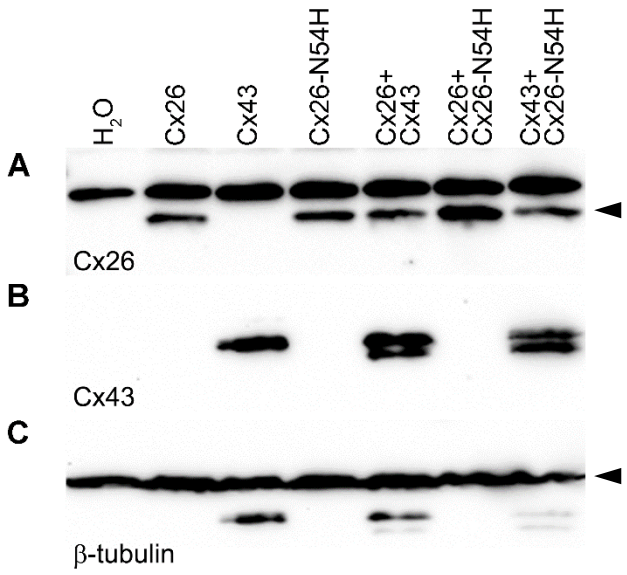

Figure 6-3. Wild-type and mutant connexin proteins were expressed in Xenopus oocytes. (A) Immunoblot analysis of oocytes expressing wild-type Cx26 and mutant Cx26-N54H showed equal expression of Cx26 proteins in all lanes. (B) Cx43 levels were the same in each condition. (C) The blot was re-probed for β-tubulin to confirm equal loading qualitatively.

Chapter 7 . Concluding Remarks and Future Directions

Connexin proteins are expressed ubiquitously in vertebrate tissues. They play an integral role in the maintenance of cellular homeostasis by facilitating intercellular communication. Connexins bind together to form a hexameric structure called a connexon or hemichannel. This structure can be composed of different connexin subunits leading to alterations in the voltage gating and permselectivity of the channel (Alexander et al., 2004; Bedner et al., 2006; Niessen, Harz, Bedner, Kramer, & Willecke, 2000; Weber, Chang, Spaeth, Nitsche, & Nicholson, 2004). Hemichannels can dock on cellular membranes and form nonjunctional channels that allow for exchange of material between the cell and the extracellular milieu. Intercellular channels are formed when two connexons dock on apposed cells resulting in the creation of a gap junction. Once considered non-selective, aqueous pores, it is now known that gap junctions are highly dynamic and selective in terms of passage of ions and small molecules (Veenstra, 1996).

There are twenty-one known connexin isoforms (Sohl & Willecke, 2004), which can interact to form heteromeric channels. However, the compatibility of connexins varies considerably and is dependent on sequence homology (Beyer et al., 2001; Lagree et al., 2003; Martinez et al., 2011; White & Bruzzone, 1996; White et al., 1994). The structure of connexin proteins is highly conserved and consists of two extracellular loops (E1 and E2), four transmembrane domains (TM1-4), a cytoplasmic loop (CL), a carboxy terminus (C-terminus) and an amino terminus (N-terminus) that are both located in the cytoplasm. Connexins are divided into five classes (A-E) (Sohl & Willecke, 2004), which are based

on sequence homology. The greatest variation in connexin sequence arises from the C-terminus.

The different structural domains in connexins play key roles in docking, intra-protomer interactions and gating. Study of connexin structural domains has highlighted the function of these domains in serving as voltage sensors and docking between connexons (White et al., 1995). Resolution of the structure of Cx26 validated these studies, and also provided a more detailed architecture of the charged pore (Maeda et al., 2009). The structure also revealed the residues responsible for intra- and inter-protomer interactions, and bonding between two connexons.

Connexin proteins are necessary components of intercellular communication as evidenced by the manifestation of adverse pathologies when they are mutated (Bruzzone et al., 2003; Bruzzone, White, Scherer, et al., 1994; Wei, Xu, & Lo, 2004). They are expressed in many tissues, including the epidermis (Risek et al., 1992), where nine connexins are found. These connexins are expressed in overlapping patterns, with Cx43 being the most abundant. Cx26 expression is limited to the basal keratinocytes and upregulated during wound repair or hyperkeratosis (C. A. Scott, Tattersall, O'Toole, & Kelsell, 2012). Intriguingly, Cx26 expression is absent in the epidermis of patients diagnosed with non-syndromic hearing loss, yet present in patients with syndromic hearing loss (Rouan et al., 2001). This observation suggests the native function of Cx26 in the skin is not essential and mutations conferring a gain of function result in the manifestation of skin disease.

In this dissertation three Cx26 mutations found in human patients exhibiting sensorineural hearing loss (SNHL) and palmoplantar keratoderma (PPK) were studied.

Building upon observations from previous studies (Rouan et al., 2001; Thomas et al., 2004), the channel activity of three Cx26 mutations, Cx26-H73R, Cx26-S183F and Cx26-N54H was analyzed. Since Cx26 PPK mutants have been shown to inhibit wild-type connexins, these mutants were also co-expressed with wild-type Cx26, Cx30 and Cx43. Examination of the hemichannel activity of these mutants indicated a complete loss of hemichannel function for Cx26-H73R and Cx26-S183F; Cx26-N54H displayed hemichannel currents significantly higher than control cells, but slightly less than wild-type Cx26. Co-expression of Cx26-H73R and Cx26-S183F with wild-type Cx26 demonstrated a reduction in the hemichannel activity of wild-type Cx26, however, co-expression with Cx43 showed an augmentation of Cx43 hemichannel activity. The change in Cx43 hemichannel behavior brought into focus the role of this protein in the causation of epidermal disorders. Moreover, an alteration in Cx43 hemichannel activity in the presence of non-functional proteins associated with syndromic hearing loss was also identified by another study (Garcia et al., 2015).

The modification of Cx43 hemichannel activity by null mutations, led to an exploration of gap junction channel properties. Electrophysiological studies of Cx26-H73R and Cx26-S183F demonstrated a lack of electrically coupling by the mutant proteins alone, but a distinct decline in Cx43 coupling in the presence of the mutants. An investigation of Cx43 biophysical properties when co-expressed with Cx26-H73R or Cx26-S183F exhibited a significant shift of Cx43 voltage gating towards a more asymmetric current decay similar to Cx26. This provided strong biophysical evidence for an interaction of Cx43 with the Cx26 mutants. Furthermore, these heteromeric channels showed changes in their closure kinetics, resulting in a faster time constant than

homotypic Cx43 channels. Absence of heterotypic channel formation by Cx43 and Cx26 mutants provided biophysical validation of the formation of heteromeric channels containing Cx43 and the Cx26 mutants. Aside from the biophysical data, a biochemical analysis via a co-immunoprecipitation was undertaken to confirm the formation of heteromeric channels consisting of Cx43 and Cx26 mutants. Together, these analyses demonstrated an alteration to Cx43 channel activity by Cx26 mutants as a result of a pathogenic interaction which forms a novel heteromeric channel. Unlike Cx26-H73R and Cx26-S183F, Cx26-N54H formed functional gap junctions and did not inhibit wild-type Cx26 or Cx43, suggesting it operated through a different mechanism. These studies used electrophysiological and biochemical assays to provide direct evidence of heteromeric mutant Cx26 and Cx43 channel formation, and offer an explanation for the gain of function activity conferred by these mutations, while simultaneously demonstrating potential differences from KID syndrome related mutations (Table 7-2).

These three Cx26 mutations associated with PPK exhibited channel behavior distinct from mutations linked to keratitis ichthyosis deafness (KID) syndrome (Levit et al., 2012; Mhaske et al., 2013; Sanchez et al., 2010). Therapeutic approaches targeting KID syndrome have been based largely on pharmacological channel inhibitors such as Zn^{2+} (Verselis & Srinivas, 2008), 2-Aminoethoxydiphenyl borate, 5-nitro-2-(3-phenylpropylamino)-benzoate, or flufenamic acid (Bai, del Corsso, Srinivas, & Spray, 2006; Eskandari, Zampighi, Leung, Wright, & Loo, 2002; Spray, Rozental, & Srinivas, 2002; Srinivas & Spray, 2003; Tao & Harris, 2007). KID syndrome usually occurs as a result of increased hemichannel activity creating a “leaky” channel. It has been shown *in vitro* that hemichannel currents can be attenuated by the application of Zn^{2+} and

mefloquine (Levit et al., 2015). The fundamental mechanistic difference between KID syndrome and PPK make it challenging to use broad spectrum inhibitors for PPK mutations.

Unlike KID syndrome, Cx26 mutations associated with KID syndrome must interact with wild-type connexins to cause skin disease. Any therapeutic approach needs to appreciate this phenomenon prior to focusing on a singular protein. Since Cx26 PPK mutations appear to localize to the cytoplasm, except when presented as heteromeric structures, many channel inhibitors cannot be used lest they cross the plasma membrane. It is also unlikely to change these mutants function since they are non-functional proteins alone. However, the use of siRNAs to block pathogenic Cx26 proteins via Cx43 (Valiunas et al., 2005) provides one possible solution because cells co-expressing mutant Cx26 and Cx43 still exhibit some conductance. Despite the convenience of specificity presented by this method, the fact that these mutations form heteromeric channels with Cx43 brings about a new challenge. The pathogenic heteromeric channel clearly has different channel properties from Cx43, and likely has an alteration in pore size. It is known that Cx43 has a wide pore which allows for the passage of large molecules like siRNAs, whereas hybrid channels composed of Cx26 and Cx32 are impermeable. A possible bypass to this dilemma is the use of peptide enhancers which can be conjugated to the siRNA and used for delivery (Chen et al., 2014; Hsu & Mitragotri, 2011). These peptide enhancers can be used for subcutaneous delivery due to their deep penetration across the stratum corneum into the epidermis and dermis. Since PPK results in hyperkeratosis, a peptide enhancer could serve an ideal partner to deliver any siRNA directly into afflicted keratinocytes. Aside from siRNAs, mimetic peptides can be used to

target Cx43 hemichannels. The enhancement of Cx43 hemichannel activity by Cx26 mutants is a potential target. These abnormal hemichannels can be targeted by Gap19, a mimetic peptide that selectively inhibits Cx43 hemichannels but not gap junctions (N. Wang et al., 2013). Presumably, Gap19 would inhibit Cx43/mutant Cx26 hemichannels since it targets the C-terminus of Cx43. But again, it cannot be discounted that a loss of Cx43 channel activity may lead to further disruption of the epidermis.

Cx26 mutations associated with PPK present an intriguing question about treatment options: target either Cx26 or Cx43. The lack of gap junction plaques at cellular appositions by Cx26 mutants alone make it difficult to target them with small molecule inhibitors that are impermeable to the plasma membrane. However, siRNAs, in conjunction with peptide enhancers, can be used as a possible option to block Cx26 mutants within the cytoplasm without disrupting Cx43. In theory this would allow Cx43 to form gap junctions devoid of the pathogenic properties exerted by the mutants. The use of mimetic peptides is another intriguing option since it could be used to selectively target Cx43 hemichannels, a structure that is abnormally active in the presence of Cx26 mutants. Overall, it is necessary to screen these molecules for their therapeutic benefits in disrupting the formation of Cx43/mutant Cx26 heteromeric channels.

The observation of functional channel formation by Cx26-N54H provides yet another tool to study Cx26 mutations associated with PPK. This mutation can serve as a conduit for testing drugs and small molecules, but it can also be used to study the structural changes brought to the channels by these mutations, which contribute to the formation of heteromeric channels and alter the permselectivity of the channels. Cx26-N54H likely has an altered voltage or permeability from wild-type Cx26, thus resulting in

PPK. Homotypic channels containing Cx26-N54H can be studied for their pore specificity and to understand how changes in the charge at the extracellular loop can alter permeability of the channel. Study of a skin biopsy from a patient with this mutation suggests there is co-localization of the mutant protein with Cx43. Screening of heteromeric channels containing both Cx26-N54H and Cx43 can provide insight with regards to specifically targeting PPK mutants in a mixed configuration.

Aside from therapeutic approaches, further study of other PPK mutants is critical to establish a pattern for the mode of action of this disease. Preliminary data investigating the hemichannel activity of Cx26-D66H and Cx26-G130V suggests these mutants are similar to Cx26-H73R and Cx26-S183F, and unable to form functional hemichannels (Figure 7-1). These mutant proteins may also be able to slightly enhance the hemichannel activity of Cx43, but more work is required for a conclusive demonstration. Analysis of Cx26-R75W has demonstrated the formation of functional hemichannels in cells expressing it alone (Figure 7-1), similar to Cx26-N54H hemichannels, thereby providing a potential second Cx26 PPK mutation that may be used as a tool for studying palmoplantar keratoderma linked with Cx26.

Understanding the mechanism whereby PPK associated with Cx26 arises provides the groundwork for therapeutic approaches. The mechanistic heterogeneity demonstrated by these mutations can be considered a factor in the phenotypic heterogeneity observed in patients. Identification of functional mutant proteins presents the opportunity to use these mutations as tools for studying different molecules and drugs targeting PPK and also can be used to gain key insights in the inherent differences between PPK and KID syndrome. Although a majority of identified KID syndrome

associated mutations form functional channels, resulting in disease, few PPK associated mutations have been shown to form functional channels. A hypothesis for why functional channels may result in disease is based on the theory that these result in changes in Ca^{2+} concentration which would affect the development of keratinocytes (Fairley, 1991). These functional mutations can be used to target the regulation of Ca^{2+} to better understand PPK and its mechanistic difference from KID syndrome.

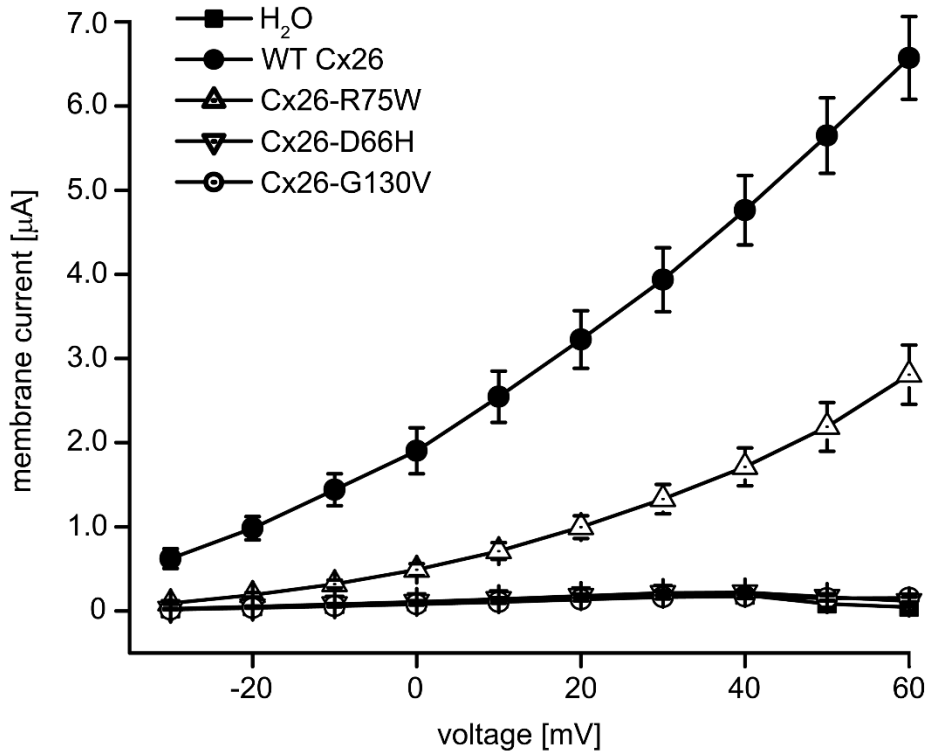

Figure 7-1. Cx26 mutants associated with PPK display varied hemichannel activity. Steady-state membrane current from each step was plotted as a function of membrane voltage. Steady-state currents for H_2O injected control cells (■) were negligible at all membrane voltages (n=21). Wild-type Cx26 (●) steady-state currents increased at each membrane voltage pulse (n=8). Both mutant Cx26-D66H (▽) and Cx26-G130V (⊙) injected cells failed to show increasing steady-state currents, rather their currents were negligible and resembled control cells (n=29 and n=13, respectively) Mutant Cx26-R75W (△) steady-state currents were higher than control cells, but lower than wild-type (n=17). Data are the means ± SE.

Table 7-1. Overview of Cx26 mutations associated with KID syndrome and PPK

Cx26 mutation	Electrophysiological assay	Dye transfer	Co-immunoprecipitation	Reference
G12R	Yes			(Lee et al., 2009)
N14K	Yes			(Lee et al., 2009)
S17F	Yes	Yes		(Lee et al., 2009; Richard et al., 2002)
A40V	Yes			(Gerido et al., 2007; Sanchez et al., 2010)
ΔE42	Yes			(Rouan et al., 2001)
G45E	Yes			(Gerido et al., 2007; Lee et al., 2009; Sanchez et al., 2010)
D50A	Yes			(Mhaske et al., 2013)
D50N	Yes			(Lee et al., 2009)
N54H	Yes			Figures 6-1 and 6-2

G59A		Yes		(Thomas et al., 2004; J. Zhang, Scherer, & Yum, 2011) (Marziano et al., 2003)
D66H	Yes	Yes		(Rouan et al., 2001; Thomas et al., 2004), Figure 7-1
H73R	Yes		Yes	Figures 3-1, 3-2, 3-3, 4-2, 4-3, 4-5, 4-6, 5-3
R75W	Yes	Yes		(Marziano et al., 2003; Rouan et al., 2001; J. Zhang et al., 2011), Figure 7-1
A88V	Yes			(Mhaske et al., 2013)
G130V	Yes			Figure 7-1
S183F	Yes		Yes	Figures 3-1, 3-2, 3-3, 4-1, 4-3, 4-5, 4-6, 5-3

Table 7-2. Summary of biophysical properties of Cx26 mutations associated with KID syndrome and PPK

Cx26 mutation	Syndrome	Hemichannels	Gap Junctions	Change Cx43 Hemichannels	Inhibit WT Connexins Gap Junctions	Alter Cx43 Gating	Reference
G12R	KID	Yes	No	Yes		?	(Garcia et al., 2015; Lee et al., 2009)
N14K	KID	Yes	Yes	?		?	(Lee et al., 2009)
S17F	KID	No	No	Yes		?	(Garcia et al., 2015; Lee et al., 2009; Richard et al., 2002)
A40V	KID	Yes	Yes	?		?	(Sanchez et al., 2010)
ΔE42	PPK	?	No	?	Cx26, Cx43	?	(Rouan et al., 2001)

G45E	KID	Yes	Yes	?	No	?	(Gerido et al., 2007)
D50A	KID	Yes	?	?		?	(Mhaske et al., 2013)
D50N	KID	Yes	No	?		?	(Lee et al., 2009)
N54H	PPK	Yes	Yes	?	No	?	Figures 6-1 and 6-2
G59A	PPK	?	No	?	Cx26, Cx30, Cx32, Cx43	?	(Marziano et al., 2003; Thomas et al., 2004)
D66H	PPK	No	No	?	Cx26, Cx30, Cx43	?	(Marziano et al., 2003; Rouan et al., 2001; Thomas et al., 2004), Figure 7-1

H73R	PPK	No	No	Yes	Cx43	Yes	Figures 3-1, 3-2, 3-3, 4-2, 4-3, 4-5, 4-6, 5-3
R75W	PPK	Yes	No	?	Cx26, Cx30, Cx43	?	(Rouan et al., 2001) (Marziano et al., 2003), Figure 7-1
A88V	KID	Yes	?	?		?	(Mhaske et al., 2013)
G130V	PPK	No	No	?	?	?	Figure 7-1
S183F	PPK	No	No	Yes	Cx26, Cx43	Yes	Figures 3-1, 3-2, 3-3, 4-1, 4-3, 4-4, 4-5, 4-6, 5-3

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